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The dorsal column-medial lemniscal projection of anuran amphibians

5.1

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Based on: European Journal of Morphology 32 (1994) 283-287

Key words: amphibians, dorsal column nucleus, medial lemniscus thalamus, torus semicircularis.

ABSTRACT

The efferent connections of the dorsal column nucleus (DCN) of the anuran amphibians *Rana ridibunda* and *Xenopus laevis* have been studied by means of bidirectionally transported tracres. Efferent projections from the DCN innervate the spinal cord, tegmentum of the brain stem, cerebellum, torus semicircularis and thalamus. The pattern of connectivity of the anuran DCN is largely comparable to that of amniotic vertebrates although some peculiarities are found.

The dorsal column-medial lemniscal system (DC-ML) i.e. the primary afferent spinal projection via the dorsal funiculus to the dorsal column nucleus (DCN), which gives rise to the medial lemniscal projection to the thalamus, represents a main ascending spinal projection in vertebrates (see Willis & Coggeshal, 1991). The existence of such a projection in anurans has been a much debated question and the connectivity of the DCN in amphibians is only partially known. The termination of the primary afferents to the DCN shows a somatotopic organization in relation to the rostrocaudal spinal level of their origin (Antal et al., 1980; Nikundiwe et al., 1982; Urbán & Székely, 1982). An efferent DCN projection to the contralateral thalamus has been obseved in ranid frogs in physiologycal stimulation (Vesselkin et al., 1971; Urbán & Székely, 1982) and tracing studies (Neary & Wilczynski, 1977). Also a medial lemniscal innervation of the torus semicircularis (Comer & Grobstein, 1981; Wilczynski, 1981; Wilczynski & Neary, 1986; Muñoz et al. 1993) has been demonstrated, and retrogradely labeled cells were observed in the DCN after cerebellar (González et al., 1984; Grover & Grüsser-Cornehls, 1984) and tectal tracer applications (Zittlau et al, 1988). In the pressent study the main ascending projections of the anuran DCN will b discussed.

In 15 adults of *Rana ridibunda* and 10 young adults of *Xenopus laevis* under anesthesia with MS222, HRP crystals or biotinylated dextran amine (BDA, Mollecular Probes) recrystallized from destilled water onto sharp tungsten needles were applied to the DCN area, or BDA or rhodamine dextran amine (RDA, Lollecular Probes) were applied as crystals or iontophoretically injected into the thalamus, torus semicircularis and cerebellum. After 2-10 days of survival time the animals were reanesthetized and perfused through the heart with saline followed by 4% paraformaldehide (2.5% paraformaldehide with 2% glutaraldehide for HRP experiments). The brain and spinal cord were then removed and stored overnight in a 30% saccharose solution. Frontal, 40 µm thick sections were cut on a frezzing microtome. HRP material was reacted with DAB as chromogen, usually intensified with nickel. To visualize BDA, a Vectastain ABC elite kit (Vector) was used, followed by the HRP reaction with DAB. Although the iontophoretic injections of the tracers were rather small, particularly those with BDA, it was always hardly possible to restrict the tracer to the area of the DCN. Medially located injections often involved partially the nucleus of the solitary tract, whereas more lateral injections included part of the nucleus of the descending trigeminal tract. Thus, solely with this type of tracer application it is not possible to fully discriminate the connections of the DCN. Therefore, subsequent retrograde tracing experiments were used to confirm the projections from the DCN. Since the results in both species are largely comparable we will describe the general pattern of the connections of the DCN. Only when species differences are found will then be mentioned separately.

Small unilateral applications of HRP or BDA that involved the DCN labeled anterogradely a distinct contralateral ascending system from the DCN, i.e. the medial lemniscus (Fig.1). Its axons could be traced from the injection site and course ventrally and medially, cross to the contralateral side beneath the central canal, and then turn rostralwards into the ventral tegmentum. As the medial lemniscus ascends in the rhombencephalon, smoothly swings to more

dorsolateral positions. All through the medulla, the medial lemniscus gives off thin fibers to various parts of the reticular formation. A few fibers course dorsally into the octavolateral area and some enter the granular layer of the cerebellum (Fig.1E). At caudal mesencephalic levels the fibers turn dorsally along the lateral aspect of the midbrain and most of them bend medially where they terminate in the torus semicircularis (Fig. 1D). The principal, magnocellular and commissural nuclei receive only a sparse DCN projection but the laminar nucleus is densely innervated, particularly its lateral portion. A few fibers cross to the contralateral commissural and principal nuclei of the torus semicircularis. A significant difference between the two species studied is that while in *R. ridibunda* DCN efferent fibers do not reach the tectum, in *X. laevis* intermediate and deep layers are innervated all through the mediolateral tectal extent by collaterals of the toral innervating medial lemniscal fibers. In both species, at more rostral mesencephalic levels (Fig. 1C), the anterodorsal and anteroventral tegmental nuclei including the red nucleus and the nucleus of the fasciculus longitudinalis medialis are innervated by labeled fibers, predominantly contralateral to the DCN injected. At meso-diencephalic transition levels scattered labeled fibers distribute in the pretoral gray. Beyond the midbrain, both dorsal and the ventral thalamic areas are innervated by DCN efferent fibers. The ventral part of the posterior and central dorsal thalamic nuclei are reached by a few thin, varicose fibers whereas the ventromedial thalamic nucleus and the nucleus of the posterior tubercle are far more densely innervated (Fig. 1A,B). No labeling was found more rostrally in the anterior diencephalon or the telencephalon in any of the cases.

Extralemniscal ipsilateral projections ascending all through the rhombencephalon up to the cerebellum were observed. In order to confirm the putative ascending projections of the DCN, HRP, BDA, or RDA injections in both anuran species were placed into the thalamus, torus semicircularis and the cerebellar region. In experiments that affected both the dorsal and the ventral thalamus bilaterally, but mostly contralaterally, irregular large and small round cells with dorsally and ventrolaterally directed processes were observed in the DCN. Their axons could be followed to the contralateral medial lemniscus. When the injection sites were restricted to the torus semicircularis, dorsomedial and lareroventral components of retrogradelly labeled cells were observed in the contralateral DCN. A few ipsilateral cells were also present. The dorsomedial component contains large and small cells with processes directed dorsally, whereas the large bipolar neurons of the lateroventral component posses several processes directed into the dorsal but mainly dorsolateral funiculus. In experiments with applications into the cerebellum, bilaterally but mostly ipsilaterally, retrogradelly labeled cells were seen in the DCN. Their axons seem to course together with the ascending primary afferents from the dorsal roots up to the cerebellum.

As in other vertebrates the dorsal column-medial lemnical projection relays ascendig spinal somatosensory information from the spinal ganglionar cells to different supraspinal targets. Together with the direct spinothalamic projection, recently described (Muñoz et al., 1994), the medial lemniscal innervation of the central and posterior nuclei of the dorsal thalamus represents the anatomical basis of a direct somatosensorial projection, already demonstrated

physiologically (Vesselkin et al., 71). In the ventral thalamus, primaryly the ventromedial nucleus, and the posterior tuberculum are the main diencephalic targets of the medial lemniscal fibers as well as those of the spinal lemniscus (Muñoz et al., 1994). A main component of the anuran medial lemnicus innervates various parts of the torus semicircularis. Our experiments pointed to the lateral aspect of the laminar nucleus as the most densely innervated part of the torus although the principal, magnocellular and in lesser extent the commissural nuclei also receive medial lemniscal innervation. Two different neuronal components in the DCN, dorsomedial and latroventral, were observed to give rise to this projection. The differences in the medial lemniscal innervation of the optic tectum in the species studied agree with previous data in wich DCN cells were descrived after tectal HRP applications in *Xenopus laevis* (Zittlau et al., 1988) but not in *Rana* (Wilczynski & Northcutt, 1977; Hofmann et al., 1990; Masino and Grobstein, 1990). Labeled varicose fibers were observed ipsilaterally at the granular layer of the cerebellum after DCN tracer application. This projection is corroborated by retrograde labeling from cerebellar regions and these results are in line with previous tracing studies both in *Xenopus* (González et al., 1984) and *Rana* (Grover & Grüsser Cornels, 1984).

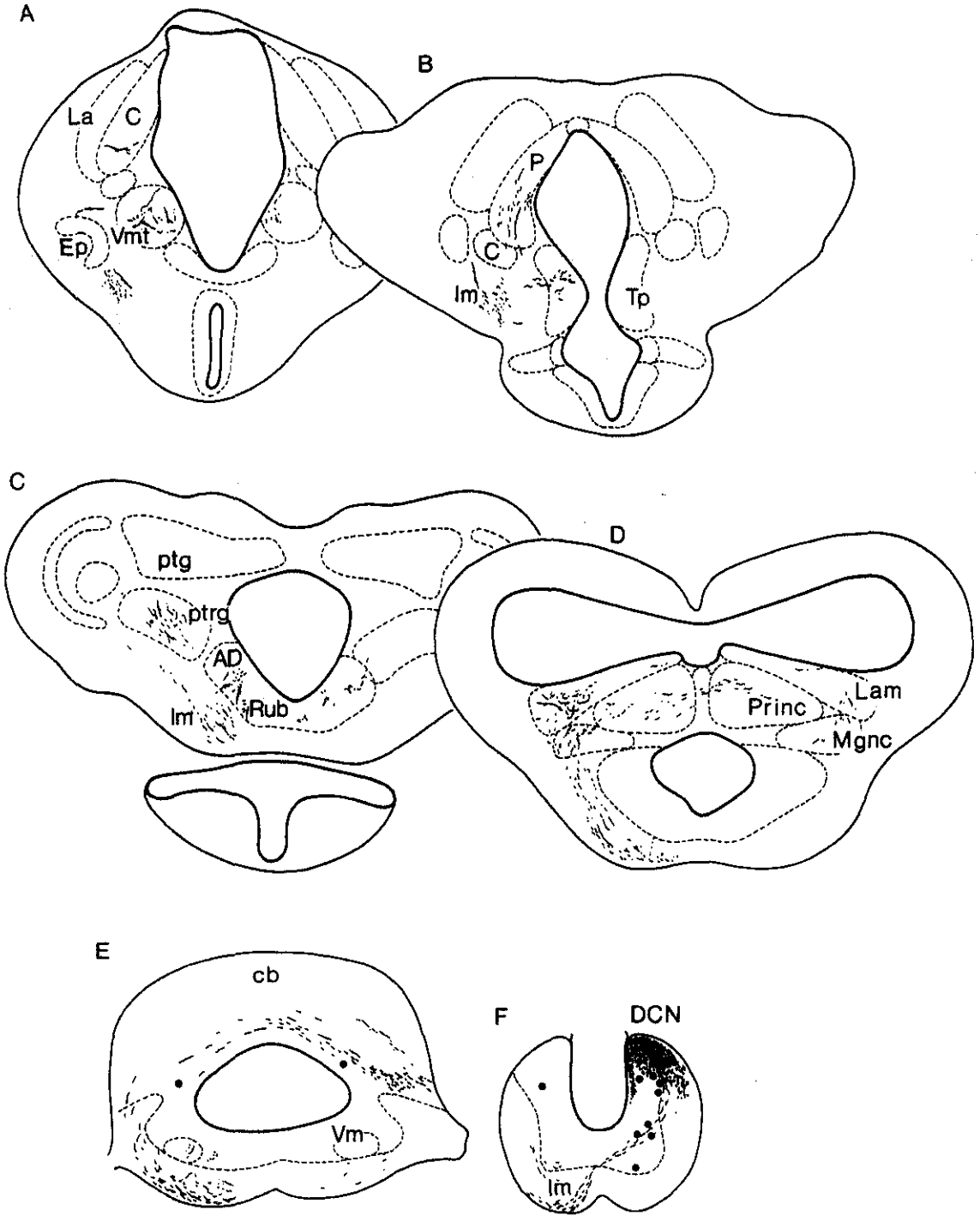

Figure 1: Schematic drawings of transverse sections through the brain of *Rana ridibunda* showing the medial lemniscal targets at diencephalic (A-B), mesencephalic (C-D) and cerebellar (E) levels after a BDA application into the DCN area (F). Abbreviations: AD: anterodorsal tegmental nucleus; C, La, P, Vmt: central, lateral anterior, posterior and ventromedial thalamic nucleus; cb: cerebellum; DCN: dorsal column nucleus; Ep: posterior entopudencular nucleus; Lam, Mgnc, Princ: laminar, magnocellular and principal nucleus of the torus semicircularis; lm: medial lemniscus; prtg: pretoral gray; ptg: pretectal gray; Rub: red nucleus; Tp: nucleus of posterior tubercle; Vm: trigeminal motor nucleus.

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The anuran dorsal column nucleus: Organization, immunohistochemical characterization, and fiber connections in Rana perezi and Xenopus laevis

5.2

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Based on: Journal of Comparative Neurology (1995) 363:1-25

Key words: amphibians, somatosensory system, medial lemniscus, thalamus, torus semicircularis

ABSTRACT

As part of a research programme on the evolution of somatosensory systems in vertebrates, the dorsal column nucleus (DCN) was studied with (immuno)histochemical and tract-tracing techniques in anurans (the large green frog, *Rana perezi*, and the clawed toad, *Xenopus laevis*). The anuran DCN contains some nicotinamide adenine dinucleotide phosphate diaphorase-positive neurons, very little calbindin D-28k, and a distinct parvalbumin-positive cell population. The anuran DCN is innervated by primary and non-primary spinal afferents, by primary afferents from the Vth, VIIth, IXth and Xth cranial nerves, by serotonin-immunoreactive fibers, and by peptidergic fibers. Non-primary DCN afferents from the spinal cord appear to arise throughout the spinal cord, but particularly from the ipsilateral dorsal grey. The present study focused on the efferent connections

of the DCN, more in particular the targets of the medial lemniscus. The medial lemniscus could be traced throughout the brainstem, and into the diencephalon. Along its course, the medial lemniscus gives off collaterals to various parts of the reticular formation, to the octavolateral area, and to the granular layer of the cerebellum. At mesencephalic levels, the medial lemniscus innervates the lateral part of the torus semicircularis as well as various tegmental nuclei. A striking difference between the two species studied is that while in *Rana perezi* medial lemniscal fibers do not reach the tectum mesencephali, in *Xenopus laevis*, intermediate and deep tectal layers are innervated. Beyond the midbrain, both dorsal and ventral thalamic areas are innervated by the medial lemniscus. The present study shows that the anuran 'lemniscal pathway' is basically similar to that of amniotes.

INTRODUCTION

In terrestrial vertebrates, two basic systems of ascending spinal projections are found (see Willis and Coggeshall, 1991): 1) a *primary* afferent ascending spinal projection via the dorsal funiculus to the dorsal column nucleus, giving rise to the medial lemniscal pathway to the thalamus, and 2) a *secondary* afferent projection via the lateral funiculus – i.e., the spinal lemniscus – to the reticular formation, mesencephalon and thalamus.

In anurans, anterograde degeneration studies (e.g., Ebbesson, 1969, 1976; Hayle, 1973a,b) did not demonstrate a spinothalamic tract, and the existence of a dorsal column-medial lemniscal system remained a much debated question until the early 1980's. A recent anterograde tracer study showed a distinct direct spinothalamic projection in anurans (A. Muñoz et al., 1994). The anuran dorsal column nucleus (DCN) is somatotopically arranged in such a fashion that its medial ('gracile') compartment is innervated by dorsal root fibers from lumbar and thoracic segments, whereas those of the cervical enlargement project to the lateral ('cuneate') compartment (Antal et al., 1980; Nikundiwe et al., 1982; Jhaveri and Frank, 1983). In *Xenopus laevis*, also, a non-primary afferent projection to the DCN or postsynaptic dorsal column system was demonstrated (ten Donkelaar and de Boer-van Huizen, 1991). In ranid frogs, Vesselkin and co-workers (Vesselkin et al., 1971; Vesselkin and Kovacevic, 1973), Silvey et al. (1974), as well as Neary and Wilczynski (1977) described a contralateral projection of the DCN (or perisolitary band) to thalamic nuclei. More recent cobalt labeling studies in *Rana esculenta* (Antal et al., 1980; Urbán and Székely, 1982), horseradish peroxidae (HRP) tracing in *Rana perezi* (M. Muñoz et al., 1991) and in *Xenopus laevis* (ten Donkelaar and de Boer-van Huizen, 1991; A. Muñoz et al., 1993) suggest that the amphibian DCN-medial lemniscal system in fact might closely resemble the system found in amniotes. With electrophysiological techniques, Urbán and Székely (1982) showed a rather extensive, contralateral thalamic somatosensory projection following stimulation of either the 2nd dorsal root, the dorsal funiculus or the dorsal column nucleus. The anuran medial lemniscus also innervates the lateral part of the torus semicircularis (Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986).

The present study is part of a research programme on the evolution of somatosensory systems in vertebrates. The development, chemical neuroanatomy and circuitry of somatosensory systems is being studied in amphibians: urodeles as well as anurans. In the present study, the connectivity of the

dorsal column nucleus in two anuran amphibians, the Spanish green frog, *Rana perezi* (formerly *R. ridibunda*) and the clawed toad, *Xenopus laevis* was analyzed, using mainly HRP and biotinylated dextran amine (BDA) tracing techniques. Little is known about the chemical neuroanatomy of the anuran DCN. The mammalian cuneate and gracile nuclei are characterized by the presence of GABAergic (inter)neurons, and are innervated by substance P-positive and many other peptidergic fibers (see Rustioni and Weinberg, 1989, for review). Recent studies (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menétrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994) also showed a certain preferential distribution of calcium-binding proteins like calbindin D-28k and parvalbumin for somatosensory structures including the dorsal column nuclei. Nitric oxide synthase (NOS) possibly marks a population of local circuit neurons within the DCN (Valtschanoff et al., 1993). No such data are available for anurans. Therefore, the existence of different cell populations within the anuran dorsal column nucleus was studied using nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry (NADPH diaphorase being a marker for nitric oxide synthase), calbindin D-28k, parvalbumin, GABA and glycine immunohistochemistry. Additionally, data on the serotonergic and peptidergic innervation of the DCN will be discussed. It will be shown that the anuran dorsal column–medial lemniscus system is basically similar to that of amniotes.

MATERIALS AND TECHNIQUES

The animals (60 adult specimens of *Rana perezi* and 45 young adult *Xenopus laevis*) were obtained from laboratory stock of the Department of Cell Biology, University Complutense of Madrid *(R. perezi)*, and the Department of Animal Physiology, University of Nijmegen *(X. laevis)*. For a cytoarchitectonic analysis of the obex region, Nissl (cresylecht violet)-stained series of both anurans were available, cut either transversally, horizontally or sagittally at a thickness of 20 µm. Adjacent sections were stained with silver proteinate, either according to Bodian's (1936) or according to Klüver and Barrera's (1953) technique. The histochemical, immunohistochemical and tract-tracing techniques used in this study are discussed below. The nomenclature used is based on studies by Opdam et al. (1976) and Nikundiwe and Nieuwenhuys (1983) on the brain stem, and by Neary and Northcutt (1983) on the anuran diencephalon.

NADPH-diaphorase histochemistry

Four adult frogs *(Rana perezi)* were anesthetized in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz), and subsequently perfused transcardially with a 0.9% saline solution followed by a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were taken out and further fixed in the same fixative for six to eight hours at room temperature. They were subsequently immersed in a 30% sucrose phosphate buffer solution at 4¤C, embedded in a 15% gelatin and 30% sucrose solution, and stored for five hours in a 4% formaldehyde solution at room temperature. On a freezing microtome, 30 or 40 µm frontal sections were cut and collected in phosphate buffer. Free-floating sections were incubated in a medium containing 1mM ß-NADPH, 0.8mM nitroblue tetrazolium and 0.06% Triton X-100 in

0.1M phosphate buffer (pH 7.6) at 37¤C for one to two hours. After incubation, the sections were thoroughly rinsed in phosphate buffer, mounted on gelatin-coated glass slides, and, after drying overnight, coverslipped. Selected sections were counterstained with 1% cresyl violet. In two cases, the sections were also processed for tyrosine hydroxylase immunohistochemistry after rinsing.

Immunohistochemical procedures

For the immunohistochemical procedures used, animals were anesthetized with an overdose of MS222, and transcardially perfused with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were removed and postfixed for 4-7 hours in the same fixative, embedded in 15% gelatin with 30% sucrose *(Rana perezi)* or in polyacrylamide *(Xenopus laevis)*. Brains were cut frontally on a freezing microtome or on a vibratome at 40 µm, and the sections were collected in a Tris-saline (TBS) buffer (0.05M, pH 7.6). All antibodies were diluted in 0.1% normal serum of the species in which the secondary antibody was raised, in TBS with 0.1% Triton X-100 (Sigma). The sections were preincubated for 1-2 hours in TBS-containing 3% normal serum and 0.1% Triton X-100 and subsequently incubated in the primary antibody-containing solution for 24-36 hours at 4¤C. Controls for the immunohistochemistry experiments included: 1) staining some selected sections with pre-immune mouse serum (1:1,000 for tyrosine hydroxylase, calbindin D-28k and parvalbumin immunohistochemistry), or with rabbit serum (1:500 for glycine; 1:1,000 for GABA, neuropeptide Y and serotonin; 1:2,000 for calcitonin-gene related peptide, substance P, and Leu-enkephalin immunohistochemistry) instead of the primary antibody (e.g., Fig. 5C), and 2) controls in which primary antibody, secondary antibody or the peroxidase-antiperoxidase complex was omitted. As an additional control for the specificity of the labeling of calcium-binding proteins, some sections were stained using antibodies that had been pre-absorbed in an excess of parvalbumin or calbindin. These procedures revealed a light, diffuse background. No stained cells or fibers were found in any of the cases. The sections were processed according to the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979) in a series of incubations with the following antisera:

1) *tyrosine hydroxylase (TH) immunohistochemistry* (8 cases): a) mouse anti-TH (Incstar), diluted 1:1,000, for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for 3-5 hours, and c) rat peroxidase-antiperoxidase (PAP) complex (Incstar), diluted 1:500, for 2 hours;

2) *calbindin D-28k and parvalbumin immunohistochemistry* (12 cases): a) mouse anti-calbindin D-28k (Sigma) and mouse anti-parvalbumin (Sigma), diluted 1:1,000, for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for 3-5 hours, and c) PAP, diluted 1:500, for 2 hours;

3) *GABA immunohistochemistry* (8 cases) after colchicine (Sigma) injections (3 µl, containing 20 µg/µl) into the fourth ventricle of deeply anesthetized animals, and perfusion after survival times of 5-12 hours, the following procedure was used: a) rabbit anti-GABA (Sigma), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) PAP-rabbit (Dakopatts), diluted 1:600, for 2 hours;

4) *glycine immunohistochemistry* (5 cases): a) rabbit anti-glycine (Chemicon), diluted 1:200-1:500, for 24 hours; b) goat anti-rabbit (Sigma), diluted

1:50-1:200, and c) rabbit PAP-complex (Dakopatts), diluted 1:600, for 2 hours;

5) *calcitonin-gene related peptide (CGRP) immunohistochemistry* (5 cases): a) rabbit anti-CGRP (Amersham), diluted 1:2,000, for 12-24 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours;

6) *substance P (SP) (5 cases) and Leu-enkephalin (L-Enk) immunohistochemistry* (4 cases): a) rabbit anti-SP or rabbit anti-L-Enk (CRB), diluted 1:2,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours;

7) *neuropeptide Y immunohistochemistry* (4 cases): a) rabbit anti-NPY serum (gift from Dr. J.D. Mikkelsen), diluted 1:1,000, for 36 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for 1 hour, and c) rabbit PAP complex (Dakopatts), diluted 1:800, for 1 hour;

8) *serotonin (5-HT) immunohistochemistry* (5 cases): a) rabbit anti 5-HT (gift from Dr. H.W.M. Steinbusch), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours.

In all cases, after rinsing, the sections were incubated with 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in phosphate buffer, for 10-15 minutes. After another rinsing, the sections were mounted on glass slides (mounting medium 0.25% gelatin in Tris buffer), dried overnight, and coverslipped. In most cases, visualization of the immunostaining was improved by processing the sections with nickel-enhanced DAB (0.05% DAB, 0.01% H_2O_2, 0.04% ammonium nickel sulphate in phosphate buffer).

Tract-tracing experiments

In vivo technique. All experiments were carried out under surgical anesthesia with MS222. The following tracers were used as retrograde and anterograde tracers: horseradish peroxidase (HRP, Boehringer), biotinylated dextran amine (BDA, 10kD-Molecular Probes D-1956), and rhodamine dextran amine (RDA, Molecular Probes D-1817). HRP was applied iontophoretically (a 15% HRP solution in phosphate buffer injected for 10 minutes using 5-8 μA positive pulsed current, 7 secs on/7 secs off), or as dry crystals onto a fine tungsten needle, to the DCN (five cases), to the most lateral part of the torus semicircularis (two cases), or to the thalamus (three cases). BDA was recrystallized from distilled water onto fine tungsten needles and applied to the proximal stumps of cut trigeminal nerves (four cases) or third spinal dorsal roots (three cases). Previous experiments (Nikundiwe et al. 1982, *Xenopus laevis*; M. Muñoz et al. 1991, *Rana perezi*) in which HRP was applied to thoracic and lumbar dorsal roots were used for the analysis of DCN projections of the more caudal dorsal roots. Additionally, material in which HRP or BDA was applied to the proximal stumps of the facial, glossopharyngeal, and vagal nerves, could be analyzed. Alternatively, BDA was injected iontophoretically as a 10% solution in phosphate buffer, into the DCN (three cases), cervical dorsal horn (three cases), the cerebellum (three cases), and thalamus (four cases). Survival times varied from 5 to 10 days. Subsequently, the animals were re-anesthetized and perfused through the heart with isotonic saline followed by a fixative containing 4% paraformaldehyde for the BDA experiments, 1.5% paraformaldehyde and 2% glutaraldehyde for the HRP cases. The brain and spinal cord were removed,

postfixed for 2-4 hours, and embedded in gelatin or polyacrylamide. Sections were cut transversally or horizontally at 40 µm on a freezing microtome. Histochemistry for HRP followed the heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product (Adams, 1981). For visualizing BDA, an avidine biotine complex (Vectastain ABC Elite Kit, Vector Laboratories) was used. Some BDA-reacted sections were rinsed and further processed for calbindin D-28K or parvalbumin immunohistochemistry as described above. The black colour of the BDA labeling contrasts with the calbinding protein labeling stained brown by using the diaminobenzidine reaction *without* heavy metal intensification. RDA, recrystallized from distilled water onto sharp tungsten needles, was applied to the thalamus and to the torus semicircularis. After survival times of 2-4 days, animals were re-anesthetized with an overdose of MS222, and perfused with 0.1M phosphate buffer (pH 7.4) followed by a fixative containing 4% paraformaldehyde in phosphate buffer. The brain and (rostral) spinal cord were taken out, embedded in polyacrylamide, left overnight in 15% saccharose in 0.1M phosphate buffer, and cut transversally on a freezing microtome at 40 µm. They were mounted in glycerin-gelatin and viewed with a Zeiss fluorescence microscope with appropriate filter combinations.

