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# Localization of NADPH Diaphorase/Nitric Oxide Synthase and Choline Acetyltransferase in the Spinal Cord of the Frog, *Rana perezi*

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ABSTRACT

The localization of nitrergic cells and fibers and cholinergic cells has been analyzed in the spinal cord of the anuran amphibian *Rana perezi*. Histochemistry for nicotinamide adenine dinucleotide phosphate–diaphorase and nitric oxide synthase immunohistochemistry revealed a concurrent pattern of labeled structures. A large population of nitrergic spinal neurons was found from the level of the obex to the filum terminale. They are abundant in the dorsal horn and intermediate gray matter, but also occur in territories of the ventral horn and, only occasionally, in somatic motoneurons. Numerous nitrergic fibers were present in the spinal white matter, particularly in the dorsal and dorsolateral funiculi. A special arrangement of nitrergic axons is present in Lissauer's tract, where a collateral system is formed. Cholinergic cells, revealed by choline acetyltransferase immunohistochemistry, were observed throughout the spinal cord. The somatic motoneurons were the most conspicuously immunoreactive cells. A large population of cholinergic cells forms a discontinuous column in the intermediate gray, from the third spinal segment to lumbar segments. These cells were organized in a medially located or intercalated cell group, and a laterally located intermediolateral group. Numerous scattered cholinergic cells were present in the central zone of the ventral horn and were absent in the dorsal horn. Double-labeling experiments revealed a high degree of codistribution of nitrergic and cholinergic cells, mainly in the intermediate gray, but colocalization of both markers in the same neurons was not found. This result contrasts with the situation found in mammals and raises the question of whether coexpression of both substances was acquired in spinal cord neurons through evolution only in amniotes or, even, only in mammals. J. Comp. Neurol. 419:451–470, 2000. 

**Indexing terms: acetylcholine; immunohistochemistry; preganglionic autonomic cells; amphibians**

The evolutionary history of nitric oxide (NO) systems in the central nervous system of vertebrates has received increasing attention in the last few years, and comprehensive studies on the distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) and/or nitric oxide synthase (NOS)–positive structures in the brain of representative species of each main vertebrate class are now available (Brüning, 1993; Schober et al., 1993, 1994; Brüning et al., 1994, 1995; Holmqvist et al., 1994; Panzica et al., 1994; Arévalo et al., 1995; Villani and Guarnieri, 1995; Brüning and Mayer, 1996; González et al., 1996; M. Muñoz et al., 1996; Smeets et al., 1997). Recent pharmacological studies have implicated NO production in the spinal cord in mechanisms mediating nociception, central sensibilization, and hyperanalgesia (Meller et al., 1992; Meller and Gebhart, 1993; McMahon et al., 1993). The

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localization of nitrergic neurons in the spinal cord of several mammalian species has been investigated either by NADPHd histochemistry (Leight et al., 1990; Anderson, 1992; Brüning, 1992; Valtschanoff et al., 1992a; Spike et al., 1993; Vizzard et al., 1994a; Wetts and Vaughn, 1994; Smithson and Benarroch, 1996; Vizzard et al., 1997; Marsala et al., 1999) or NOS immunohistochemistry (Blottner and Baumgarten, 1992; Dun et al., 1992; Valtschanoff et al., 1992b; Dun et al., 1993; Terenghi et al., 1993; Egberongbe et al., 1994; Saito et al., 1994; Vizzard et al., 1994d; Kanda, 1996). The distribution of labeled neurons appears to differ among species, and discrepancies exist between results obtained with different techniques. In all of these studies, the majority of nitrergic neurons were found in the superficial dorsal horn and in the autonomic nuclei. In addition, nitrergic cells were reported in the dorsal root ganglia (Aimi et al., 1991; McNeill et al., 1992; Vizzard et al., 1993a, 1994a), and abundant fibers were described in Lissauer's tract (LT) and a collateral system formed by the axons of this tract (Dun et al., 1993; Vizzard et al., 1993a,b, 1994a,b,c,d; Pullen and Humphreys, 1995; Vizzard et al., 1997; Marsala et al., 1999). This distribution pattern strengthened the notion of NO involvement in somatosensory processing and autonomic transmission.

Very few studies, however, have dealt with the distribution of NADPHd/NOS cells and fibers in the spinal cord of nonmammalian vertebrates (Radmilovich et al., 1997; Smeets et al., 1997). Nevertheless, these studies have shown that the presence of labeled spinal cells seems to be a characteristic feature shared among vertebrates. Previous studies in amphibians have reported that NADPHd-reactive cells are widely distributed in the spinal cord, at least in the upper segments (M. Muñoz et al., 1996; González et al., 1996). NADPHd histochemistry was used as an adjunct to characterize sympathoadrenal preganglionic neurons in some spinal segments of the bullfrog (Peruzzi and Forehand, 1993) and to study developmental changes in the spinal cord of *Xenopus laevis* (Crowe et al., 1995).

The distribution of NADPHd/NOS cells in the spinal cord has been frequently related to the spinal cholinergic cell groups, particularly the preganglionic neurons (Blottner and Baumgarten, 1992; Dun et al., 1992, 1993; Spike et al., 1993; Wetts and Vaughn, 1994; Vizzard et al., 1994a; Papka et al., 1995; Vizzard et al., 1997). Cholinergic neurons were identified reliably by choline acetyltransferase (ChAT) immunohistochemistry, and colocalization studies in the spinal cord of the rat demonstrated that certain spinal populations may be heterogeneous in regard to intracellular messenger phenotypes involving acetylcholine and NO (Blottner and Baumgarten, 1992; Dun et al., 1993; Spike et al., 1993; Wetts and Vaughn, 1994). In a recent study using ChAT immunohistochemistry, the organization of the cholinergic system in the brain of amphibians has been reported (Marín et al., 1997), but a comprehensive analysis of ChAT-positive cell groups in the spinal cord is still lacking.

The aim of the present study was, therefore, threefold: (1) to provide a detailed analysis of NADPHd/NOS-containing neuronal structures in the spinal cord of anuran amphibians; (2) to describe the distribution of cholinergic cell bodies throughout the spinal cord; and (3) to investigate possible colocalization of NADPHd/NOS with ChAT in anuran spinal cord cells. By comparing the results of this study with those obtained in mammals and other nonmammalian vertebrates, it may be discovered to what extent spinal cord organization has been conserved during evolution.

*Abbreviations*

| | |
|---|---|
| cc | central canal |
| DF | dorsal funiculus |
| dh | dorsal horn |
| DLF | dorsolateral funiculus |
| IC | intercalated cell column |
| IML | intermediolateral cell column |
| LCP | lateral collateral pathway |
| LDT | laterodorsal tegmental nucleus |
| LF | lateral funiculus |
| LT | Lissauer's tract |
| MCP | medial collateral pathway |
| SMN | somatic motoneurons |
| VF | ventral funiculus |
| vh | ventral horn |

## MATERIALS AND METHODS

For the present study, a total of 47 adult Iberian green frogs (*Rana perezi*) were used. The original research reported herein was performed under guidelines established by the Spanish Royal decree 223/1988. The animals were obtained from the laboratory stock of the Department of Cell Biology, University Complutense of Madrid. All animals were anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz; pH 7.3) and perfused transcardially with saline followed by 150–200 ml 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4). The brain and spinal cord were removed and kept in the same fixative for 2–3 hours. Subsequently, they were immersed in a solution of 30% sucrose in PB for 3–5 hours at 4°C until they sank, embedded in a solution of 15% gelatin with 30% sucrose in PB, and then stored for 5 hours in a 4% formaldehyde solution at 4°C. The complete spinal cords were cut on a freezing microtome at 40 μm in the frontal, sagittal, or horizontal plane and collected in PB. They were then rinsed twice in PB, treated with 1% $H_2O_2$ in PB saline (PBS, pH 7.4) for 15 minutes to reduce endogenous peroxidase activity, and rinsed again three times in PBS.

### NADPHd histochemistry (n = 10)

Free-floating sections were rinsed in Tris-buffered saline (TBS), 0.1 M, pH 8.0 and then were incubated in a medium made up of 1 mM β-NADPH, 0.8 mM nitro blue tetrazolium, and 0.08% Triton X-100 in 0.1 M TBS (pH 8.0), at 37°C for 1–2 hours. The reaction was stopped by successive rinses in cold TBS. Some sections were incubated in a medium without β-NADPH. A second group of control sections was heated in TBS to 70°C for 10 minutes. In both cases, no reaction was observed. All sections were then mounted on slides (mounting medium: 0.25% gelatin in 0.1 M Tris buffer, pH 7.6), dried overnight, and coverslipped. In some cases, sections were counterstained with 0.1% cresyl violet.

### NOS immunohistochemistry (n = 6)

After rinsing in PB, free-floating sections were incubated in a sheep antiserum against neuronal NOS (K205 antibody, donated by Dr. P.C. Emson), diluted 1:20,000 in TBS containing 0.5% Triton X-100 (TBS-T), for 48–60

hours at 4°C. Rhodamine-conjugated donkey anti-sheep secondary antiserum (Chemicon, Temecula, CA) diluted 1:100 in TBS-T was applied for 1.5 hours at 20°C, or fluorescein-conjugated rabbit anti-sheep secondary antibody (Vector, Burlingame, CA) diluted 1:100 in TBS-T was used. The sections were mounted on glass slides and coverslipped with Vectashield (mounting medium for fluorescence, Vector).

Immunohistochemical control experiments involved parallel incubation of alternate sections either with antiserum raised against different antigens, normal serum, or with the omission of primary antiserum. No residual immunostaining was detected. Furthermore, the specificity of the antiserum has been previously described (Herbison et al., 1996). This antibody recognizes neuronal NOS using Western blotting, and the immunoreactivity is abolished by absorption of the K205 antiserum with recombinant neuronal NOS protein (1 mM overnight at 4°C).

### Double labeling for NADPHd and NOS (n = 9)

NOS-immunoreacted sections as described above were mounted on glass slides and studied, plotted, and photographed, without drying. They were then collected in TBS and rinsed profusely for 1 hour. The histochemical technique for NADPHd was subsequently performed. The comparative study of both stainings was accomplished throughout the spinal cord, after plotting and photographing the NADPHd-reactive elements, analyzing all sections at all segments.

### ChAT immunohistochemistry (n = 12)

Spinal cord sections were processed for ChAT immunohistochemistry by the peroxidase antiperoxidase (PAP) method (Sternberger, 1979). This included a first incubation of the sections in a goat anti-ChAT serum (Chemicon), diluted 1:100 in PBS containing 0.5% Triton X-100, 15% normal rabbit serum (NRS), and 2% bovine serum albumin (BSA), for 40 hours at 4°C. Subsequently, the sections were rinsed three times in PBS for 10 minutes and incubated for 1 hour in rabbit anti-goat serum (1:50, Chemicon). After rinsing again three times for 10 minutes, the sections were incubated for 90 minutes in goat PAP (1:600, Chemicon). Secondary antiserum and PAP complex were diluted in PBS containing Triton X-100, NRS, and BSA in the same concentrations as used for the primary antiserum. Finally, the sections were rinsed three times for 10 minutes in PBS and twice in 0,05 M Tris-HCl buffer (TB, pH 7.6), and subsequently stained in 0.5 mg/ml 3,3-diaminobenzidine (DAB) with 0.01% $H_2O_2$ in TB for 5–15 minutes. A series of sections was stained according to the glucose oxidase method (Shu et al., 1988), which specifically enhances the staining of nerve fibers and terminals. Briefly, after rinsing in PBS, the sections were rinsed in 0.1 M acetate buffer (AB, pH 6.0) for 10 minutes and, subsequently, incubated in a medium containing 0.5 mg/ml DAB, 0.027 mg/ml glucose oxidase (Sigma, type VII), 25 mg/ml nickel ammonium sulfate (Merck), 2 mg/ml D-glucose (Merck), and 0.4 mg/ml ammonium chloride (Merck) in AB for 5–10 minutes. The sections were rinsed twice in AB and another three times in TB. The sections were then mounted (mounting medium: 0.25% gelatin in TB) and, after drying overnight, coverslipped. Some sections were counterstained with cresyl violet to facilitate the analysis of the results.

