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Descending Brain-Spinal Cord Projections in a Primitive Vertebrate, the Lamprey: Cerebrospinal Fluid-Contacting and Dopaminergic Neurons

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ABSTRACT

We used Neurobiotin as a retrograde tract tracer in both larval and adult sea lampreys and observed a number of neuronal brainstem populations (mainly reticular and octaval populations and some diencephalic nuclei) that project to the spinal cord, in agreement with the results of previous tracer studies. We also observed small labeled neurons in the ventral hypothalamus, the mammillary region, and the paratubercular nucleus, nuclei that were not reported as spinal projecting. Notably, most of the labeled cells of the mammillary region and some of the ventral hypothalamus were cerebrospinal fluid-contacting (CSF-c) neurons. Combined tract tracing and immunocytochemistry showed that some of the labeled neurons of the mammillary and paratubercular nuclei were dopamine immunoreactive. In addition, some CSF-c cells were labeled in the caudal rhombencephalon and rostral spinal cord, and many were also dopamine immunoreactive. Results with other tracers (biotinylated dextran amines, horseradish peroxidase, and the carbocyanine dye DiI) also demonstrated that the molecular weight or the molecular nature of the tracer was determinant in revealing diencephalic cells with very thin axons. The results show that descending systems afferent to the spinal cord in lampreys are more varied than previously reported, and reveal a descending projection from CSF-c cells, which is unknown in vertebrates. The present results also reveal the existence of large differences between agnathans and gnathostomes in the organization of the dopaminergic cells that project to the spinal cord. J. Comp. Neurol. 511:711–723, 2008.
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Indexing terms: hypothalamus; paratubercular nucleus; mammillary nucleus; cerebrospinal fluid-contacting cells; rhombencephalon; agnathans; tract-tracing; Neurobiotin; dextran amine; dopamine; horseradish peroxidase; DiI

Lampreys are important models for analyzing the structure and function of the central nervous system because they are living agnathans, the most primitive group of vertebrates. The lamprey spinal cord has been fundamental for revealing the neural mechanisms underlying the control of locomotion (Grillner and Wallén, 2002). In addition, lampreys have been an important model in studies of regeneration in the nervous system (Wood and Cohen, 1979; Davis and McClellan, 1994; Zhang et al., 2005). Knowledge of the descending spinal projections from the brain is therefore essential in understanding both locomotion pattern generation and axonal regeneration. Experimental studies with horseradish peroxidase (HRP) revealed that various neuronal populations of the reticular formation, the descending trigeminal tract, the intermediate and posterior octavomotor nuclei, and the nucleus of the posterior tubercle project to the lamprey spinal cord

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(Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994). Although most of these descending projections appear to be glutamatergic, a spinal projection from serotonin-immunoreactive neurons of the brainstem has been observed in lamprey by use of combined retrograde tracing and immunohistochemistry (Brodin et al., 1986).

In lampreys, dopamine-immunoreactive (DAir) cells were observed in the spinal cord and some brain regions (McPherson and Kemnitz, 1994; Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005). In the brain, most dopaminergic neurons are located in the hypothalamus and are of the cerebrospinal fluid-contacting (CSF-c) type (Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005). In the spinal cord, small DAir neurons located slightly ventral to the central canal give rise to a sparse plexus of DAir fibers (McPherson and Kemnitz, 1994; Abalo et al., 2005; Rodicio et al., 2008). Several studies have addressed the functions of dopamine in the spinal cord of lampreys and have shown that it is involved in the modulation of spinal neurons (McPherson and Kemnitz, 1994; Schotland et al., 1996; Kemnitz, 1997). Dopamine is also involved in the function of higher brain centers (Thompson et al., 2008). On the other hand, descending catecholaminergic projections to the spinal cord have been reported in some vertebrates (Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006). However, it is not known whether dopaminergic populations of the lamprey brain contribute to the dopaminergic innervation of the spinal cord.

In the present study, we used Neurobiotin and low molecular weight dextran amine in order to re-evaluate the descending projections from the brain. We report here on previously unidentified CSF-c cell populations that give rise to descending spinal projections in lampreys. A combination of tract tracing with immunohistochemistry against dopamine was also used with the aim of searching for spinal cord-projecting DAir neurons, which are reported here for the first time. We also compare this descending system in lampreys with those reported in other vertebrates in order to obtain further information about the evolution of the dopaminergic descending projections.

Abbreviations

BDA	biotinylated dextran amine
CSF-c	cerebrospinal fluid-contacting cell
Di	diencephalon
DHyp	dorsal hypothalamus
DiI	1,1′-dioctadecyl 3,3,3′,3′ tetramethylindocarbocyanine perchlorate
DTh	dorsal thalamus
HRP	horseradish peroxidase
HY	hypophysis
Ith	olfactory nerve
IVth	trochlear nerve
IXth	glossopharyngeal nerve
M	mesencephalon
M1-M2	Müller cells 1 and 2
MD	medial diencephalic nucleus
Mm	mammillary population
NB	Neurobiotin
nMLF	nucleus of the medial longitudinal fasciculus
OB	olfactory bulb
PT	posterior tubercle
Pt	paratubercular nucleus
PVO	paraventricular organ
Rh	rhombencephalon
T	telencephalon
VHyp	ventral hypothalamus
VHyp D	dorsal subpopulation of the ventral hypothalamus
VHyp V	ventral subpopulation of the ventral hypothalamus
VIIth	facial nerve
VIIIth	octaval nerve
Vth	trigeminal nerve
VTh	ventral thalamus
Xth	vagal nerve

MATERIALS AND METHODS

Animals

Larval (n = 60; body length between 50 and 150 mm) and upstream migrating adult (n = 12) sea lampreys (*Petromyzon marinus*) were used. All experiments conformed with the European Community guidelines on animal care and experimentation. Before experiments, the animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water.

Retrograde tract tracing

In the larvae, the spinal cord was exposed by a transverse incision made in the dorsal region of the body. Crystals of Neurobiotin (NB; Vector, Burlingame, CA) were applied with a minute pin (00) at one of three different levels of the spinal cord: the level of the second branchiopore (8.7% of the body length), the level of the fourth branchiopore (15% of the body length), and just caudal to the seventh branchiopore (20% of the body length). The incision was closed with Histoacryl tissue glue (B. Braun Surgical, Tuttlingen, Germany). The larvae were maintained at 4°C, with appropriate aeration conditions, in lamprey Ringer's solution of the following composition (in mM): 137 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 2 HEPES. In six larvae, the tracer was applied to only one side of the spinal cord to assess the laterality of the projections, and the cord was sealed with tissue glue. After a period ranging between 1 and 5 days, the larvae were deeply anesthetized and the heads were fixed by immersion in one of the following fixatives: 1) 4% paraformaldehyde in 0.4 M Tris buffer pH 7.4 (TBS) for 5–6 hours, or 2) 5% glutaraldehyde/1% sodium metabisulfite in 0.05 M TBS for 17 hours. The heads were rinsed in TBS, then cryoprotected with 30% sucrose in TBS, embedded in Tissue Tek (Sakura, Torrance, CA), frozen by using liquid nitrogen-cooled isopentane, and cut in the transverse or sagittal planes (20 μm thickness) on a cryostat.

For application of tracer to the spinal cord of adults, NB crystals were inserted with a minute pin (00) in the rostral spinal cord at the level of the second branchiopore of previously dissected brain/spinal cord wholemounts (n = 6). The brain/spinal cord wholemounts were maintained in the same conditions as larval samples for 3 days and then processed as above.

Indirect detection of NB

For detection of NB, the sections were incubated at room temperature with fluorescein isothiocyanate (FITC)-labeled avidin D (Vector) diluted 1:1,000 in TBS containing 0.3% Triton X-100, for 4 hours. The sections were rinsed in distilled water and coverslipped with mounting medium for fluorescence (Vectashield; Vector). In addition, some sections of heads of untreated larvae fixed as above were treated with FITC-labeled avidin-D to rule out the possibility that staining was due to endogenous brain biotin. No FITC staining was observed in these sections (not shown).