In vitro technique. In 10 young adult *Xenopus laevis*, an *in vitro* approach was used, based on Cochran et al. (1987). The animals were deeply anesthetized with a 0.2% solution of MS222 and perfused with iced Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose; pH 7.4). The brains were removed, submerged in the same iced Ringer solution, and cut at middiencephalic or midmesencephalic levels. Applications of 3kD BDA (Molecular Probes, D-7135), recrystallized at the tip of sharp tungsten needles, were made at the ventral thalamus (5 cases) or the torus semicircularis (5 cases). The brains were kept for 5-18 hours at room temperature in continuously oxygenated Ringer solution (pH 7.4) with carbogen, and subsequently processed as described for the *in vivo* BDA experiments.

RESULTS

Delineation and (immuno)histochemical characterization of the anuran dorsal column nucleus

Cytoarchitecture. No distinct dorsal column nucleus (DCN) or nucleus funiculi dorsalis could be distinguished in most cytoarchitectonic studies of the anuran brain stem (e.g., Ariëns Kappers and Hammer, 1918; Zeehandelaar, 1921; Opdam et al., 1976). Therefore, since Woodburne's (1939) Marchi studies, the anuran DCN is defined as the site of termination of dorsal funicular fibers in the caudal brain stem, rather than as a cytoarchitectonic entity. Nevertheless, Nissl-stained sections of the brain stem at obex levels allow the delineation of a DCN (Fig. 1), although labeling of spinal primary afferent projections to the obex level by cobalt staining (Antal et al., 1980), HRP (Nikundiwe et al., 1982) or BDA (see Fig. 6) much more clearly delineates the DCN.

In *Xenopus laevis*, medial ('gracile') and lateral ('cuneate') compartments of the DCN can be distinguished above and lateral to the distinct solitary tract (Nikundiwe et al., 1982; Nikundiwe and Nieuwenhuys, 1983). A dorsal indentation suggests such a subdivision. Medially, the DCN is difficult to

distinguish from the nucleus of the solitary tract, and laterally it is very poorly segregated from the nucleus of the descending tract of the trigeminal nerve. Both compartments of the DCN consist of small (8-10 μm) and medium-sized (15 μm) multipolar elements. The medial, gracile part begins at the level of the second spinal nerve and extends into the brain stem, where it is situated dorsal and dorsolateral to the solitary tract (Fig. 1A,B). The lateral, cuneate part extends further rostrally than the gracile part, and borders on the nucleus of the descending trigeminal tract. In *Rana perezi*, like in *R. esculenta* (see Antal et al., 1980), the segregation of the DCN from the surrounding cell structures such as the nucleus of the descending trigeminal tract and the nucleus of the solitary tract is also rather poor. In *R. perezi*, the cell area dorsal and lateral to the solitary tract only occasionally shows an indentation allowing the distinction of medial and lateral compartments in the DCN (Fig. 1C-F). These gracile and cuneate subdivisions rostrally extend to the level of the glossopharyngeal nucleus where they are slightly more laterally located since in that position the most medial part of the rhombencephalic alar plate is occupied by the caudal pole of the vestibular nuclear complex and related descending vestibular root fibres (Fig. 1C,D). Lateral to the DCN cells, the cells in the dorsolateral position of the alar grey are mingled with afferent fibers of the Vth, VIIth, IXth and Xth cranial nerves forming the descending tract of the trigeminal nerve. These dorsolateral alar grey cells can be regarded as a component of the nucleus of the descending trigeminal tract.

In *Xenopus laevis* and to a lesser extent also in *Rana perezi*, the cell area above and lateral to the solitary tract at the obex level is composed of two DCN compartments, the lateral of which fades into the nucleus of the descending trigeminal tract.

Chemical neuroanatomy

NADPH-diaphorase histochemistry

In the caudal part of the rhombencephalic alar plate in *Rana perezi*, NADPHd-positive neurons were observed in the DCN, in the adjacent descending trigeminal nucleus, and in the nucleus of the solitary tract (Fig. 2A,B). In *Xenopus laevis*, the caudal lateral line nucleus was labeled as well. In the DCN, a cluster of NADPHd-positive neurons was found, the dendrites of which are directed dorsally toward the dorsal funiculus. It should be noted, however, that NADPHd-positive neuron populations are more distinct in the nucleus of the solitary tract, mainly in its medial and ventromedial parts below the solitary tract, and in the nucleus of the descending trigeminal tract (see Fig. 2A,C). By combining NADPHd staining with immunohistochemistry against tyrosine hydroxylase (TH), the NADPHd-positive part of the nucleus of the solitary tract was shown to be intermingled with the catecholaminergic cells (see González and Smeets, 1991) situated ventral to the solitary tract. In the caudal part of the rhombencephalic alar plate of both anuran species studied, NADPHd-positive fibers were found predominantly in two bundles, i.e., the descending trigeminal tract and the solitary tract. In the solitary tract, NADPHd-positive fibers were observed in its most dorsal part. Dorsal to the DCN the rostral continuation of labeled dorsal and dorsolateral (tract of Lissauer) funicular fibers can be observed. Heavy staining within these funiculi is present in the spinal cord.

Immunohistochemical data

The distribution of the calcium-binding proteins calbindin D-28k (Calb) and parvalbumin in the DCN is shown in figures 3 and 4. In *Rana perezi*, in the DCN area two Calb-positive neuron populations were distinguished (Fig. 3A-C): the first group coincides with the nucleus of the solitary tract; the second group is located in the dorsolateral grey, and is composed of round or oval-shaped, small and medium-sized cells with processes directed dorsolaterally into the dorsolateral funiculus. The highest density of this cell population was observed at the obex level and in the rostral spinal cord. This cell population forms part of the nucleus of the descending trigeminal tract, which was verified in experiments in which BDA was applied to the trigeminal nerve. A close relationship was observed between labeled trigeminal afferents and this Calb-positive cell group at the obex level. In young adult *Xenopus laevis*, a similar pattern of Calb-immunoreactivity was observed (Fig. 4A,D,E), although caudal to the obex the number of Calb-positive neurons in the nucleus of the descending trigeminal tract was much higher. In both anuran species, hardly any Calb-positive neurons were found in the DCN. In the dorsal horn of the spinal cord, however, an abundant Calb-positive cell population was observed.

In striking contrast to the lack of Calb-positive neurons in the DCN, in both anuran species a distinct parvalbumin (Parv)-positive DCN cell population was observed, particularly in *Xenopus laevis* (Fig. 4A-C). Apart from the DCN, Parv-positive neurons were observed in the reticular formation, octavolateral area, rostral part of the nucleus of the solitary tract, trigeminal nuclear complex, and dorsal and ventral horn of the spinal cord. In *Rana perezi*, relatively few Parv-positive DCN neurons were observed, spread throughout the nucleus. The dendrites of these rather large cells are mainly directed dorsally or laterally into the adjacent white matter. In some sections more dorsally located and smaller Parv-positive cells were observed as well. In young adult specimens of *X. laevis*, many more Parv-positive neurons were observed in the DCN. Even a clear segregation into a medial and a lateral component could be observed (see Fig. 4A-C). More ventrally located neurons probably form part of the nucleus of the solitary tract. In experiments in which the immunostaining against parvalbumin was combined with a BDA application to the third dorsal root, BDA-labeled endings close to the somata of Parv-positive neurons were observed, suggesting that they receive primary spinal afferents.

The data presented suggest that parvalbumin can be used as a marker for the DCN in anurans, and that calbindin D-28k almost certainly can not. However, since calbindin D-28k is abundantly present in the nucleus of the solitary tract as well as in the nucleus of the descending trigeminal tract, it can help to delineate the lateral and medial borders of the DCN.

In the caudal part of the medulla oblongata, scattered GABAergic neurons were observed in the alar plate. They are more densely grouped in two different locations: one in the nucleus of the solitary tract, the other in the DCN and the adjacent nucleus of the descending trigeminal tract. In the DCN, small, round or oval-shaped, neurons were found (see Fig. 5A,B). Figure 5C shows a control experiment. In the DCN of the anuran species studied no glycine-immunoreactive neurons were observed. In *Rana perezi*, however, some large glycinergic neurons were observed just ventrolateral of the DCN (see Fig. 5A,D). These bipolar neurons are oriented

dorsoventrally with processes directed into dorsal and ventral directions.

Tract-tracing experiments

In order to characterize the afferent and efferent connections of the DCN in *Rana perezi* and *Xenopus laevis*, horseradish peroxidase (HRP) histochemistry and biotinylated dextran amine (BDA) immunodetection were used. Additionally, the fluorescent tracer rhodamine amine (RDA) was used as a retrograde tracer. Even though the iontophoretic injections of the tracers were rather small, particularly the BDA injections, it was nearly impossible to restrict the application site to the DCN. Medially located injections often partially involved the nucleus of the solitary tract, whereas more lateral injections included part of the nucleus of the descending trigeminal tract. It is therefore not possible to fully discriminate the connectivity of the DCN using only this type of tracer application. Subsequent retrograde tracing experiments (HRP, BDA and RDA) were used to confirm the projections from the DCN, whereas anterograde tracing experiments were accomplished to corroborate and describe the terminal fields of the efferent connections of the DCN. Since the results in both species are largely comparable, in the following section the general pattern of the connections of the anuran DCN will be described. Only when differences are found between the species, this will be mentioned separately.

Afferent connections

The afferent projections to the DCN were studied by application of tracers to the cervical, thoracic or lumbar spinal dorsal roots, and to the roots of the trigeminal, facial, glossopharyngeal and vagal nerves. Additionally, after injections of tracers into the DCN, the cells of origin of non-primary afferent projections could be studied. Immunohistochemical data on afferent connections of the DCN will be discussed.

Ascending primary afferents. In all experiments of tracer application to a dorsal root, the labeled afferent fibers bifurcate into ascending and descending tracts upon entering the spinal cord. Two components are present, i.e., a medially located bundle of thick fibers that enters the dorsal funiculus, and a more laterally situated group of fibers within the dorsal portion of the dorsolateral funiculus. Both fiber systems project to widespread spinal and supraspinal regions. In the obex region the fibers in the dorsal funiculus are somatotopically organized. Fibers originating at lumbar segments occupy positions medial to those of thoracic origin. The most laterally located fibers in the dorsal funiculus enter at cervical levels. In the experiment shown in figure 6, BDA was applied to the proximal end of the cut third dorsal root. In such experiments the lateral compartment of the DCN could be clearly delineated (see Fig. 6A,D). Ascending collaterals from the large medial component of the cervical dorsal roots ascend in the lateral part of the dorsal funiculus. Those of Lissauer's tract reach the obex level via the dorsolateral funiculus. Lissauer's tract could be traced as far rostrally as the rostral pole of the nucleus of the solitary tract. By comparing the labeling pattern obtained after BDA application of the proximal end of the cut trigeminal nerve (Fig. 6A,B) to the pattern after labeling the third dorsal root, it seems likely that the sites of termination of cervical dorsal root fibers and trigeminal afferents are largely segregated. At DCN levels, however, thin trigeminal fibers not only innervate the nucleus of the descending trigeminal

tract, but also the DCN as delineated by dorsal root projections.

These ascending spinal projections clearly outline the DCN. In *Xenopus laevis*, the terminal fields marking the DCN were found from the level of the second spinal nerve up to the entrance of the vagal nerve. In *Rana perezi*, a similar rostral extent was observed but its caudal end coincides with the end of the hypoglossal nucleus. The terminal fields in this area largely resemble the organization of the fibers in the dorsal funiculus. Thus, medially situated axons from lumbar and thoracic dorsal root ganglion cells terminate on medial cells in the DCN, while laterally located fibers arising from cervical dorsal root ganglion cells end on more lateral cells, with a certain degree of overlap in the projection. This gracile- and cuneate-like organization of the DCN is similar in both anuran species.

Immunohistochemical studies showed that substance P- and CGRP-immunoreactive fibers are present in the tract of Lissauer. Some of these peptidergic fibers leave this tract and innervate the lateral part of the DCN (Fig. 7A). In addition to this peptidergic primary afferent projection to the DCN, the DCN is innervated by Leu-enkephalin, neuropeptide Y (NPY) and serotonin-immunoreactive fibers. Leu-enkephalin immunoreactive terminal structures were found in the dorsal grey at obex levels and in a thin rim around the solitary tract. NPY-immunoreactive fibers enter the DCN from the dorsal aspect of the lateral funiculus and form varicose fibers and terminal boutons. Serotonin-immunoreactive fibers rather strongly innervate the DCN and adjacent structures such as the nucleus of the solitary tract and the nucleus of the descending trigeminal tract (Fig. 7B).

Afferents from the Vth, VIIth, IXth and Xth cranial nerves. The application of anterogradely transported tracers to the proximal stumps of severed trigeminal, facial, glossopharyngeal and vagal nerve roots resulted in the labeling of their primary afferent projections. Apart from projections to their specific targets, all these nerves share a component of afferent fibers which, on entering the medulla, turn caudally in the lateral aspect of the rhombencephalon and continue into the spinal cord. These fibers course in the descending trigeminal tract and they constitute thin varicose fibers at DCN levels, which project not only to the laterally located nucleus of the descending trigeminal tract or to the commissural nucleus of the solitary tract but also to the DCN itself (Fig. 6B,C).

Non-primary DCN afferents. Following HRP or BDA injections into the DCN (see Figs. 8, 9), various non-primary DCN afferents were demonstrated. Non-primary ascending spinal projections arise throughout the spinal cord. Most of these cells were found ipsilaterally in the dorsal grey (Fig. 7C), but also some contralateral cells, as well as some cells in the intermediate and ventral grey were found (Fig. 7D). It should be noted that medially located injections resulted in the labeling of a higher population of lumbar and thoracic spinal cells, whereas more laterally placed injections labeled mainly cervical spinal cells. Other afferent projections to the DCN arise from the brain stem. After injections of retrograde tracers into the DCN, labeled cells were found, ipsilaterally, in the nucleus of the solitary tract, and, bilaterally, in the nucleus cerebelli. Bilaterally labeled cells were found in the reticular formation at levels between the VIIth and IXth motor nuclei, and in the ventral nucleus of the VIIIth nerve. A small population of labeled cells was present in the

raphe zone at levels between the entrance of the VIIIth and the IXth nerve roots. This projection from the raphe nucleus, presumably serotonergic, is in line with the rather strong serotonin-immunoreactive innervation of the DCN (see Fig. 7B).

Efferent projections: ascending

Anterograde tracing experiments

1) *The medial lemniscus.* In a first set of experiments, unilateral applications of the tracers HRP or BDA were made dorsally into the obex region (Figs. 8, 9). These applications clearly involved the DCN and the anterogradely labeled fiber projections were easily observed. In all cases, a distinct contralateral ascending system from the DCN was present, i.e., the medial lemniscus. Its axons could be traced from the injection site ventrally and medially, decussating to the contralateral side beneath the central canal (Figs. 8L, 9M), then turning rostrally into the ventral tegmentum. As the medial lemniscus ascends in the rhombencephalon, it smoothly swings to more dorsolateral positions. All through the medulla, the medial lemniscus gives off thin varicose fibers to various parts of the rhombencephalic reticular formation (Figs. 8J,K, 9I-L). A few smooth fibers run dorsally into the octavolateral area, and some enter the granular layer of the cerebellum (Figs. 8I, 9I). Rostrally, fine varicose fibers are observed ventrolateral to the isthmic nucleus. At caudal mesencephalic levels, the fibers turn dorsally along the lateral aspect of the midbrain and most of them bend medially, where they terminate in the torus semicircularis (Figs. 8F,G, 9F,G, 10C,D). The principal, magnocellular, and commissural nuclei receive only a sparse DCN projection, but the laminar nucleus is densely innervated, mainly in its lateral portion. A few fibers pass to the contralateral commissural and principal nuclei of the torus semicircularis. In *Rana perezi* medial lemniscal fibers do not reach the mesencephalic tectum, while in *Xenopus laevis* the intermediate and deep tectal layers are innervated. These rather thick medial lemniscal fibers innervating the tectum mesencephali often give off thin collaterals that terminate in the laminar nucleus of the torus semicircularis (Figs. 9E-G, 10C). In both species, at more rostral mesencephalic levels, the anterodorsal and anteroventral tegmental nuclei as well as the red nucleus and the interstitial nucleus of the fasciculus longitudinalis medialis are innervated by medial lemniscal fibers (Figs. 8E,F, 9E,F).

At rostral mesencephalic levels, scattered labeled fibers distribute to the pretoral grey, and, in *Xenopus laevis*, also to the pretectal grey (Figs. 8E, 9E). Beyond the midbrain, both the dorsal and ventral thalamic areas are innervated by medial lemniscal fibers (Figs. 8A-D, 9A-D). A few thin, varicose fibers innervate the ventral parts of the posterior and central dorsal thalamic nuclei, whereas the ventromedial thalamic nucleus and the posterior tubercle are far more densely innervated. The fibers reaching the ventromedial nucleus pass through the dorsal and ventral parts of the ventrolateral thalamic nucleus and varicosities are also found among its cells. Apart from a few fibers reaching the anterior nucleus of the dorsal thalamus (in two cases in *Rana perezi*), no labeling was found more rostrally in the anterior diencephalon or in the telencephalon in any of the cases.

2) *Extralemniscal ascending projections.* Apart from the medial lemniscus, the DCN gives rise to a distinct ipsilateral ascending projection (Figs. 8I-K, 9I-L). Due to their proximity, the ascending primary afferent spinal fibers bypassing the injection site were

most likely to be involved. Such fibers are known to project to the octavolateral area and the cerebellum (see Antal et al., 1980; Nikundiwe et al., 1982). Additionally, adjacent cell groups such as the nucleus of the descending trigeminal nucleus and the nucleus of the solitary tract might have incorporated the tracer from the injection sites. Therefore, ipsilateral projections from the DCN are difficult to demonstrate in anterograde experiments.

Retrograde tracing experiments

In order to verify whether these ascending projections really arise in the DCN, in both anuran species injections of HRP, BDA or RDA were placed into the thalamus, torus semicircularis and cerebellar region.

1) *Thalamic applications.* In this group of experiments, a retrograde tracer was applied to the thalamus in such a way that both the dorsal and ventral thalamus were implicated. Retrogradely labeled cells in the region of the DCN formed a mixed population of irregular, large cells, and round, small cells (Fig. 11A,D). Although the majority of the cells were located contralaterally, a minor component of ipsilateral cells was also present. The dendrites of these cells are long and directed both dorsally and ventrolaterally, reaching the dorsal and the dorsolateral funiculi, respectively. Their axons were followed into the contralateral medial lemniscus. In addition, a few cells were labeled bilaterally in the dorsolateral descending trigeminal tract. In *in vitro*-BDA experiments in young adult *Xenopus laevis* a similar pattern of labeling was observed (Fig. 12A,C).

2) *Toral applications.* When the injection sites were limited to the torus semicircularis, neurons were retrogradely labeled within the DCN. They were particularly found on the contralateral side, although an ipsilateral component was present as well. Two distinct cell groups were observed in *Rana perezi* (Fig. 11B,E). The first one is made up of large cells with a minor component of small cells located in the dorsalmost grey. They possess several processes extending into the dorsal fiber layer. Their axons course ventromedially, cross the midline and form part of the medial lemniscus. The second group of labeled cells is located in the lateral marginal zone of the dorsal grey from the level of the obex to the second spinal segment. These are large bipolar and irregular cells with long processes directed mainly to the dorsal part of the lateral funiculus and into the dorsal funiculus while their axons do participate in the medial lemniscus. In addition, retrogradely labeled cells were always found in the ipsilateral descending nucleus of the trigeminal nerve following toral injections. After similar toral injections in *Xenopus laevis*, retrogradely labeled cells in the DCN form a band positioned from dorsomedial to ventrolateral above the solitary tract. The most dorsally located cells possess dendrites extending into the dorsal funiculus, whereas more ventrolateral cells have dendrites reaching the dorsal aspect of the lateral funiculus. Some neurons were observed with dendrites reaching both the dorsal and dorsolateral funiculi (Fig. 12D). In *in vitro* experiments, BDA was applied to the torus semicircularis of *Xenopus laevis*. The pattern of labeling in the DCN is shown in Fig. 12B,D,E.

3) *Cerebellar applications.* In experiments with tracer applications into the lateral portion of the cerebellar plate, the underlying cerebellar nucleus and the adjacent grey were mostly implicated as well. At the obex region, these applications resulted in the

labeling of three cell groups. Most labeled cells were found at the ventromedial margin of the caudal extent of the fasciculus solitarius, on both sides of the medulla, probably due to the uptake of the tracer by fibers projecting from the nucleus of the solitary tract to the nucleus visceralis secundarius (parabrachial region). In the nucleus of the descending trigeminal tract labeled cells were found as well, mainly ipsilateral to the application site. The third group of retrogradely labeled cells was found, bilaterally, in the DCN (Fig. 11C,F). These cells were mainly found ipsilaterally. Their axons seem to run together with the ascending primary afferent fibers from the spinal dorsal roots.

Efferent projections: descending

In experiments with BDA or HRP applications into the DCN region anterogradely labeled axons could be followed from the injection site caudalwards into the spinal cord. These fibers course via the ipsilateral dorsal funiculus, and form fine arborizations of thin varicose fibers terminating among the cells in the dorsal horn throughout the cord, but particularly at cervical levels. A sparse bilateral innervation of the intermediate and ventral zones was also observed. These fibers could, however, represent fibers by-passing the injection site. Therefore, spinal injections with retrograde tracers were studied. A small population of cells, scattered in the area of the ipsilateral DCN, was always labeled after injection of the various spinal segments (Fig. 10G).

DISCUSSION

In the present study the organization, immunohistochemical characterization, and more particularly, the fiber connections of the anuran DCN were investigated. Although it is obvious that the anuran DCN remains a rather ill-defined area in the caudal part of the rhombencephalic alar plate, and no selective markers for the DCN other than its labeling by primary afferents from the spinal cord are available, NADPH-diaphorase staining and immunohistochemical staining of calcium-binding proteins and various neurotransmitters certainly help in delineating and characterizing the DCN. Since no clear cytoarchitectonic separation of the DCN into a medial, 'gracile' nucleus and a lateral, 'cuneate' nucleus is obvious, the term 'dorsal column nucleus' is preferred.

The NADPH-diaphorase (NADPHd) histochemical technique, known to stain specific neurons (Thomas and Pearse, 1964), can selectively stain particular populations of neurons in a Golgi-like manner (Scherer-Singler et al., 1983). Throughout the brain NADPHd and nitric oxide synthase (NOS) localizations are identical (Bredt and Snyder, 1992). Therefore, NADPHd can be used as a marker for NOS. Nitric oxide probably plays a major role as a neuronal messenger (Bredt and Snyder, 1992; Meller and Gebhart, 1993; Schuman and Madison, 1994). The presence of NADPHd-positive cells and fibers in the mammalian spinal cord (Valtschanoff et al., 1992) suggests that nitric oxide may be involved in spinal sensory processing. In the rat DCN, Valtschanoff et al. (1993) found that most NOS-positive neurons are also immunoreactive for GABA, but not for the excitatory transmitters glutamate and aspartate. Moreover, since NOS-positive neurons could not be labeled retrogradely from the thalamus or spinal cord, they are probably local circuit neurons (Valtschanoff et al., 1993). In the anuran species studied, NADPHd-positive neurons were found in the DCN, but

especially in the adjacent nucleus of the solitary tract and the descending nucleus of the trigeminal nerve in keeping with data in mammals (e.g., Leight et al., 1990; Vincent and Kimura, 1992; Dohrn et al., 1994; Takemura et al., 1994). Since no double labeling studies for GABA or excitatory transmitters were carried out, it remains to be analyzed whether these NADPHd-positive neurons are local circuit neurons or give rise to efferent projections such as the medial lemniscus.

Calcium-binding proteins such as calbindin D-28k, calretinin, and parvalbumin are found in certain subpopulations of neurons in the central and peripheral nervous system (Baimbridge et al., 1982; Garcia-Segura et al., 1984; Braun, 1990; Celio, 1990; Ren and Ruda, 1994). They even label entire pathways, sometimes whole functional systems (Celio, 1990; Andressen et al., 1993). In mammals, calcium-binding proteins like calbindin and parvalbumin show a preferential distribution in somatosensory structures, including the DCN (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menetrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994). Parvalbumin (Parv) appears to be abundant in the pathway for epicritic sensibility, i.e., the dorsal column-medial lemniscal system, calbindin D-28k (Calb) occurs in the whole taste pathway of rats (Celio, 1990). In rats, Calb-positive neurons are found in certain laminae of the dorsal horn (see Antal et al., 1990; Ren and Ruda, 1994) including the cells of origin of ascending spinal projections (Menétrey et al., 1992b), in the sensory trigeminal nuclei as well as in the gracile and cuneate nuclei (Celio, 1990). In rats, Menétrey et al. (1992a) showed that Calb-positive neurons form a major part of the solitary and trigeminal projection systems. In the trigeminal system of monkeys, both proteins are differentially expressed in the ascending trigeminothalamic projections to the ventral posteromedial (VPM) nucleus (Rausell and Jones, 1991a,b). Antisera to parvalbumin and calbindin mark VPM rods and matrix which receive principal and spinal trigeminal input, respectively. A similar segregation has been demonstrated for the ascending somatosensory projections from the spinal cord (Rausell et al., 1992): A non-nociceptive Parv-positive dorsal column-medial lemniscal projection terminates in cytochrome oxidase (CO)-rich domains of the ventral posterolateral thalamic nucleus (VPL) where Parv-positive neurons are found. Nociceptive Calb-positive spinothalamic fibers terminate in CO-poor domains of the VPL where Calb-positive cells are present (Rausell et al., 1992).

Against this background, the presence of calbindin D-28k and parvalbumin in the anuran DCN was studied. It appeared that in the alar plate of the caudal rhombencephalon, Calb-positive neurons were found particularly in the nucleus of the solitary tract and in the descending nucleus of the trigeminal nerve continuing into the dorsal horn of the spinal cord. In both anuran species studied, hardly any Calb-positive neurons were found in the DCN itself. This pattern of distribution of Calb-positive neurons suggests that in anuran amphibians, as in mammals, Calb could be restricted to the nociceptive part of the somatosensory system including neurons in the dorsal horn of the spinal cord, and the descending nucleus of the trigeminal nerve. In striking contrast, in both anuran species a distinct parvalbumin (Parv)-positive DCN population was observed, particularly in *Xenopus laevis*. Parv-positive neurons were found throughout the DCN, their dendrites were mainly directed dorsally or laterally into the adjacent dorsal funiculus. Immunostaining of parvalbumin can therefore be used

to delineate the anuran DCN. It should be noted, however, that the pattern of distribution of calcium-binding proteins in the rat DCN is quite different. Maslany et al. (1992) found both Calb- and Parv-positive neurons in the cuneate and gracile nuclei, although Parv-positive DCN cells were more numerous. The distribution of Parv-cells appeared to be similar to the known distribution of thalamic projection neurons.