The specificity of the antisera was tested previously (Shiromani et al., 1987; Medina and Reiner, 1994; Grosman et al., 1995). As a further control, the primary antiserum was omitted from some sections in each experiment, which resulted in no specific labeling of somata or fibers.

### Double labeling for NADPHd and ChAT (n = 15)

Two sets of experiments were performed to stain ChAT and NADPHd in the same sections throughout the spinal cord. In the first group (n = 6), ChAT-immunoreacted sections in which fluorescein-conjugated rabbit anti-goat second antiserum (diluted 1:100, Chemicon) was used were studied, plotted, and photographed and, subsequently, the histochemical technique for NADPHd was performed. In the second set of experiments (n = 9), NADPHd-reacted sections were incubated in goat anti-ChAT antiserum (Chemicon) diluted 1:100 in TBS-T for 48–60 hours, at 4°C. The sections were subsequently processed with the PAP technique. This included incubation in goat anti-goat IgG (Dakopatts) diluted 1:50 in TBS-T, for 1 hour at room temperature, and then in goat PAP (Chemicon) diluted 1:500 in TBS-T, for 1 hour at room temperature. They were reacted with 0.5 mg/ml DAB (Sigma) with 0.01% $H_2O_2$ in TBS, for 10–20 minutes. Some sections that were mounted, coverslipped, and photographed were then immersed in xylene (until the coverslip was detached) and subsequently in 100% diethyl amide (Sigma), at 60°C, for 5–7 days to eliminate the blue reaction product of the NADPHd histochemistry (Alonso et al., 1995).

### Evaluation and presentation of the results

The pattern of distribution of NADPHd activity in the spinal cord of *R. perezi* matches that of NOS immunolabeling. However, the clear picture of well-defined cells and fiber structures yielded by NADPHd histochemistry was not obtained by NOS immunohistochemistry. Therefore, the description of the cell groups and fiber systems with putative NOS activity were based on the material stained for NADPHd.

The demonstration of NADPHd activity was achieved by means of a direct method using exogenous NADPH (Alonso et al., 1995). In this variant, the activity of endogenous NADPHd reduced NADPH in the presence of the dye nitro blue tetrazolium to form a blue insoluble reaction product that labels neuronal elements in the brain. The distribution of NADPHd-positive cell bodies and fibers and ChAT immunoreactive (ChATi) neurons in the spinal cord of *R. perezi* was studied throughout the length of the spinal cord attending to brachial (segments 3–4), thoracic (segments 5–7), lumbar (segments 8–9), sacral (segments 10–11), and filum terminale levels (ten Donkelaar, 1998). The pattern of labeling was charted in representative transverse sections at different spinal segments. Drawings were made by means of camera lucida in which the sections counterstained with cresyl violet facilitated the interpretation of the localization of the reactive structures.

## RESULTS

For clarity, we first describe the distribution of nitrergic cell bodies and fibers and, subsequently, we analyze the

location of ChATi neuronal elements throughout the spinal cord of the frog. Finally, we describe the results of the double labeling experiments used to assess colocalization of NADPHd/NOS and ChAT.

## NADPHd/NOS in the spinal cord

The pattern for the distribution of NOS immunoreactive neuronal elements in the spinal cord of the frog usually appeared identical to that obtained for NADPHd histochemistry, suggesting a colocalization of these markers (Fig. 1a–f). To corroborate this, horizontal sections of two animals were first immunolabeled for NOS and photographed. Subsequently, the sections were restained for NADPHd and the sections were rephotographed. We have analyzed the photographs corresponding to the same section and have observed a one-to-one correlation between NOS- and NADPHd-labeled neurons and fibers in all spinal segments (Fig. 1c,f). Because our results demonstrated that these techniques yield nearly identical labeling of the putative nitrergic structures, in the following description we will refer to nitrergic cells and fibers independently of the technique used. However, it should be noted that because of the simplicity of the histochemical technique to reveal NADPHd and the Golgi-like images of cell profiles and fiber processes that it yields, the drawings and most of the pictures presented were based on NADPHd-stained material.

***Nitrergic neurons.*** In the frog spinal cord, a large population of nitrergic neurons was found from the level of the obex to the filum terminale (Fig. 2). The morphology of the cells varies depending on their location within the spinal cord. The size was also variable and, in general, we have subdivided the labeled neurons into three size categories: small (7–20 μm), medium (20–35 μm), and large (35–50 μm). In addition, a mixed population of large and small nitrergic cells was found in the dorsal root ganglia, at all spinal segments (Fig. 3a). In the spinal cord of amphibians, it is not possible to recognize a laminar organization of the gray matter (Ebbesson, 1976) and, therefore, the distribution of nitrergic elements will be framed within the dorsal horn, intermediate gray, ventral horn, and filum terminale.

*Dorsal horn.* Prominent staining of numerous cells was found in the dorsal horn in all spinal segments (Figs. 2, 3). At brachial levels, a cluster of labeled cells is present in the medial portion of the dorsal horn corresponding the dorsal column nucleus (Muñoz et al., 1995). These small neurons have abundant processes directed dorsally toward the dorsal funiculus (DF). Similarly, at the lateral aspect of the dorsal horn, the caudal extent of the nucleus of the descending trigeminal tract contained nitrergic neurons whose dendrites arborized laterally among the descending fibers of the trigeminal tract.

In the dorsal horn of the spinal cord nitrergic cells formed a conspicuous population arranged amidst a densely labeled neuropil (Figs. 2, 3b,c). Labeled neurons located laterally were small to medium-sized cells with round to oval perikarya and long processes that extended toward the DF, dorsolateral funiculus (DLF), and lateral funiculus (LT). Some short processes of these cells coursed ventrally and medially in the gray matter. Medium-sized, multiform cells possessing radially oriented dendrites were observed in the dorsal neuropil (Figs. 2, 3b). The highest number of labeled cells was found at the ventral limit of the dorsal neuropil. These cells generally were larger than those located more dorsally. Their long processes were directed dorsally and some entered the DLF and LT, whereas short processes arborized ventrolaterally (Figs. 2, 3c–e). Processes from neurons located close to the midline extended dorsally into the ipsilateral and contralateral dorsal horns (Figs. 2, 3d).

*Intermediate gray.* Scattered cells of very different morphology were labeled in discrete clusters located at periodic intervals in the intermediate gray matter (Fig. 4). These neurons emanate numerous processes in a longitudinal axis, which interconnect discrete groups of neurons along the rostrocaudal axis of the cord. Nitrergic cells of the intermediate gray matter occupied positions from the central canal to the lateral gray border, and some cells were located in the LF and DLF (Figs. 1a,b, 2, 3c, 4a). The neurons located medially showed numerous long radial processes that reached the region around the central canal (medially) and the white matter (laterally) (Figs. 3c, 4c). Nitrergic cells in the lateral aspect of the gray possessed long processes that intermingle with the fibers of the DLF and LF, and shorter dendrites that extended medially (Figs. 1a,b, 3c, 4a,d). In some cases, labeled cells were found at the margin between gray/white matters, and their long processes outlined the dorsal and ventral borders of the gray matter (Figs. 2, 4e).

At cervical levels, some neurons were located at the lateral border of the gray or in the LF, and their long processes were directed ventrolaterally (Figs. 2, 4f). Because of their position and morphology, they might correspond to cells of the anuran lateral cervical nucleus (A. Muñoz et al., 1996).

Some nitrergic cells located medially in the intermediate gray at thoracic segments showed long processes that extended into ventromedial territories. Many of these processes crossed in the ventral commissure to reach the contralateral ventral funiculus (VF) (Fig. 2).

*Ventral horn.* As in other spinal gray regions, abundant nitrergic cells were distributed in the ventral horn at all spinal segments (Figs. 2, 3c, 5). In general, they were polymorphic medium-sized cells. Neurons located dorsally in the ventral horn had numerous long processes preferentially extending to the LF. Some of these cells showed dendritic arborizations that reached the contralateral ventral horn and even the LF (Figs. 2, 3c, 5a). At the lateral border of the horn, labeled cells with long processes oriented medially and laterally were observed. In some cases, these processes outlined the limit of the horn (Figs. 2, 5a). Nitrergic cells were also present in a medial position close to, and sometimes within, the VF and whose long processes were radially directed, frequently reaching the contralateral side (Figs. 2, 5a–c). In some cases, they formed a net in the medial margin of the ventral horn.

The rest of the nitrergic neurons in the ventral horn were loosely distributed and showed radial dendritic processes that reached the white matter and intermingled with the fibers of the ventral, lateral, or occasionally, the dorsolateral funiculi (Figs. 2, 3c, 5a,d,e). Staining of somatic motoneurons in the ventral horn was generally not observed, although numerous small cells were always labeled. Only in two cases of the 34 animals whose spinal cord were stained for NADPHd were one and four (respectively) big multipolar cells labeled among the somatic motoneurons at lumbar levels (Fig. 5d,e). However, these cases belonged to the group of experiments in which only NADPHd histochemistry was carried out in the whole