Dopamine immunofluorescence combined with tract tracing

After the application of NB in the rostral spinal cord, as above, and the corresponding survival times, some larval heads (80–150 mm in body length, n = 15) and adult brains (n = 3) were fixed by immersion in freshly prepared 5% glutaraldehyde/1% sodium metabisulfite in 0.05 M TBS for 17 hours, and prepared for sectioning as above.

Sections were pretreated with 0.2% $NaBH_4$ in distilled water for 45 minutes at room temperature. The sections were then incubated with a rabbit polyclonal anti-dopamine antibody (dilution 1:750; H.W.M. Steinbusch, Maastricht, The Netherlands). This anti-dopamine antibody was raised against dopamine-glutaraldehyde-bovine serum albumin (BSA) conjugates. For detection of the immunocomplex, a rhodamine isothiocyanate (RICT)-conjugated swine anti-rabbit antibody (dilution 1:30; Dako, Glostrup, Denmark) was used (incubation time: 1 hour at room temperature). All antibodies were diluted with TBS containing 1% metabisulfite, 10% normal swine serum, and 0.2% Triton X-100 as detergent. The NB was then revealed with FITC-labeled avidin D as above.

Control sections for the dopamine antibody were processed as above, except for omission of the primary antiserum. No staining was observed in these controls. We tested the specificity of this antibody in lamprey with preadsorption experiments; immunostaining was completely abolished after preadsorption of the diluted anti-dopamine antibody with dopamine-glutaraldehyde-BSA conjugates. However, staining was not modified after preadsorption of the primary antiserum with BSA (Sigma). Western blotting of lamprey brain protein extracts with this anti-dopamine antibody showed that it does not recognize native proteins (Fig. 1A). As a positive control for Western blot we used an anti-calretinin antibody (dilution 1:500; SWant, Bellinzona, Switzerland; code 7699/4; lot 18299; immunogen: recombinant human calretinin), which recognizes a band of 29 kDa corresponding to lamprey calretinin (Fig. 1A; Villar-Cheda et al., 2006). This antibody was used previously to demonstrate specific populations of cells in the spinal cord (Megías et al., 2003) and retina (Villar-Cheda et al., 2006) of the sea lamprey.

Additional experiments

Most of the new spinal-projecting cells described in the present study were CSF-c cells. The following controls were included to ensure that the tracer was taken up via the axons, and not from the cerebrospinal fluid, blood or other routes:

1. NB was injected into the fourth ventricle of three larval and three adult lampreys (dissected brains plus the rostral spinal cord) under deep anesthesia, either with a minute pin through the choroid plexus, or by injection with a hypodermic syringe (NB diluted 10% in distilled water). No CSF-c cells were labeled in the brain or spinal cord in these experiments (not shown).
2. Adult lamprey brain show blood capillaries that have an endothelial blood-brain barrier to macromolecules like HRP or microperoxidase (Bundgaard, 1982). Instead, larval lamprey brains lack blood vessels (Rovainen, 1979; present results), and uptake of tracer from the blood during experiments therefore appears unlikely. However, to preclude this route of entry of

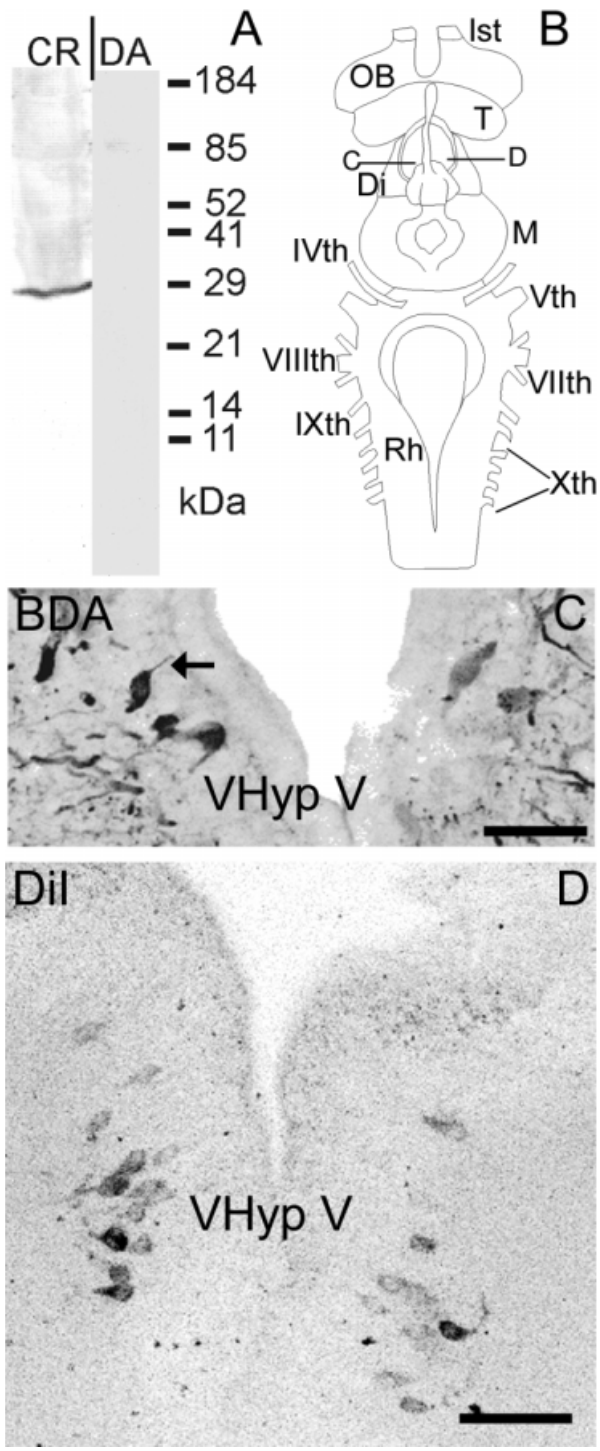

Fig. 1. **A:** Western blot of lamprey protein extracts immunostained with antisera to calretinin (used as positive control) and DA. Note the protein band of 29 kDa stained in the calretinin lane and the absence of labeling in the DA lane. **B:** Schematic drawing showing a dorsal view of the sea lamprey brain. The black lines indicate the approximate levels of the transverse sections C and D. **C:** Photomicrograph of transverse section of the ventral hypothalamus showing cells labeled with BDA applied in the rostral spinal cord. The arrow indicates the dendrite of a CSF-c cell. **D:** Photomicrograph of transverse section of the ventral hypothalamus showing cells labeled with DiI applied in the rostral spinal cord. For abbreviations, see list. Scale bar = 50 μm in C; 75 μm in D.

tracer, NB crystals were inserted intraperitoneally into three larvae and after 3 days, the larvae were processed as above. No cells were labeled in the brain and spinal cord, although meningeal vessels were labeled (not shown).