In mammals, the presence of small GABAergic interneurons within the DCN has been extensively described (for reviews see Mugnaini and Oertel, 1985; Rustioni and Weinberg, 1989). Also glycinergic inhibitory effects within the DCN were observed. Does the anuran DCN contain GABAergic interneurons? In the present study small, round or oval-shaped GABA-immunoreactive neurons were observed. The pattern of labeling in other parts of the brain stem is comparable to that described by Franzoni and Morino (1989, *Rana esculenta*), who, unfortunately, did not include the most caudal part of the brain stem in their analysis. Double labeling studies, i.e. combinations with tract-tracing or NADPH-diaphorase, are needed to demonstrate whether these GABA-immunoreactive neurons in the anuran DCN are actually interneurons. In this respect, it should be noted that Pritz and Stritzel (1989a) suggested that the reptilian *(Caiman crocodilus)* DCN lacks glutamic acid decarboxylase (GAD)-immunoreactive neurons, indicating that the reptilian DCN – like the dorsal thalamus (see Pritz and Stritzel, 1988) – lacks local circuit neurons. A few glycinergic neurons were found at the ventrolateral border of the DCN. In the lamprey, such glycinergic neurons are known to inhibit reticulospinal neurons (Dubuc et al., 1993a,b). In mammals, glycinergic cells of different sizes have been observed in the gracile and cuneate nuclei (Porucho et al., 1992).

Even though cytoarchitectonic studies do not clearly define the anuran DCN, this nucleus is characterized by its somatotopic organization of primary afferent projections from the spinal cord (Antal et al., 1980, *Rana esculenta*; Nikundiwe et al., 1982, *Xenopus laevis*). Data in *Rana perezi* (M. Muñoz et al., 1991) indicate a similar pattern of arrangement, whereby primary afferents from lumbar and thoracic dorsal root ganglia innervate the medial, 'gracile' compartment of the DCN, while those from cervical ganglia innervate its lateral, 'cuneate' compartment as well as the spinal or descending trigeminal nucleus. The dorsal funicular projection continues rostrally to innervate the vestibular nuclear complex and, rather abundantly, the granular layer of the cerebellum (Antal et al., 1980; Székely et al., 1980). Fibers terminating in the vestibular nuclei and in the cerebellum arise from limb-innervating spinal ganglia (Antal et al., 1980; González et al., 1984). The non-primary spinal afferents or postsynaptic dorsal column system (PDCS) also appears to be arranged somatotopically. The presence of such a PDCS has now been demonstrated throughout terrestrial vertebrates (e.g., Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni and Kaufman, 1977; Bennett et al., 1984; Giesler et al., 1984; Funke, 1988; ten Donkelaar and de Boer-van Huizen, 1991; Pritz and Stritzel, 1994). In mammals, the cells of origin of these non-primary afferent projections to the DCN, or postsynaptic dorsal column neurons, have been shown to transmit nociceptive information (Uddenberg, 1968; Angaut-Petit, 1975b; Bennett et al., 1984; Kamogawa and Bennett, 1986), at least in cats.

The lateral part of the anuran DCN is innervated by fibers from the descending tract of the trigeminal nerve, arising from the descending part of the trigeminal, facial, glossopharyngeal and vagal nerves (Fig. 12; see also Rubinson and Friedman, 1977; Matesz and Székely, 1978; Fuller, 1979; Lowe and Russell, 1982; Altman and Dawes, 1983; Stuesse et al., 1984; Oka et al., 1987; González et al., 1993; M. Muñoz et al., 1994). In contrast, lateral line nerve projections, present in permanently aquatic species such as *Xenopus laevis*, strictly avoid the DCN (see Lowe and Russell, 1982; Altman and Dawes, 1983; Fritzsch et al., 1984; Will et al., 1985a).

The most lateral part of the anuran DCN is also innervated by substance P- and CGRP-immunoreactive fibers passing via the tract of Lissauer (see also Rosenthal and Cruce, 1985; Adli et al., 1988; Petkó and Sánta, 1992). In addition to this peptidergic primary afferent projection to the DCN, the anuran DCN is innervated by Leu-enkephalin, neuropeptide Y and serotonin-immunoreactive fibers in line with data by Ueda et al. (1984), Merchenthaler et al. (1989), and Lázár et al. (1990). This serotonergic and peptidergic innervation of the DCN is in line with immunohistochemical data in mammals (e.g., Steinbusch, 1981; Westman et al., 1984; Halliday et al., 1988; Ibuki et al., 1989; Tamatani et al., 1989; Conti et al., 1990; Fabri and Conti, 1990; Blomqvist and Broman, 1993). Since after tracer applications to the DCN retrogradely labeled neurons were observed in the (serotonergic – see Ueda et al., 1984) raphe nucleus, it seems likely that this nucleus is the source of the serotonergic innervation of the DCN. In rats, Willcockson et al. (1987) observed serotonergic terminals in apposition to neurons of the DCN that project to the thalamus, whereas in cats and monkeys, Blomqvist and Broman (1993) observed serotonergic input to DCN neurons projecting to various brainstem areas including pretectum, superior colliculus and pontine nuclei, related to motor processing.

Descending control of the DCN, so prominent in mammals (see Willis and Coggeshall, 1991 for review), seems to be rather restricted in anurans. After injections of tracers into the DCN, labeled cells were found bilaterally in the cerebellar nucleus, in the ventral nucleus of the VIIIth nerve and in the reticular formation at levels between the VIIth and IXth motor nuclei including the inferior raphe nucleus. In mammals, the transmission of sensory information through the dorsal column-medical lemniscus pathway is controlled by pathways from the cerebral cortex (e.g., Kuypers, 1958; Kuypers and Tuerck, 1964), the red nucleus (Edwards, 1972; Weinberg and Rustioni, 1989), vestibular nuclei (Weinberg and Rustioni, 1989), the cerebellum (Sotgiu and Cesa-Bianchi, 1972), and the reticular formation (Willcockson et al., 1987; Weinberg and Rustioni, 1989). Therefore, with the possible exception of the red nucleus, a comparable brain stem 'control' of the DCN is found in anurans.

A major part of the present study focused on the efferent connections of the DCN, more in particular on the targets of the medial lemniscus. The existence of a dorsal column-medial lemniscal system in amphibians remained a much debated question until the early 1980's. Subsequently, Vesselkin and co-workers (Vesselkin et al., 1971; Vesselkin and Kova´cevi´c, 1973), Silvey et al. (1974) as well as Neary and Wilczynski (1977), described a contralateral projection of the DCN or 'perisolitary band' (Neary and Wilczynski, 1977) to thalamic nuclei. With electrophysiological techniques, Urbán and Székely

(1982) noted slow negative potentials from the posterocentral nucleus of the thalamus in response to stimulation of the 2nd dorsal root, the dorsal column and the dorsal column nucleus. In the present study the course and site of termination of the medial lemniscus was shown by anterograde tracing (Figs. 8, 9), and its cells of origin by retrograde labeling of the DCN from its main targets, i.e., the ventral thalamus, the lateral part of the torus semicircularis and the cerebellar cortex (Figs. 11, 12). The use of a new and powerful anterograde tracer like BDA made it possible to identify even fine terminal fields and the scattered fibers in the thalamus. The data obtained are summarized in Fig. 13. Since it was hardly possible to restrict tracer applications to the DCN, in such injections the adjacent nucleus of the solitary tract and the descending nucleus of the trigeminal nerve as well as fibers of passage (e.g., spinal primary afferents to the cerebellum) might be involved. By retrograde labeling the origin of the medial lemniscal projections was verified. It should be emphasized that the ventrolateral part of the DCN found to project to the thalamus and, more in particular, to the torus, extends caudally as far as the second spinal segment. The dendrites of these cells are mainly directed to the dorsolateral funiculus, whereas their axons join the contralateral medial lemniscus. A certain similarity to the mammalian lateral cervical nucleus, known to receive somatosensory information via the spinocervical tract and projecting contralaterally via the medial lemniscus (see Willis and Coggeshall, 1991), seems likely.

The medial lemniscus could be traced throughout the brain stem and into the diencephalon. Along its course, the medial lemniscus gives off collaterals to various parts of the reticular formation, to the octavolateral area, and to the granular layer of the cerebellum. At mesencephalic levels, the medial lemniscus primarily innervates the lateral part of the torus semicircularis (also noted by Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986; Neary, 1988), and the anterodorsal and anteroventral tegmental nuclei as well as the red nucleus and the interstitial nucleus of the fasciculus longitudinalis medialis. While in *Rana perezi* medial lemniscal fibers do not reach the tectum mesencephali, in *Xenopus laevis* intermediate and deep tectal layers are innervated in agreement with retrograde tracer data (Wilczynski and Northcutt, 1977; Zittlau et al., 1988; Hofmann et al., 1990; Masino and Grobstein, 1990). Beyond the midbrain, both dorsal and ventral thalamic areas are innervated by the medial lemniscus. The ventral parts of the posterior and central nuclei of the dorsal thalamus are reached by a few thin, varicose, fibers, but the ventromedial thalamic nucleus and the nucleus of the posterior tubercle are far more densely innervated. In two cases in *R. perezi*, a few fibers also reached the anterior nucleus of the dorsal thalamus. No projections beyond the diencephalon were observed. Extralemniscal projections were found to the ipsilateral cerebellar cortex, confirming retrograde tracer data (González et al., 1984), and bilaterally to the spinal cord. The ipsilateral spinal projection from the DCN was previously observed in *Xenopus laevis* (ten Donkelaar et al., 1981).

Hence, the present study not only further substantiated the presence of a rather well-developed dorsal column-medial lemniscus system in anurans, but also showed that its mesencephalic and diencephalic targets are much more extensive and diverse than suggested in previous studies (Vesselkin et al., 1971; Silvey et al., 1974; Neary and Wilczynski, 1977; Comer and Grobstein, 1981;

Wilczynski, 1981; Forehand and Farel, 1982; Urbán and Székely, 1982; Neary, 1988). The anuran 'lemniscal pathway' appears to be basically similar to that of amniotes (reptiles: Ebbesson, 1978; Siemen and Künzle, 1994a; birds: Wild, 1989; mammals: e.g., Hazlett et al., 1972; Hand and van Winkle, 1977; Feldman and Kruger, 1980; Berkley et al., 1986; see also Willis and Coggeshall, 1991 for a summary of mammalian studies), although in mammals the widespread thalamic projections should be particularly emphasized. In the red-eared turtle, *Pseudemys scripta elegans*, Siemen and Künzle (1994a,b) noted a direct ascending projection from the most medial part of the DCN area, by-passing the thalamus, to the basal part of the telencephalon.

At first sight, the mesencephalic target of the anuran medial lemniscus seems to be quite different from the amniote mesencephalic target. For the opossum, RoBards et al. (1976) introduced the term 'intercollicular terminal zone' for the common target of projections from the dorsal column nuclei, spinal cord and sensorimotor cortex in the central midbrain. In reptiles, Ebbesson (1967, 1969) introduced the term 'intercollicular nucleus' for the mesencephalic target of ascending spinal projections. In this nucleus, a projection from the dorsal column nucleus terminates as well (Ebbesson, 1978; see also Belekhova et al., 1985; Pritz and Stritzel, 1989b). It seems likely that this intercollicular zone, nucleus or 'midbrain somatosensory area' (Pritz and Stritzel, 1989b), characterized by at least an input from the spinal cord and DCN, is a major integrative center of the somatosensory system. Pritz and Stritzel (1990) showed that the medialis complex in the dorsal thalamus is the thalamic target of the midbrain somatosensory intercollicular area. In anurans, the main midbrain target of the medial lemniscus is formed by the lateral part of the torus semicircularis (Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986; Neary, 1988; the present study). The anuran torus semicircularis is a major integrating center for a number of sensory and non-sensory afferents in addition to its auditory input, and may well serve a role similar to the one the tectum mesencephali serves for the visual system (Wilczynski and Capranica, 1984). It includes a laminar nucleus, a principal nucleus, a magnocellular nucleus, and two smaller nuclei (Potter, 1965), each of which receives a particular set of afferents (e.g., Wilczynski, 1988; Feng and Lin, 1991). Physiological studies (Comer and Grobstein, 1981) in *Rana pipiens* suggest a certain overlap of tactile and auditory information: the very dorsolateral torus is almost exclusively concerned with tactile information; auditory activity is most often found to be localized in central parts of the torus, but in between the two, multimodal (tactile and auditory) activity is found. Toral afferents also arrive from the vestibular (Wilczynski, 1981), and, in *Xenopus laevis*, from the lateral line system (Will et al., 1985b; Lowe, 1986; Zittlau et al., 1986). The laminar toral nucleus not only receives DCN efferents but also spinal (Ebbesson, 1976; A. Muñoz et al., in preparation) and trigeminal afferents (Comer and Grobstein, 1981; M. Muñoz et al., 1994), and so – at least partly – represents a midbrain somatosensory area. The multimodal laminar nucleus as well as the mainly auditory magnocellular nucleus extensively innervate the central and posterior nuclei (Frontera's nucleus posterocentralis; see Frontera, 1952) of the dorsal thalamus, whereas the ascending projections of the principal nucleus are restricted to the caudal part of the posterior thalamic nucleus (Hall and Feng, 1987; Feng and Lin, 1991). The laminar nucleus also innervates the ventromedial thalamic nucleus (Feng

and Lin, 1991; A. Muñoz and ten Donkelaar, unpublished observations), i.e., the main diencephalic target of the medial lemniscus (present study) as well as of the spinothalamic tract (A. Muñoz et al., 1994). The central thalamic nucleus extensively projects to the ipsilateral striatum (Vesselkin et al., 1980; Wilczynski and Northcutt, 1983a; Neary, 1988). Hence, this DCN – torus – central thalamic nucleus – striatal pathway is one way by which somatosensory information may reach the telencephalon.

Although the anuran dorsal column-medial lemniscus system is basically similar to that of amniotes, large differences are found with regard to the telencephalic targets of this pathway. Therefore, a few remarks on the telencephalic structures receiving somatosensory information in anurans seem appropriate. Physiological studies revealed somatosensory activity within the medial pallium (e.g., Supin and Gusel'nikov, 1964; Karamian et al, 1966; Northcutt, 1970; Vesselkin and Kova´cevi´c 1973), possibly relayed in the dorsal thalamus. Since the anterior thalamic nucleus is the only thalamic nucleus innervating the medial pallium (Scalia and Colman, 1975; Vesselkin and Ermakova, 1978; Kicliter, 1979; Neary, 1984; Northcutt and Ronan, 1992), somatosensory information to this pronounced telencephalic structure, also known as the archipallium (Ariëns Kappers et al., 1936; Clairambault and Derer, 1968) or the primordium hippocampi (Herrick, 1910; Hoffman, 1963), must relay in the anterior nucleus. However, since spinal afferents to the anterior thalamic nucleus – either via the spinothalamic tract (A. Muñoz et al., 1994) or via the dorsal column-medial lemniscal pathway (Neary and Wilczynski, 1977; present study) – are rather limited, alternative routes must be available, possibly via the posterior thalamic nucleus known to project to the anterior thalamic nucleus (Neary and Wilczynski, 1979; see also Neary, 1990; Northcutt and Ronan, 1992). It should also be noted that the dendrites of cells in the anterior thalamic nucleus to penetrate the central nucleus (Neary, 1990). Therefore, somatosensory information could reach the medial pallium via multisynaptic routes.

Another telencephalic structure in which somatosensory activity was recorded is the striatum (see Vesselkin et al., 1971; Vesselkin and Kova´cevi´c 1973). The anuran striatum receives a major thalamotelencephalic input from nuclei relaying sensory information from the midbrain roof, from the torus semicircularis, and from ventral diencephalic structures, receiving spinal and DCN-medial lemniscal afferents (Scalia and Colman, 1975; Vesselkin et al., 1980; Wilczynski and Northcutt, 1983a; Lázár and Kozicz, 1990). The striatum in amphibians appears to be able to influence the midbrain roof via the pretectum and various midbrain and isthmal nuclei (Wilczynski and Northcutt, 1983b). The anuran striatum plays a crucial role in processing sensory information as well as in coordinating all telencephalic output to lower brain stem motor centers. Both visual (Gruberg and Ambros, 1974) and auditory (Mudry and Capranica, 1980) activity was recorded from the striatum. Further, electrical stimulation of the sciatic nerve evoked potentials in the striatum (Vesselkin et al., 1971; Vesselkin and Kova´cevi´c, 1973). The sensory input to the striatum is relayed in the anterior division of the lateral thalamic nucleus (visual information: Lázár, 1969; Scalia, 1976), in the central thalamic nucleus (auditory and also somatosensory information: Hall and Feng, 1987; Feng and Lin, 1991; present study), and in the ventromedial thalamic nucleus (somatosensory information: Neary and Wilczynski,

1977; present study). In *Rana perezi*, tracer applications to the striatum showed a direct striatal projection arising from cells in the lateral aspect of the ventromedial thalamic nucleus (A. Muñoz, unpublished observations) in line with observations by Vesselkin et al. (1980) as well as by Lázár and Kozicz (1990). Vesselkin et al. (1980) also noted a direct striatal projection from the torus semicircularis, whereas Lázár and Kozicz (1990) found a few faintly labeled small cells in the nucleus of the posterior tubercle projecting to the lateral wall of the telencephalon including the striatum (see also Wilczynski and Northcutt, 1983a). Somatosensory information to the striatum may thus be relayed via the dorsal thalamus (the central thalamic nucleus), the ventral thalamus (the ventromedial thalamic nucleus), and via the nucleus of the posterior tubercle, a separate diencephalic region (see Neary and Northcutt, 1983). Which thalamic nuclei really relay somatosensory information to the striatum (and medial pallium) is now being studied in a series of double-labeling experiments.

ACKNOWLEDGMENTS

This research was supported by an ENP short-term fellowship of the European Science Foundation to A. Muñoz, a NATO Collaborative Research Grant (930542) to H.J. ten Donkelaar, and by a grant from the Spanish Government (DGICYT PB 93-0083) to A. González. The authors thank Mrs. Roelie de Boer-van Huizen and Mrs. Ine Bergervoet-Vernooy for excellent technical assistance, Mrs. Marlu Ackermans for making the final versions of the drawings, and Ms. Inge Eijkhout for secretarial assistance. The valuable comments of Dr. William L.R. Cruce and an anonymous reviewer of the manuscript are gratefully acknowledged.

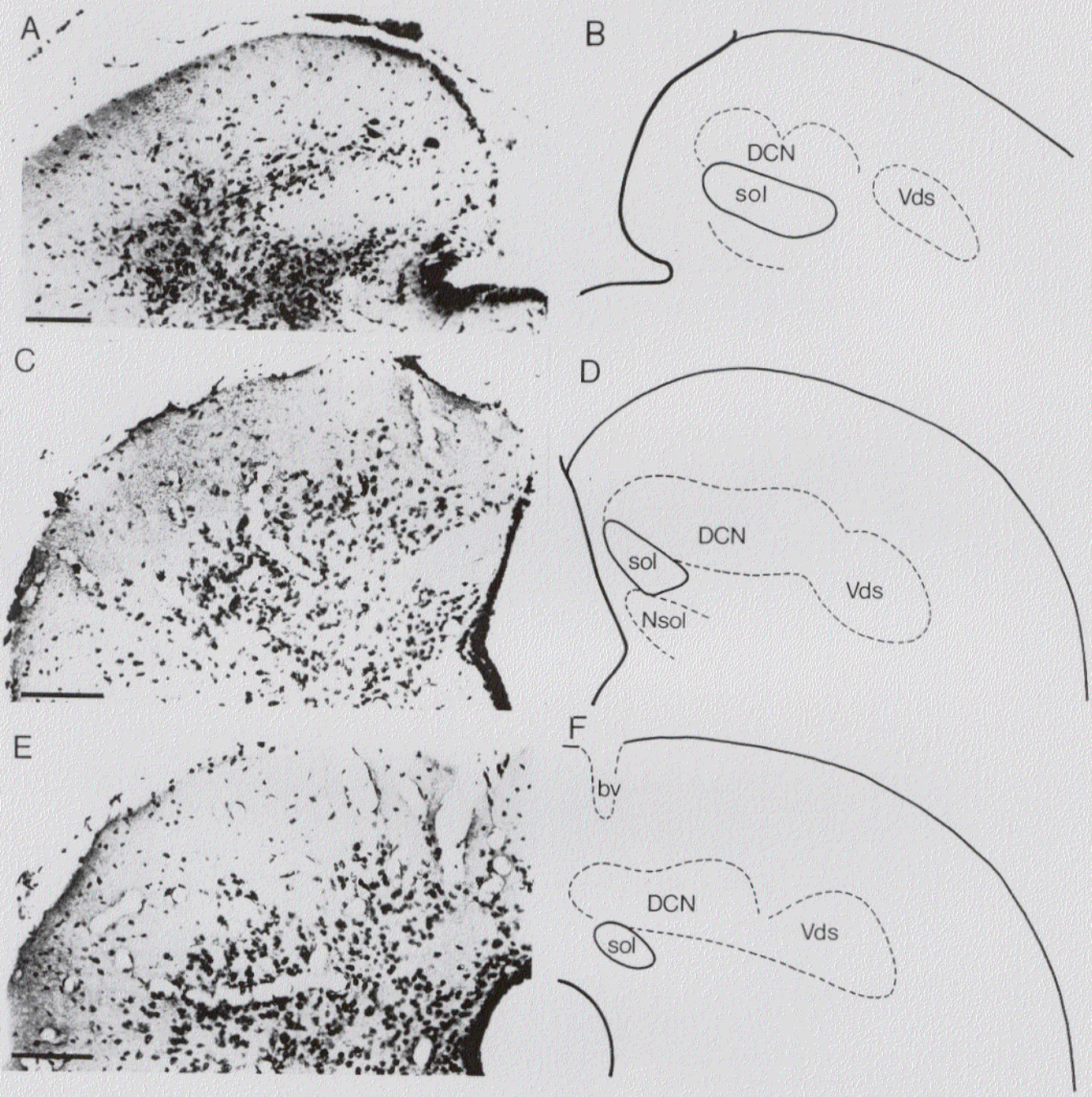

Fig. 1: Photomicrographs of Nissl-stained transverse sections and schematic drawings of the caudal part of the alar plate in *Xenopus laevis* (A, B) and in *Rana perezi* (C, F). Scale bars indicate 100 µm.

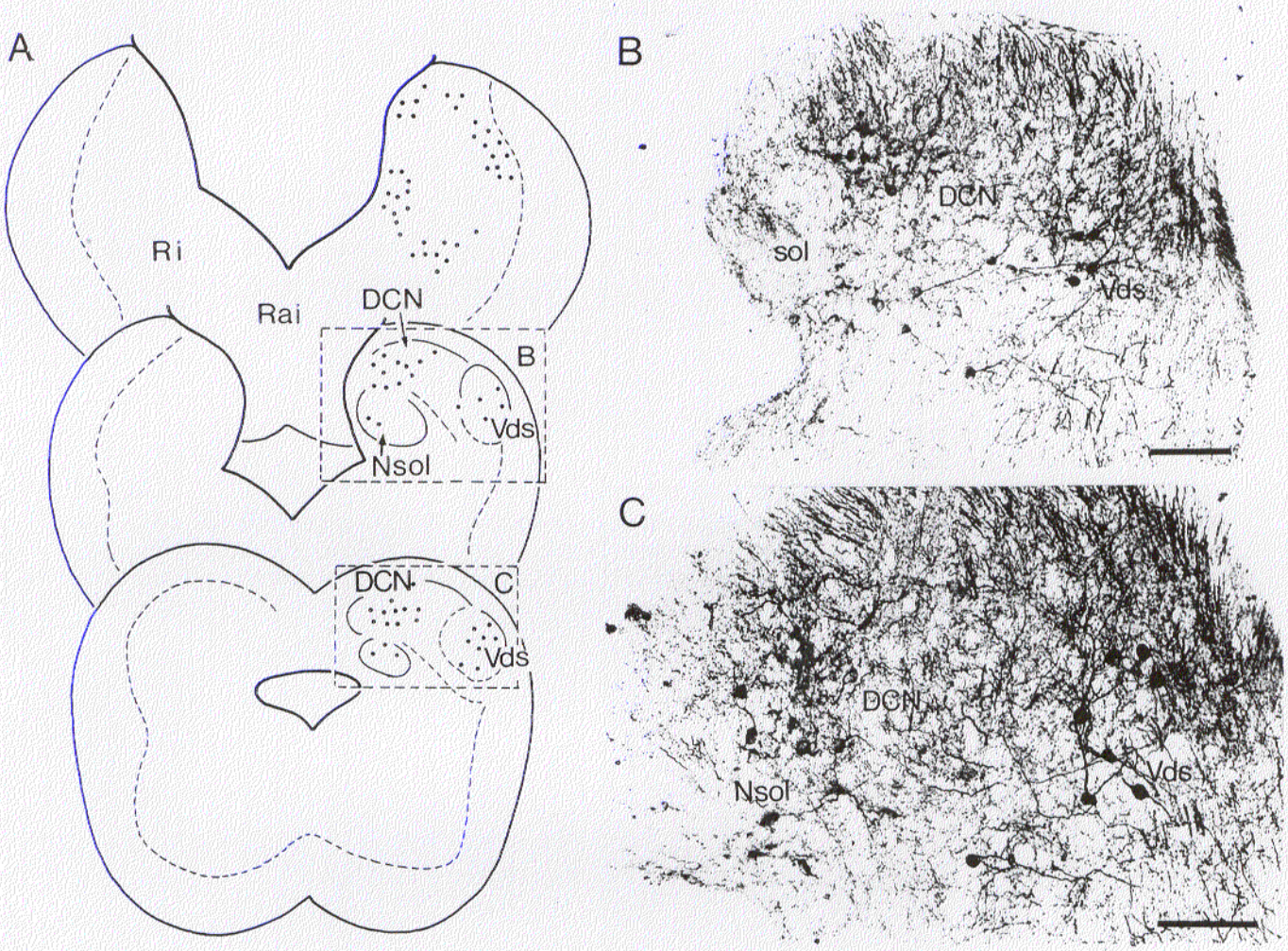

Fig. 2: A, The distribution of NADPH diaphorase-positive cells in the caudal part of the rhombencephalic alar plate of *Rana perezi*. B, C, Photomicrographs illustrating the medial (nucleus of the solitary tract), lateral (trigeminal) and dorsomedial (DCN) components. Scale bars indicate 100 µm.