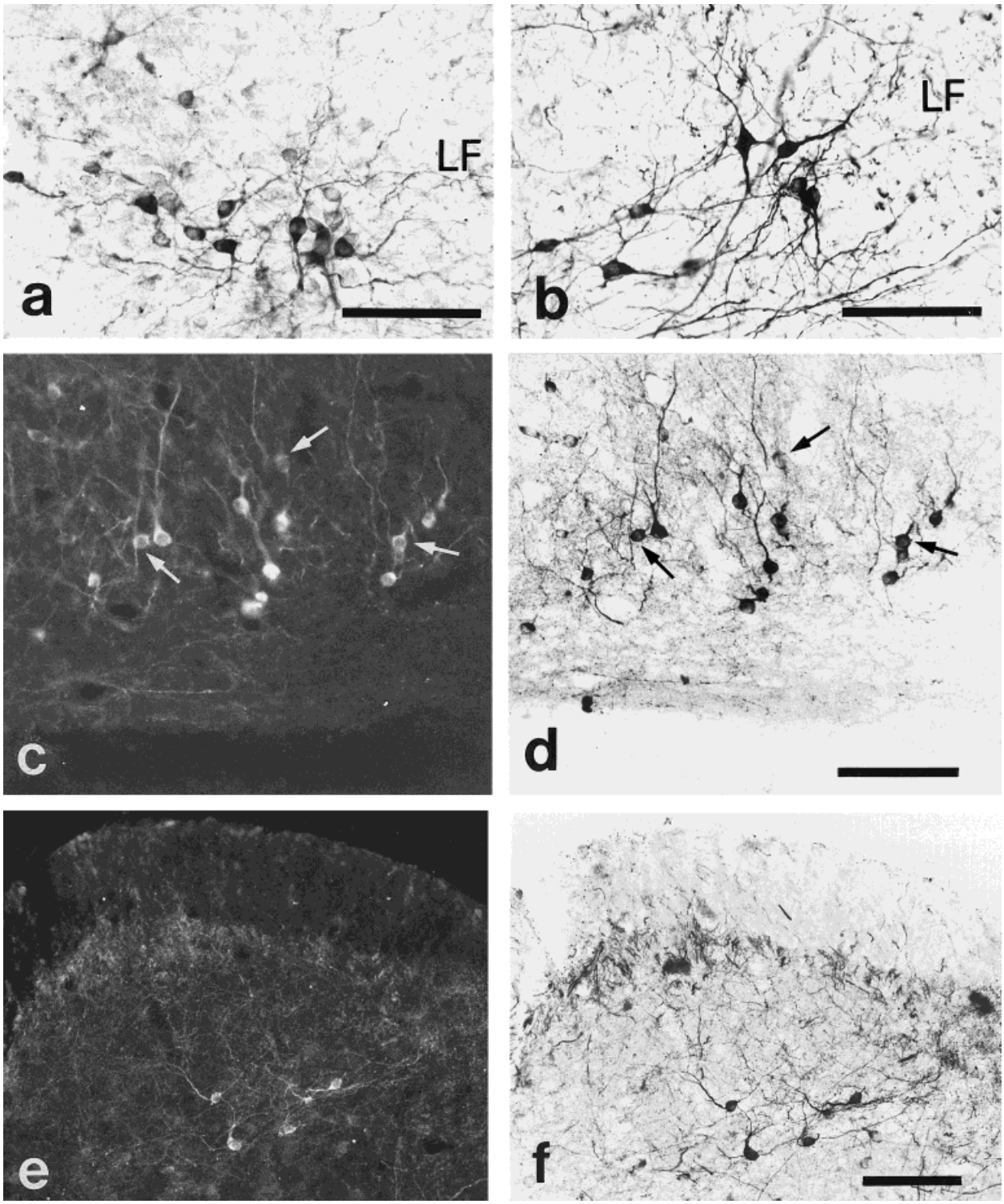



Fig. 1. Photomicrographs of transverse (a,b,e,f) and horizontal (c,d) sections through the thoracic spinal cord of the frog. A conspicuous group of nitrergic cells is labeled in the intermediate gray both with nitric oxide synthase (NOS) immunohistochemistry (**a**) and nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) histochemistry (**b**). NOS immunohistofluorescence (**c,e**) and NADPHd histochemistry performed on the same sections (**d,f**) demonstrate a complete match of stainings. Arrows are given to guide identification of common structures. Additional double-labeled neurons and fibers are present out of the plane of focus in c and e. LF, lateral funiculus. Scale bars = 100 μm.

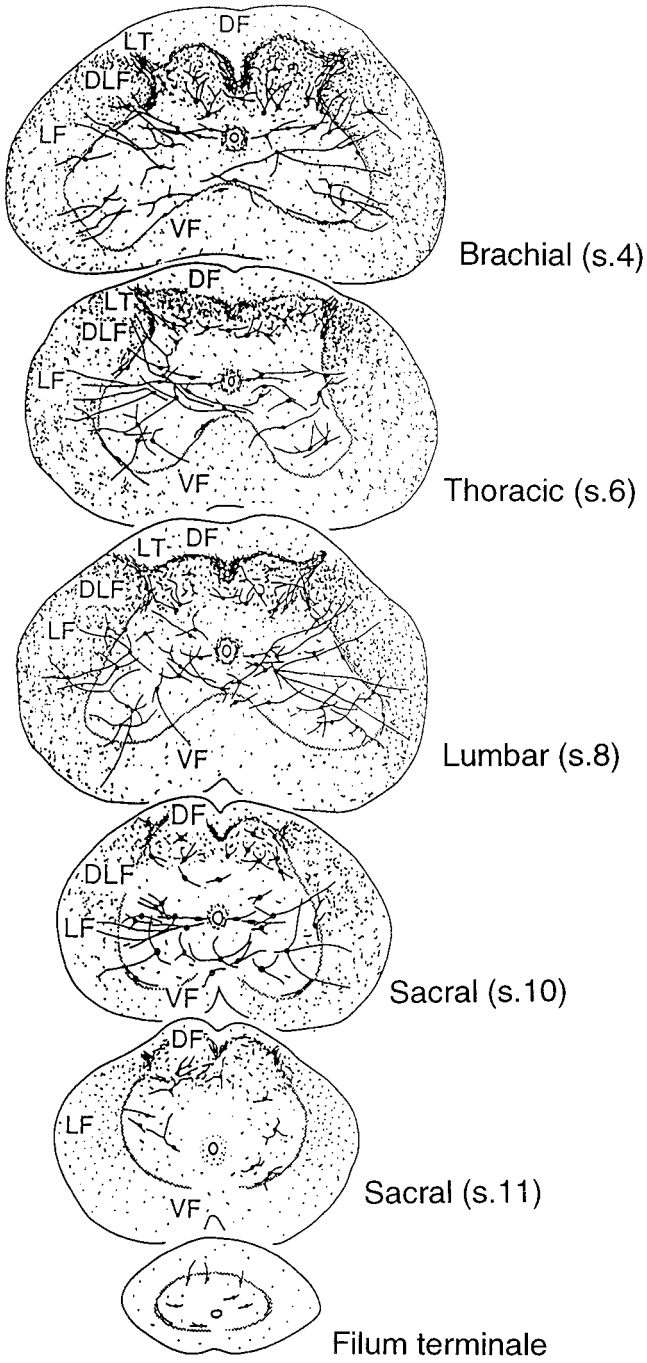



Fig. 2. Diagrams of transverse sections through the spinal cord of *Rana perezi* at representative segments. Nicotinamide adenine dinucleotide phosphate diaphorase–reactive cell bodies are indicated as large black dots, whereas fibers are drawn as small dots or wavy lines. For abbreviations, see list.

spinal cord and, therefore, it is not known whether or not these large cells were also NOS immunoreactive. Of note, in the sacral spinal cord, a group of nitrergic cells was observed medial to the somatic motoneurons, in the region where the Onuf's nucleus was described (Campbell et al., 1994). The processes of these small cells were tangential to the ventral horn border (Fig. 2).

*Filum terminale.* Transverse sections through the filum terminale revealed a thin rim of gray matter around the central canal without distinguishable horns. Even at these caudal levels, nitrergic cells were abundant. Dorsal to the canal, small cells extended their main processes toward the DF, whereas cells located lateral to the canal possessed processes that arborized in the horizontal plane. A few cells with radially oriented processes were also present and, occasionally in the border of the gray, some stained cells showed two unbranched processes (arising from opposite poles of the soma) that arched dorsally and ventrally following the curvature of the surface (Fig. 2).

***Nitrergic fibers.*** Although all dorsal root ganglia possessed nitrergic neurons, few axons were found in the dorsal root entry. Within the DF, labeled axons were mainly located at its ventral portion (Figs. 2, 3c,d, 5a). Axons coursing in LT were labeled at all spinal segments. The majority of LT axons progressed directly into the dorsal horn, whereas a few axons, or collaterals, formed a medial collateral pathway (MCP) and a lateral collateral pathway (LCP) around the dorsal horn (Figs. 2, 3c,d, 5a, 6). The fibers in the MCP coursed medially, capping the dorsal horn to enter the zone of the dorsal gray commissure. The LCP fibers at periodic intervals formed dorsoventrally oriented bundles that followed the lateral edge of the dorsal horn and intermediate gray. These fibers mainly arborized around clusters of labeled cells in the dorsal horn (Fig. 6b,c). Other LCP fibers reached more ventral positions and intermingled with dendritic processes of the nitrergic cells in the intermediate gray (Fig. 6a,c).

Apart from fibers of the LT, nitrergic fibers were abundant in the superficial area of the dorsal horn (Figs. 1, 3b,c, 4a, 5a). Because of the abundance of labeled cell processes, distinct fiber systems could not be identified, with the exception of a fine fiber network formed around the central canal (Fig. 2). Throughout the rostrocaudal extent of the spinal cord, large-caliber axons were present in all funiculi of the white matter, being most prevalent in the DLF and LF (Figs. 2, 3c, 5a). Finally, weakly labeled fibers were observed in the ventral roots.

## ChAT immunoreactive neurons

The antibodies against ChAT used in the present study revealed patterns of immunostaining that were consistent between different animals. Sections of the spinal cord were studied in series that were processed with or without nickel intensification. The results obtained by the different procedures were essentially similar, but the use of glucose oxidase–DAB–nickel technique resulted in a better staining of cell processes (Marín et al., 1997). Selected spinal transverse sections stained for ChAT immunohistochemistry are illustrated in Figure 7.

***Dorsal horn.*** ChAT immunoreactive cell bodies were not observed in the spinal dorsal horn at any level. The only exception was found at very rostral spinal segments, just caudal to the obex, where some scattered cells were labeled in the region of the dorsal column nucleus.

***Intermediate gray.*** A conspicuous population of ChATi neurons was found in the intermediate gray matter, which showed variability in number and position along the spinal cord (Figs. 7–9). Starting just caudal to the obex, small ChATi cells were found at the lateral

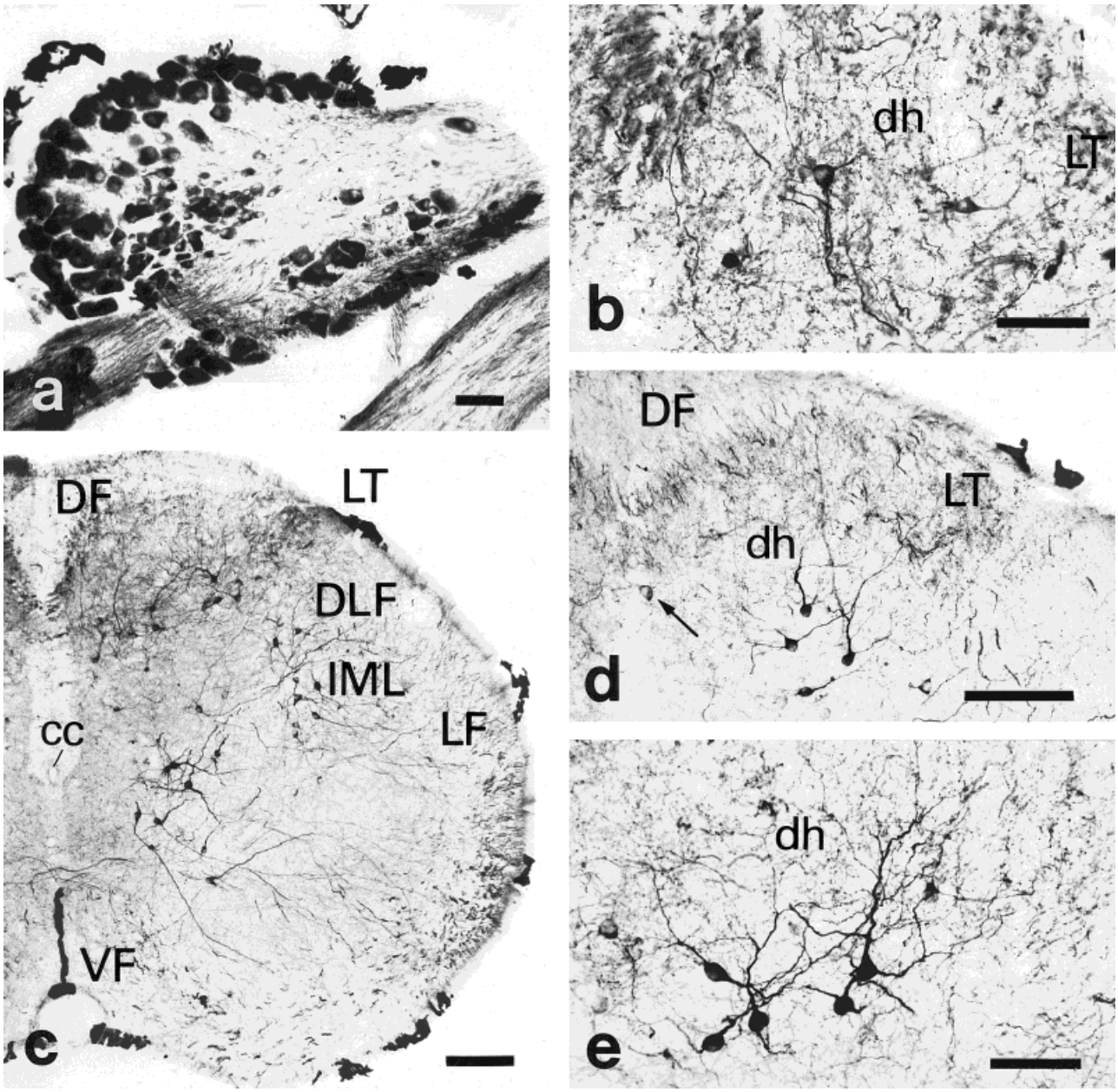



Fig. 3. Photomicrographs of nicotinamide adenine dinucleotide phosphate diaphorase–reactive large and small cells and fibers in a dorsal root ganglion (**a**), the dorsal horn at brachial segments (**b**), the spinal cord at thoracic segments (**c**), and the dorsal horn at lumbar levels (**d,e**). Arrow in d points to a medially located cell. For abbreviations, see list. Scale bars = 100 μm in a,c,d; 50 μm in b,e.