3. Larvae (n = 3) were fixed with 4% paraformaldehyde in TBS. Small crystals of a lipophilic carbocyanine dye (DiI; Molecular Probes, Eugene, OR) were applied with a minute pin to the rostral spinal cord. The larvae were maintained in darkness in frequently renewed fresh fixative for 3 months at 37°C. The brains were then dissected out, embedded in 3% agarose, and sectioned on a Vibratome (100 μm thickness).
4. Neurobiotin may also be a transneuronal tract tracer (Huang et al., 1992). To prevent CSF-c cells from being labeled transneuronally, three larvae were injected in the rostral spinal cord following the same protocol described above but with biotinylated dextran amine (BDA; 3,000 Da; Molecular Probes) instead of NB, and with an incubation time of 5 days. BDA was revealed with FITC-labeled avidin-D, as for NB.
5. In previous studies of the lamprey spinal cord, HRP was used to reveal the brain/spinal-projecting neurons (Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994), and thus the differences observed in the present results may be due to different efficiency of NB. For direct comparison of the transport efficiency of NB and HRP (Serva, Heidelberg, Deutschland), a mixture of both tracers was applied in the rostral spinal cord of three larvae. The larvae were maintained in the same conditions as above for 1 day, and then fixed and sectioned as above. HRP was revealed by sequential incubation with a mouse monoclonal anti-HRP (dilution 1:1,000 in 0.2% TST and 15% normal goat serum; Sigma; code P6-38; lot 102K4812) and a secondary goat anti-mouse antibody coupled to Alexa 546 (dilution 1:100; Interchim, Montluçon, France). The NB was then revealed as above. Omission of the anti-HRP antibody did not result in immunostaining in any sections.

Photography

Photomicrographs were taken with a spectral confocal laser microscope (Leica TCS-SP2). The photomicrographs were adjusted for brightness and contrast with Adobe Photoshop 7.0 software. For presentation in plates, some photomicrographs were inverted and converted to gray scale.

Cell measurements and counts

The sizes of the cells in the larval brains were measured with LCS (free Leica confocal software) on confocal projections. Five well-labeled larvae were used, and 30 cells of each nucleus were measured (either the long axis or the diameter in spherical cells). The total number of NB-labeled cells was counted in the spinal cord-projecting nuclei reported here for the first time in the two brain sides in these larvae. Counts were made on photomicrographs of all the transverse sections in which each nucleus was present. Neurobiotin-labeled elements with a neuronal appearance (more than 9 μm in diameter) were included in the counts. To avoid double counting of cells, series of photographs were aligned with ImageJ (free NIH software), and the positions of labeled cells in pairs of consecutive sections were compared. This unbiased approach and the low number of labeled cells encountered made it unnecessary to introduce any correction factor for possible overestimations of cell numbers. Because high variations were found in the number of labeled cells in each of the nuclei, the range of labeled cells is provided for each nucleus. In addition, the percentage of retrogradely labeled cells that were DAir in these nuclei was also calculated.

Nomenclature of brain structures

For the brain nuclei previously reported to project to the spinal cord, we adopted the nomenclature used by Ronan (1989), although the correspondence of the spinal cord-projecting nuclei to brain regions was made in the context of the lamprey prosomeric model (Pombal and Puelles, 1999) and the revision of Puelles and Rubenstein (2003). The nomenclature for the new positive cells was adopted from that used in previous studies carried out in our laboratory (Meléndez-Ferro et al., 2002, 2003; Abalo et al., 2007).

RESULTS

A schematic drawing showing a dorsal view of the lamprey brain is presented in Figure 1B. Schematic drawings showing the organization of the spinal cord-projecting cells in the brain are presented in Figure 2. Figures 3 and 4 show the new spinal cord-projecting cells reported here for the first time. Figures 5–7 show the double-labeling experiments made with the combination of HRP and NB or DA and NB.

Technical comments

In the present study, we evaluated the labeling of spinal projections in larvae maintained for between 1 and 5 days after NB application. All the new spinal cord-projecting nuclei reported here were observed after 1 day of incubation (Fig. 5B), but optimal labeling was achieved after 2 days. An increase in the survival time did not improve the labeling or reveal more cell groups. In addition, when the tracer was applied to one side of the spinal cord, the diencephalo-spinal projecting groups identified here for the first time were not labeled; these cells were only labeled when NB application involved the medial region of the spinal cord. This is probably because fibers arising from these groups ran in the ventral midline of the brain and spinal cord (Fig. 4A,B).

Control experiments

The neuronal populations revealed with NB tracing were also labeled in experiments in which BDA was used as a neuronal tracer, although fewer cells were labeled in the new populations reported here (i.e., those of the ventral hypothalamus; Fig. 1C). Moreover, DiI experiments in paraformaldehyde-fixed larval lampreys (n = 3) labeled cells in all the populations that showed labeled cells in NB experiments (i.e., those of the ventral hypothalamus; Fig. 1D). Tract-tracing experiments with a mixture of HRP and NB demonstrated that with a short incubation period, NB labeled many more cells and fibers and reached more rostral brain regions (Fig. 5A,B).

Distribution of cells projecting to the spinal cord in the brain of lamprey

The present study was mainly performed in larvae of sea lampreys (n = 60), and therefore we mainly describe the spinal cord-projecting nuclei of this stage. Analysis of the adult brains (n = 12), used as controls, showed that the organization of their nuclei projecting to the spinal cord is similar to that found in larvae. Spinal-projecting nuclei were observed in the diencephalon, mesencephalon, and rhombencephalon. The pattern of neuron labeling in the mesencephalon and rhombencephalon revealed by NB and BDA tracing was similar to that previously revealed with HRP as a tracer (Swain et al., 1993), and these well-known spinal-projecting cells will not be described here. However, our experiments revealed further groups of labeled cells situated in the caudal rhombencephalon and the diencephalon. Remarkably, all the new cell groups reported in larvae were also labeled in adults, e.g., the mammillary population (Fig. 7B).

Caudal rhombencephalon

The experiments revealed a group of between 40 and 51 CSF-c cells situated in the caudal rhombencephalon around the central canal that projects caudally (Fig. 6A,A′,B,B′). The CSF-c cells labeled in the caudal rhombencephalon, were small bipolar cells of about 18–21 μm in their major axis, with an apical dendrite that ended in a small intraventricular protrusion (Fig. 6A,A′). Cells of this type were also labeled in the spinal cord rostral to the site where the tracer was applied. In addition, occasional non-CSF-c cells of about 10–11 μm in diameter were also labeled in the caudal rhombencephalon (Fig. 6A,A′). Most of the CSF-c cells were located ventromedially in the central canal wall at levels just rostral to the site of application of the tracer; a few of the CSF-c cells had a ventrolateral location in the ependymal region of the caudal rhombencephalon. These cells were only retrogradely labeled when the tracer was applied at the level of the second branchiopore, i.e., their caudal projections only reached spinal cord levels close to the obex.

Spinal-projecting nuclei in the sea lamprey diencephalon

The application of NB in the rostral spinal cord labeled a number of small neurons in the ventral hypothalamic, mammillary, and paratubercular nuclei that were not previously reported as spinal-projecting by other authors. In addition, our experiments labeled medium-sized cells in the nucleus of the medial longitudinal fasciculus, posterior tubercle, and ventral thalamus/dorsal hypothalamus (medial diencephalic nucleus), as previously reported with other tracers (Ronan, 1989). The location of the labeled cells in the sea lamprey diencephalon is shown schematically in Figure 2A–C. The sizes of the cells in these diencephalic populations are indicated in Table 1.

Paratubercular nucleus. Between 10 and 16 labeled cells were observed in the paratubercular nucleus of the larvae studied (Figs. 3A, 4A, 6C–C′). These were small, round cells that ranged between 10 and 13 μm in diameter and showed both rostral and caudal processes. These cells were only labeled when the Neurobiotin was applied at rostral spinal cord levels (second branchiopore).

Mammillary nucleus. Between 30 and 42 labeled CSF-c cells were observed in the so-called mammillary population (Mm) in the walls of the posterior infundibular recess (Figs. 3C,D, 4C, 5B, 7A–B′) when NB was applied at the level of the second branchiopore. They were small, oval or pear-shaped cells, ranging from 9 to 10.5 μm in size. Their soma gave rise to an apical dendrite that protruded in the ventricle as a club and a basal process arising from the opposite cell pole. The density of cells in this Mm population varied; the highest density was observed in the ventrolateral corner of the recess and the lowest in its dorsal wall, where only very few labeled cells were observed. The caudodorsal region of this population partially overlaps with the paratubercular nucleus labeled cells, but the cell morphology and size allowed clear differentiation between these two spinal-projecting cell populations.