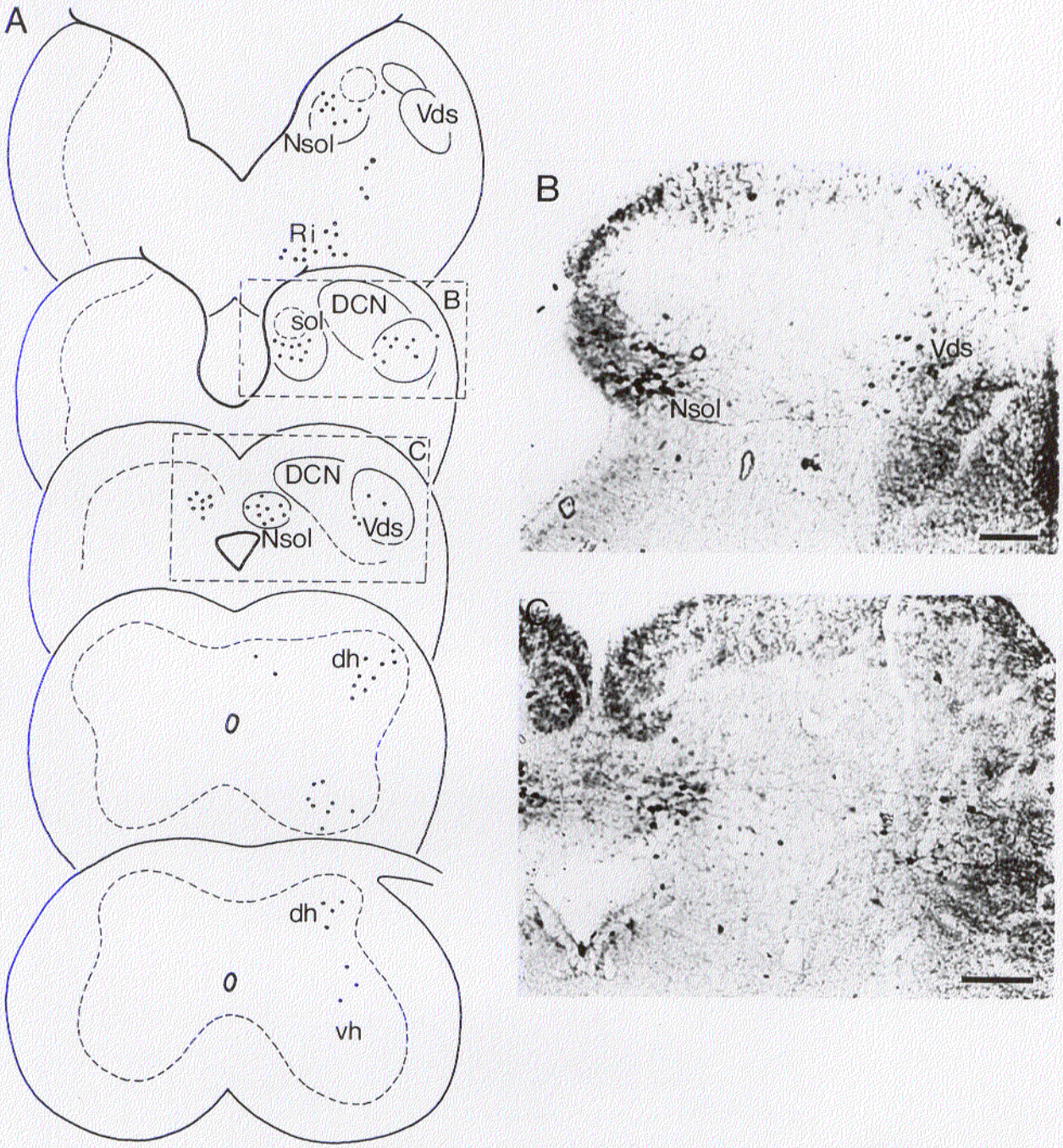

Fig. 3: A, B, C, The distribution of calbindin D-28k-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Rana perezi*. Scale bars indicate 100 µm.

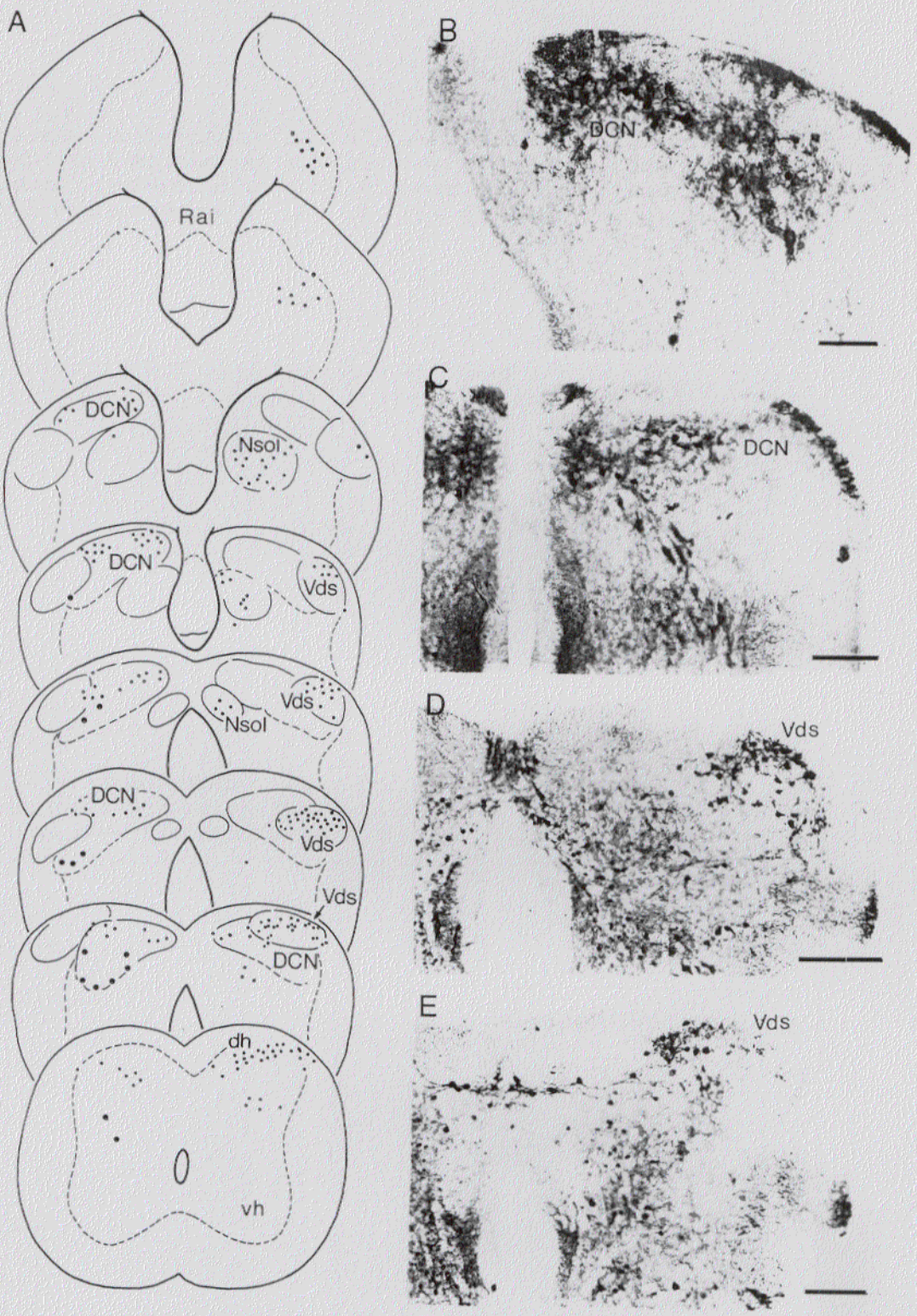

Fig. 4: A, The distribution of calcium-binding proteins in the caudal part of the rhombencephalon and most rostral part of the spinal cord of *Xenopus laevis:* on the left the distribution of parvalbumin-immunoreactive neurons, on the right the distribution of calbindin D-28k immunoreactive neurons. B-E, Photomicrographs of examples of parvalbumin (B, C) and calbindin (D, E) labeling. Scale bars indicate 100 µm.

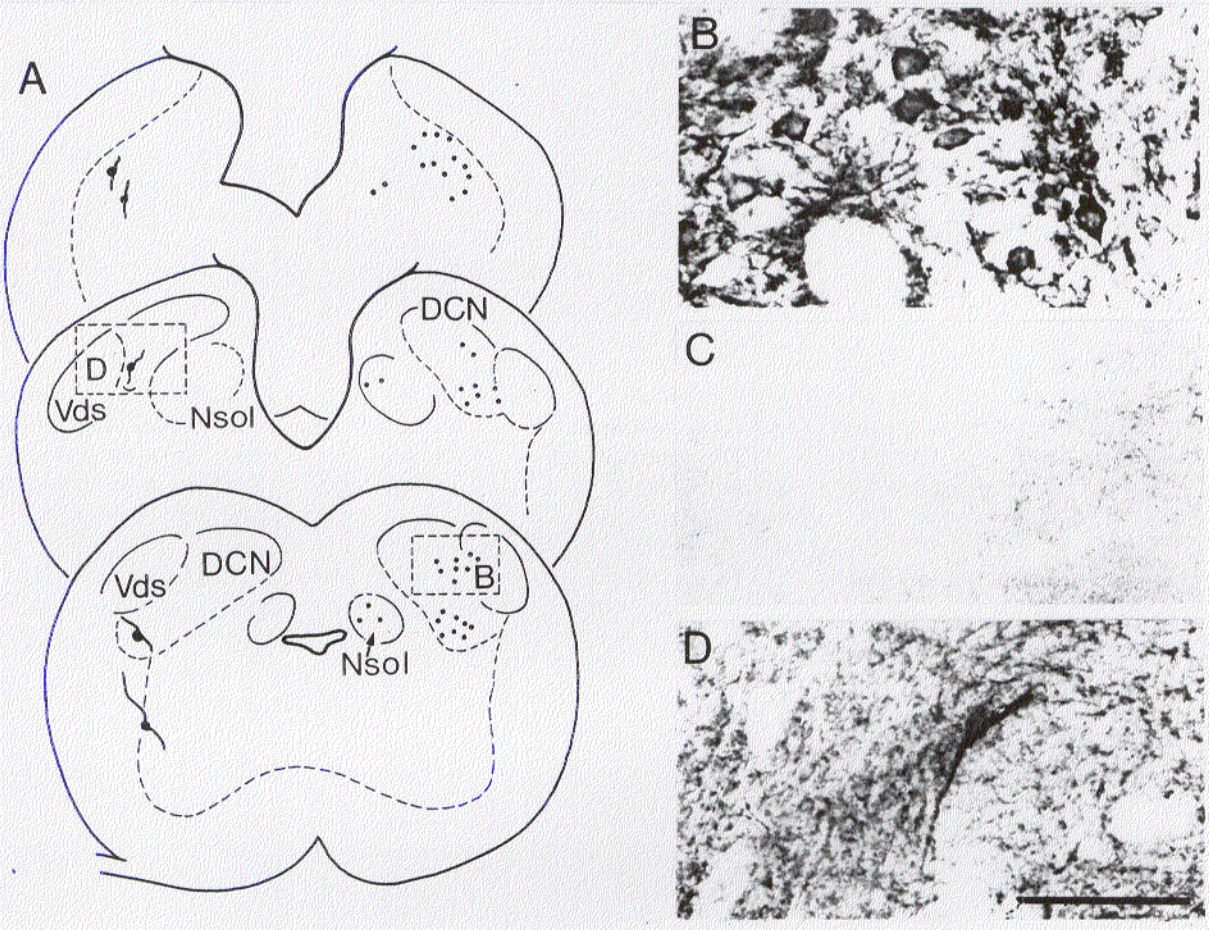

Fig. 5: A, The distribution of GABA-immunoreactive (on the right) and glycine-immunoreactive (on the left) neurons. In B, some GABA-immunoreactive neurons are shown, in C, the DCN area in a control experiment in which sections were stained with a solution of preimmune rabbit serum (1:1,000) instead of the rabbit anti-GABA antiserum, in D a glycinergic neuron. Scale bar for the photomicrographs indicates 100 µm.

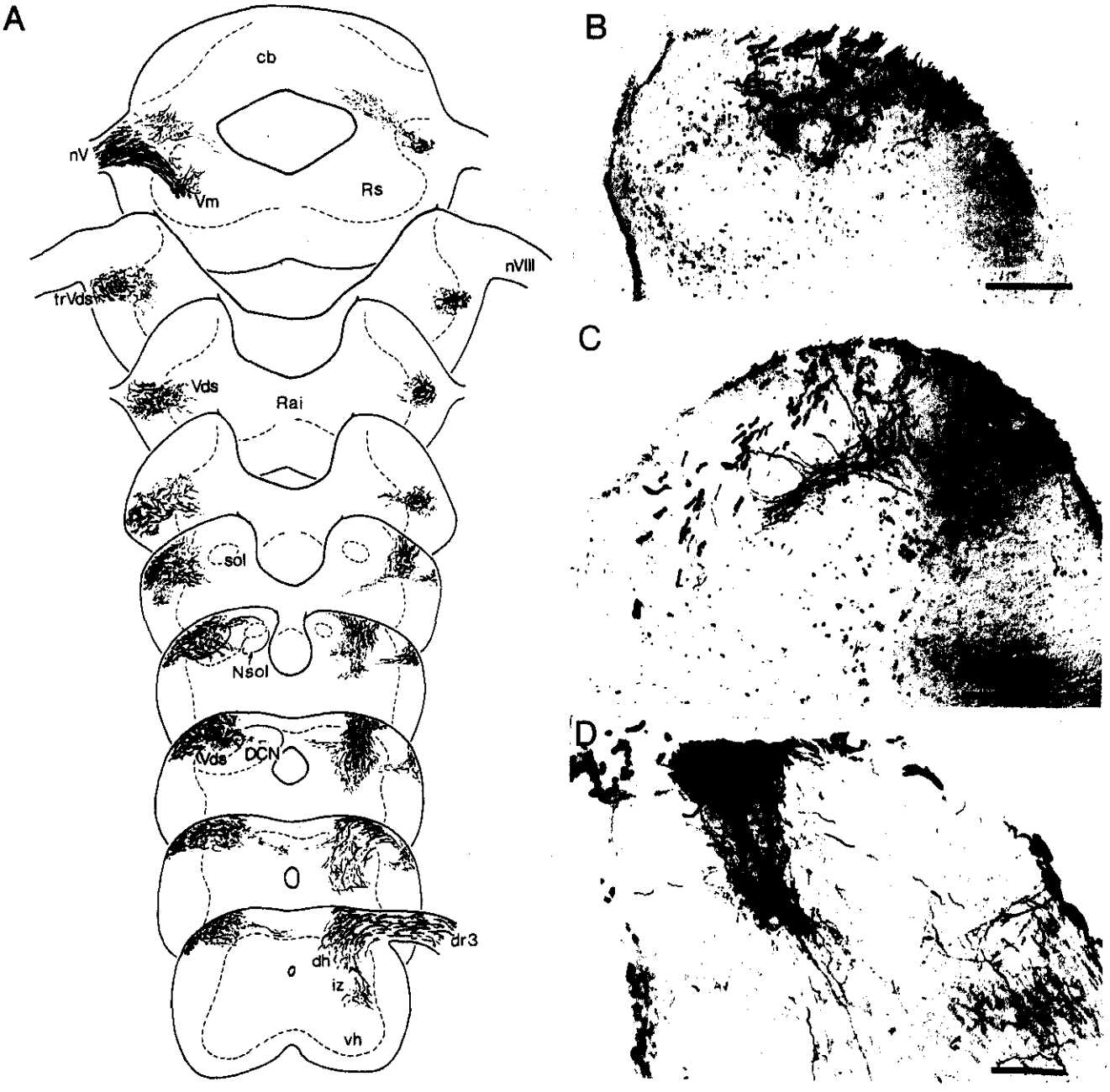

Fig. 6: A, Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Rana perezi* showing the distribution of biotinylated dextran amine (BDA)-labeled trigeminal (left side) and third dorsal root (right side) afferent fibers. B, C, Photomicrographs showing ipsilateral trigeminal (B) and glossopharyngeal (C) afferents to the DCN of *R. perezi*. D, Photomicrograph showing the termination pattern of BDA labeled brachial afferents of the third dorsal root at the rostral DCN of *R. perezi*. Scale bars indicate 100 μm.

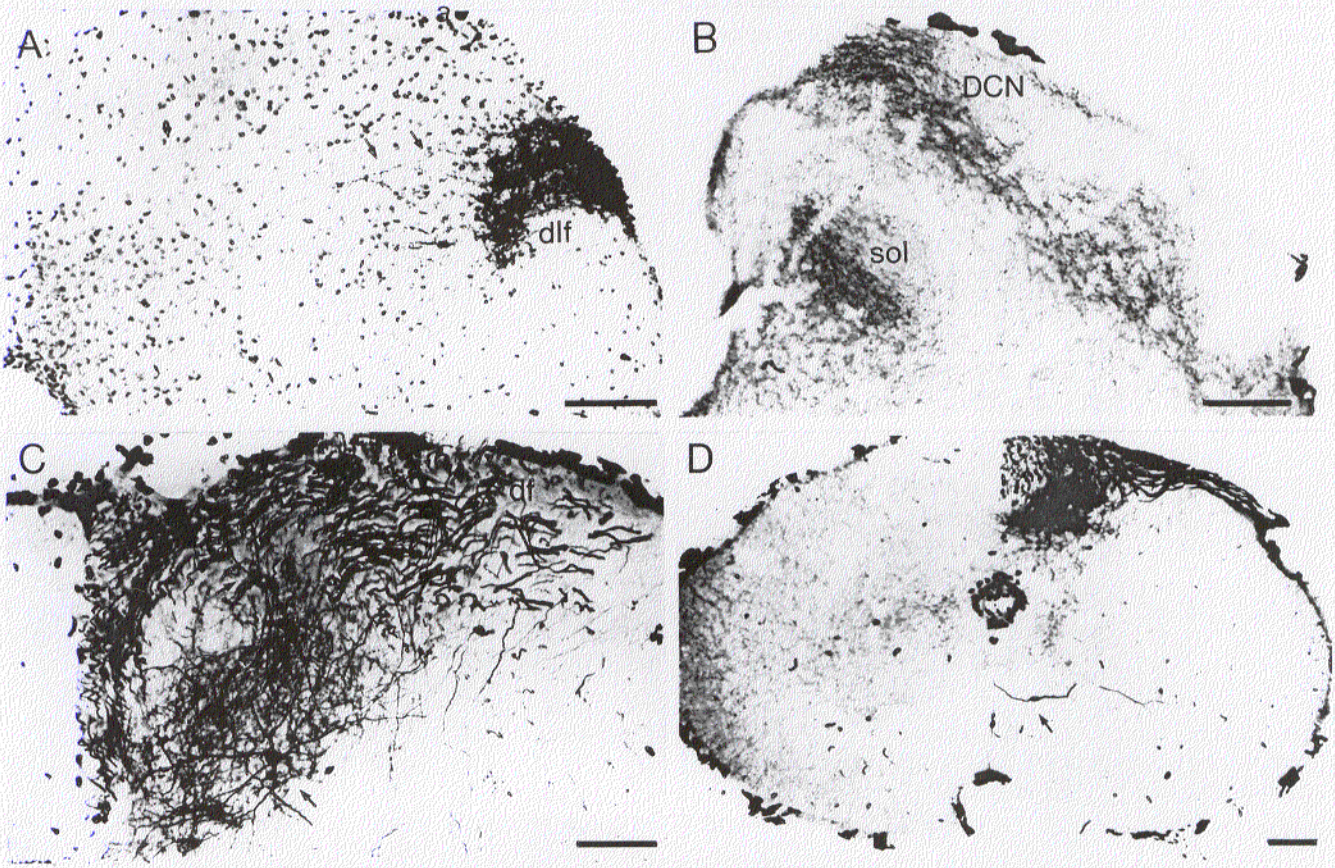

Fig. 7: A, B, Photomicrographs showing the substance P- (A, arrows) and serotonin-immunoreactive (B) innervation of the DCN in *Xenopus laevis* and *Rana perezi*, respectively. C, D, Photomicrographs showing (arrows) neurons of the postsynaptic dorsal column system in the cervical dorsal horn (C), and in the thoracic ventral horn (D) of *R. perezi*. Scale bars indicate 100 µm.

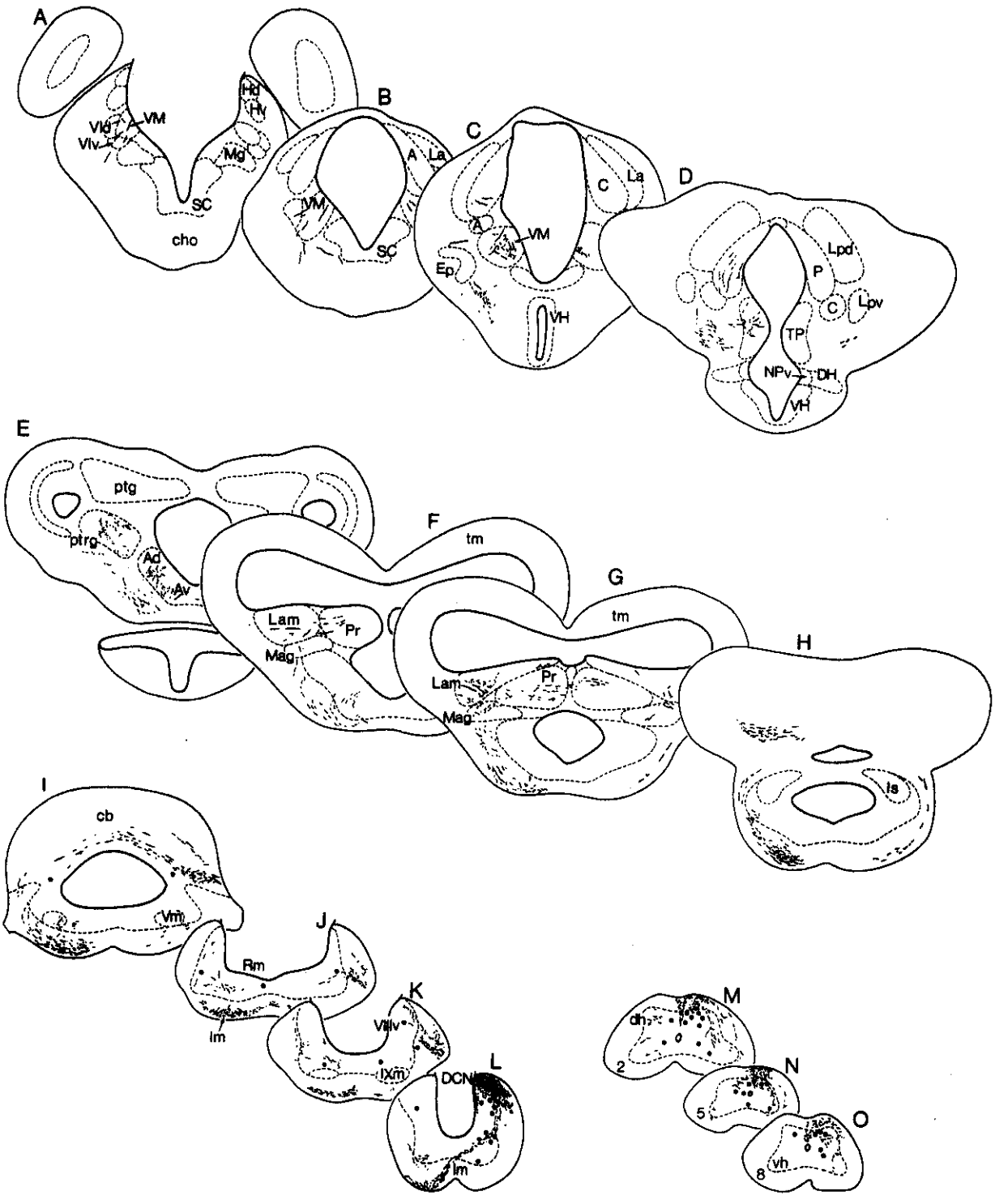

Fig. 8: Labeling observed after a BDA injection into the DCN of *Rana perezi* (for injection site see also Fig. 10A). In a series of transverse sections through the diencephalon (A-D), brain stem (E-L) and spinal cord (M-O) the pattern of anterogradely labeled fibers and retrogradely labeled cells (black dots) is shown.

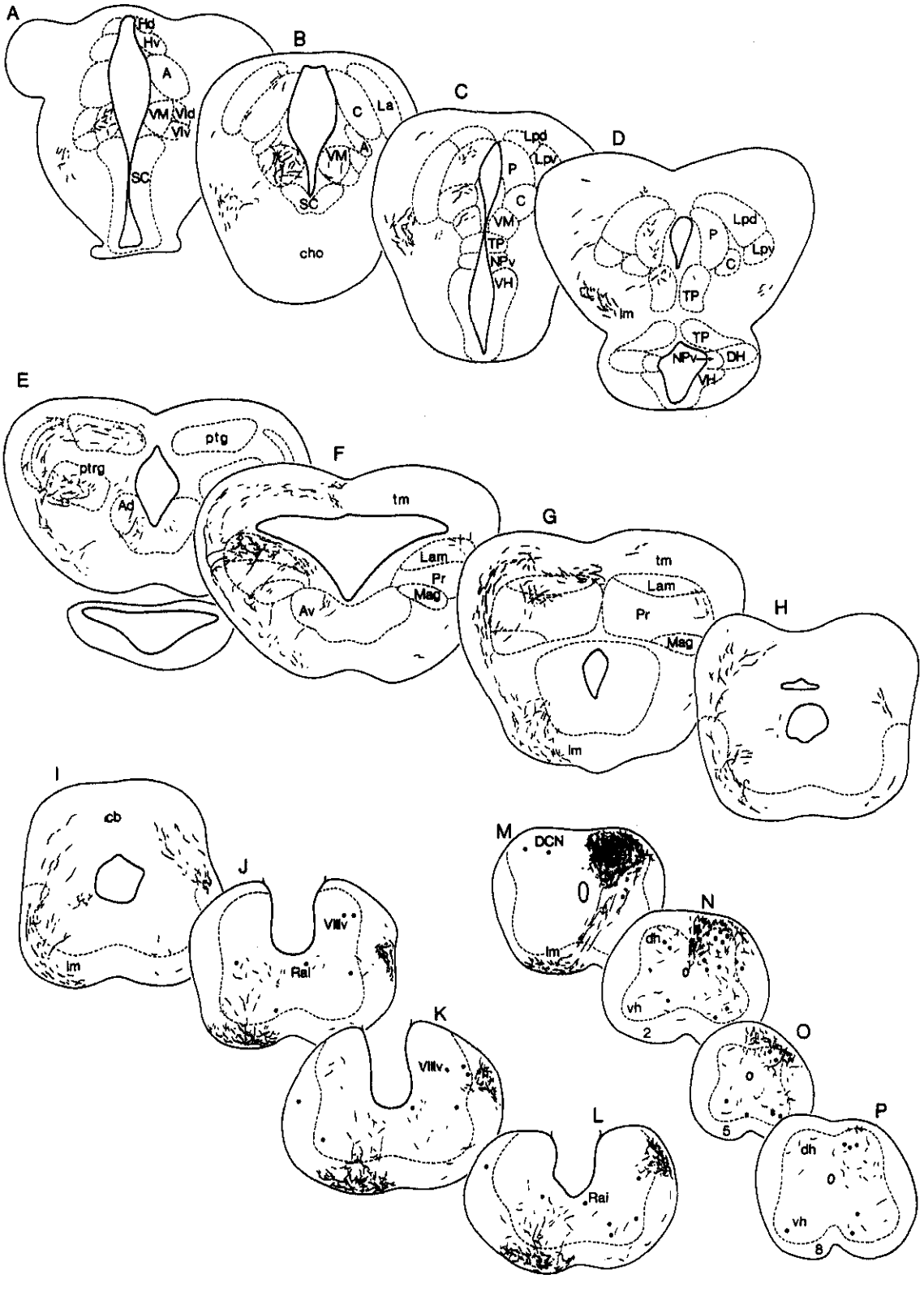

Fig. 9: Labeling observed after a BDA injection into the DCN of *Xenopus laevis*. In a series of transverse sections through the diencephalon (A-D), brain stem (E-M) and spinal cord (N-P) the pattern of anterogradely labeled fibers and retrogradely labeled cells (black dots) is shown.