border of the gray. This population was particularly numerous immediately caudal to the brachial enlargement, at the level of the third and fourth spinal segments, and continued caudally along the thoracic spinal cord. This column of fusiform or multiform ChATi cells was organized into two conspicuous groups, lateral (intermediolateral, IML) and medial (intercalated, IC) (Figs. 7, 8b, 10a, 11e–h). Longitudinally, this population was discontinuous, forming separated cell clusters interconnected by numerous dendritic processes (Fig. 9a–c). The neurons in the IC group, located lateral and dorsal to the central canal, showed two main sets of dendritic processes organized in the horizontal plane. One set was directed medially, crossing the midline and proceeding to the contralateral IC group, whereas the second set of processes extended laterally toward the laterally located ChATi cells (Figs. 7, 8b, 10a, 11e). The IML group was labeled in the lateral border of the intermediate gray, and its cells showed short dendritic processes dorsoventrally and medially oriented, and long processes that entered the LF and DLF (Figs. 7, 8b). At caudal thoracic levels (posterior to the sixth spinal nerve), the lateral group of ChATi neurons in the intermediate gray was prominent, whereas the medial group gradually disappeared (Figs. 8c, 11g).

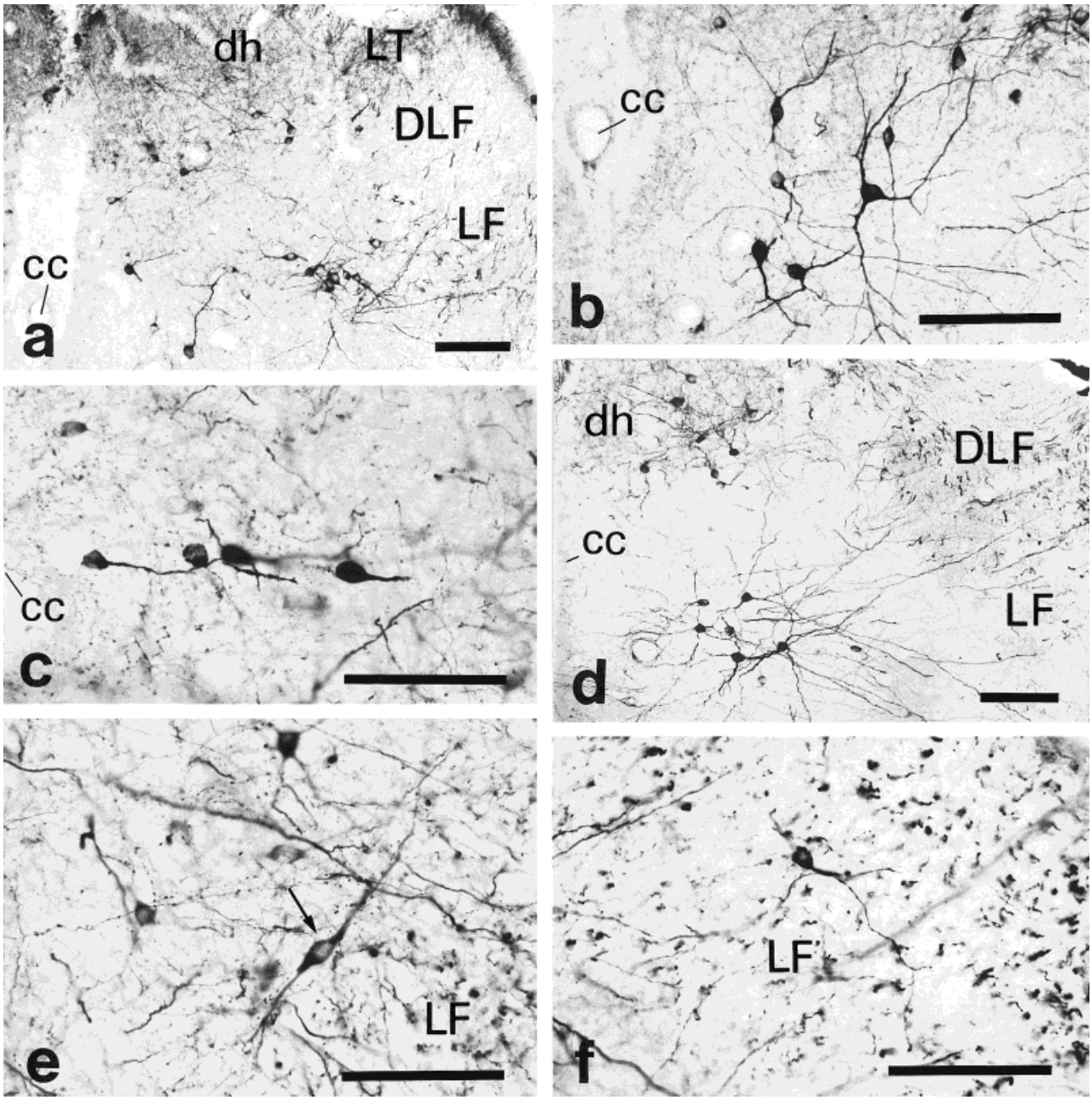



Fig. 4. Photomicrographs of transverse sections through the spinal cord illustrate localization and morphology of nicotinamide adenine dinucleotide phosphate diaphorase–reactive neurons in the intermediate gray (**a–d**). Cells in the margin between the intermediate gray and the lateral funiculus (LF) (**e**, arrow) and within the LF (**f**) are also illustrated. For abbreviations, see list. Scale bars = 100 μm.

At lumbar spinal segments, the number of ChATi cells in the intermediate gray was very limited. Scattered medium-sized cells with radially oriented processes were observed in mediolateral positions (Fig. 7). In the sacral spinal segments, numerous ChATi neurons were positioned more dorsally than at rostral levels (Fig. 11h). A clear separation between IML and IC groups could not be distinguished. Some dendritic processes of these cells in the lateral border of the dorsal horn were seen to intermingle with LCP fibers.

Throughout the spinal cord, occasional ChATi cell bodies were observed among the fibers of the LF. These multipolar or fusiform cells showed radially oriented dendritic processes. Remarkably, at caudal sacral segments, a group of large ChATi neurons were present at the lateral margin of the LF (Fig. 7).

***Ventral horn.*** The most conspicuous ChATi cells constituted the somatomotor column in the ventral horn (Fig. 8). They were large neurons with an extremely wide, fanlike dendritic arborization, which occupied almost the

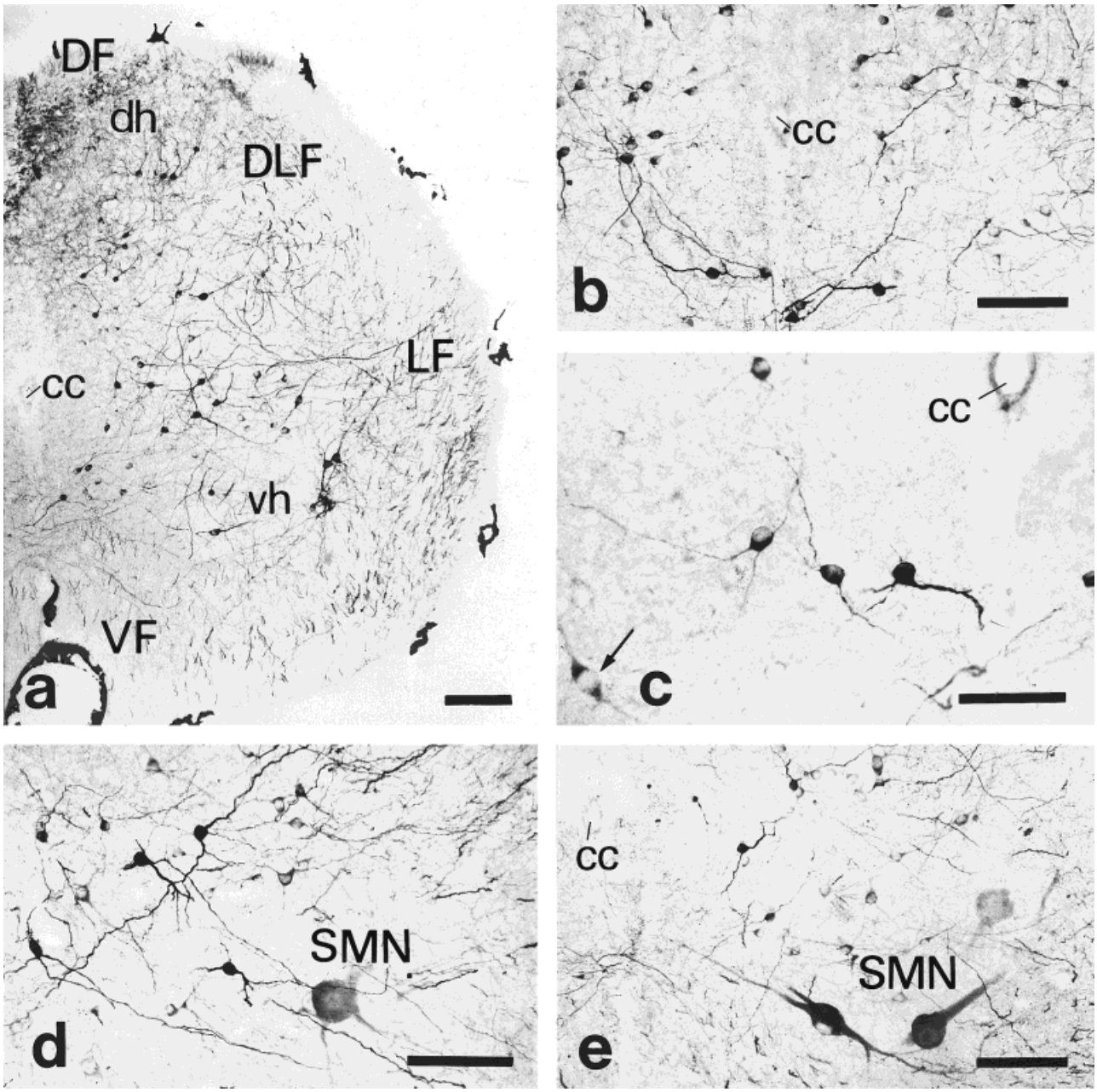
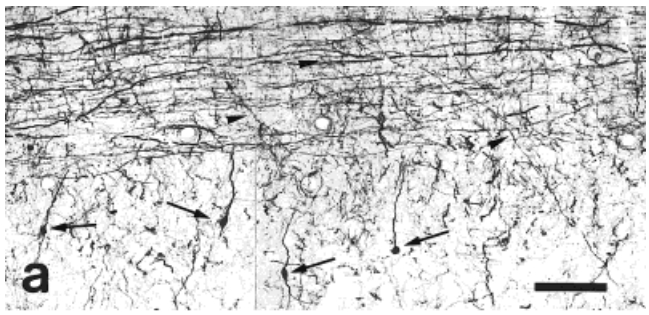