Ventral hypothalamic population. A large population of bipolar cells was labeled in the ventral hypothalamus (VHyp) in the walls of the infundibular recess (Figs. 2A,B, 3B, 4D, 5C). As regards the size and location of cells, and the lengths of their projections, this hypothalamic group can be subdivided into two subpopulations: dorsal (VHypD) and ventral (VHypV). Dopaminergic immunohistochemistry allowed assessment of the location of these cells in the diencephalon in comparison with that of the dopaminergic paraventricular organ, which is situated in the dorsal hypothalamus (Fig. 5C). The spinal cord-projecting cells of the VHyp were located ventral to this organ. The dorsal subpopulation was formed by scattered medium-sized cells (between 10 and 20) of diameter between 13 and 16 μm, and cells of this subpopulation were labeled when the NB was applied rostrally (second branchiopore level) or more caudally (at the level of the fourth or seventh branchiopores), but the number of labeled cells decreased when the point of application of tracer was more caudal. Most, if not all, of the dorsal subpopulation cells were not CSF contacting. The ventral subpopulation was formed by a compact group of numerous (between 40 and 60) small, CSF-c round cells (10–12 μm in diameter). Cells of the ventral subpopulation were only labeled when the tracer was applied at the level of the second branchiopore. Most of the labeled VHypV cells were of CSF-c type, with bipolar perikarya showing a ventricular dendrite and a basal process coursing to the lateral area. The VHyp is situated rostral to the Mm; these nuclei were separated by a gap where no labeled cells were observed in any experiment (Fig. 3C).

Dopaminergic neurons projecting to the spinal cord

Combined experiments revealed that some spinal cord-projecting CSF-c cells of the caudal rhombencephalon/rostral spinal cord (Fig. 6A–B″) and mammillary nucleus (Fig. 7) and some cells of the paratubercular nucleus (Figs. 6C–C″), were also DAir. These spinal cord-projecting DAir cells were observed both in larval and adult lampreys, and appeared well developed in 80-mm larvae.

Caudal rhombencephalic DAir CSF-c cells. In the caudal rhombencephalon and rostral spinal cord, DAir CSF-c cells formed a longitudinal cell column extending in the ventral margin of the central canal (Fig. 6A,A″,B,B″). A high proportion of the NB-labeled cells in this region were DAir CSF-c cells (between 45% and 50% of the cells). Very few non-CSF-c DAir cells located in the ventral midline were also double-labeled from the rostral spinal cord (Fig.

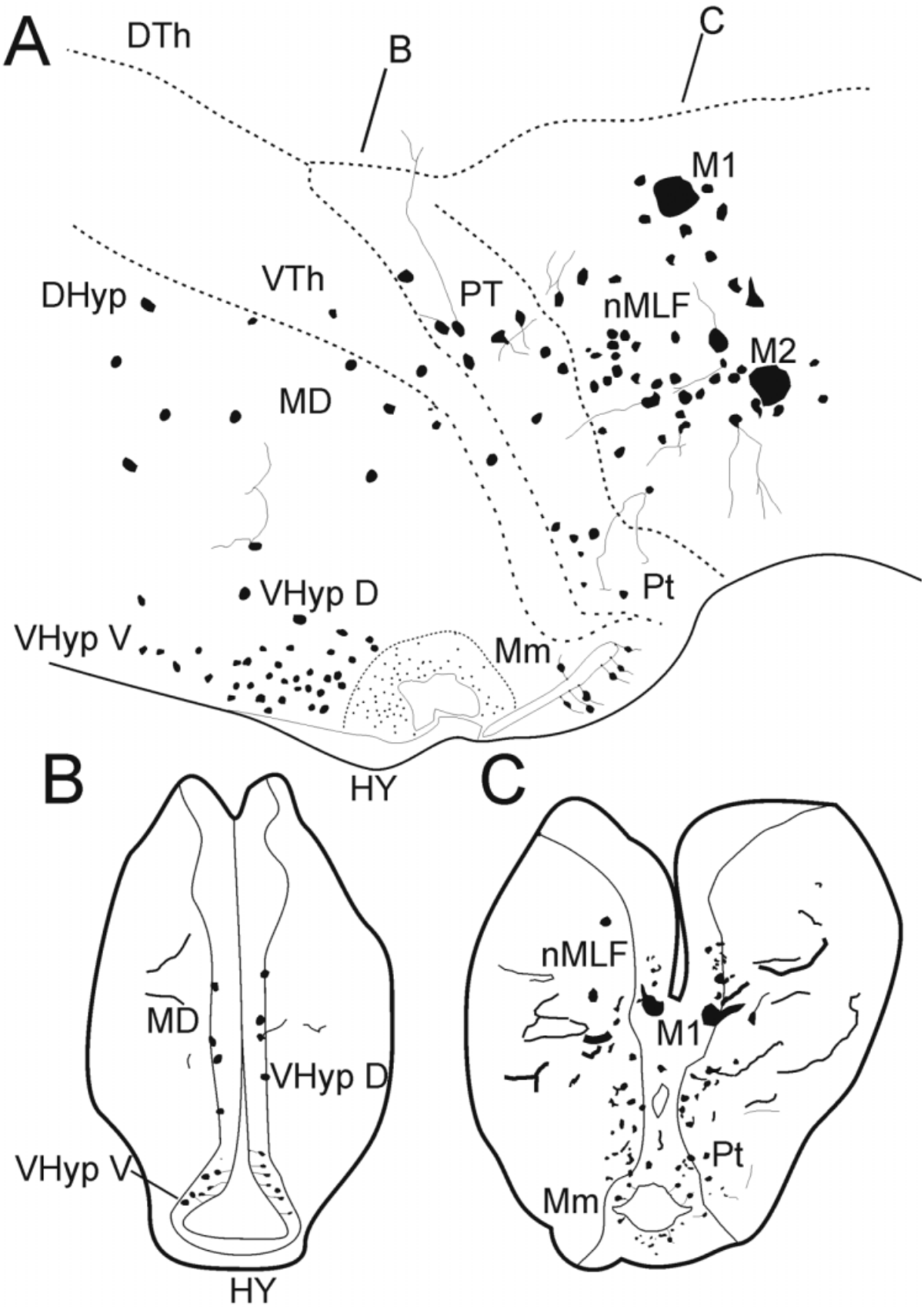

Fig. 2. **A:** Schematic drawing representing the distribution and organization of the spinal cord projecting populations in the larval sea lamprey diencephalon in lateral view. Oblique bars at the top indicate the approximate levels of the transverse schematic drawings B and C. **B,C:** Schematic drawings of transverse sections showing the location of the diencephalic spinal cord projecting cells. For abbreviations, see list.

TABLE 1. Range of Cell Sizes of the Different Diencephalic Spinal Cord-Projecting Nuclei

Nuclei	Range of sizes (μm)
nMLF	11.5–40
PT	15–20
MD	13.5–26
Pt	10–13
Mm	9–10.5
VHyp D	13–16
VHyp V	10–12

For abbreviations, see list.

6A–A″). Ventral processes of these cells coursed toward a ventromedial DAir plexus of the caudal rhombencephalon and spinal cord.

Paratubercular nucleus. After application of the tracer at rostral spinal cord levels, occasional rounded or pear-shaped DAir paratubercular cells were double-labeled (Fig. 6C–C″). Neurons of this nucleus showed processes ascending in a dorsorostral direction or coursing in a caudal direction, as was observed by immunofluorescence in sagittal brain sections. However, the low number of double-labeled DAir neurons observed in this nucleus indicates that most cells do not project to the spinal cord.