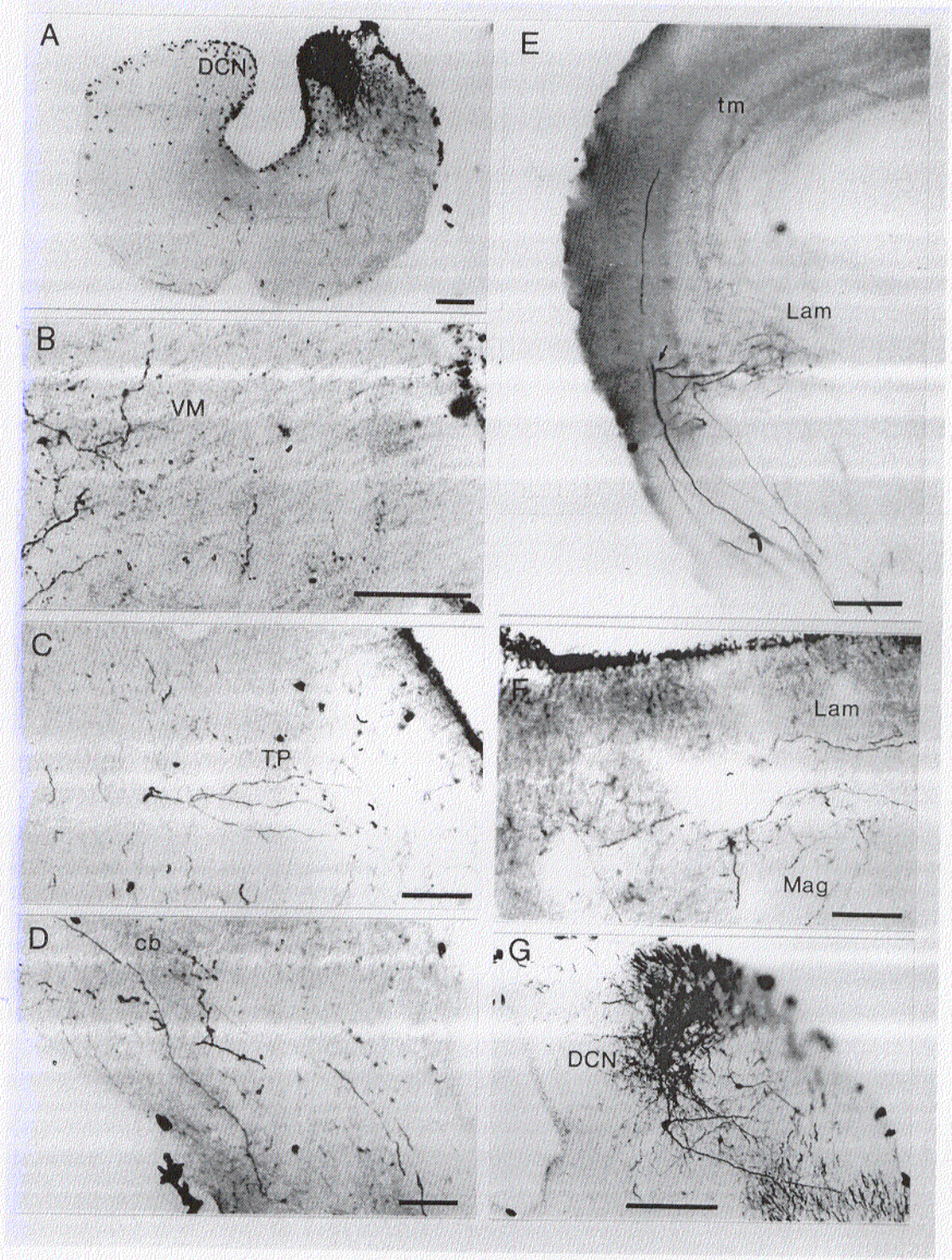

Fig. 10: Photomicrographs illustrating the labeling observed after BDA injections into the DCN (A-D, F) or spinal cord (G) of *Rana perezi*. In E anterogradely HRP labeled fibers from the DCN to torus and tectum in *Xenopus laevis* are shown; arrow points to a tectal axon giving off collaterals to the laminar nucleus of the torus. A: BDA injection site of the experiment shown in Fig. 8. B: Anterograde labeling in the contralateral ventromedial thalamic nucleus; C: Ibid., in the contralateral nucleus of the posterior tuberculum; D: Ibid., in the ipsilateral granular layer of the cerebellum; F: Ibid., in the laminar and principal nuclei of the contralateral torus semicircularis. In G: Retrogradely labeled cells in the DCN following a BDA application into the fourth spinal segment. Scale bars indicate 100 µm.

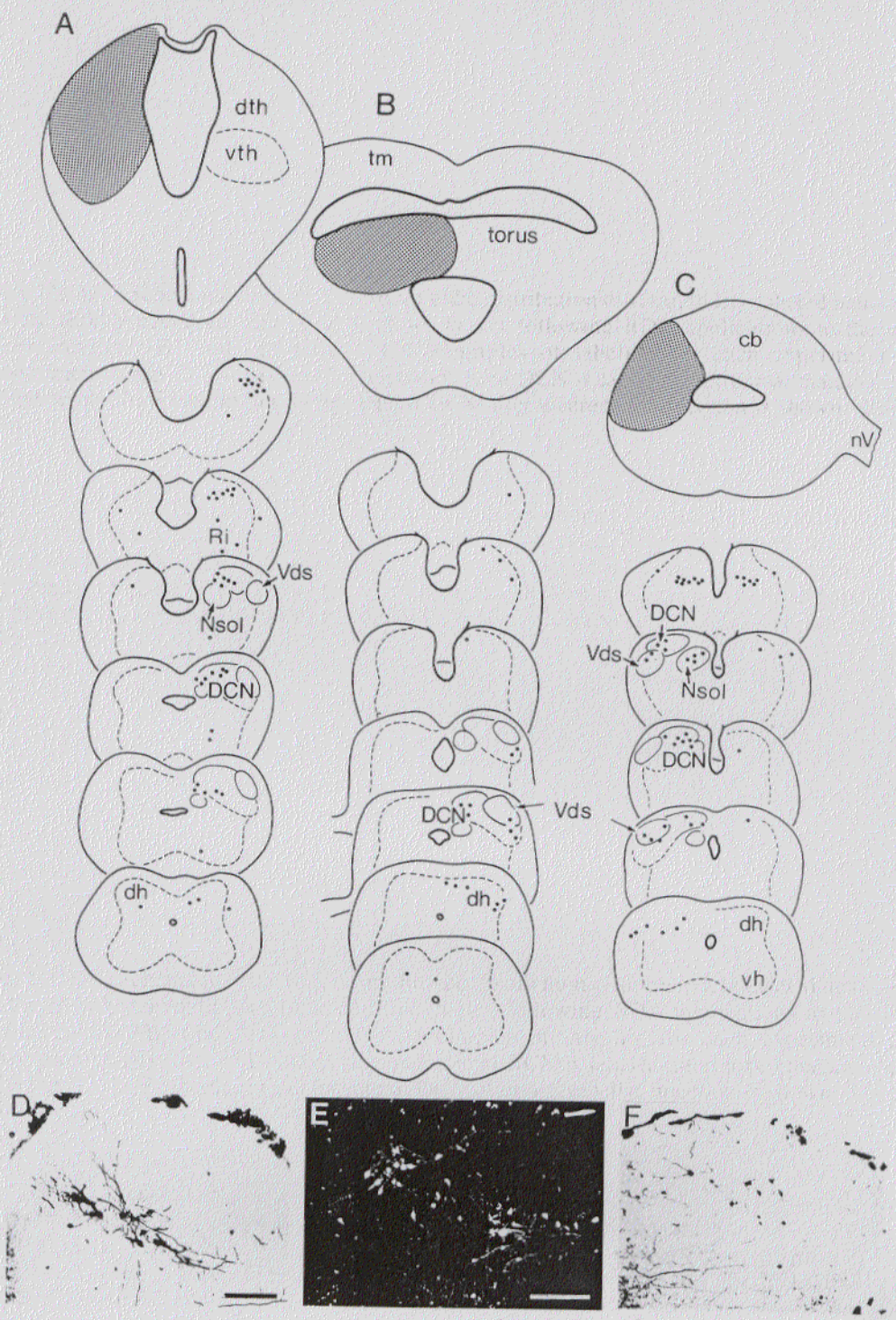

Fig. 11: A-C, Schematic drawings illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalic alar plate of *Rana perezi* following BDA applications to the thalamus (A), torus semicircularis (B) and cerebellum (C). Examples of labeling for each experiment are shown in the photomicrographs D-F. In D and E the contralateral DCN is labeled after dorsal thalamic and toral injections, respectively. In F labeling in the ipsilateral DCN after a cerebellar injection is shown. Scale bars indicate 100 µm.

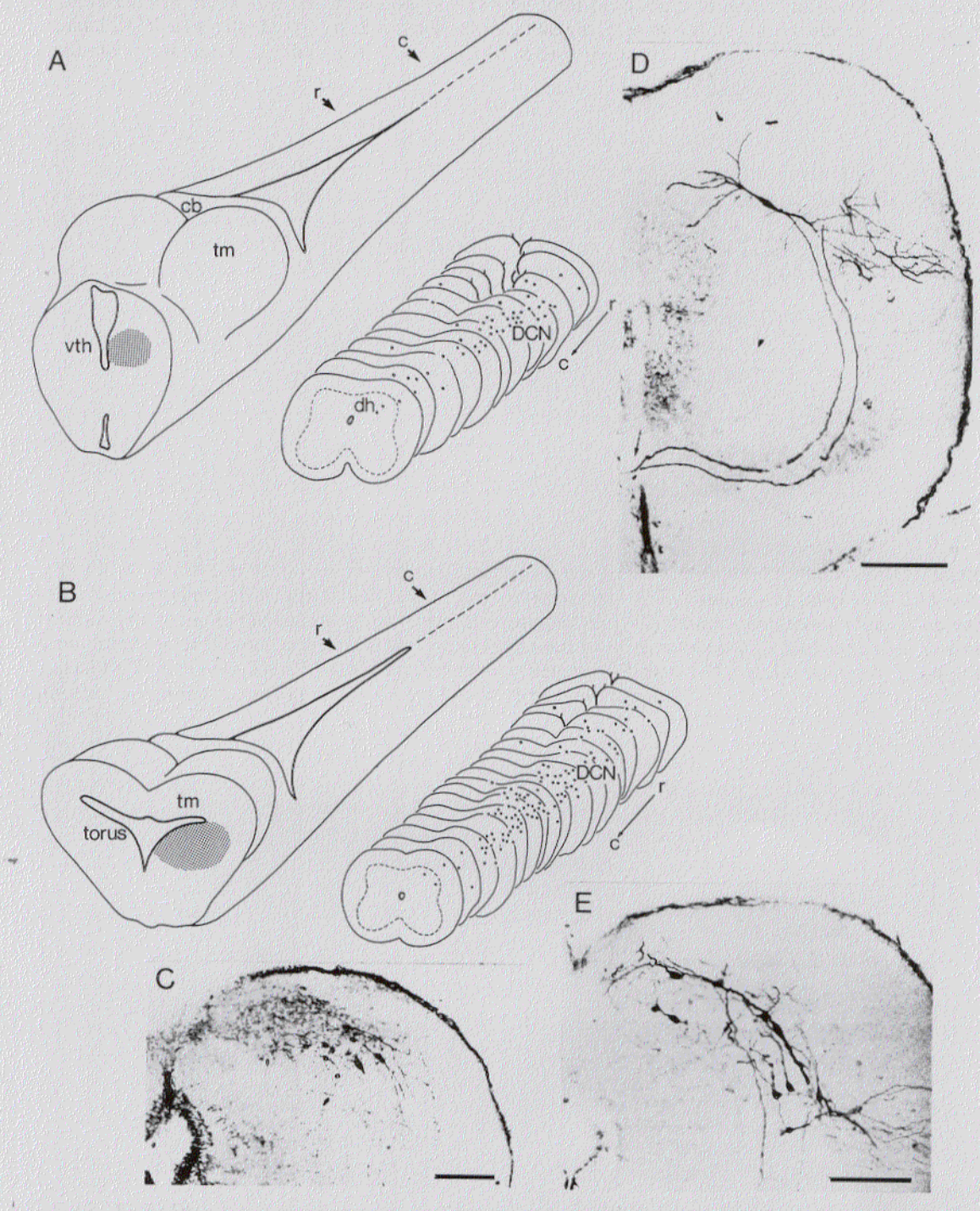

Fig. 12: A, B, Two *in vitro* experiments in *Xenopus laevis*. BDA was applied to the ventral thalamus (A) and torus semicircularis (B), respectively. In the photomicrographs C-E examples of labeling are shown: C, DCN neurons projecting to the contralateral ventral thalamus; D, E, DCN neurons projecting to the contralateral torus semicircularis. In D, one contralaterally projecting toral projection neuron at the ventrolateral aspect of the caudal DCN area with dorsally and ventrally oriented dendrites is shown; the arrow marks two axons crossing the midline to join the medial lemniscus. Scale bars indicate 100 μm.

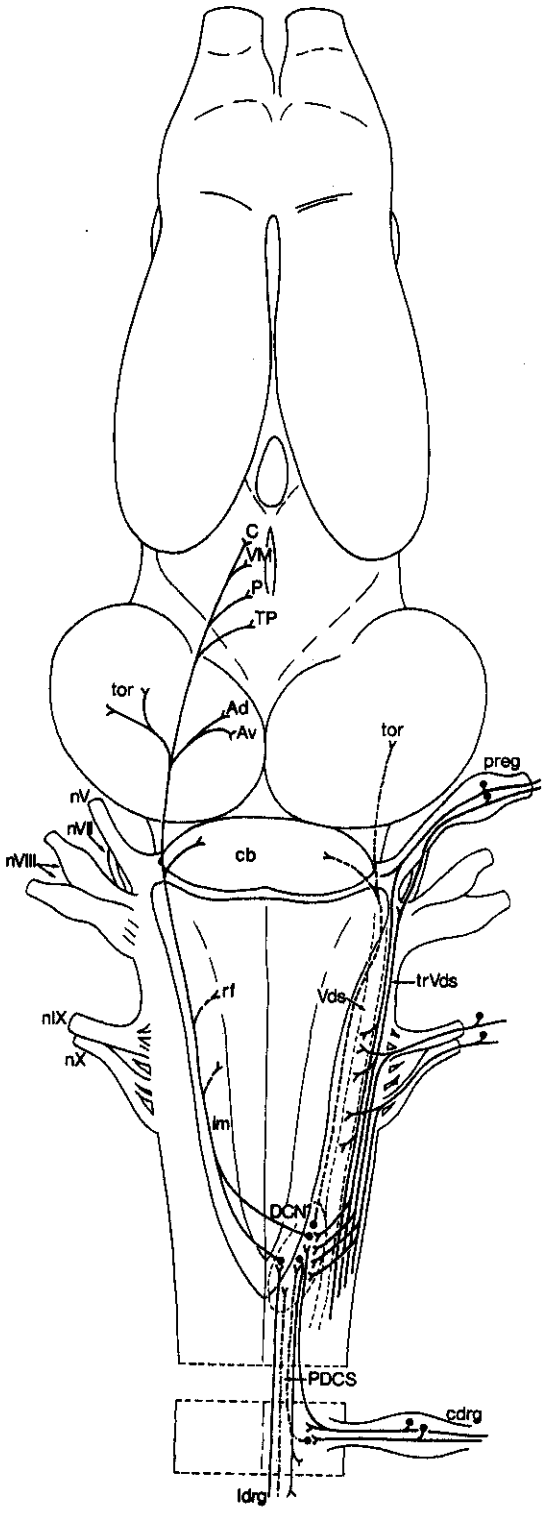

Fig. 13: Diagram summarizing the fiber connections of the anuran dorsal column nucleus shown in a dorsal view of the brain of *Rana perezi*.

LIST OF ABBREVIATIONS

A	anterior thalamic nucleus
Ad	anterodorsal tegmental nucleus
Av	anteroventral tegmental nucleus
bv	blood vessel
C	central thalamic nucleus
c	caudal
cb	cerebellum
cdrg	cervical dorsal root ganglion
cho	chiasma opticum
DCN	dorsal column nucleus
df	dorsal funiculus
DH	dorsal hypothalamic nucleus
dh	dorsal horn
dlf	dorsolateral funiculus
dr3	third dorsal root
dth	dorsal thalamus
Ep	posterior entopeduncular nucleus
Hd	dorsal habenular nucleus
Hv	ventral habenular nucleus
Is	nucleus isthmi
IXm	motor nucleus of the glossopharyngeal nerve
iz	intermediate zone
La	lateral thalamic nucleus: anterior division
Lam	laminar nucleus of the torus semicircularis
ldrg	lumbar dorsal root ganglion
lm	lemniscus medialis
Lpd	lateral thalamic nucleus: posterodorsal division
Lpv	lateral thalamic nucleus: posteroventral division
Mag	magnocellular nucleus of the torus semicircularis
Mg	magnocellular preoptic nucleus
Nsol	nucleus of the solitary tract
NPv	nucleus of the periventricular organ
nV	trigeminal nerve
nVII	facial nerve
nVIII	vestibulocochlear nerve
nIX	glossopharyngeal nerve
nX	vagal nerve
P	posterior thalamic nucleus
PDCS	postsynaptic dorsal column system
Pr	principal nucleus of the torus semicircularis
preg	preotic ganglion
ptg	pretectal grey
ptrg	pretoral grey
r	rostral
Rai	nucleus raphes inferior
Ri	nucleus reticularis inferior
Rm	nucleus reticularis medius
Rs	nucleus reticularis superior
SC	suprachiasmatic nucleus
sol	solitary tract
tm	tectum mesencephali
tor	torus semicircularis
TP	nucleus of the posterior tubercle
trVds	descending tract of the trigeminal nerve
VH	ventral hypothalamic nucleus
vh	ventral horn
Vds	nucleus of the descending tract of the trigeminal nerve
VIIId	descending nucleus of the VIIIth nerve
VIIIv	ventral nucleus of the VIIIth nerve
Vld	ventrolateral thalamic nucleus: dorsal part
Vlv	ventrolateral thalamic nucleus: ventral part
VM	ventromedial thalamic nucleus
Vm	motor trigeminal nucleus

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Evidence for an anuran homologue of the mammalian spinocervicothalamic system: An in vitro tract-tracing study in Xenopus laevis

5.3

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Based on: European Journal of Neuroscience (Submitted)

Key words: amphibians, spinal cord, lateral cervical nucleus, thalamus, torus semicircularis, somatosensory projections

ABSTRACT

Evidence is presented for an anuran homologue of the mammalian spinocervicothalamic system. *In vitro* tract-tracing experiments with biotinylated dextran amine in *Xenopus laevis* show that ascending spinal fibers from all levels of the spinal cord, passing via the dorsolateral funiculus, terminate in a cell area ventrolateral to the dorsal column nucleus. This cell area can be considered a possible homologue of the mammalian lateral cervical nucleus. After tracer applications to the ventral thalamus or to the torus semicircularis (both targets for somatosensory projections), the anuran lateral cervical nucleus was retrogradely labeled contralateral to the application sites. Tracer applications to the dorsolateral funiculus at the obex level and rostral spinal cord resulted in labeling of the cells of origin of the anuran spinocervical tract. These were found, mainly ipsilaterally, in the ventral part of the dorsal horn, and

were rather evenly distributed throughout the spinal cord. These data suggest the presence of an anuran homologue of the mammalian spinocervicothalamic system. A brief survey of the literature shows that such a system is much more common in vertebrates than previously thought.

INTRODUCTION

The presence of a bisynaptic spinocervicothalamic pathway composed of spinocervical and cervicothalamic tracts has been demonstrated for mammals (Morin, 1955; Nijensohn and Kerr, 1975; Boivie, 1983). The first physiological studies suggested that this pathway forms a rapidly conducting system carrying information from the skin to the cerebral cortex (Catalano and Lamarche, 1957). Subsequent tract-tracing studies in several mammalian species demonstrated the spinocervical tract as an ipsilateral projection from dorsal horn neurons throughout the spinal cord ascending via the dorsolateral funiculus to the lateral cervical nucleus (LCN). The LCN is a special group of neurons within the white matter just ventrolateral to the dorsal horn in the uppermost cervical segments (C1-C3). The projections from the LCN decussate and pass via the contralateral medial lemniscus towards mesencephalic and thalamic somatosensory areas and form the cervicomesencephalic and cervicothalamic tracts, respectively (see Willis and Coggeshall, 1991). So far, a definitive spinocervical tract has not been demonstrated in nonmammalian vertebrates. Only fragmentary data are available (e.g., Ebbesson, 1967; Finger, 1981; Forehand and Farel, 1982; Ito *et al.*, 1986; Necker, 1989; Ronan and Northcutt, 1990).

In anurans, the presence of a spinal lemniscus passing via the ventral brain stem and innervating the rhombencephalic and mesencephalic parts of the reticular formation in particular was demonstrated using anterograde degeneration techniques (Ebbesson, 1969, 1976; Hayle, 1973a,b). Recently, we demonstrated a distinct spinothalamic component in amphibians (A. Muñoz *et al.*, 1994a). Moreover, well-established ascending projections from a dorsal column nucleus present at the obex and upper spinal segments, via the medial lemniscus were shown for anurans (A. Muñoz *et al.*, 1993; 1994b; 1995a). The possible existence of an ascending pathway from spinal cells comparable to the LCN of mammals was considered briefly since injections of retrograde tracers into the thalamus or the mesencephalic torus semicircularis revealed a distinct cell population situated in the dorsolateral part of the spinal cord at upper cervical segments (A. Muñoz *et al.*, 1995a). The location, morphology and ascending projections of these cells suggested the presence of an anuran homologue of the LCN of mammals, and prompted the present study. A separate nucleus in the dorsolateral part of the upper cervical spinal cord or at the obex is not distinguishable as a cytoarchitectonic entity (Ebbesson, 1976; Nikundiwe and Nieuwenhuys, 1983).

The aim of the present study is to characterize this cell group in the lateral aspect of the spinal dorsal horn by studying its afferent and efferent connections. The powerful and fast tracer low-weight (3kD) biotinylated dextran amine (BDA) was used to label ascending spinal projections, to trace possible LCN projections retrogradely from the ventral thalamus and torus semicircularis (the mesencephalic somatosensory target in anurans), and to analyze the cells of origin of the possible spinocervical tract. BDA can be used for anterograde as well as retrograde tracing (Fritzsch, 1993; A. Muñoz *et al.*, 1995a). An *in vitro* approach

was used in the clawed toad, *Xenopus laevis:* an isolated preparation of the central nervous system (CNS) well suited for a variety of neuroanatomical tracing techniques (Luksch *et al.*, 1995). Part of this study was published in abstract form (A. Muñoz *et al.*, 1995b).

MATERIALS AND METHODS

In the present study a total of 25 young adult *Xenopus laevis* were used in tracing experiments under *in vitro* conditions (Luksch *et al.*, 1995; based on Cochran *et al.*, 1987). The animals were deeply anesthetized with a 0.2% solution of MS222 and cooled to a body temperature of about 5°C. The heart was exposed by rapid thoracotomy in order to perfuse the animal transcardially with approximately 40 ml iced Ringer's solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose) that had been oxygenized with carbogen (95% O_2, 5% CO_2) to a pH of 7.3 (Straka and Dieringer, 1993). The brain and spinal cord were isolated by a dorsal approach by removing the overlaying bony tissue of the skull and vertebral column. After isolation, the CNS was tranferred into a dish with fresh iced Ringer. Subsequently, the dura mater and the choroid plexus were removed to facilitate oxygen diffusion into the tissue. Applications of BDA (3kD, Molecular Probes D-7135), recrystallized at the tip of glass micropipettes or sharp tungsten needles, were made at the dorsal horn of cervical, thoracic or lumbar spinal levels (10 cases), the ventral thalamus (5 cases) and the torus semicircularis (5 cases). In the latter cases, the CNS was cut transversally at midthalamic and midmesencephalic levels, respectively. In addition, small applications of 3kD BDA were made into the dorsolateral funiculus at cervical (2 cases) and obex (3 cases) levels. The brains were kept for 15-18 hours at about 15°C in continuously oxygenated Ringer's solution with carbogen. They were then immersed for 3-5h in 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 (PB), embedded in polyacrylamide (see ten Donkelaar and de Boer-van Huizen, 1991), and cryoprotected overnight in a 15% sucrose solution in PB. Sections were cut transversally at 40 µm on a freezing microtome and collected in 0.05 M Tris buffer (pH 7.6). For visualizing BDA, an avidine biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) was used, followed by heavy metal intensification of the diaminobenzidine (DAB)-based peroxidase reaction product (Adams, 1981). Selected sections were counterstained with 1% cresyl violet solution. The sections were mounted on gelatin coated glasses and coverslipped with glycerin-gelatin.

The nomenclature used is based on studies by Ebbesson (1976) on the subdivision of the spinal grey and by Nikundiwe and Nieuwenhuys (1983) on brainstem structures.

RESULTS

In the present study, BDA was applied to the dorsal part of lumbar (Fig.1), thoracic (Fig. 2) and cervical spinal segments. As a general feature, after tracer application to the spinal cord, distinct ascending fiber tracts were noted in the dorsal funiculus and in the dorsolateral funiculus, ipsilaterally, and mainly contralaterally in the ventral and ventrolateral funiculi. The course of the contralaterally ascending pathway coincides with that observed in previous studies (Ebbesson, 1976; M. Muñoz *et al.*, 1991; A. Muñoz *et al.*, 1994a). Its main targets and cells of origin will be discussed in a companion paper (A. Muñoz *et al.*, in preparation). Here, only the ascending fibers passing

via the dorsal and the dorsolateral funiculi will be discussed. In all experiments, a small contralateral component of ascending fibers in the dorsolateral funiculus was labeled. In a second set of experiments, BDA was used as a retrograde tracer to identify the possible anuran homologue of the mammalian lateral cervical nucleus (LCN). BDA was applied to the torus semicircularis (Fig. 4) and the thalamus (Fig. 5). Finally, the cells of origin of the possible spinocervical tract were studied after small BDA applications to the dorsolateral funiculus at the rostral spinal cord (Fig. 6).