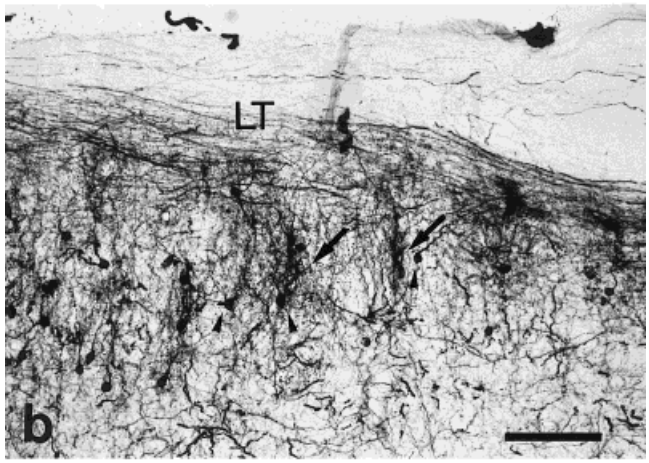

Fig. 5. Photomicrographs of transverse sections through the spinal cord at different segments illustrate particularly the localization of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd)-positive cells in the ventral horn territories. Scattered cells with some grouping at the ventrolateral aspect of the ventral horn are present at lumbar segments (**a**). Abundant cells located close to the midline beneath the central canal are present at sacral segments (**b,c**). Occasionally, at lumbar levels, very large cells are NADPHd-positive among somatic motoneurons (**d,e**). Arrow in c points to a labeled cell at the margin of the ventral gray. For abbreviations, see list. Scale bars = 100 μm.

whole ventrolateral aspect of the spinal cord. Their axons formed fascicles toward the ventral surface that exited in the ventral roots (Figs. 7, 8, 9a,b). The organization of these ChATi neurons was the same as that observed in tracing studies in anurans where the subgroups of somatic motoneurons have been delineated (ten Donkelaar, 1998). Of note, ChATi neurons at sacral levels occupied the region of Onuf's nucleus (Fig. 7). Scattered dorsal to the somatic motoneurons, a population of small ChATi cells was usually present at central positions of the ventral horn (Figs. 7, 11c,d). Although these cells were found throughout the spinal cord, they were more abundant in cervical segments.

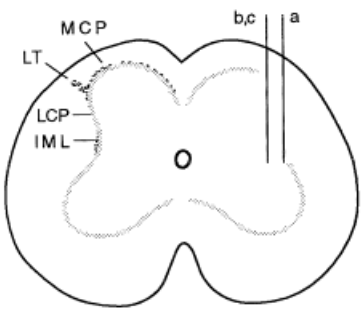
***Filum terminale.*** ChATi cell bodies were found scattered up to the very end of the filum terminale. They progressively occupied dorsal positions, and their den-

dritic processes coursed into the DF. Occasionally, elongated cells were present at the ventrolateral margin of the gray with two unbranching processes in parallel to the gray border (Fig.7).








## Codistribution of nitrergic and cholinergic neurons

The comparative analysis of the patterns observed for the distribution of ChATi neurons and NOS/NADPHd cells in the spinal cord of the frog pointed to an extensive codistribution of both cell types. In particular, in the region of the conspicuous ChATi cell groups in the intermediate gray matter, abundant nitrergic cells were found, and although most of them were smaller than the ChATi neurons, some cells were of identical size and shape. In the dorsal and central regions of the ventral horn, numerous nitrergic and ChATi cells of similar size and morphology were also codistributed, whereas nitrergic cells among the somatic motoneurons were smaller than the ChATi cells and were morphologically different. Only at caudal sacral spinal levels, nitrergic and ChATi neurons of similar size and shape occupied the area of Onuf's nucleus (Campbell et al., 1994), but the lack of ChAT immunoreactivity of the nitrergic cells in this position indicated that they were not somatic motoneurons.

The study of sections stained for ChAT immunoreactivity and NADPHd histochemistry revealed that there was no colocalization in the same neurons. Two methods corroborated this result, i.e., first, using ChAT immunohistofluorescence followed by NADPHd histochemistry (Fig. 10), and second, the method in which ChAT was revealed by DAB in sections previously labeled for NADPHd (Fig. 11). With the latter technique, the blue product of the NADPHd reaction in nitrergic cells and the brown DAB reaction product of ChATi cells were distinguished in separate populations of neurons. However, sometimes the two labels were difficult to discern, mainly in areas where dark-blue neurons could mask the putative brown staining. Because double fluorescent immunolabeling for ChAT and NOS could not be combined due to cross-reactivity of the antibodies, we have clarified the actual lack of colocalization by a simple method in which double-stained sections were photographed and then the blue reaction product of the NADPHd histochemistry was dissolved in diethyl amide. The subsequent, careful comparison of the same sections revealed the presence of masked double-labeled cells in the medulla (Fig. 11a,b) or the absence of colocalization in spinal neurons (Figs. 11c,d). The analysis of sections at all spinal cord levels showed that in spite of the high degree of codistribution observed (Fig. 11e–h), colocalization of ChAT and NADPHd was absent in all spinal neurons of the frog.

Fig. 6. Photomicrographs show the distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) staining in sagittal sections of the lumbar spinal cord. **a:** Longitudinally coursing axons in Lissauer's tract (LT) branch and give off fibers of the lateral collateral pathway (LCP, arrowheads) that reach NADPHd-positive neurons in the intermediate gray (arrows). **b,c:** Rostrocaudal bundles of NADPHd axons in LT form periodic dorsoventral projecting fiber bundles of the LCP (arrows in b) that intermingle with NADPHd-positive cells of the dorsal horn that have also periodic distribution (arrowheads in b). Less conspicuously, LCP axons reach more ventral regions (arrowheads in c) among abundant labeled cells of the intermediate gray (arrows in c). The orientation scheme of a transverse section of the spinal cord (drawing at bottom) shows the approximate location of the sections (a–c). For abbreviations, see list. Scale bars = 100 μm.

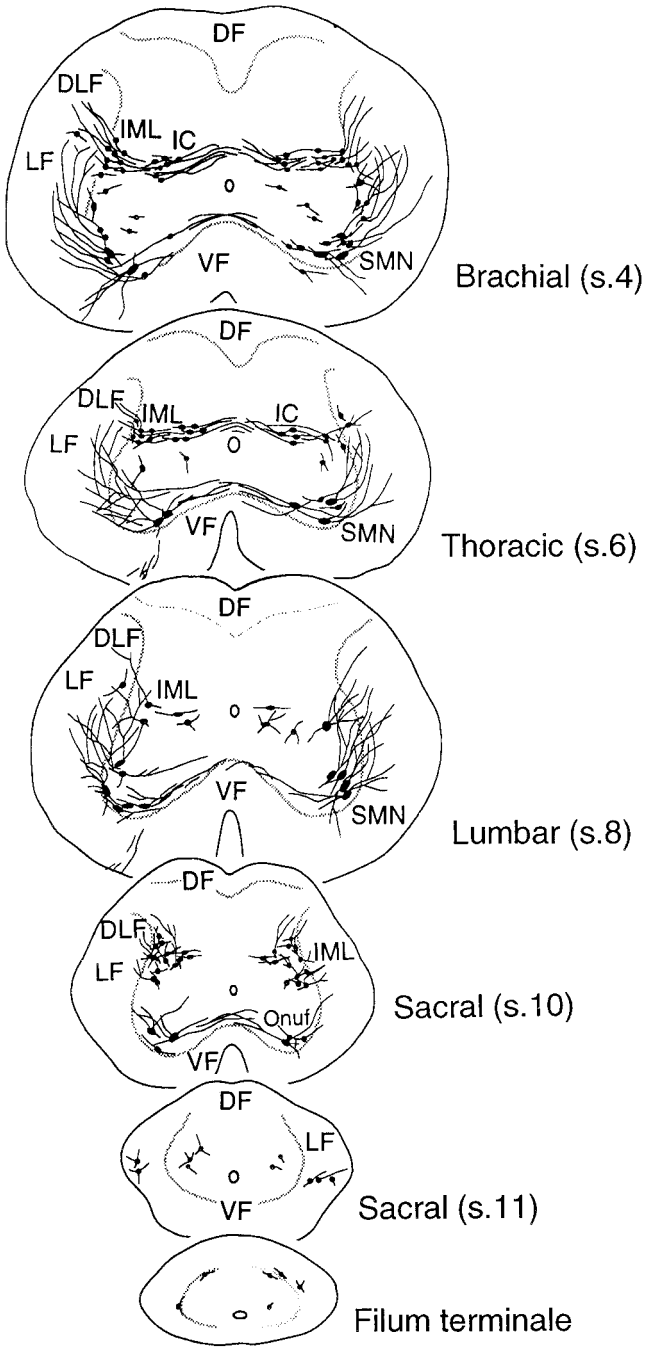



Fig. 7. Diagrams of transverse sections through the spinal cord of *Rana perezi* at representative segments. Choline acetyltransferase–immunoreactive cell bodies and their main processes are depicted. For abbreviations, see list.

## DISCUSSION

### Nitrergic neurons in the spinal cord

The antibody against NOS used in the present study yields a one-to-one correlation between NADPHd histochemistry and neuronal NOS immunoreactivity, as was also the case in the brain of urodele amphibians (González et al., 1996). Our results in *R. perezi* show that the histochemical procedure used to reveal NADPHd activity is highly sensitive and specific for neuronal structures. Thus, cells of the endothelial wall and glial structures were never stained, in contrast to the results obtained in amphibians using different histochemical protocols (Crowe et al., 1995).

In amphibians, as in other nonmammalian vertebrates (Arévalo et al., 1995; Brüning et al., 1995; Radmilovich et al., 1997; Smeets et al., 1997), numerous cells were observed in deep regions of the dorsal horn at all spinal segments and, frequently, stained neurons were present above the central canal, in the dorsal gray commissure. In particular, cells located at the midline with processes extending bilaterally are largely comparable to those "bitufted cells" described in the turtle (Radmilovich et al., 1997). Tracing studies in the spinal cord of the frog suggest that cells located in the deep dorsal horn could be spinal tract neurons and intersegmental connecting cells (A. Muñoz et al., 1996, 1997).

The nitrergic cells found in the dorsal horn are included in a dense neuropil of labeled fibers and terminals that resemble the terminal fields of primary afferent fibers, in the spinal enlargements (Jhaveri and Frank, 1983; Rosenthal and Cruce, 1985). In the frog, a large number of neurons of all dorsal root ganglia were NADPHd reactive, as has been reported for other amphibians (Crowe et al., 1995; González et al., 1996). These results suggest a major role of NO in spinal somatosensory processing, analogous to that found in mammals (Moore et al., 1991; Haley et al., 1992; Kitto et al., 1992).