Mammillary nucleus. A periventricular DAir cell group, the mammillary nucleus, was observed around the

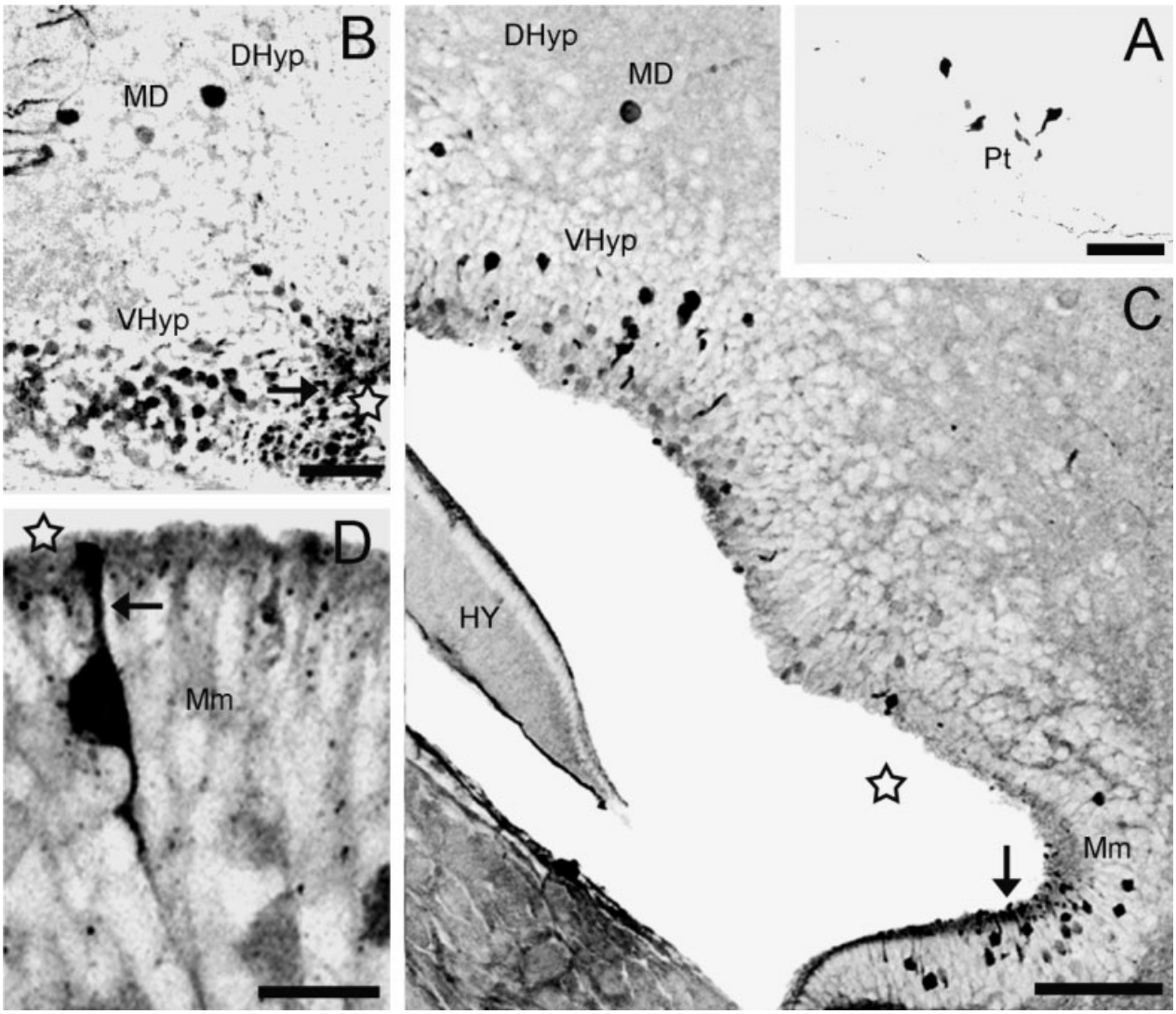

Fig. 3. Photomicrographs of sagittal sections of larval brains and spinal cord showing the cell populations labeled after application of NB to the rostral spinal cord. **A:** Photomicrograph of a sagittal section of the paratubercular nucleus of a larva showing the presence of small labeled cells. **B:** Photomicrograph of a sagittal section of the hypothalamic region of a larva showing numerous spinal cord-projecting cells in the VHyp population. **C:** Photomicrograph of a sagittal section of the hypothalamic region of a larva showing labeled CSF-c cells of the Mm and Vhyp populations. **D:** Detail of a CSF-c cell spinal cord-projecting cell situated in the ventral part of the mammillary recess. The arrows indicate the ventricular dendrite. For abbreviations, see list. In all photomicrographs, rostral is on the left. The outlined stars (in B, C, and D) indicate the ventricle. Scale bar = 50 μm in A,B; 75 μm in C; 12.5 μm in D.