Lumbar spinal cord applications

In this set of experiments, BDA was placed unilaterally into the dorsal spinal cord. The application sites included the dorsal horn, but also the dorsal and dorsolateral funiculi (Fig. 1). The applications were made at the lumbar level between dorsal roots 9 and 10. Both descending and ascending projections were observed. Rostral to the application, anterogradely labeled fibers were observed in the ipsilateral dorsal and dorsolateral funiculi. The fibers in the dorsolateral funiculus give off thin collaterals that innervate the lateral aspect of the grey in the deep part of the dorsal field and in the lateral field between the levels of the spinal dorsal roots 9 and 5. The more rostral part of the spinal grey is not innervated by lumbar dorsolateral funiculus fibers. Fibers from the dorsal funiculus reach the superficial part of the dorsal field, while others course more ventrally into the lateral field and even the ventral fields. This pattern of innervation is present up to the 5th spinal segment. A few scattered fibers cross to innervate the contralateral superficial part of the dorsal field. Rostrally, the ascending fibers form a compact bundle in the medial part of the dorsal funiculus, and just a few fibers turn laterally and enter the grey matter of the rostral spinal segments.

At upper cervical segments and at the level of the obex, most of the labeled fibers in the dorsolateral funiculus turn dorsomedially and massively innervate the neurons in the dorsolateral grey (Figs. 1, 3A). At these levels, the fibers that course in the dorsal funiculus massively innervate the medial portion of the ipsilateral dorsal column nucleus (DCN) and less densely the caudal aspect of the nucleus of the solitary tract. Only a few fibers terminate in the contralateral DCN. Just caudal to the obex a band-shaped area is present in the grey where the projections from the dorsal and dorsolateral funiculi overlap (Figs. 1, 3A). This band marks the ventral border of an area known to be occupied by fibers of the descending trigeminal tract and the cells related to them (González *et al.*, 1993).

Thoracic spinal cord applications

After tracer applications to the dorsal part of the spinal cord at various thoracic levels, ascending and descending projections were observed (Fig. 2). Caudal to the application site, labeled fibers course in the dorsal funiculus and, especially, in the dorsolateral funiculus and innervate the dorsal field and, to a lesser extent, the lateral and ventral fields up to the lumbar segments. Rostral to the BDA application the labeling of ascending fibers prevails in the dorsolateral funiculus. The innervation of the deep part of the dorsal field continues up to the level of the DCN. At the obex level and slightly caudally, the labeled fibers in the dorsal funiculus innervate the ipsilateral DCN, with a minor contralateral component, while the fibers coursing in the dorsolateral funiculus terminate around a group of neurons along the ventrolateral border of the dorsal horn in the two first spinal segments (Figs. 2,

3B). At these levels, the terminal fields of both systems form a band-shaped area with an overlapping zone where terminals from fibers in the dorsal and dorsolateral funiculi intermingle.

Cervical spinal cord applications

Following tracer application to the dorsal part of the cervical spinal cord, a similar pattern of labeled fibers as described above for the thoracic and lumbar cases was observed. When the BDA application site was restricted to the dorsal and lateral fields of the spinal grey in cervical segments 3-4, only a few labeled fibers passing caudalwards via the dorsal funiculus were noted, whereas labeled fibers in the dorsolateral funiculus could be observed up to lower lumbar spinal segments, innervating the dorsal and lateral fields throughout their course. Rostral to the application site, numerous labeled fibers were found in the dorsolateral funiculus and in the lateral part of the dorsal funiculus. Fibers from both funiculi innervate the dorsal field of the spinal grey at cervical segments. More rostrally, the fibers from the dorsal funiculus innervate the lateral aspect of the DCN, whereas the fibers in the dorsolateral funiculus innervate the group of neurons along the ventrolateral border of the dorsal horn (Fig. 3C). This innervation zone overlaps with the innervation of the DCN.

Thalamic and toral BDA applications

Experiments with BDA application to the ventral thalamus or the torus semicircularis in the mesencephalon were used to study whether the particular group of neurons along the ventrolateral border of the dorsal horn in rostral spinal segments gives rise to ascending projections to the mesencephalon and the thalamus. In a previous study (A. Muñoz *et al.*, 1995a) tracer applications that included the ventral thalamus resulted in retrogradely labeled cells in what was identified as the DCN. However, the most ventrolaterally located cells of this population extend their dendrites laterally and were seen to be more closely related with the dorsolateral funiculus than with the dorsal funiculus. A similar pattern was observed when the tracer was applied to the lateral aspect of the torus semicircularis. The *in vitro* experiments illustrated in figures 4 and 5 clearly demonstrate that the cell population previously labeled as dorsal column nucleus, in fact, is composed of two more or less separate cell groups: the medial DCN and a more lateral cell group in the area where fibers passing via the dorsolateral funiculus massively terminate. Due to its position and connections, the name *lateral cervical nucleus* (LCN) will be introduced for this cell group. It should be emphasized that in anurans this cell group is not recognizable as a cytoarchitectonic entity (see Fig. 3D). This neuronal population extends slightly more caudally than the DCN, i.e. from the obex level up to the second cervical segment. The cells in the LCN project to the torus semicircularis and the thalamus via the medial lemniscus (Figs. 4, 5). The cells in the LCN occupy a position in the lateral aspect of the spinal grey and their dendrites extend profusely into the dorsolateral funiculus (Figs. 4, 5). Only a few scattered labeled cell bodies were observed within the dorsolateral funiculus itself. The fibers ascending in the dorsolateral funiculus richly innervate the area of the LCN.

Tracer applications into the cervical part of the dorsolateral funiculus

In order to identify the cells of origin of the fibers ascending in the dorsolateral funiculus towards the lateral cervical nucleus, small applications of BDA

were made to the dorsolateral funiculus at the obex level (Fig. 6A) and at the second cervical spinal segment. By means of retrograde tracing, the cells of origin of this spinocervical tract were demonstrated. A large population of cells was labeled, mainly ipsilaterally to the application site, although a small number of contralateral cells were present in the ventral fields of the grey at thoracic and lower cervical segments (Fig. 6A). The ipsilateral population is predominantly located in the dorsal horn from cervical (Fig. 6B,C) to sacral segments. The majority of the cells are found in the deeper part of the dorsal field of the spinal grey. More sparsely distributed neurons are present in the superficial part of the dorsal horn. Most of the labeled neurons have round-to-oval somata with dorsally or ventrolaterally directed processes that often enter the dorsolateral funiculus (Fig. 6C). Larger, triangular or irregular cells are more rarely labeled. A small number of cells are ipsilaterally located in the lateral spinal field at thoracic levels.

DISCUSSION

In the present study the organization of the ascending projections in the dorsal and dorsolateral funiculi was studied in the clawed toad, *Xenopus laevis*. In particular, the presence of a spinocervical tract and of a possible anuran homologue of the mammalian lateral cervical nucleus were investigated.

The relatively new tracer BDA was used in an *in vitro* approach. In previous studies the suitability of isolated preparations of the anuran central nervous system has been discussed (Luksch *et al.* 1995; A. Muñoz *et al.* 1995a). BDA was first described to be very successfully transported anterogradely by neuron processes (Veenman *et al.*, 1992). However, BDA can also be used effectively as a retrograde tracer (A. Muñoz *et al.*, 1995a). When BDA is applied iontophoretically, it will be transported primarily anterogradely. In contrast, application in dry form, both *in vivo* and *in vitro*, results in an effective retrograde transport. The present study shows that BDA, when applied as dry crystals, is also an effective anterograde tracer. It should be emphasized, however, that even the smallest BDA application to the spinal cord also results in retrograde labeling of fibers and cells.

Tracer applications to the dorsal part of the spinal cord at cervical, thoracic and lumbar levels, once more demonstrated a somatotopical arrangement of the fibers ascending in the dorsal funiculus including their pattern of termination in the DCN as previously noted in anurans (Antal *et al.*, 1980; Nikundiwe *et al.*, 1982; M.Muñoz *et al.*, 1991; A.Muñoz *et al.*, 1995a). These fibers include primary afferents as well as second-order projections towards the DCN, i.e. the postsynaptic dorsal column system (ten Donkelaar and de Boer van Huizen, 1991; A.Muñoz *et al.*, 1995a).

A well-developed system of ascending fibers in the dorsolateral funiculus was demonstrated. The targets of these fibers include the dorsal and lateral spinal fields of the grey and, especially, the area of the lateral cervical nucleus. Similar observations were made in the Spanish green frog, *Rana perezi* (A. Muñoz *et al.* 1995b). Other brainstem projections will be discussed in a companion paper (A. Muñoz, in preparation).

In experiments where the tracer application site included the dorsal and lateral spinal fields as well as the dorsolateral funiculus some fibers were found leaving that funiculus to innervate the dorsal and lateral spinal fields rostral and caudal to the spinal

segment involved. Some of these fibers may be spinal primary afferents running in Lissauer's tract (Antal *et al.*, 1980; Nikundiwe *et al.*, 1982; M.Muñoz *et al.*, 1991; A.Muñoz *et al.*, 1995a). However, also non-primary intersegmental intraspinal projections through the dorsolateral funiculus may exist in amphibians, as is the case in mammals in which the spinocervical tract neurons give off collateral branches to various targets of the spinal grey at different spinal levels (Snow *et al.*, 1976; Brown *et al.*, 1977; Rastad et al, 1977; Jankowska *et al.*, 1979; Maxwell and Koerber, 1986; Cao *et al.*, 1993). Some of the dorsolateral funiculus fibers that innervate the different spinal fields caudal to the application site may belong to descending projections from different spinal and supraspinal sources, including the LCN and the lateral reticular formation. In these structures, retrogradely labeled neurons were observed. Close to the obex, the dorsolateral funiculus massively gives off thin fibers, directed dorsomedially towards a region located along the ventral border of the dorsal horn in rostral spinal segments. This zone represents the anuran homologue of the mammalian LCN.

The tract-tracing experiments presented in this study suggest that the spinal projections to the LCN arise in cells located throughout the spinal cord, mainly in the ipsilateral deep dorsal field. The spinocervical tract in mammals is an excitatory glutamatergic tract (Broman *et al.*, 1990; Kechagias and Broman, 1994) known to arise in spinal cells that receive an input from the periphery (see Willis and Cogheshall, 1991). The distribution of these spinal cells was studied in the rat, cat and dog (Baker and Giesler, 1984; Craig, 1976, 1978; Craig *et al.*, 1992). They are distributed predominantly in the ipsilateral nucleus proprius, substantia gelatinosa and lamina IV, with fewer cells in lamina V. At cervical levels, scattered cells in laminae I, VI and VII also contribute to the spinocervical tract. A small contralateral component from laminae I, VII and VIII was described in cats (Brown *et al.*, 1980). A possible spinocervical tract was also demonstrated in other non-mammalian vertebrates (see Table I). In birds, van den Akker (1970) showed a "dorsolateral ascending bundle" in the dorsolateral funiculus that arises in neurons found in the deep part of the dorsal horn. At cervical levels, this bundle innervates the deep dorsal and central spinal grey. More recent tract-tracing studies showed various ascending non-primary spinal projections in the dorsolateral funiculus of the pigeon (Funke and Necker, 1986; Funke 1988; Necker, 1991), most likely including the spinocervical tract. The ascending spinal projections through the dorsolateral funiculus in birds arise from neurons located in laminae I, IV, V and in Clarke's column (Funke and Necker, 1986; Funke 1988; Necker, 1991). In reptiles, the only evidence for the presence of a spinocervical tract comes from an anterograde degeneration study in the tegu lizard, *Tupinambis teguixin*. Ebbesson (1967) noted that at caudal brainstem levels some collateral fibers leave the dorsolateral funiculus and innervate an area located dorsal to the hypoglossal nucleus and ventral to the DCN. In other anamniotes, evidence for a spinocervical tract projecting to a lateral cervical nucleus is at least suggestive. In agnathans (Northcutt and Ebbesson, 1980; Ronan and Northcutt, 1990) as well as in cartilaginous (Hayle, 1973a,b; Ebbesson and Hodde, 1981; Smeets *et al.*, 1984) and bony fishes (Hayle 1973a,b; Finger, 1981), ascending spinal projections were demonstrated via the dorsal part of the lateral funiculus. No separate site of termination, reminiscent of an LCN, was noted.

The mammalian LCN extends from the obex level to the second spinal segment, and is formed by

neurons close to and extending into the dorsolateral funiculus. Their axons pass via the medial lemniscus, and mainly reach the mesencephalic somatosensory intercollicular zone and the ventrobasal complex of the thalamus, and form the cervicomesencephalic and cervicothalamic tracts, respectively (Willis and Coggeshall, 1991). There is substantial evidence for the presence of an LCN in non-mammalian vertebrates (Table I). In birds, the available data on the existence of the spinocervicothalamic pathway and the LCN are sparse. With a silver impregnation technique, Ramón y Cajal (1911) already noted an interstitial nucleus in the chick embryo, formed by triangular cells located throughout the spinal dorsolateral funiculus, but preferentially at cervical levels. In the pigeon, the LCN was defined as a cytoarchitectonic entity at upper cervical levels (Karten and Hodos, 1967). However, van den Akker (1970) could not distinguish an LCN, although he found some cells located within the dorsolateral funiculus at high spinal levels. Also in the pigeon, although more caudally, Necker (1990) described a lateral spinal nucleus in the dorsolateral funiculus at the level of the cervical intumescence. Necker (1989) noted some neurons located in the lateral neck of the dorsal horn close to the dorsolateral funiculus in the first cervical segment that were retrogradely labeled from the contralateral thalamus. This cell population is located in a position comparable to that of the mammalian (see Willis and Coggeshall, 1991) and amphibian (present study) thalamic and midbrain projecting LCN neurons. It may represent the origin of the cervicothalamic tract in birds. In reptiles, an LCN has not been described as a cytoarchitectonic entity (Ebbesson 1967, 1969; Kusuma, 1979; Künzle and Woodson, 1982; Pritz and Stritzel, 1986). Retrograde tracer studies (Hoogland, 1981, 1982; Pritz and Stritzel, 1989, 1990) did not focus on the possible presence of an LCN.

In anurans, no separate LCN was noted in Nissl (Opdam *et al.*, 1976; Nikundiwe and Nieuwenhuys, 1983; see also Fig. 3D) or Golgi (Ebbesson, 1976) studies, but even the anuran DCN was not clearly defined until recently (A. Muñoz *et al.*, 1995a). The existence of an anuran LCN was suggested in a developmental study (Forehand and Farel, 1982). In *Rana catesbeiana* tadpoles, HRP was applied to the lateral aspect of the reticular formation at rhombencephalic levels between the Vth and the Xth nerves and to the tectum. Some contralateral neurons were retrogradely labeled at cervical levels in the marginal zone, just outside the intermediate grey. Moreover, in a recent study focussed on the anuran medial lemniscus (A. Muñoz *et al.*, 1995a) it was noted that some neurons, then called the ventrolateral component of the DCN, were retrogradely labeled from the ventral thalamus and, more conspicuously, from the lateral aspect of the torus semicircularis. These cells are intermingled with the proper DCN neurons in *Xenopus laevis*, but somewhat more segregated in *Rana perezi* (A. Muñoz *et al.*, 1995b), and extend more caudally than the DCN, up to the second spinal segment. This group of cells represents the anuran homologue of the LCN in mammals. Although only a few cells are actually present in the dorsolateral funiculus itself, the dendrites of the LCN neurons are mainly directed ventrolaterally and extend throughout the dorsolateral funiculus. Nevertheless, at the obex level, where the DCN and the LCN coexist, it is difficult to define neurons at intermediate locations as belonging to the DCN or the LCN. Only the morphology of their dendrites, mainly oriented dorsally or ventrolaterally to the dorsal funiculus or the dorsolateral funiculus, respectively, and the more rostral extent of the DCN, allow to distinguish the neurons of the DCN and the LCN.

In other anamniotes, data on the presence of an LCN are sparse (Table I). In teleosts, a comparable nucleus was observed at the obex level and the first cervical spinal segments (Finger, 1981; Ito *et al.*, 1986). In *Sebasticus marmoratus*, Ito *et al.* (1986) demonstrated that an LCN projects to the ventromedial thalamic nucleus. Additionally, in lampreys, experiments with diencephalic or mesencephalic HRP applications resulted in retrogradely labeled neurons at the obex level and in the first cervical spinal segments suggesting the presence of a putative LCN in agnathans (Ronan and Northcutt, 1990).

Broman (1994) reviewed the chemoarchitecture of the area of the mammalian LCN. In short, GABA-, catecholamine- (see Doyle and Maxwell, 1994), serotonin- and substance P- positive terminals were found within the LCN. The GABA-positive terminals are thought to belong to intrinsic LCN neurons, but the substance P-positive and serotonergic innervation of the LCN is thought to have a spinal and supraspinal origin, respectively. Glutamatergic terminals within the LCN are thought to belong to spinocervical tract neurons and also to cervicothalamic tract collateral axons that terminate within the LCN itself (Broman, 1994). Within the limits of the anuran LCN, GABA-, glycine- and parvalbumin-positive cells are present, as well as terminals immunopositive for CGRP, substance P, neuropeptide Y and serotonin (A. Muñoz *et al.* 1995a). Additionally in anurans, processes of catecholaminergic neurons in the vicinity of the nucleus of the solitary tract extend to the area where LCN neurons are located (González and Smeets, 1994; A. Muñoz *et al.*, 1995a).

ACKNOWLEDGEMENTS

This research was supported by a grant from the Spanish Government to A. González (DGICYT PB 93-0083) and by a NATO Collaborative Research Grant (930542) to H.J. ten Donkelaar. The authors thank Mrs. Roelie de Boer-van Huizen for excellent technical assistance and Ms. Inge Eijkhout for secretarial assistance.

TABLE I

The distribution of a spinocervicothalamic system in vertebrates

Vertebrate group	Spinocervical tract	Lateral cervical nucleus	Cervico-mesencephalic tract	Cervico-thalamic tract
Agnathans				
Lampreys				
• Silver lamprey *(Ichthyomozon unicuspis)*	*[21]	?	?	?
• Sea lamprey *(Petromyzon marinus)*	*[20,21]	+[21]	+[21]	+[21]
Hagfishes				
• Pacific hagfish *(Eptatretus stouti)*	*[21]	?	?	?
Gnathostomes				
Cartilaginous fishes				
• Spotted dogfish *(Scyliorhinus canicula)*	*[7]	–[22]	?	?
• Nurse shark *(Ginglymostoma cirratum)*	*[2]	?	?	?
Bony fishes				
• Sea robin *(Prionotus carolinus)*	+[3]	?	?	?
• *Sebasticus marmoratus*	?	+[10]	?	+[10]
• Rudd *(Scardinius erythophthalmus)*	*[7,8]	?	?	?
Amphibians				
• Tiger salamander *(Ambystoma tigrinum)*	+[9]	?	?	?
• Ribbed newt *(Pleurodeles waltl)*	+[16]	+[16]	+[16]	+[16]
• Clawed toad *(Xenopus laevis)*	+[15]	+[15]	+[15]	+[15]
• Bullfrog *(Rana catesbeiana)*	?	+[4]	+[4]	?
• Large green frog *(Rana perezi)*	+[14]	+[13,14]	+[14]	+[14]
Reptiles				
• Red-eared turtle *(Pseudemys scripta elegans)*	*[12]	?	?	*[12]
• Tegu lizard *(Tupinambis teguixin)*	+[1]	+[1]	?	?
Birds				
• Pigeon *(Columba livia)*	+[5,6,19,23]	+[11,17]	?	+[18]
Mammals				
• Rodents, carnivores, primates	+[24]	+[24]	+[24]	+[24]

Symbols used: + present; - not reported or present; * indirect or suggestive evidence; ? unknown.
References: 1 - Ebbesson (1967); 2 - Ebbesson and Hodde (1981); 3 - Finger (1981); 4 - Forehand and Farel (1982); 5 - Funke and Necker (1986); 6 - Funke (1988); 7 - Hayle (1977a); 8 - Hayle (1973b); 9 - Herrick (1930); 10 - Ito et al. (1986); 11 - Karten and Hodos (1967); 12 - Künzle and Woodson (1982); 13 - A. Muñoz *et al.* (1995a); 14 - A. Muñoz *et al.* (1995b); 15 - A. Muñoz *et al.* (present study); 16 - A. Muñoz *et al.* (unpublished observations); 17 - Necker (1989); 18 - Necker (1990); 19 - Necker (1991); 20 - Northcutt and Ebbesson (1980); 21 - Ronan and Northcutt (1990); 22 - Smeets *et al.* (1984); 23 - van den Akker (1970); 24 - Willis and Coggeshall (1990).

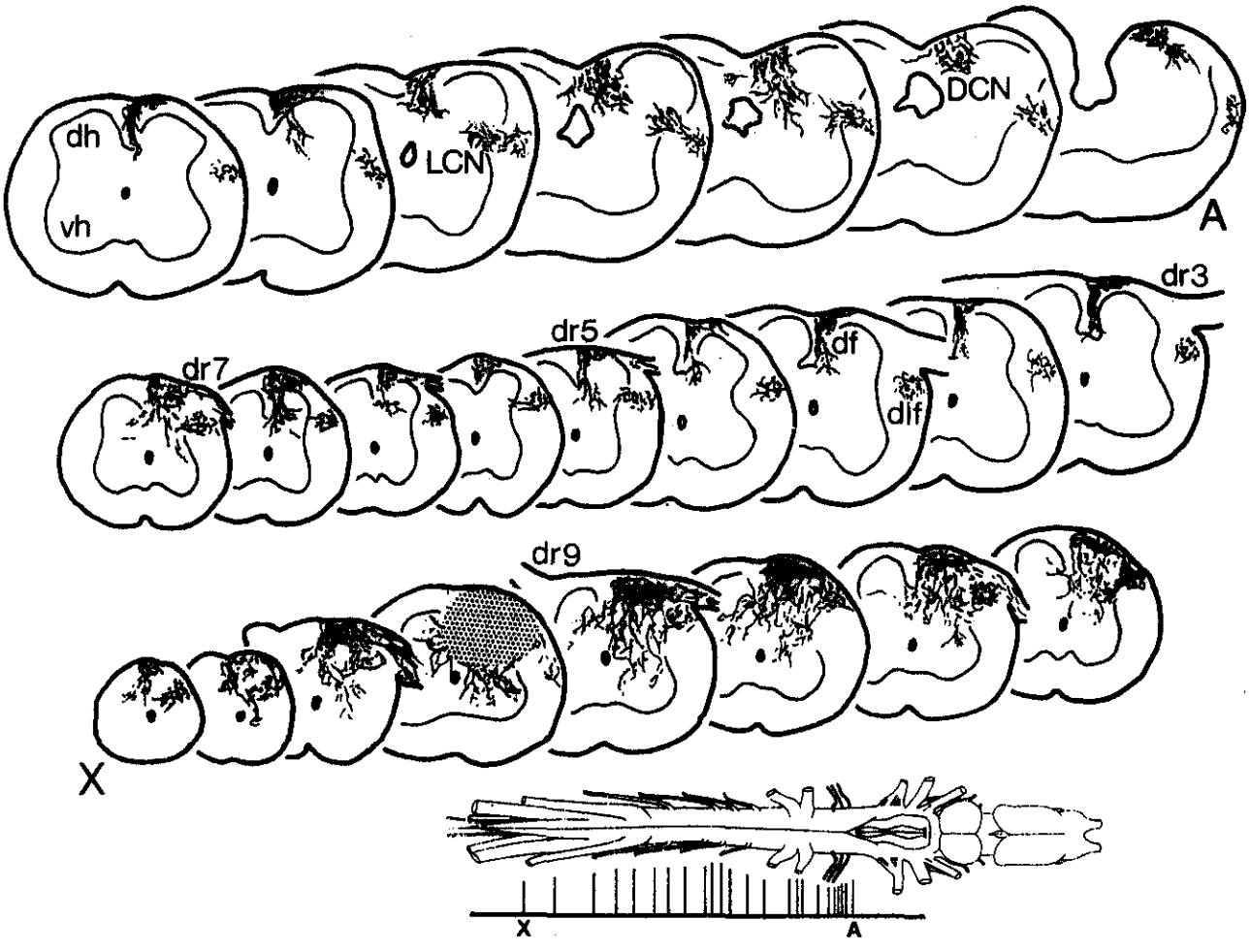


Fig. 1. Schematic drawing of transverse sections of the brain stem and spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* application of 3kD BDA into the lumbar spinal cord, between the 9th and 10th dorsal roots. The level of the sections in this and other figures is indicated along a dorsal view of the central nervous system.

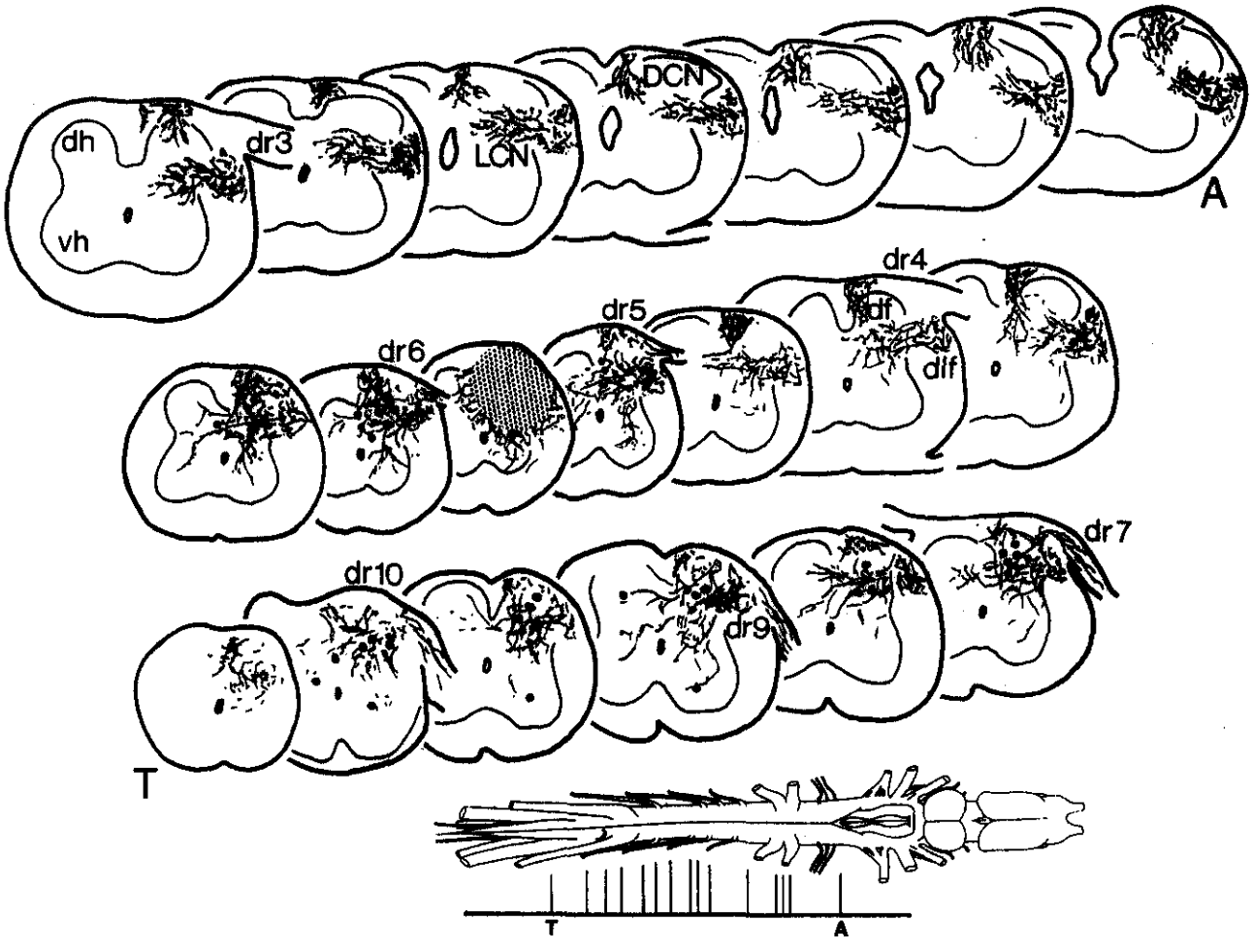


Fig. 2: Schematic drawing of transverse sections of the brain stem and the spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* 3kD BDA application into the thoracic spinal cord between the 5th and 6th dorsal roots.