Numerous nitrergic cells have been found in the spinal intermediate gray of the frog. These cells have been described in the regions of autonomic sympathetic and parasympathetic nuclei of the anuran spinal cord (Robertson, 1987; Horn and Stofer, 1988; Peruzzi and Forehand, 1993; Campbell et al., 1994). The location of the nitrergic cells, however, did not fully match that of the autonomic neurons. Similar experiments in amphibians using the NADPHd technique reported contradictory results and thus, whereas in the bullfrog preganglionic neurons at segments 5–8 were not labeled (Peruzzi and Forehand, 1993), in *X. laevis* sympathetic and parasympathetic neurons were found to be NADPHd positive (Crowe et al., 1995). Species differences could account for these discrepancies, although differences in the technical procedure could also be responsible for the contradictory results (Alonso et al., 1995). Numerous studies have demonstrated nitrergic cells in the intermediate gray of the spinal cord of diverse mammalian and nonmammalian species (e.g., Anderson, 1992; Blottner and Baumgarten, 1992; Brüning, 1992, 1994; Valtschanoff et al., 1992a; Dun et al., 1993; Terenghi et al., 1993; Saito et al., 1994; Vizzard et al., 1994a,Vizzard et al., 1994d; Brüning et al., 1995; Smithson and Benarroch, 1996; Marsala et al., 1999). However, discrepancies in observed levels of NOS expression raise the question of whether autonomic neurons have nitrergic activity (Mizukawa et al., 1989; Anderson, 1992; Brüning, 1992; Dun et al., 1992, 1993; Vizzard et al., 1993a; Saito et al., 1994). Pharmacological studies in the rat have suggested that NO may be involved in transmission in sympathetic ganglia (Briggs, 1992), and this would be consistent with the presence of nitrergic cells in the autonomic spinal nuclei and the observation of NADPHd-positive axons in the thoracic ventral roots (Aimi et al., 1991).

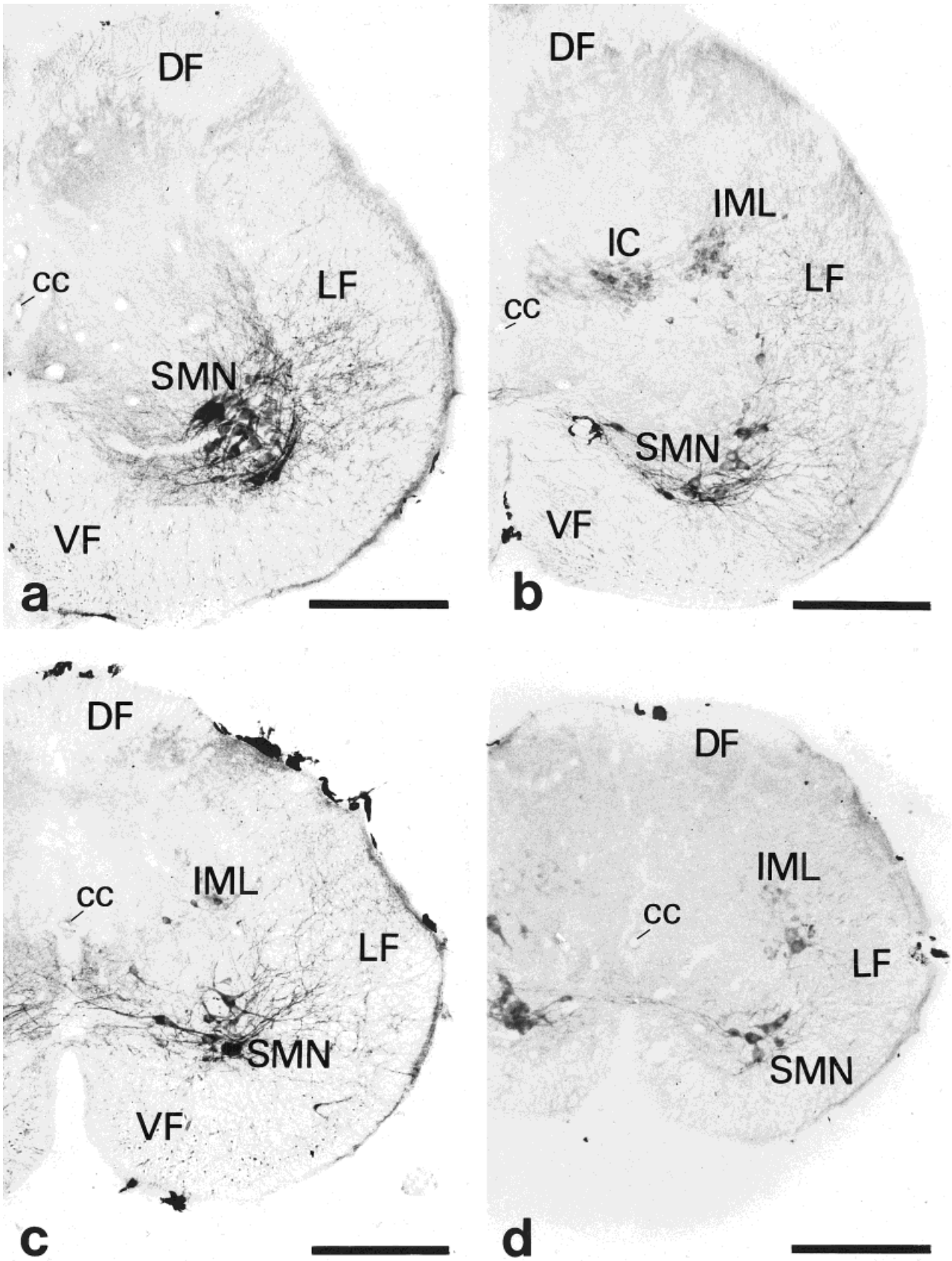

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Figure 8

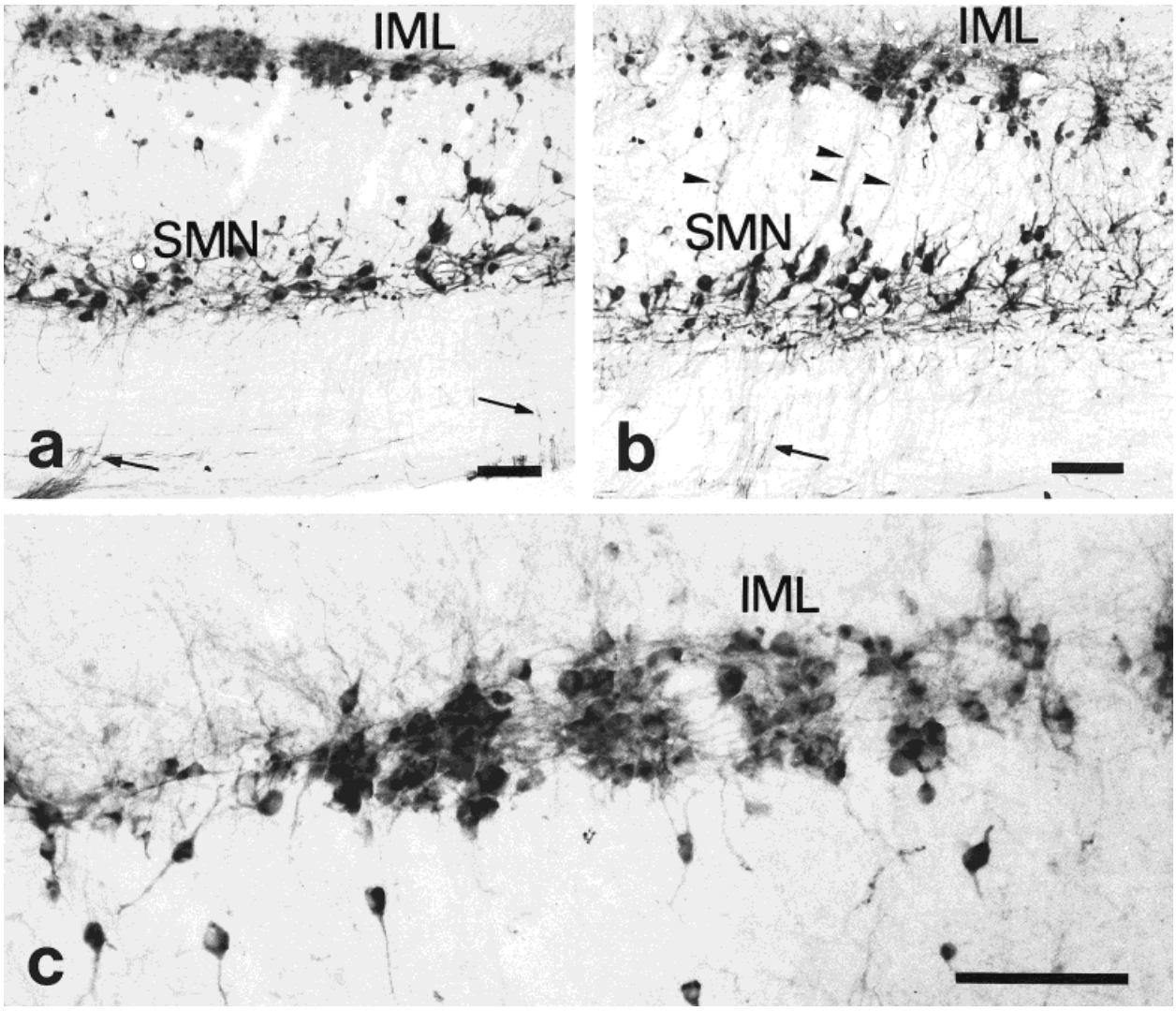


Fig. 9. Photomicrographs of sagittal sections through the thoracic spinal cord show the distribution and topography of choline acetyltransferase (ChAT)–immunoreactive cells and fibers. In the sagittal plane, the staining pattern indicates that the preganglionic intermediolateral cell column (IML) contains periodically clustered ChAT-positive neurons. **a,b:** low-power magnification of the distribution of IML and somatic motoneuron (SMN) columns and numerous scattered cells between them. Arrows point to ventral root fibers, and arrowheads point to axons of IML cells. The periodic organization of IML cells with profuse interconnecting processes is readily visible in higher magnification (**c**). Scale bars = 100 μm.

A large population of nitrergic cells is present in the ventral horn of the frog spinal cord. Because of their size, location, and morphology, these cells most likely represent spinal interneurons. Similar cells have been reported for mammalian (e.g., Valtschanoff et al., 1992a; Dun et al., 1993; Brüning, 1994; Vizzard et al., 1994d, 1997; Marsala et al., 1999) and nonmammalian (Radmilovich et al., 1997; Smeets et al., 1997; Brüning, 1994; Brüning et al., 1995; Arévalo et al., 1995; M. Muñoz et al., 1996; González et al., 1996) species. However, contradictory results have been obtained concerning the possible nitrergic nature of somatic motoneurons. In *R. perezi*, we have found only in two cases one and four, respectively, large NADPHd-positive cells in the ventral horn. Because of their large size and position, they are likely to be somatic motoneurons. However, because in these two experiments NOS or ChAT immunohistochemistry were not carried out, we cannot be sure of the presence of nitrergic somatic motoneurons in the ventral horn. In contrast, somatic motoneurons were NADPHd positive throughout the spinal cord of *X. laevis* (Crowe et al., 1995). Discrepancies have been reported also for other vertebrates. In reptiles and birds, ventral horn somatic motoneurons seem to be di-

Fig. 8. Photomicrographs of spinal cord transverse hemisections at different segments illustrate choline acetyltransferase immunoreactivity. **a:** Brachial level just rostral to spinal nerve 3. **b:** Upper thoracic segment 4. **c:** Lumbar segment 8. **d:** Sacral segment 10. For abbreviations, see list. Scale bars = 250 μm.