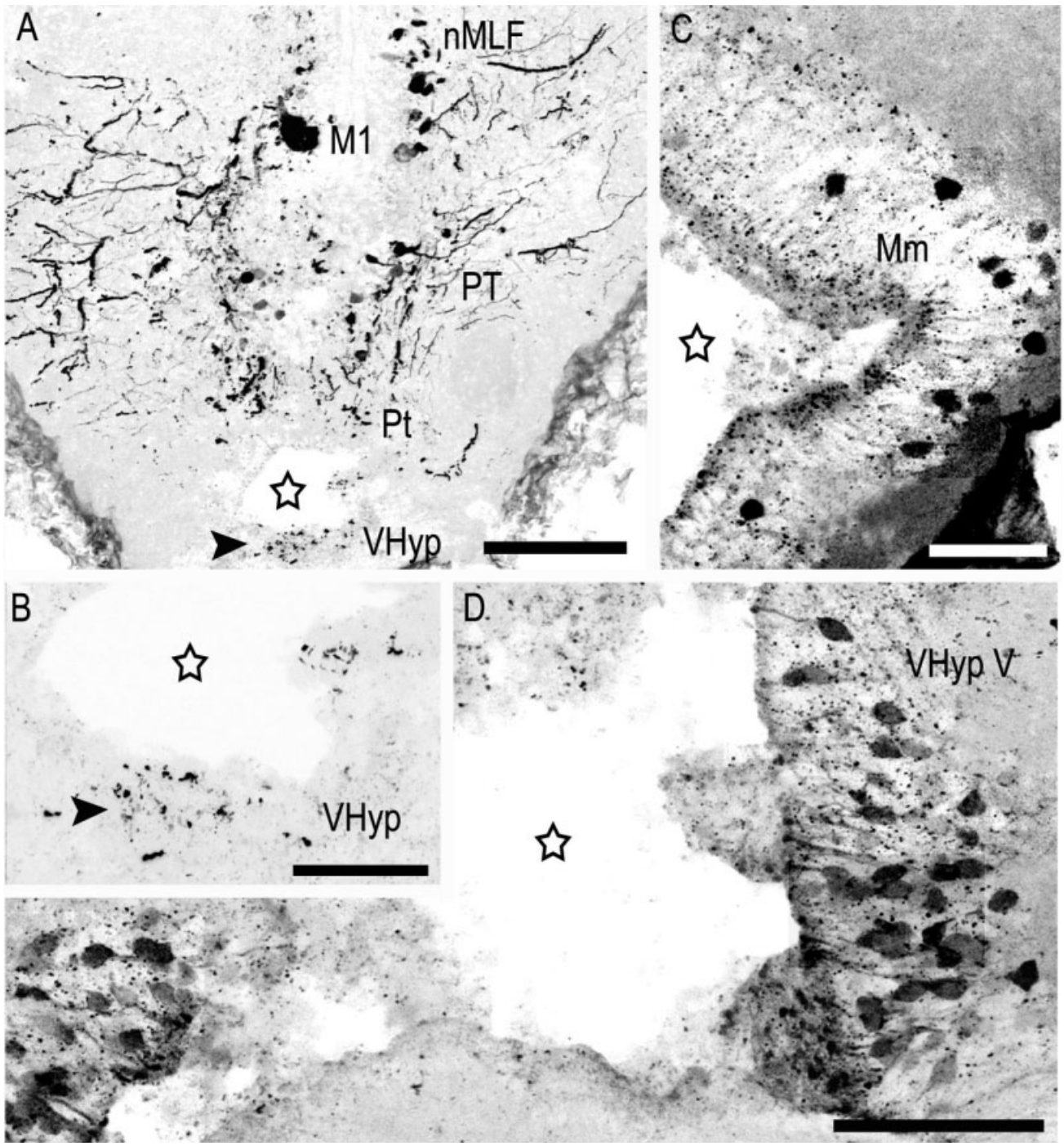

Fig. 4. Photomicrographs of transverse sections of the larval diencephalon after application of NB to the rostral spinal cord. **A:** Photomicrograph of a transverse section of the caudal diencephalon showing labeled cells of the MLF (including the M1 giant neuron), posterior tubercle, and paratubercular nucleus. Note the presence of descending fibers in the ventral midline of the hypothalamus. **B:** Detail of Figure 5A showing thin labeled fibers running in the ventral hypothalamus. **C:** Photomicrograph of a section of the mammillary nucleus showing labeled CSF-c cells. **D:** Photomicrograph of a transverse section of the rostral diencephalon showing CSF-c cells of the VHyp V. The arrowheads in A and B indicate thin labeled fibers coursing in the ventral midline of the hypothalamus. The outlined stars indicate the ventricle. Small dots in C and D are autofluorescent lipidic droplets present in ependymal cells of lampreys. For abbreviations, see list. Scale bar = 150 μm in A; 100 μm in B,D; 50 μm in C.

posterior infundibular recess. The majority, if not all, of the DAir double-labeled cells of the mammillary nucleus were CSF-c neurons (Fig. 7). After application of the tracer in the rostral spinal cord, between 30% and 35% of NB-labeled cells of the mammillary population were DAir. Fibers arising from the ventral pole of these cells formed a

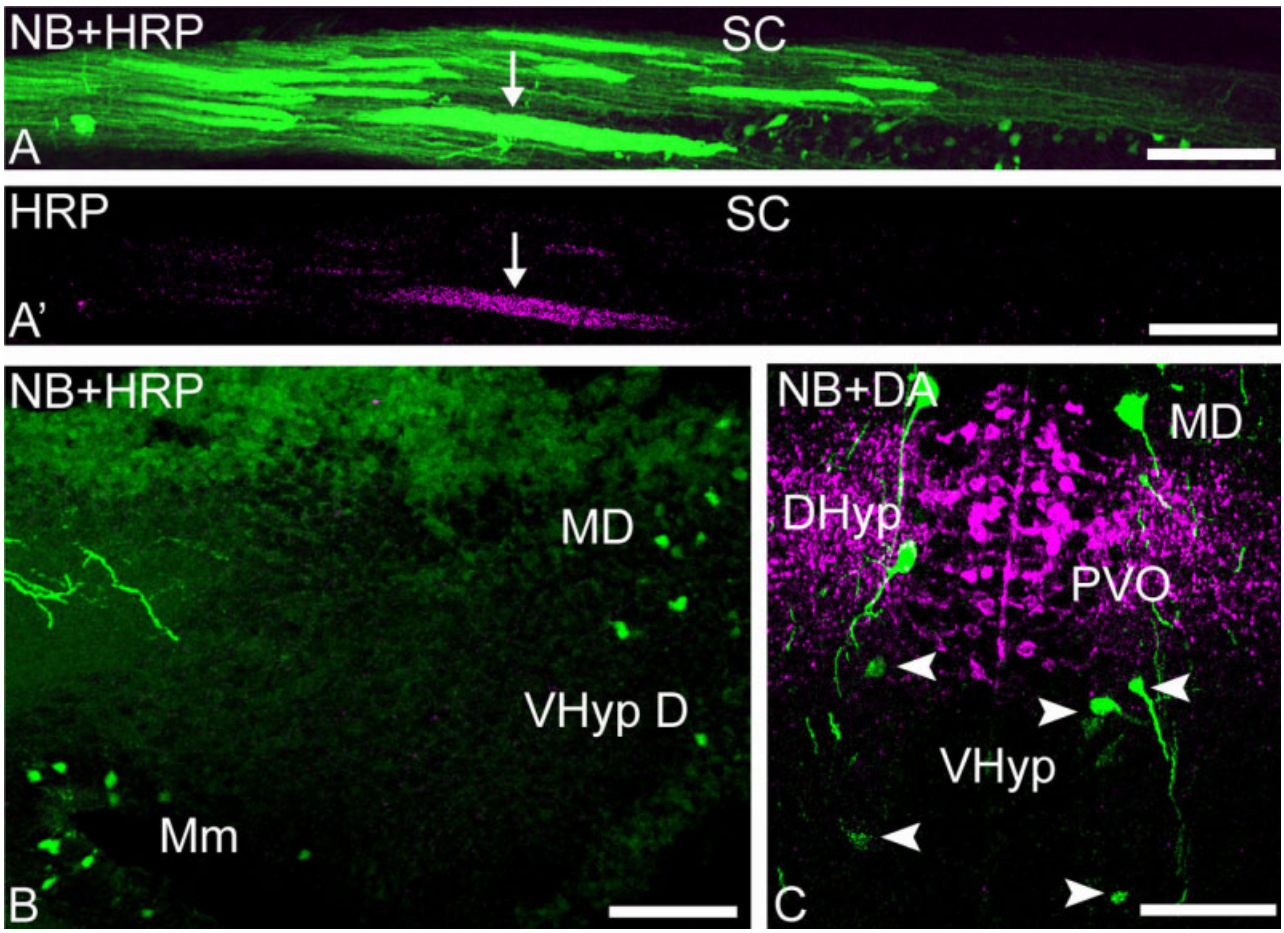

Fig. 5. **A,A′:** Photomicrographs of a sagittal section of the caudal diencephalon showing cells and descending fibers labeled with NB (green channel) applied in the spinal cord after 1 day of incubation. Only the large fibers were labeled with HRP (magenta channel). **B:** Photomicrograph of a sagittal section of the diencephalon of the same larval sample in A,A′ showing NB-labeled cells in the mammillary and ventral hypothalamic nuclei. Note that HRP (magenta channel) did not label any cell or fiber in the lamprey diencephalon. **C:** Photomicrographs of a transverse section of the rostral diencephalon showing the location of spinal-projecting cells of the medial diencephalic and ventral hypothalamic nuclei labeled with NB (green channel) with respect to the dopaminergic (magenta channel) paraventricular organ, which is located in the dorsal hypothalamus. The arrows indicate a large fiber labeled with NB and HRP in the spinal cord. The arrowheads indicate cells labeled with NB in the ventral hypothalamus. For abbreviations, see list. Scale bar = 100 μm in A,A′,B; 50 μm in C.

ventromedial dopaminergic plexus that coursed caudally (Fig. 7A–A″). Most of the double-labeled DAir cells were situated in the caudoventral walls of the mammillary recess.

DISCUSSION

Methodological considerations

The present study increases the number of known brain populations that project to the spinal cord in lampreys by four, three of which consist of CSF-c neurons. The present results showed that application of NB (322.8 Da) to the rostral spinal cord led to the retrograde labeling of a greater size range of cells than in previous studies with HRP (molecular weight, 40,000 Da) (Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994), and demonstrated a number of small CSF-c spinal-projecting neurons in regions not mentioned by these authors. Differences between present and previous studies in lampreys may be explained in part for the different molecular weight/chemical nature of the tracers used and the different point of tracer application (8.7% of the body length [present results]; third muscle segment [Ronan, 1989; level of the second branchiopore?]; 10–20% of the body length [Swain et al., 1993; Davis and McClellan, 1994]). The various additional experiments performed, especially the positive staining of these newly populations by alternative tracers such as BDA applied in vivo or DiI applied to fixed tissue (see Materials and Methods), clearly indicate that this was not due to transneuronal labeling or to diffusion of NB through the CSF or the blood.