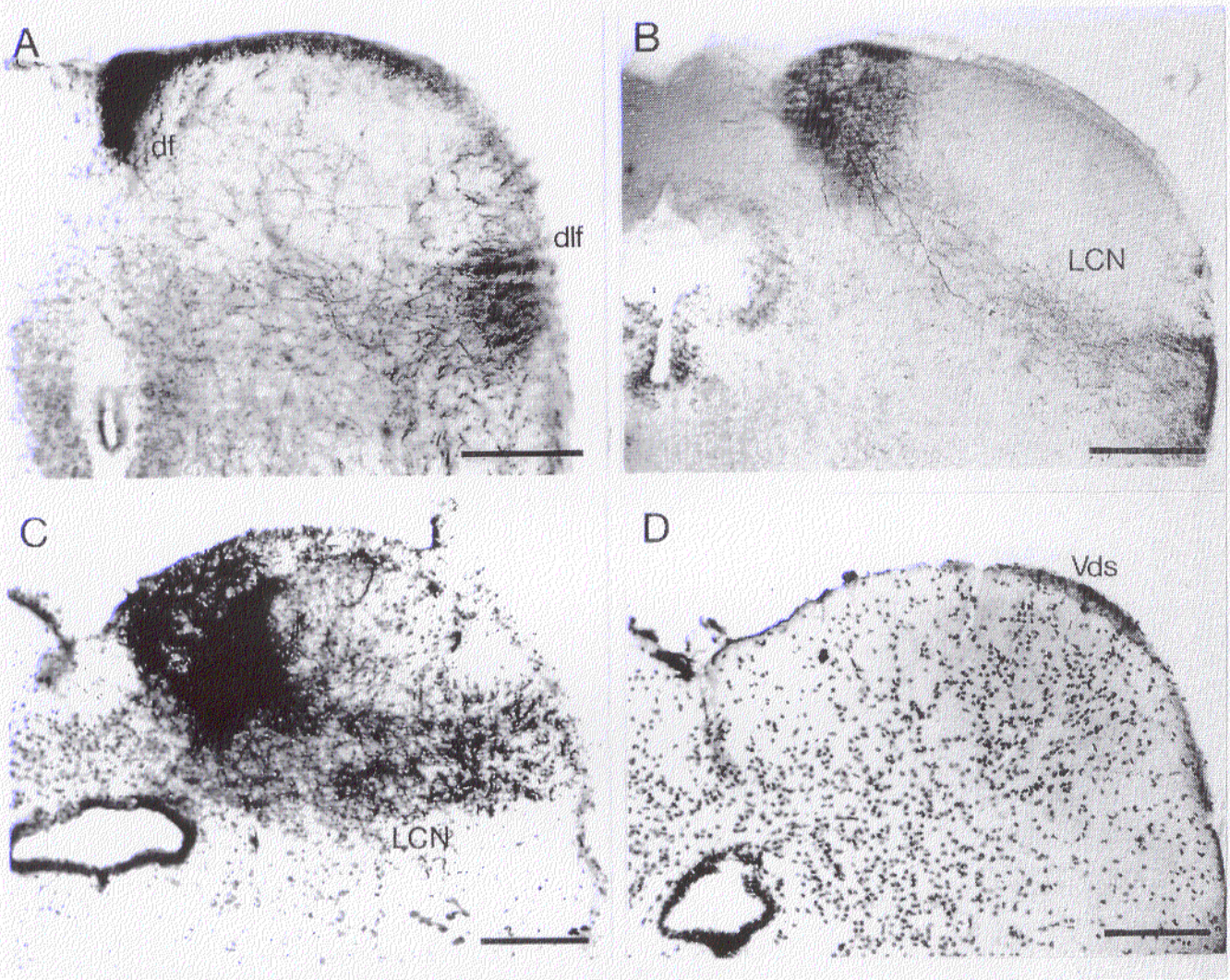


Fig. 3: Photomicrographs showing anterogradely labeled fibers in the dorsal column nucleus and in the lateral cervical nucleus (A-C), and a Nissl-stained section at the same level. A, BDA-labeling after lumbar application; B, BDA-labeling after a thoracic application; C, BDA-labeling after a cervical application; D, Nissl-stained section. Note the presence of a distinct nucleus of the descending tract of the trigeminal nerve in the lateral corner. The dorsal column and lateral cervical nuclei are ill-defined. Scale bars indicate 100 µm.

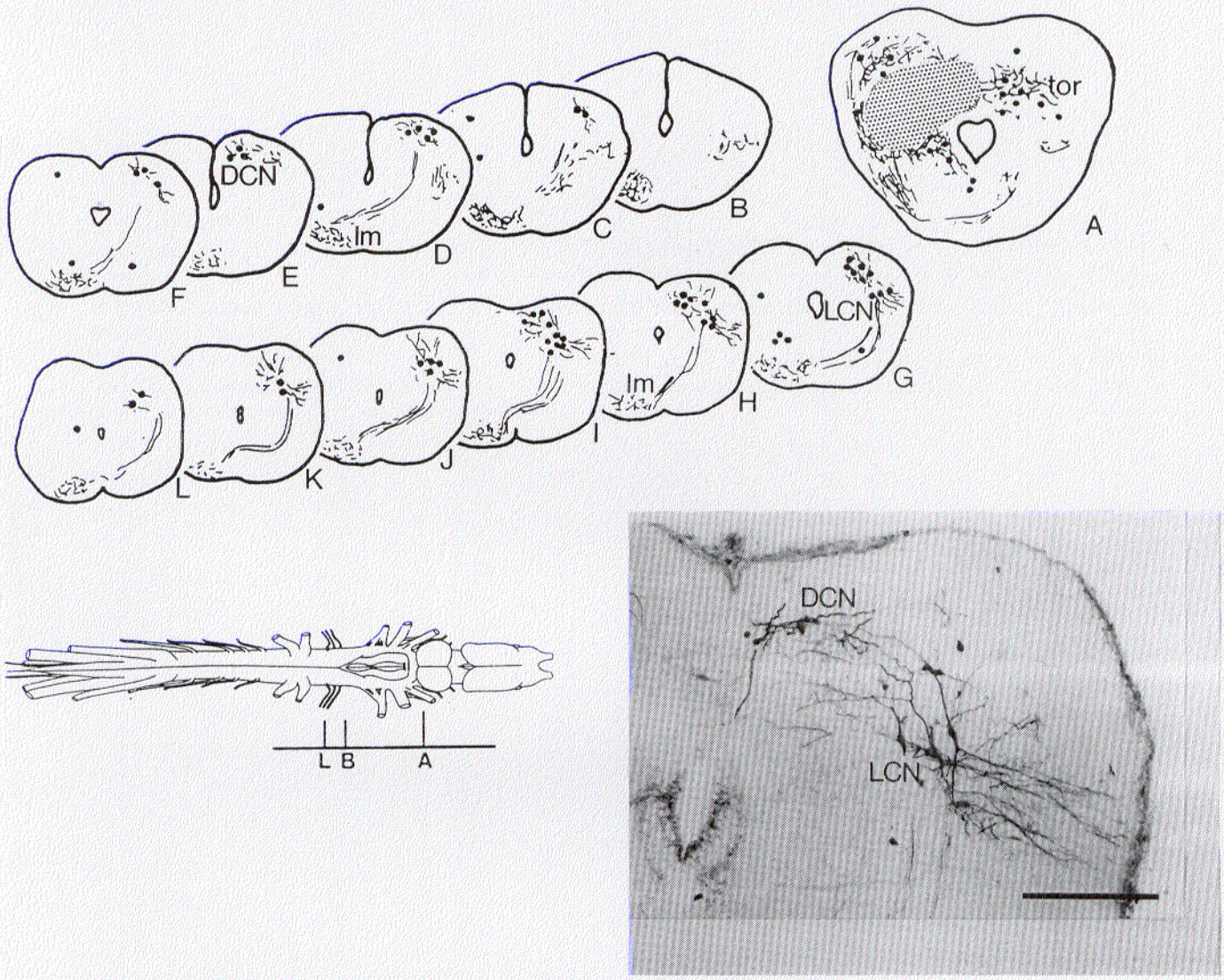
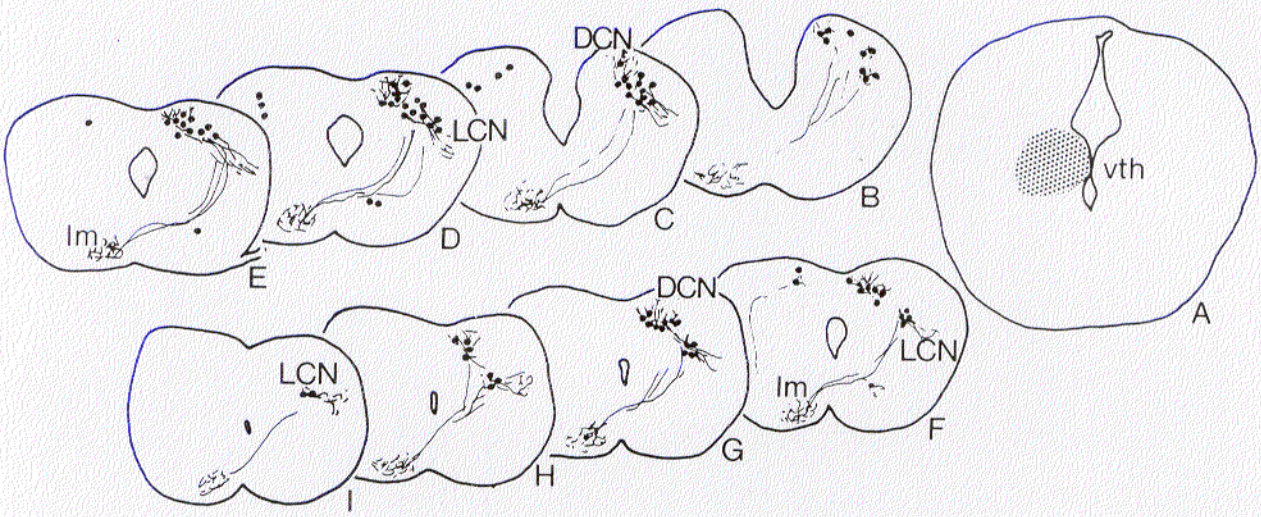
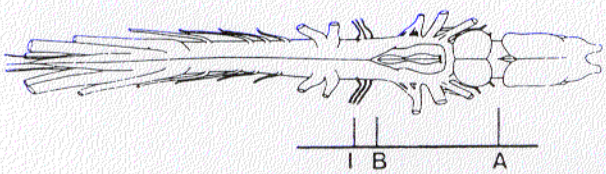


Fig. 4: Schematic drawing illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalon and in the rostral part of the spinal cord of *Xenopus laevis*. BDA was applied to the torus semicircularis. Inset shows an example of the labeling in the dorsal column nucleus and in the lateral cervical nucleus. LCN neurons possess dorsally oriented dendrites as well as ventrolaterally directed dendrites aimed at the dorsolateral funiculus. Scale bar indicates 100 µm.





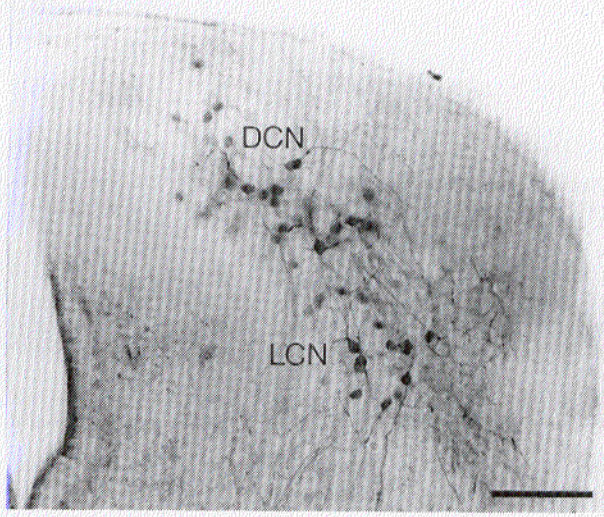


Fig.5: Schematic drawing illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalon and in the rostral part of the spinal cord of *Xenopus laevis*. BDA was applied to the ventral thalamus. Inset shows an example of the labeling in the dorsal column nucleus and in the lateral cervical nucleus. Scale bar indicates 100 µm.

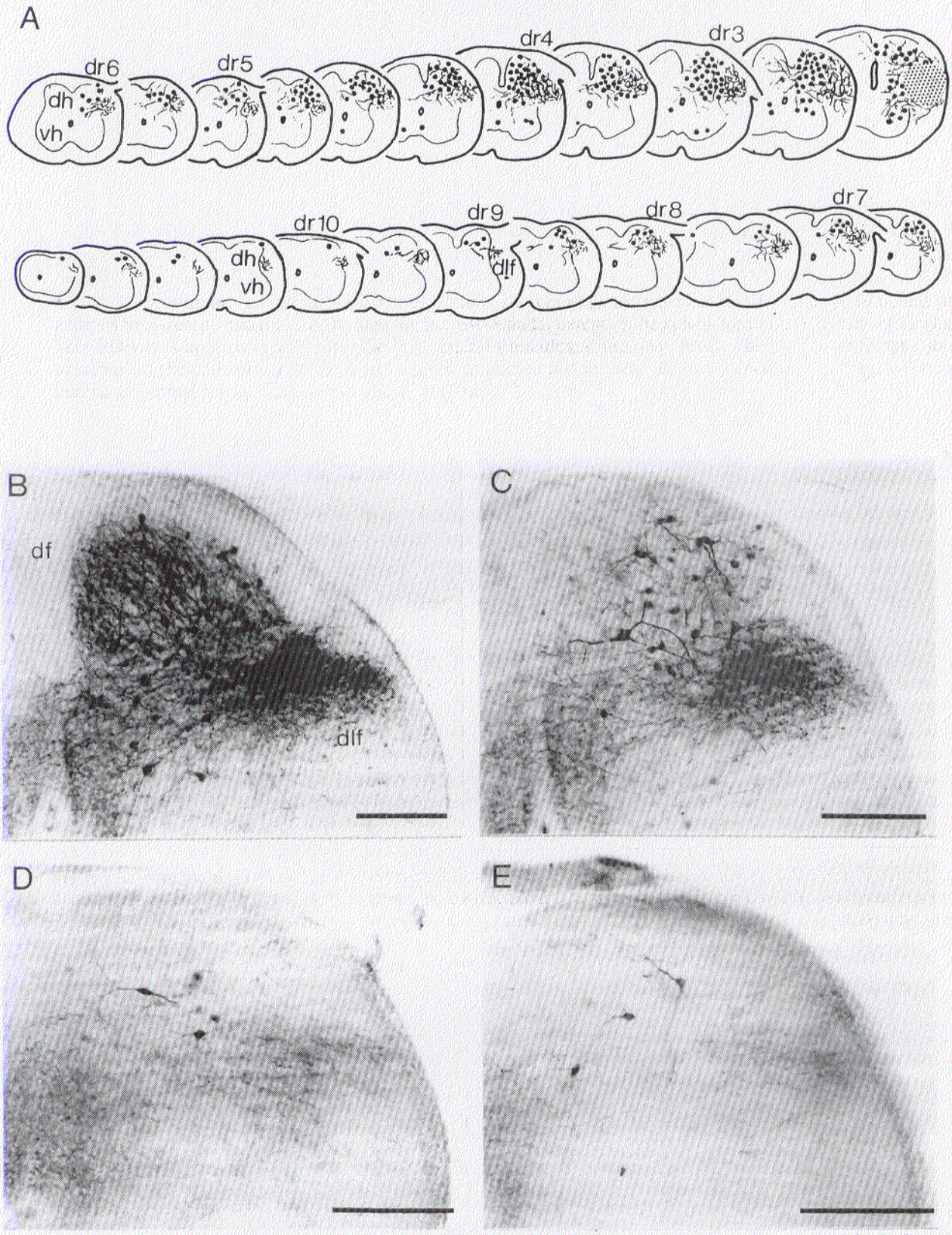


Fig. 6: A, Schematic drawing of transverse sections showing labeled fibers in the dorsolateral funiculus and neurons in different spinal fields throughout the rostrocaudal extent of the spinal cord in an experiment in which 3kD BDA was applied *in vitro* into the dorsolateral funiculus at the obex level. The photomicrographs show examples of the cells of origin of the spinocervical tract at midcervical (B), low cervical (C), thoracic (D) and lumbar (E) spinal levels. Scale bars indicate 100 µm.

ABBREVIATIONS

DCN	dorsal column nucleus
df	dorsal funiculus
dh	dorsal horn
dlf	dorsolateral funiculus
dr(3-10)	third-tenth dorsal root
LCN	lateral cervical nucleus
lm	lemniscus medialis
tor	torus semicircularis
Vds	nucleus of the descending tract of the trigeminal nerve
vh	ventral horn
vth	ventral thalamus

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The dorsal column-medial lemniscal projection of anuran amphibians

The anuran dorsal column nucleus: Organization, immunohistochemical characterization, and fiber connections in Rana perezi and Xenopus laevis

Evidence for an anuran homologue of the mammalian spinocervicothalamic system: An in vitro tract-tracing study in Xenopus laevis

COMENTARIOS

5.4

En vertebrados terrestres básicamente existen dos sistemas de proyecciones espinales ascendentes (Willis y Coggeshall, 1991): 1) Sistema columna dorsal-lemnisco medial formado por proyecciones espinales aferentes primarias y no primarias que, a través del funículo dorsal, llegan hasta los núcleos de la columna dorsal, los cuales dan origen a la vía del lemnisco medial que asciende hasta el tálamo. 2) Sistema anterolateral formado por proyecciones aferentes secundarias que, a través del funículo ventrolateral, ascienden para alcanzar la formación reticular, el techo mesencefálico y el tálamo.

Igualmente se ha descrito la presencia de una tercera vía bisináptica denominada sistema espino-cervico-talámico, formado por el tracto espinocervical que termina en el núcleo cervical lateral (LCN), el cual proyecta contralateralmente, a través del lemnisco medial, hacia regiones somatosensoriales mesencefálicas y talámicas, originando los tractos cervicomesencefálico y cervicotalámico respectivamente (Willis y Coggeshall, 1991).

En el presente capítulo se presentan tres artículos en los que se ha estudiado la posible presencia de estos sistemas en anfibios. En el segundo, se ha logrado diferenciar, mediante la utilización de técnicas histoquímicas e inmunohistoquímicas, los distintos componentes celulares de la placa alar de la región del óbex que se encuentran pobremente diferenciados citoarquitectónicamente.

Citoarquitectura

En la mayoría de los estudios citoarquitectónicos del tronco cerebral en los anuros no se distinguió el núcleo de la columna dorsal (DCN) o del funículo dorsal (Ariëns Kappers y Hammer, 1918; Zeehandelaar, 1921; Opdam y cols., 1976). A partir de los estudios de Woodburne (1939) con técnicas de Marchi, el DCN de los anuros se ha considerado como el sitio de terminación de fibras funiculares dorsales en el rombencéfalo caudal, más que como una entidad citoarquitectónica (Antal y cols., 1980; Nikundiwe y cols., 1982). Nuestros resultados muestran que el DCN se localiza en la placa alar en la región celular que rodea dorsal y lateralmente al polo caudal del tracto solitario, en niveles de transición entre el rombencéfalo y la médula espinal, segregado escasamente de los núcleos del tracto solitario y del tracto descendente del nervio trigémino, situados medial y lateralmente al DCN respectivamente. Sin embargo, una escotadura dorsal sugiere la subdivisión del DCN en un componente medial (*gracilis*) y otro lateral (*cuneatus*) de acuerdo con observaciones previas (Nikundiwe y cols., 1982; Nikundiwe y Nieuwenhuys, 1983).

Quimioarquitectura

En el presente trabajo se han caracterizado diferentes poblaciones celulares en el núcleo de la columna dorsal y en los núcleos adyacentes, mediante la utilización de marcajes histoquímicos e inmunohistoquímicos, en algunos casos combinados con técnicas de trazado neuronal, y se ha realizado la comparación de nuestros resultados con los datos existentes en otros vertebrados.

En mamíferos la sintasa del óxido nítrico (NOS), el cual probablemente juega un papel importante como mensajero neuronal (Bredt y Snyder, 1992; Meller y Gebhart, 1993; Schuman y Madison, 1994), marca una población de neuronas en el DCN que podrían establecer circuitos locales dentro de sus límites (Valtschanoff y cols., 1993). La distribución de la NOS y de la diaforasa neuronal del dinucleótido-fosfato de

nicotinamida y adenina (NADPDd) son idénticas (Bredt y Snyder, 1992), por lo que la NADPHd puede usarse como un marcador para NOS. Nuestros resultados en las especies de anuros estudiadas, demuestran la presencia de neuronas positivas para NADPHd en el DCN, núcleo del tracto solitario y en el núcleo del tracto descendente del nervio trigémino, coincidiendo con datos obtenidos en mamíferos (Leight y cols., el 1990; Vincent y Kimura, 1992; Dohrn y cols., 1994; Takemura y cols., 1994). Igualmente en nuestro materal hemos observado fibras positivas para NADPHd en los tractos descendente del trigémino y solitario, así como en los funículos dorsal y dorsolateral.

Las proteínas ligantes de calcio, como la calbindina D28k (Calb) y la parvoalbúmina (Parv), se expresan en determinadas subpoblaciones neuronales en el sistema nervioso central y periférico (Baimbridge y cols., 1982; Garcia-Segura y cols., 1984; Braun, 1990; Celio, 1990; Ren y Ruda, 1994), e incluso marcan vías enteras y sistemas funcionales completos (Celio, 1990; Andressen y cols., 1993). Estudios recientes (Celio, 1990; Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a,b; Maslany y cols., 1992; Ren y Ruda, 1994), han demostrado una distribución preferencial de proteínas ligantes de calcio, como la Calb y la Parv en estructuras somatosensoriales.

En la rata existen neuronas positivas para Calb en determinadas láminas del asta dorsal espinal (Antal y cols., 1990; Ren y Ruda, 1994), incluyendo las células de origen de las proyecciones ascendentes espinales (Menétrey y cols., 1992b), en los núcleos sensitivos trigeminales y en menor medida en los núcleos *gracilis* y *cuneatus* (Celio, 1990; Maslany y cols., 1992). En la rata Menétrey y cols. (1992a) demostraron que las neuronas positivas para Calb constituyen una parte importante de los sistemas de proyección trigeminales y del núcleo del tracto solitario, y Celio (1990) sugirió que la Calb se expresa en toda la vía gustativa. En el mono la Calb se expresa en las proyecciones nociceptivas ascendentes trigeminotalámicas (Rausell y Jones, 1991a,b) y espinotalámicas (Rausell y cols., 1992). En nuestros experimentos, en ambas especies de anuros, se observaron en la región del óbex dos poblaciones neuronales positivas para Calb. La primera en el núcleo del tracto solitario, y la segunda en el núcleo del tracto descendente del nervio trigémino en relación con afarencias primarias trigeminales. En la médula espinal se observaron neuronas positivas para Calb en el asta dorsal. Sin embargo, apenas se observaron neuronas positivas para Calb en el DCN.

Dicho patrón de distribución de neuronas positivas para Calb sugiere que en anfibios anuros, como en mamíferos, la Calb podría estar restringida a la parte nociceptiva del sistema somatosensorial, incluyendo neuronas en el asta dorsal de la médula espinal, y en el núcleo del tracto descendente del trigémino.

La Parv en mamíferos se expresa abundantemente en la vía de la sensibilidad no nociceptiva, es decir, en el sistema columna dorsal-lemnisco medial (Celio, 1990; Rausell y cols., 1992) y en el componente no nociceptivo de las proyecciones trigeminotalámicas (Rausell y Jones, 1991a,b). En nuestros experimentos en anuros, se observó la presencia de una población neuronal diferenciada de células positivas para Parv, que se relacionan con las aferencias primarias espinales y que se asemeja a la población de neuronas de proyección talámica (ver más adelante).

Asimismo, el DCN de los anuros se caracteriza por su contenido en neuronas GABAérgicas, coincidiendo con los datos publicados en mamíferos que describen la presencia de interneuronas GABAérgicas dentro del los núcleos de la columna dorsal (Mugnaini y Oertel, 1985; Rustioni y Weinberg, 1989). Sin embargo, Pritz y Stritzel (1989a) sugirieron que el DCN del reptil (*Caiman crocodilus*) no posee neuronas inmunoreactivas para la descarboxilasa del ácido glutámico (GAD), e indicaron que el DCN en reptiles, al igual que el tálamo dorsal (ver Pritz y Stritzel, 1988), carece de neuronas para la elaboración de circuitos locales.

Igualmente, en el presente estudio se han encontrado algunas neuronas glicinérgicas en el borde ventrolateral del DCN de *Rana perezi*, en línea con datos obtenidos en la lamprea, en la que se han descrito neuronas glicinérgicas que inhiben a neuronas reticuloespinales (Dubuc y cols., 1993a,b), y en mamíferos en los que se han observado células glicinérgicas de distintos tamaños en los núcleos *gracilis* y *cuneatus* (Porucho y cols., 1992).