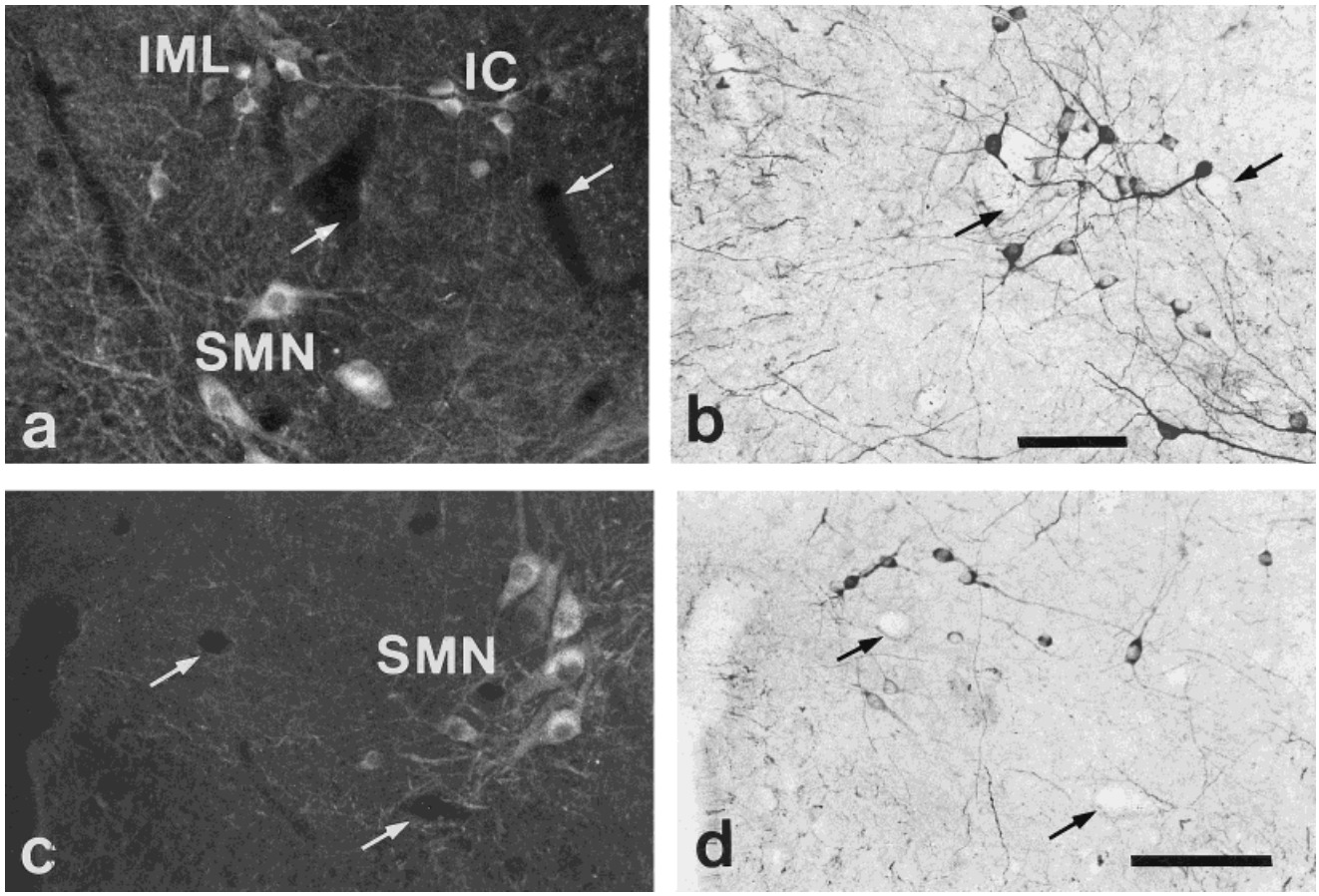


Fig. 10. Photomicrographs of transverse sections through the thoracic (**a,b**) and lumbar (**c,d**) spinal cord of the frog. Choline acetyltransferase immunohistofluorescence (a,c) and nicotinamide adenine dinucleotide phosphate diaphorase histochemistry performed on the same sections (b,d) demonstrate a complete lack of colocalization. Arrows are given to guide identification of common structures. For abbreviations, see list. Scale bars = 100 μm.

aphorase negative (Brüning, 1994; Brüning et al., 1995; Radmilovich et al., 1997; Smeets et al., 1997), whereas in mammals, somatic motoneurons have been reported to show both negative (Brüning, 1992, 1994; Dun et al., 1992, 1993; Valtschanoff et al., 1992a; Spike et al., 1993; Vizzard et al., 1994d; Saito et al., 1994; Wetts and Vaughn, 1994; Marsala et al., 1999) and positive staining (Terenghi et al., 1993; Pullen and Humphreys, 1995; Marsala et al., 1998), even within the same species. A few scattered NADPHd-positive somatic motoneurons have been identified in aged but not young rats, suggesting NADPHd reactivity may be associated with neuronal death (Kanda, 1996). Because we have not controlled the age of the frogs used in our study, we cannot assert this possibility for amphibians. Interestingly, in the rat, a subset of somatic motoneurons, located in the ventrolateral corner of a few caudal segments of the spinal cord, expressed diaphorase activity transiently during development but gradually became negative by birth (Kalb and Agostini, 1993; Wetts et al., 1995). In a developmental study of NADPHd reactivity in the spinal cord of *X. laevis*, it was found that somatic motoneurons were positive from early developmental stages (Crowe et al., 1995). However, in that study, somatic motoneurons were also NADPHd reactive in the adult. Recent experimental evidence from our laboratory indicates lack of NADPHd activity in spinal somatic motoneurons during development in the frog *R. perezi*, thus suggesting differences with *X. laevis* or technical discrepancies.

Fig. 11. **a–d:** Photomicrographs of double-labeled sections for detection of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) and choline acetyltransferase (ChAT) (a,c) and after selective elimination of the NADPHd reaction product (b,d). In the upper medulla, the cells of the laterodorsal tegmental nucleus (LDT) were double labeled and the strong reaction for NADPHd masked the diaminobenzidine (DAB)-based reaction for ChAT detection (a). After dissolving the NADPHd reaction product, the brown DAB staining was observed, proving double labeling of LDT cells (arrows are meant to help identification of the same structures in a and b). Similar treatment applied to spinal cord sections showed that NADPHd and ChAT cells are intermingled in many regions, but they are separated populations (arrowheads in d point to ChAT cells; note the lack of labeling in their surrounding, where NADPHd cells were labeled in c). Double-labeled sections demonstrated overlapping of NADPHd and ChAT cells in several regions, as in the intermediolateral cell column (IML) and somatic motoneuron groups at caudal brachial segments (**e**), particularly in the IML (**f**), at caudal thoracic segments (**g**), and at sacral segments (**h**). For abbreviations, see list. Scale bars = 100 μm.

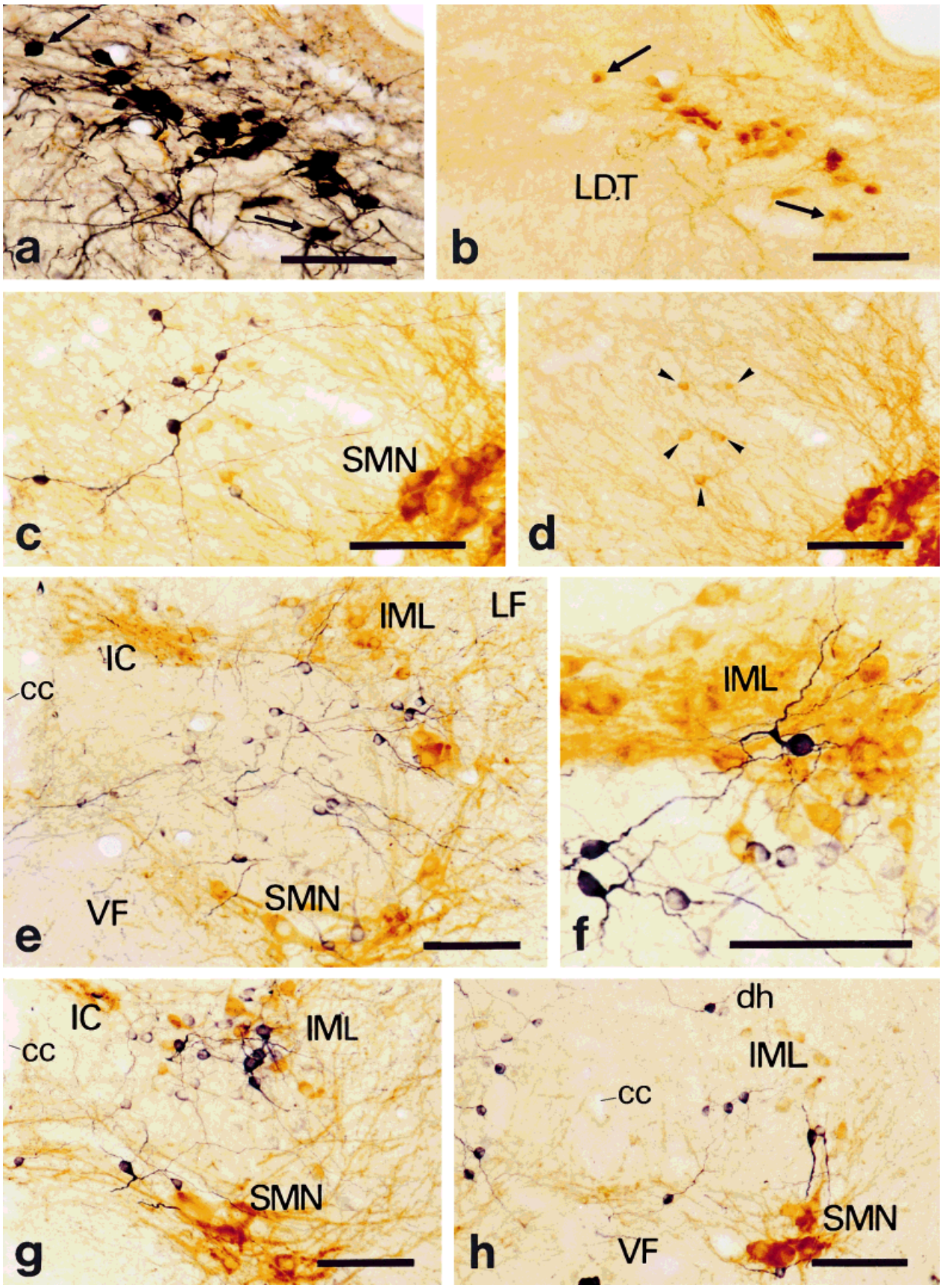
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Figure 11

## Nitrergic fiber systems

Afferent and efferent projections in the spinal cord of the frog are organized in essentially the same funicular arrangement as in amniotes (Ebbesson, 1976; ten Donkelaar, 1982), facilitating the comparison between vertebrate classes. In the frog, nitrergic fibers course in all funiculi, although the highest density of labeled fibers was seen in the dorsal, dorsolateral, and lateral funiculi. However, the most conspicuously labeled fibers occupy a ventromedial position within the dorsal funiculus that corresponds to lumbosacral ascending fibers (Rosenthal and Cruce, 1985; Muñoz et al., 1995). Similar results have been reported in the spinal cord of amniotes (e.g., Valtschanoff et al., 1992a; Smeets et al., 1997) and even in the larval lamprey (Schober et al., 1994).