Some hypothalamic CSF-c cells project to the spinal cord in lampreys

The most remarkable new finding from the present investigation was the direct demonstration of a prominent hypothalamo-spinal projection arising from CSF-c cells of the walls of the infundibular and mammillary recesses,

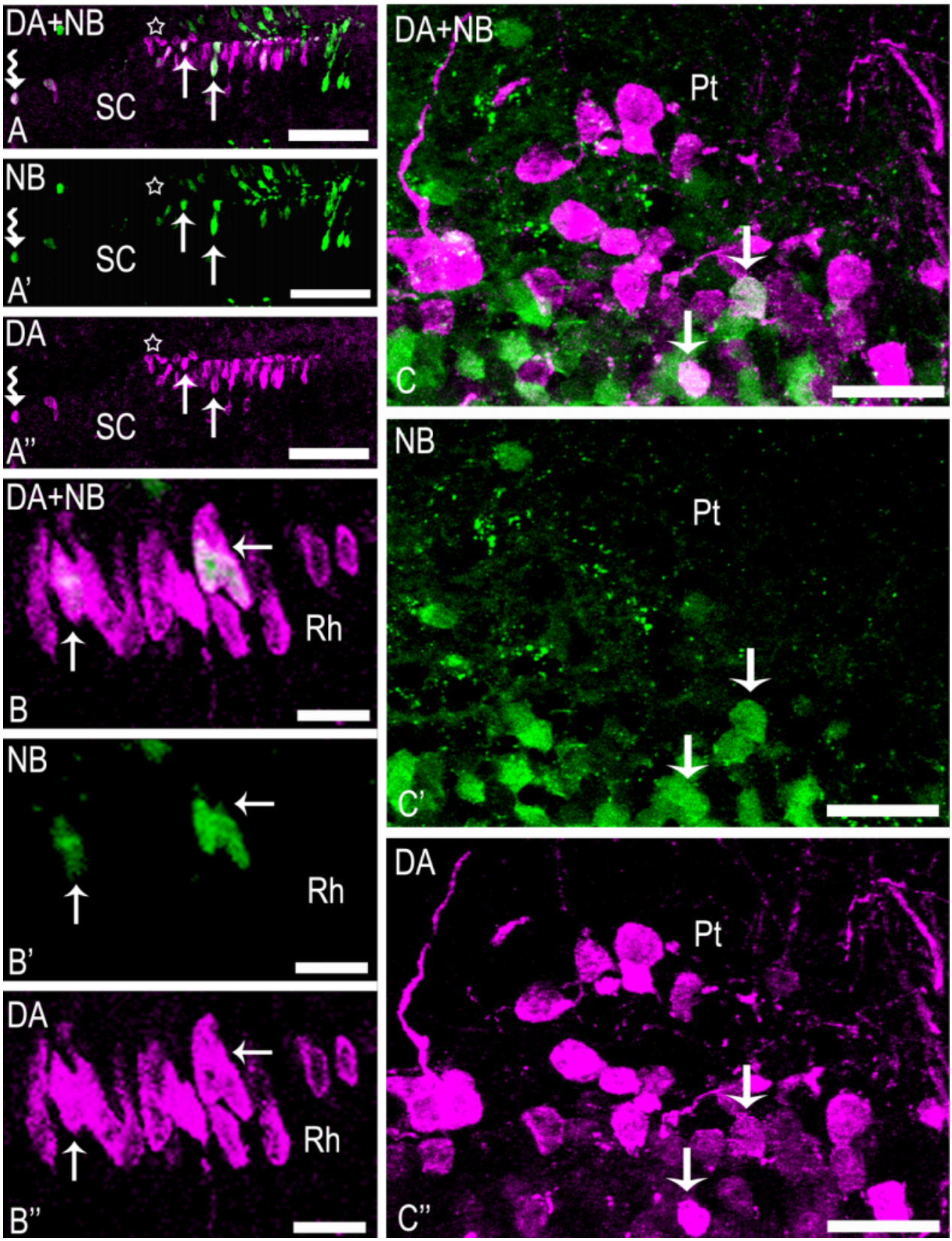

Fig. 6. **A–A″, B,B″:** Photomicrographs of sagittal sections showing dopaminergic cells (magenta channel) located in the ventral midline of the rostral spinal cord (A) and caudal rhombencephalon (B) labeled with NB (green channel) applied in the rostral spinal cord. **C–C″:** Photomicrographs of sagittal brain sections showing dopaminergic spinal cord-projecting cells of the paratubercular nucleus. The arrows indicate double-labeled neurons. Curved arrows indicate non-CSF cells of the ventral midline of the spinal cord or caudal rhombencephalon. The outlined stars indicate the ventricle (B) and the central canal (A). For abbreviations, see list. Scale bar = 75 μm in A–A″; 50 μm in B–B″; 25 μm in C–C′.