NADPHd labeling of fibers in LT is a feature shared by all vertebrates studied (e.g., Dun et al., 1993; Pullen and Humphreys, 1995; Smeets et al., 1997; Vizzard et al., 1997; Marsala et al., 1999). In the frog, the well-organized LT (Antal et al., 1980; Rosenthal and Cruce, 1985) is densely labeled with NADPHd histochemistry and NOS immunohistochemistry. In mammals, the axons in LT organize an MCP in the superficial dorsal horn laminae and a LCP in the lateral border of the dorsal horn that, periodically, send branches to the preganglionic autonomic neurons (Morgan et al., 1981; Mawe et al., 1986). Different techniques have shown that in the cat, the MCP and LCP are stained for NOS and NADPHd (Dun et al., 1993; Pullen and Humphreys, 1995). However, also in the cat (Vizzard et al., 1994a,b), rat (Vizzard et al., 1994b,c, 1995b), and dog (Vizzard et al., 1997), the fibers in the LCP were NADPHd reactive but NOS immunonegative. As discussed by Vizzard et al. (1997), this mismatch between the two markers might be related to technical factors. However, previous experiments suggest that a real biochemical difference exists between NADPHd activity and NOS immunoreactivity in the LCP. In the normal rat, only a few cells in the L6–S1 dorsal root ganglia are NOS immunopositive (Vizzard et al., 1995a; Vizzard and de Groat, 1996; Vizzard, 1997), which is reflected in the absence of NOS staining in the central projections of these cells. However, after nerve injury (Verge et al., 1992; Zhang et al., 1993), chemical injury (Vizzard et al., 1995b), chronic bladder irritation (Vizzard and de Groat, 1996), or chronic spinal cord injury (Vizzard, 1997), the levels of NOS immunoreactivity and/or NOS mRNA are increased in the lumbosacral DRG. These results further support the notion that the expression of neuronal NOS is plastic, as suggested in experiments in which NADPHd or NOS staining was increased after axotomy in motoneurons (Gonzales et al., 1987; Wu et al., 1994) and in some parasympathetic preganglionic neurons (Vizzard et al., 1995a).

In the LT of anurans, a collateral system was not observed in tracing studies (Rosenthal and Cruce, 1985; Muñoz, et al., 1995), although fibers comparable to the LCP of mammals have been described at sacral spinal segments in *X. laevis* (Campbell et al., 1994). The observations of the present study have shown that a collateral system organized as in mammals might be present and would be NADPHd reactive and NOS immunopositive. Taken together, the results of the present study in the frog suggest that nitric oxide may mediate in autonomic transmission, because nitrergic cells have been localized in visceral ganglia and autonomic spinal nuclei, and NOS/NADPHd-labeled visceral afferent fibers have been observed in LT and its collateral system.

A dense neuropil of small caliber nitrergic fibers and terminals is present in the spinal dorsal horn of the frog. This seems to be a characteristic feature of the spinal nitrergic systems in all vertebrates (Valtschanoff et al., 1992a,b; Brüning, 1994; Vizzard et al., 1994d; González et al., 1996; M. Muñoz et al., 1996; Smeets et al., 1997). Our results in the frog suggest that both primary afferents and intraspinal fibers form the dorsal neuropil. The dense labeling of dorsal root ganglion cells would explain the presence of labeled fibers in the terminal fields of primary afferents in the dorsal horn. In addition, extensive intraspinal connections between cells in the dorsal horn of the frog have been demonstrated, and the morphology of these interneurons is largely similar to that of the nitrergic cells observed in the deep dorsal horn (A. Muñoz et al., 1996, 1997).

## ChAT-immunoreactive neurons in the spinal cord

In the frog spinal cord, ChAT immunohistochemistry has revealed different cell types in the intermediate gray matter and in the ventral horn, but has failed to stain neurons in the dorsal horn. Cholinergic cells in the dorsal horn seem to be a common feature of the spinal cord in amniotes (Barber et al., 1984; Phelps et al., 1984; Borges and Iversen, 1986; Kosaka et al., 1988; Ribeiro-da-Silva and Cuello, 1990; Thiriet et al., 1992; Medina et al., 1993; Medina and Reiner, 1994; Wetts and Vaughn, 1994). These cells have been demonstrated to correspond to interneurons connecting dorsal horn territories, and thus they are responsible for the high density of cholinergic terminals located in laminae I and III (Gillberg et al., 1990). In amphibians, the lack of ChATi neurons in the dorsal horn is also accompanied by absence of ChATi fibers or terminals in these territories. Data about the situation in other anamniotic vertebrates are needed to establish whether the presence of a cholinergic interneuron system in the spinal dorsal horn is a characteristic shared only by amniotes.

In the intermediate gray matter, ChATi neurons are arranged in a manner that clearly corresponds to the autonomic spinal system previously studied in anurans with tracing techniques (Robertson, 1987; Horn and Stoffer, 1988; see also Smith, 1994). Thus, the entire sympathetic preganglionic pool of cells was labeled in a column that extends between the levels of the brachial spinal nerve 3 and a point just rostral to lumbar nerve 8. The organization of these cells into medial and lateral groups is readily comparable to the intercalated and intermediolateral sympathetic preganglionic neurons, described in *Rana pipiens* after retrograde axonal transport of horseradish peroxidase (HRP) from the first sympathetic ganglion (Robertson, 1987). However, this organization contrasts with other descriptions of a continuous rostrocaudal column of cells (ten Donkelaar, 1998). In addition to the ChATi neurons in the intermediate gray, the ChATi neurons found within the lateral funiculus at different spinal levels in the frog could correspond to HRP-labeled cells found in similar position and thought to represent displaced autonomic neurons (Robertson, 1987). At caudal thoracic and lumbar segments, the morphology and arrangement observed in the ChATi cells in the intermediate gray vary compared to more rostral levels. This could

reflect the fact that sympathetic preganglionic neurons that control the vasculature lie in a position caudal to those that control nonvascular targets (Horn and Stoffer, 1988) and both types exhibit significant differences in cell morphology (Peruzzi and Forehand, 1993).

At sacral spinal levels, ChATi neurons are abundant in the intermediate gray, although they are more dorsally located than in other spinal segments. Their disposition makes them comparable to the cells of the dorsal and lateral bands of HRP-filled preganglionic neurons described in sacral parasympathetic nucleus of *X. laevis* (Campbell et al., 1994). The location of these cells, their morphology, and the relationship with the fibers in the LCP system make them readily comparable to the parasympathetic neurons of the mammalian spinal cord (Morgan et al., 1979, 1981; de Groat et al., 1981; Mawe et al., 1986; Nadelhaft et al., 1986; Vizzard et al., 1997; Marsala et al., 1999).

The most conspicuous ChATi neurons found in the frog spinal cord were the somatic motoneurons of the ventral horn. This is in agreement with all previous studies in different vertebrate classes. It is noteworthy that the antibody used in the present study densely labeled these cells, although the enzyme activity in the frog somatic motoneurons has been shown to be one-tenth of the activity found in warm-blooded animals (Kato and Murashima, 1985). The pattern of organization of somatic motoneurons observed with ChAT immunoreactivity is similar to that observed in experimental tracing studies, where it was shown that somatic motoneurons innervating forelimb and hindlimb muscles are somatotopically arranged in clusters as longitudinal motor columns (Rubin and Mendell, 1980; Frank and Westerfield, 1982; Hulshof et al., 1987; Oka et al., 1989). Many dendritic processes from somatic motoneurons extend into the region of the ChATi sympathetic cells in the lateral group of the intermediate gray, making it possible to receive similar afferent connections, as has also been observed in mammals (Light and Metz, 1978; de Groat, 1976; Morgan et al., 1981; Mawe et al., 1986).

A significantly large population of scattered, small ChATi cells occupy central and dorsal regions in the ventral horn of the frog. These cells most likely correspond to intraspinal connecting cells. If the situation is comparable to that of mammals, the cholinergic puncta and terminal structures observed in the spinal cord would be of intraspinal origin, because they are not derived from other brain regions (Sherriff et al., 1991) or from the periphery (Barber et al., 1984). Of note, in the rat, ChATi partition cells in laminae VII have been found to be mainly responsible for intraspinal cholinergic circuits (Sherriff and Henderson, 1994). Experimental tracing studies are needed in the frog to demonstrate a similar condition for the small ChATi neurons in the ventral horn.

## Colocalization of NADPHd/NOS and ChAT

The results of the present double-labeling experiments failed to identify colocalization of NADPHd/NOS and ChAT.

In the rat, about 70% of the nitrergic cells in the dorsal horn also stained for ChAT (Wetts and Vaughn, 1994). In preliminary experiments, a similar situation has been found in the avian spinal cord (Brüning and Hauswedell, 1998). Strikingly, abundant nitrergic cells have been found in the dorsal horn of the frog, where no ChATi cells could be demonstrated. Whether this situation is a general characteristic of the spinal cord of anamniotic vertebrates awaits confirmation with double-labeling techniques in fish.

Neurons that express ChAT and NOS in the intermediate gray of the frog are often intermingled, but correspond to distinct subpopulations. This situation contrasts with that observed in several mammalian species, including the rat, cat, mouse, and squirrel monkey (Blottner and Baumgarten, 1992; Dun et al., 1993), in which NOS immunoreactive neurons of the IML comprise a distinct subpopulation of cholinergic preganglionic neurons. In fact, studies using methods similar to those used in the present study have shown that up to 62% of the autonomic ChATi cells coexpress NOS in the mammalian spinal cord (Blottner and Baumgarten, 1992; Dun et al., 1993; Spike et al., 1993; Wetts and Vaughn, 1994). To date, our study in the frog and a preliminary report in the spinal cord of the chicken (Brüning and Hauswedell, 1998) provide the only data about lack of colocalization in the intermediate gray along the spinal cord in nonmammalian vertebrates. However, in both cases, the relatively high degree of codistribution of ChAT- and NOS-containing cells might suggest that these cells would participate in related spinal functions. In amphibians, a further support for the lack of NOS expression in preganglionic neurons has been provided for cells innervating the adrenal gland, the celiac ganglion, or the sympathetic chain at ganglion 9 (Peruzzi and Forehand, 1993).

In the ventral horn, the scattered cells in the central and dorsal regions that stain for NOS/NADPHd or ChAT form totally distinct populations, although they intermingle profusely. In contrast, in a comparable position of the rat spinal cord, cells colocalizing both enzymes have been reported (Wetts and Vaughn, 1994). Lack of colocalization was found in the somatic motoneurons of the frog. Of note, several studies in mammals also failed to find colocalization in somatic motoneurons in experiments using double-labeling techniques (Blottner and Baumgarten, 1992; Dun et al., 1993; Spike et al., 1993; Wetts and Vaughn, 1994).

Cholinergic and nitrergic systems in the spinal cord of the frog seem to be essentially segregated, whereas in birds they are incompletely segregated (Brüning and Hauswedell, 1998), and in mammals they can be extensively overlapping in certain regions (Wetts and Vaughn, 1994). Different vertebrate species need to be studied to test whether these systems are primitively separated and whether the occurrence of neurons with both NOS and ChAT is acquired in amniotes or is specific to mammals.

## ACKNOWLEDGMENTS

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