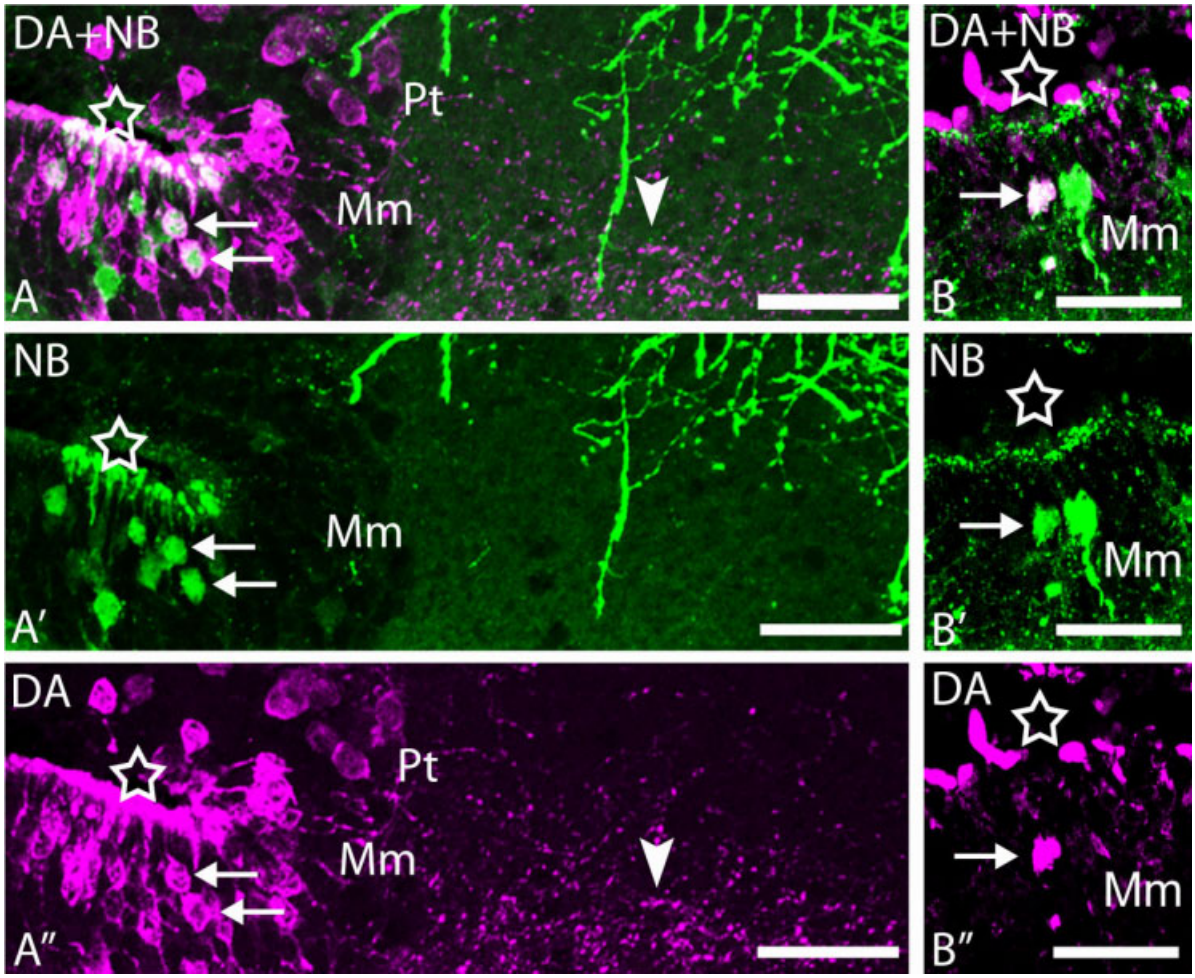

Fig. 7. Photomicrographs of sagittal sections of the caudal hypothalamus showing the presence of dopaminergic cells (magenta channel) of the mammillary nucleus labeled with NB (green channel) applied in the rostral spinal cord in larval (**A**) and adult (**B**) lampreys. The arrows indicate double-labeled neurons. The arrowheads indicate the descending dopaminergic plexus in the caudal hypothalamus, which probably arises from the mammillary nucleus. The outlined stars indicate the ventricle. For abbreviations, see list. Scale bar = 50 μm in A–A′,B–″.

which has not previously been described in lampreys or other vertebrates. Hypothalamic CSF-c cells were first described in the late 1960s in most vertebrates, including lampreys (Nakai et al., 1979), although they decrease in number from fishes to mammals (Vigh et al., 2004). In lampreys, some hypothalamic CSF-c neurons were shown to project to the striatum (Pombal et al., 1997), torus semicircularis (González et al., 1999), and optic tectum (de Arriba and Pombal, 2007), but as far as we are aware no projections from diencephalic CSF-c neurons to the spinal cord have been reported. A study in lungfish, in which cobalt-lysine was applied intraocularly, revealed a number of brain targets attributed to projections of CSF-c cells of the paraventricular organ, including fibers that extend to the spinal cord (von Bartheld and Meyer, 1990). No projections from CSF-c cells of similar circumventricular regions were reported in zebrafish (McLean and Fetcho, 2004a,b) or amphibians (Sánchez-Camacho et al., 2001). It is not clear why similar spinal connections have not been reported in other vertebrates, because a paraventricular organ is present in all nonmammalian vertebrates (Vigh et al., 2004). It is possible that most tracers used in studies of spinal connections were taken up in very low amounts by fibers of these cells, or were transported very slowly.

In the lamprey, hypothalamic CSF-c cells are neurochemically heterogeneous, including cells immunoreactive to dopamine (Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005), noradrenaline (Steinbusch et al., 1981), serotonin (Steinbusch et al., 1981; Abalo et al., 2007), histamine (Pombal and Puelles, 1999), γ-aminobutyric acid (GABA; Meléndez-Ferro et al., 2002; Robertson et al., 2007), and various neuropeptides, but their projections are largely unknown. The present results with combined tracing and immunofluorescence methods indicate that axons from some dopaminergic CSF-c neurons reach the spinal cord. These cells may indicate changes in the CSF composition (Vigh et al. 2004), and en passant or terminal release of dopamine by their fibers may modulate the activity of the regions through which axons course, including the rostral spinal cord, in response to variations in the third ventricle.

Dopaminergic projections to the spinal cord in vertebrates

The present results showed that the sea lamprey dopaminergic brain neurons that project to the spinal cord are located in the diencephalon (paratubercular nucleus and mammillary nucleus) and the caudal rhombencephalon. Some experimental studies of descending dopaminergic projections performed in other vertebrates were carried out with tyrosine hydroxylase (TH) immunohistochemistry (Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006). These studies reported the presence of diencephalic catecholaminergic cells that project to the spinal cord in some mammals and amphibians (zona incerta: Blessing and Chalmers, 1979; Sánchez-Camacho et al., 2001; dorsal hypothalamus: Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006), which show no DAir spinal projecting cells in lamprey. Because TH is the common enzyme of synthesis of catecholamines, the presence of this enzyme does not identify unequivocally the catecholamine contained by these cells. Immunohistochemical studies in wholemount developing zebrafish also reveal the origin in the brain of early catecholaminergic fibers observed in the spinal cord, and dopaminergic fibers (THir and dopamine-β-hydroxylase negative) putatively originate from cells of the posterior tubercle, whereas noradrenergic fibers (dopamine-β-hydroxylase immunoreactive) arise from the locus coeruleus (McLean and Fetcho 2004a).

The most conserved catecholaminergic projection to the spinal cord in lower vertebrates appears to be the projection from cells of the posterior tubercle nucleus (larval zebrafish: McLean and Fetcho, 2004a,b; amphibians: Sánchez-Camacho et al. 2001). This nucleus was proposed to be equivalent to the paratubercular nucleus of lampreys (Abalo et al., 2005). The dopaminergic projection to the spinal cord from the paratubercular nucleus appears to be less developed in lampreys than in other vertebrates studied, because only very few DAir cells of this nucleus became double-labeled after the application of NB. The paratubercular region has been considered by Baumgarten (1972) and Pombal et al. (1997) to be homologous to the substantia nigra of mammals on the basis of the cytoarchitectural organization and striatal projections respectively. Although in mice the substantia nigra does not contain spinal cord-projecting cells, some catecholaminergic neurons of the midbrain ventral tegmental area (A10 group) project to the spinal cord (Qu et al., 2006). As regards the locus coeruleus projections observed in other vertebrates, their projections are noradrenergic (McLean and Fetcho, 2004a,b). Moreover, no DAir cells were observed in the rostral rhombencephalon of larval sea lamprey (Abalo et al., 2007; present results).

Projections from catecholaminergic cells of the nucleus of the solitary tract were observed in some amphibians (Sánchez-Camacho et al., 2002) and in mammals (Pindzola et al., 1988; Maisky and Dorosenko, 1991), but a similar DAir population projecting to the spinal cord was not observed in lamprey.

Functional implications of the dopaminergic projections to the spinal cord

Dopamine is involved in a variety of functions in the brain of vertebrates, including locomotion, emotion, learning, and autonomic and neuroendocrine modulation (Riederer et al., 1985; Kobayashi and Sano, 2000). In the lamprey spinal cord, dopamine modulates the cycle period of fictive swimming and motoneuron physiology (McPherson and Kemnitz, 1994). However, owing to the abundance of intraspinal DA-ir neuronal populations (Schotland et al., 1996; Pombal et al., 1997; Abalo et al., 2007; Rodicio et al., 2008), it is not possible to infer any possible contribution of the descending dopaminergic projections to the modulation of locomotor circuitry. It has also been suggested that the hypothalamic-spinal cord dopaminergic projection plays a role in the regulation of the sympathetic outflow in mammals (Qu et al., 2006). Lampreys do not possess sympathetic ganglia, and it has even been suggested that they lack a sympathetic nervous system (Gibbins, 1994; Funakoshi and Nakano, 2007). Accordingly, any relation between dopaminergic descending spinal innervation from hypothalamic cells in lampreys and autonomic functions appears unlikely. At the present, any functional consideration of this descending system in lampreys remains speculative.

CONCLUSIONS

The most important novel finding of the present study was the existence in lampreys of a spinal cord projection system arising from CSF-c cells of the ventral hypothalamus and caudal rhombencephalon, not previously reported in any vertebrate species. Important differences between the sea lamprey and other previously studied vertebrates were also observed in the organization of the dopaminergic projection system to the spinal cord.

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