En experimentos inmunohistoquímicos hemos podido comprobar que la parte más lateral del DCN en anuros, está inervada por fibras inmunoreactivas para sustancia P y CGRP, que ascienden a través del tracto de Lissauer, al igual que en trabajos previos (Rosenthal y Cruce, 1985; Adli y cols., 1988; Petkó y Sánta, 1992). Además de esta proyección peptidérgica, el DCN en anuros está inervado por fibras inmunoreactivas para Leu-encefalina, neuropéptido Y y serotonina, de acuerdo con datos publicados por Ueda y cols. (1984), Merchenthaler y cols. (1989), y Lázár y cols. (1990) en anuros, así como con los resultados obtenidos en mamíferos (Steinbusch, 1981; Westman y cols., 1984; Halliday y cols., 1988; Ibuki y cols., 1989; Tamatani y cols., 1989; Conti y cols, 1990; Fabri y Conti, 1990; Blomqvist y Broman, 1993). Debido a que en nuestros experimentos con aplicaciones de trazadores en el DCN (ver apartado de conectividad) se observaron neuronas retrógradamente marcadas en el núcleo del rafe, rico en neuronas serotoninérgicas (Ueda y cols., 1984), parece probable que este núcleo sea la fuente de la inervación serotoninérgica del DCN, como es el caso en mamíferos (Willcockson y cols., 1987; Blomqvist y Broman, 1993)

Conectividad

Los artículos primero y segundo del presente capítulo se centran en el estudio del sistema columna dorsal-lemnisco medial, y de las proyecciones extralemniscales del núcleo de la columna dorsal (DCN), mediante técnicas de trazado neuronal tanto anterógrado como retrógrado, y demuestran su similitud con el mismo sistema presente en vertebrados amniotas.

Nuestros resultados en anuros corroboran, de acuerdo con estudios previos (Antal y cols., 1980; Nikundiwe y cols., 1982; Jhaveri y Frank, 1983), la existencia un sistema de aferencias primarias espinales, somatotópicamente organizado, que alcanza y delimita el DCN, de forma que la región medial (*gracilis*) está inervada por fibras procedentes de segmentos corporales lumbares y torácicos, mientras que las fibras de segmentos cervicales proyectan a la región lateral (*cuneatus*). En el caso de las aferencias braquiales las fibras primarias continúan rostralmente para inervar el complejo nuclear vestibular y, en mayor número, la capa granular del cerebelo (Antal y cols., 1980; Székely y cols., 1980).

La presencia en el funículo dorsal de aferencias espinales no primarias a los núcleos de la columna dorsal, o sistema postsináptico de la columna dorsal (PDCS), se ha demostrado en mamíferos (Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni y Kaufman, 1977; Bennett y cols., 1984; Giesler y cols., 1984), aves (Funke, 1988) y reptiles (Pritz y Stritzel, 1994). En algunos mamíferos el PDCS transmite información nociceptiva (Uddenberg, 1968; Angaut-Petit, 1975b; Bennett y cols., 1984; Kamogawa y Bennett, 1986). En anbibios ten Donkelaar y de Boer-van Huizen, (1991) sugirieron su existencia por primera vez en un estudio realizado en larvas de *Xenopus laevis*. Nuestros resultados en experimentos con aplicaciones de trazadores al DCN confirman, en adultos tanto de *Xenopus laevis* como *Rana perezi*, la presencia de aferencias espinales no primarias al DCN, las cuales se originan mayoritariamente en la sustancia gris dorsal ipsilateral, en toda la extensión rostrocaudal de la médula espinal, y se organizan de manera somatotópica, como en el caso de las aferencias primarias.

Igualmente hemos observado que la parte lateral del DCN de los anuros está inervada por fibras del tracto descendente del nervio trigémino, procedentes de los nervios trigémino, facial, glosofaringeo y vago, de acuerdo con resultados obtenidos en estudios previos (Rubinson y Friedman, 1977; Matesz y Székely, 1978; Fuller, 1979; Lowe y Russell, 1982; Altman y Dawes, 1983; Stuesse y cols., 1984; Oka y cols., 1987; González y cols., 1993).

En mamíferos la transmisión de información sensitiva, mediante el sistema columna dorsal-lemnisco medial, es controlada por vías procedentes de la corteza cerebral (Kuypers, 1958; Kuypers y Tuerck, 1964), núcleo rojo (Edwards, 1972; Weinberg y Rustioni, 1989), núcleos vestibulares (Weinberg y Rustioni, 1989), cerebelo (Sotgíu y Cesa-Bianchi, 1972), y formación reticular (Willcockson y cols., 1987; Weinberg y Rustioni, 1989). Nuestros resultados demuestran que en anuros, con la excepción de la corteza cerebral y del núcleo rojo, existe un "control" del DCN comparable al de mamíferos. En experimentos con inyecciones de trazadores en el DCN, se observó un marcaje bilateral en células del núcleo cerebeloso, núcleo ventral del nervio VIII y en la formación reticular en niveles entre los núcleos motores de los nervios VII y IX, así como en el núcleo inferior del rafe cuya proyección, presumiblemente serotonérgica, podría ser la fuente de la fuerte inervación inmunoreactiva para serotonina del DCN, de acuerdo con los datos descritos en mamíferos (Willcockson y cols., 1987; Blomqvist y Broman, 1993).

Una parte del presente estudio se ha centrado en las conexiones eferentes del DCN. Aunque son escasos en la literatura los trabajos sobre el lemnisco medial en anuros, diversos autores presentaron evidencias anatómicas y electrofisiológicas de la existencia una proyección contralateral desde la región del DCN al tálamo (Vesselkin y cols., 1971; Vesselkin y Kovacevic, 1973; Silvey y cols., 1974; Neary y Wilczynski, 1977; Urbán y Székely, 1982), y a la región lateral del torus semicircularis (Comer y Grobstein, 1981; Wilczynski, 1981; Neary y Wilczynski, 1986; Neary, 1988), lo que sugiere que el lemnisco medial, se asemeja al presente en amniotas, si bien no existen, hasta el momento, estudios basados en técnicas de trazado anterógrado sobre la anatomía detallada del lemnisco medial, que lo confirmen.

En nuestros experimentos, con aplicaciones de trazadores en el DCN, se observó el lemnisco medial como un sistema mayoritariamente contralateral que asciende a lo largo del tronco cerebral hasta el

diencéfalo. En su recorrido origina colaterales que alcanzan diversas zonas de la formación reticular rombencefálica, el área octavolateral, y la capa granular del cerebelo. En niveles mesencefálicos, el lemnisco medial inerva la región lateral del torus semicircularis, principalmente los núcleos laminar y magnocelular, y los núcleos tegmentales anterodorsal y anteroventral, así como los núcleos rojo e intersticial del fascículo longitudinal medial. En *Xenopus laevis*, hemos observado que las capas tectales intermedias y profundas están también inervadas, de acuerdo con datos previos de trazado retrógrado (Wilczynski y Northcutt, 1977; Zittlau y cols., 1988; Hofmann y cols., 1990; Masino y Grobstein, 1990). En niveles rostrales mesencefálicos, algunas fibras marcadas se distribuyen en la sustancia gris pretoral y gris pretectal. En el diencéfalo, diversas áreas talámicas, tanto dorsales como ventrales, reciben fibras del lemnisco medial. La parte ventral de los núcleos posterior, central y, en menor medida, del núcleo anterior del tálamo dorsal están inervadas, mientras que los núcleos ventromedial y ventrolateral talámicos así como el núcleo del tubérculo posterior reciben una inervación más densa.

Debido a la escasa diferenciación de los distintos componentes presentes en la placa alar de la región del óbex, y con el fin de confirmar si las citadas proyecciones ascendentes realmente se originan en el DCN, se realizaron aplicaciones de diversos trazadores en el tálamo ventral, torus semicircularis y en el cerebelo en ambas especies de anuros.

En los experimentos con aplicaciones de trazadores en el tálamo se observaron células marcadas retrógradamente en la región del DCN, mayoritariamente en el lado contralateral. Las dendritas de dichas neuronas son largas y se dirigen tanto dorsal como ventrolateralmente, alcanzando los funículos dorsal y dorsolateral, respectivamente. Sus axones se pueden seguir en el lemnisco medial contralateral. Las aplicaciones realizadas en el torus semicircularis permitieron marcar neuronas dentro del DCN, principalmente contralaterales. Se observaron dos grupos celulares distintos cuya segregación es más patente en *Rana perezi* que en *Xenopus laevis*. El primero está constituído por células localizadas en la región más dorsal de la sustancia gris, con dendritas que se extienden en la zona de fibras situada dorsalmente a ellas. Sus axones se dirigen ventromedialmente, para cruzar la línea media y formar parte del lemnisco medial. El segundo grupo de células marcadas se localiza en la zona lateral marginal de la sustancia gris dorsal, desde el nivel del óbex al segundo segmento espinal, sus dendritas se dirigen principalmente a la parte dorsal del funículo lateral y al funículo dorsal, y sus axones ingresan en el lemnisco medial.

Además, en los experimentos con aplicaciones tanto en el tálamo como en el torus semicircularis, se marcaron bilateralmente algunas células en el núcleo del tracto descendente del nervio trigémino, coincidiendo con datos previos (Comer y Grobstein, 1991; M.Muñoz y cols., 1994).

El componente ventrolateral del DCN proyecta al tálamo y al torus semicircularis y se extiende caudalmente hasta el segundo segmento espinal. Las dendritas de sus neuronas se dirigen principalmente al funículo dorsolateral, mientras que sus axones se incorporan al lemnisco medial contralateral. Se podría establecer una comparación con el núcleo cervical lateral de los mamíferos, el cual recibe información somatosensorial a través del tracto espinocervical y proyecta, contralateralmente a través del lemnisco medial, a regiones somatosensoriales mesencefálicas y talámicas (Willis y Coggeshall, 1991).

El presente estudio confirma la presencia en anuros del sistema columna dorsal-lemnisco medial cuyas dianas rombencefálicas, mesencefálicas y diencefálicas son mucho más diversas y extensas de lo que se había sugerido en estudios previos (Vesselkin y cols., 1971; Silvey y cols., 1974; Neary y Wilczynski, 1977; Comer y Grobstein, 1981; Wilczynski, 1981; Forehand y Farel, 1982; Urbán y Székely, 1982; Neary, 1988). La vía lemniscal en anuros parece ser, en líneas generales, similar a la de amniotas (reptiles: Ebbesson, 1978; Siemen y Künzle, 1994a; aves: Wild, 1989; mamíferos: Hazlett y cols., 1972; Hand y van-Winkle, 1977; Feldman y Kruger, 1980; Berkley y cols., 1986; ver también Willis y Coggeshall, 1991)

Además del lemnisco medial, en nuestro material con aplicaciones de trazador en el DCN, observamos que éste núcleo origina proyecciones extralemniscales ipsilaterales a la corteza cerebelosa, y proyecciones bilaterales a la médula espinal. En experimentos de trazado retrógrado, con aplicaciones en el cerebelo, observamos neuronas marcadas bilateralmente en el DCN, aunque en mayor número en el lado ipsilateral de acuerdo con datos previos (González y cols., 1984). Los axones de dichas neuronas parecen discurrir junto con las fibras primarias espinales que ascienden igualmente hasta la capa granular del cerebelo. Las neuronas del DCN que proyectan a la médula espinal envían sus axones a través del funículo dorsal ipsilateral, y terminan en el asta dorsal y de forma más dispersa en los campos lateral y ventral, principalmente en niveles cervicales. Dicha proyección fue igualmente confirmada en experimentos de trazado retógrado, con aplicaciones en diversos niveles espinales, en los que se marcó una población celular en el DCN ipsilateral.

En el tercer artículo de este capítulo se presentan evidencias en favor de la existencia en anuros del sistema espino-cervico-talámico, descrito en mamíferos y formado por el tracto espinocervical que termina en el núcleo cervical lateral (LCN), y los tractos cervicomesencefálico y cervicotalámico respectivamente que forman parte del lemnisco medial (ver Willis y Coggeshall, 1991). Hasta el momento, el sistema espino-cervico-talámico no ha sido descrito en vertebrados no mamíferos, en los que únicamente se dispone de datos aislados que sugieren su existencia (Ebbesson, 1967; Finger, 1981; Forehand y Farel, 1982; Ito y cols., 1986; Necker, 1989; Ronan y Northcutt, 1990).

En anfibios el tracto espinocervical no ha sido descrito, aunque en estudios basados en técnicas degenerativas se observó la presencia de proyecciones espinales ascendentes en el funículo dorsolateral (Ebbesson, 1976). Algunas de dichas proyecciones corresponden a aferencias primarias del tracto de Lissauer (Antal y cols., 1980; Nikundiwe y cols., 1982), si bien podrían igualmente incluir fibras no primarias del tracto espinocervical.

En el presente estudio se han realizado experimentos *in vitro* de trazado anterógrado y retrógrado en *Xenopus laevis*, con objeto de caracterizar el patrón de terminación del posible tracto espinocervical, así como las células que lo originan.

En experimentos con aplicaciones de BDA, en la región dorsal de la médula espinal en segmentos lumbares, torácicos y cervicales, se observaron tractos de fibras en los funículos dorsal y dorsolateral, rostral y caudalmente a los sitios de inyección.

Las fibras que ascienden en el funículo dorsal presentan un ordenamiento somatotópico, de acuerdo con los datos descritos para las proyecciones primarias (Antal y cols., 1980; Nikundiwe y cols., 1982; Jhaveri y Frank, 1983) y no primarias (A.Muñoz y cols., 1995), y emiten colaterales que alcanzan principalmente los campos dorsal y lateral y ocasionalmente los campos ventrales en distintos segmentos espinales, dependiendo del nivel de la aplicación. En la región del óbex dichas fibras inervan el DCN ipsilateral, de acuerdo con la somatotopía mediolateral que presentan en el funículo dorsal.

Las fibras del funículo dorsolateral originan, rostral y caudalmente al nivel de inyección, colaterales que inervan los campos dorsal y lateral de distintos segmentos espinales, según el nivel de la aplicación. Algunas de estas fibras podrían ser aferencias primarias espinales pertenecientes al tracto de Lissauer (Antal y cols., 1980; Nikundiwe y cols, 1982), aunque también podrían existir en anfibios proyecciones espinales no primarias intersegmentarias a través del funículo dorsolateral, como ocurre en mamíferos, en los que las neuronas del tracto espinocervical emiten colaterales a diversas dianas en distintos niveles espinales (Snow y cols., 1976; Brown y cols, 1977; Rastad y cols, 1977; Jankowska y cols., 1979; Maxwell y Koerber, 1986; Cao y cols., 1993).

En segmentos cervicales superiores y a nivel del óbex, la mayoría de las fibras del funículo dorsolateral, procedentes de todos los niveles espinales, se tuercen dorsomedialmente para inervar masivamente las neuronas situadas en el límite ventrolateral del asta dorsal. Dicha proyección representa en anfibios un posible equivalente del tracto espinocervical, presente en mamíferos (Willis y Coggeshall, 1991). En niveles ligeramente caudales al óbex se observó una región en la sustancia gris, con forma de banda, en la que se produce un solapamiento de las proyecciones, procedentes de los funículos dorsal y dorsolateral, que inervan el DCN y el equivalente en anfibios del LCN respectivamente (ver más adelante). Dicha región delimita el borde ventral del tracto descendente del trigémino, y de las células relacionadas con él (González y cols., 1993).

El tracto espinocervical en mamíferos es una proyección glutamatérgica (Broman y cols., 1990; Kechagias y Broman, 1994) que se origina en células espinales que reciben proyecciones desde la periferia (Willis y Coggeshall, 1991). Dichas neuronas se distribuyen ipsilateralmente en el núcleo propio, la sustancia gelatinosa de Rolando, láminas IV, V, y en niveles cervicales en las laminas I, VI y VII; y contralateralmente en las láminas I, VII y VIII (Brown y cols, 1980; Baker y Giesler, 1984; Craig, 1976, 1978; Craig y cols., 1992).

En vertebrados no mamíferos el tracto espinocervical no se ha descrito como tal, si bien existen evidencias, basadas en experimentos de degeneración y de trazado anterógrado y retrógrado, en favor de su existencia en aves (van den Akker, 1970; Funke y Necker, 1986; Funke 1988; Necker, 1991) y reptiles (Ebbesson; 1967). En agnatos, (Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990), así como en peces cartilaginosos (Hayle, 1973a,b; Ebbesson y Hodde, 1981; Smeets y cols., 1984) y óseos (Hayle 1973a,b; Finger, 1981), se ha demostrado la presencia de proyecciones espinales ascendentes a través de la región dorsal del funículo lateral, aunque no se ha descrito el tracto espinocervical.

Con objeto de caracterizar la población neuronal cuyas proyecciones ascienden en el funículo

dorsolateral, se realizaron experimentos *in vitro* con pequeñas aplicaciones de BDA en el funículo dorsolateral, a nivel del óbex y en el segundo segmento espinal cervical. Dichos experimentos marcaron una extensa población de células en el asta dorsal, principalmente ipsilateral, desde niveles cervicales hasta niveles sacros. La mayoría de las células se encuentran en la región más profunda del campo dorsal de la sustancia gris espinal. Igualmente se observan neuronas marcadas, aunque en menor número, ipsilateralmente en la parte superficial del asta dorsal, y contralateralmente en los campos ventrales de la sustancia gris, en segmentos cervicales inferiores y torácicos.

El LCN en mamíferos está formado por neuronas adyacentes así como incluídas dentro del funículo dorsolateral, y se extiende desde el nivel del óbex hasta el segundo segmento espinal. Los axones de sus neuronas ascienden a través del lemnisco medial y alcanzan, principalmente, la región intercolicular mesencefálica somatosensorial, así como el complejo ventrobasal del tálamo, formando los tractos cervicomesencefálico y cervicotalámico, respectivamente (Willis y Coggeshall, 1991).

Aunque en aves no ha sido descrito como tal núcleo, existen evidencias que indican la existencia del LCN (Ramón y Cajal, 1911; Karten y Hodos, 1967; van den Akker, 1970; Necker, 1989, 1990), sin embargo, en reptiles no se ha mencionado nada respecto a la posible existencia del LCN (Ebbesson 1967, 1969; Kusuma, 1979; Künzle y Woodson, 1982; Pritz y Stritzel, 1986; Hoogland, 1981, 1982; Pritz y Stritzel, 1989, el 1990).

En anamniotas los datos sobre la presencia de un LCN son escasos. En teleósteos, se ha descrito un núcleo comparable a nivel del óbex y en los primeros segmentos espinales cervicales (Finger, 1981; Ito y cols., 1986). En *Sebasticus marmoratus* Ito y cols. (1986) describieron el LCN en base a sus proyecciones al núcleo ventromedial talámico. Adicionalmente, en lampreas, en experimentos con aplicaciones diencefálicas o mesencefálicas de HRP, se observaron neuronas marcadas retrógradamente a nivel del óbex y en los primeros segmentos cervicales espinales, sugieriéndose la presencia de un posible LCN en agnatos (Ronan y Northcutt, 1990).

En anuros, mediante estudios basados en tinciones de Nissl (Opdam y cols., 1976; Nikundiwe y Nieuwenhuys, 1983, o de Golgi (Ebbesson, 1976), no se ha descrito el LCN como una entidad citoarquitectónica definida. Sin embargo, la existencia del LCN fue sugerida en un trabajo sobre el desarrollo ontogénico de la médula espinal (Forehand y Farel, 1982), en base al marcaje retrógrado observado en experimentos en los se realizaron aplicaciones de HRP en la región lateral de la formación reticular rombencefálica, y en el mesencéfalo; asimismo A. Muñoz y cols. (1995; artículo segundo del presente capítulo), mediante aplicaciones de trazadores en el tálamo y en el torus semicircularis, marcaron retrógradamente neuronas, separadas ventrolateralmente de las del DCN en *Rana perezi*, y parcialmente entremezcladas con ellas en *Xenopus laevis*, que fueron consideradas entonces como un componente ventrolateral de este núcleo, con cierta similitud al LCN por su relación con el funículo dorsolateral.

En el tercer artículo del presente capítulo se realizaron experimentos adicionales con aplicaciones de BDA en el tálamo ventral o en el torus semicircularis. En ambos tipos de experimentos se marcó retrógradamente una población neuronal en un área de la placa alar de la región del óbex, que incluye dos

componentes no distinguibles citoarquitectónicamente: el DCN, localizado dorsomedialmente y en relación con las fibras ascendentes del funículo dorsal, y el LCN en posiciones más ventrolaterales donde terminan masivamente las fibras del funículo dorsolateral. Aunque solo algunas células del LCN están realmente localizadas dentro del propio funículo dorsolateral, las dendritas de todas ellas se dirigen mayoritariamente en dirección ventrolateral, extendiéndose a lo largo del funículo dorsolateral, a través del cuál pueden recibir información espinal ascendente. No obstante, a nivel del óbex, donde coexisten el DCN y el LCN, es difícil discernir a cuál de las dos poblaciones pertenecen las neuronas localizadas en posiciones intermedias, únicamente la orientación de sus dendritas, dorsalmente hacia el funículo dorsal en el caso de neuronas del DCN, o ventrolateralmente hacia el funículo dorsolateral en el caso de las del LCN, así como la extensión más rostral del primer núcleo, permiten establecer diferencias

En el presente capítulo se presentan evidencias que demuestran la existencia en anuros de los sistemas columna dorsal-lemnisco medial y espino-cervico-talámico, con un patrón básico de organización, similar al descrito en amniotas. Si bien existen datos que sugieren una organización similar en peces, no se han realizado estudios recientes, mediante técnicas de trazado neuronal e inmunohistoquímicas que lo demuestren, por lo que resulta necesario su estudio con objeto de establecer si existe un esquema similar en la anatomía de sistemas somatosensoriales en todos los vertebrados.

CAPÍTULO 6

Organization of the caudal rhombencephalic alar plate of the ribbed newt Pleurodeles waltl: Evidence for the presence of dorsal column and lateral cervical nuclei

6.1

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Based on: Brain Behaviour and Evolution (Submitted)

Key words: amphibians, ascending spinal projections, dorsal column nucleus, lateral cervical nucleus, medial lemniscus, torus semicircularis, thalamus

ABSTRACT

As part of a research programme on the evolution of somatosensory systems in vertebrates, the cytoarchitecture, chemoarchitecture, and fiber connections of the caudal rhombencephalic alar plate were studied in the ribbed newt, *Pleurodeles waltl*. This part of the brain stem includes ill-defined dorsal column and lateral cervical nuclei. A cytoarchitectonic analysis revealed that the caudal medullary alar plate consists of an inner and an outer cell layer. The outer cell layer at the obex level forms the dorsal column nucleus (DCN), whereas the ventrolateral part of this cell layer forms a lateral cervical nucleus (LCN). NADPH-diaphorase histochemistry and calbindin D-28k immunohistochemistry clearly delineate the main components of the compact inner cell layer, i.e. the nucleus of the solitary tract dorsally and the nucleus of the descending trigeminal tract ventrally. Neither NADPH-diaphorase-labeled nor calbindin D-28k

positive neurons were observed in DCN and LCN. With anterograde and retrograde tracing the DCN and LCN were further delineated. Labeling of ascending dorsal root afferents showed that the dorsal column and the DCN are somatotopically arranged: lumbar primary afferents terminate on medial DCN neurons, whereas cervical primary afferents terminate on lateral DCN neurons. The LCN is densely innervated by the dorsolateral funiculus. Retrograde tracing showed extensive predominantly contralateral projections of both the DCN and LCN to the torus semicircularis and the ventral thalamus.

These data show that even in the poorly segregated caudal rhombencephalic alar plate of urodeles a DCN and LCN can be distinguished with afferent and efferent projections comparable to those in anurans and other terrestrial vertebrates.

INTRODUCTION

Ascending spinal projections in the brain of vertebrates include three main systems: 1) mainly ipsilateral primary and non-primary (i.e. the postsynaptic dorsal column system) projections via the dorsal funiculus that terminate primarily in the dorsal column nucleus within the alar plate of the most caudal part of the medulla; 2) ipsilateral, non-primary projections via the dorsolateral funiculus that terminate at upper cervical segments, in the lateral cervical nucleus and in various rhombencephalic and mesencephalic targets; 3) secondary projections via the anterolateral column of ventral quadrant, i.e. the ventral and ventrolateral funiculi, to the reticular formation, mesencephalon and thalamus (A. Muñoz et al., 1995, 1996a,b). Additionally, both the dorsal column and the lateral cervical nuclei give rise to contralateral and, to a lesser extent, ipsilateral, projections to the mesencephalon and thalamus through the medial lemniscus (Willis and Coggeshall, 1991).

In urodeles, in previous studies based on silver staining (Herrick, 1914, 1930) and on anterograde degeneration (Nieuwenhuys and Cornelisz, 1971) techniques, spinal ascending fibers in the ventral and lateral funiculi were described that reach rhombencephalic and mesencephalic targets. Recent studies based on modern tract-tracing techniques demonstrated a more elaborate pattern of spinal fibers that ascend in the ventral quadrant of the spinal cord (A. Muñoz et al., 1994a). In anurans, the dorsal column nucleus (DCN) is characterized by its afferent (Antal et al., 1980; Nikundiwe et al., 1982; ten Donkelaar and de Boer van Huizen, 1991; A. Muñoz et al., 1995) and efferent (Neary and Wilczynski, 1977; Forehand and Farel, 1982; A. Muñoz et al., 1994b, 1995) connections, as well as immunohistochemically (A. Muñoz et al., 1995) and electrophysiologically (Silvey et al., 1974; Urbán and Székely, 1982). Moreover, in anurans, the presence of a spinocervical tract and other ascending spinal projections in the dorsolateral funiculus, as well as the existence of a lateral cervical nucleus (LCN) and its mesencephalic and diencephalic medial lemniscal projections were described recently (A. Muñoz et al., 1996a). In urodeles, studies based on silver staining (Herrick, 1914, 1930, 1944), anterograde degeneration (Nieuwenhuys and Cornelisz, 1971) and tract-tracing (Roth and Wake, 1985) techniques suggest that spinal primary afferent projections to the dorsal gray at the obex level are somatotopically organized. In the tiger salamander, *Ambystoma tigrinum*, Herrick (1944) reported the presence of a poorly segregated nucleus commissuralis in the obex region related to visceral afferents of the solitary tract, and a nucleus of the funiculus dorsalis probably receiving lateral line,

vestibular, trigeminal and, especially, ascending spinal afferents. Neither a DCN nor an LCN were noted in a later cytoarchitectonic study of the brain stem in the axolotl (Opdam and Nieuwenhuys, 1976).

The central nervous system of urodeles is often considered to be primitive. However, careful studies by e.g. Roth and co-workers (summarized by Roth, 1987 and Roth et al., 1993) show that the salamander brain possesses virtually all the anatomical and functional properties found in anurans, which are usually regarded as being much more evolved with respect to the guidance of comparable behavior. Recent studies (e.g., Northcutt, 1987; Roth et al., 1992, 1993) suggest that the evolution of the salamander nervous system is characterized by *secondary simplification*, which gives the impression that the brains of salamanders are more primitive than the phylogenetic position of salamanders, as tetrapods, implies (Roth et al., 1992). In the present study, an attempt is made to characterize the various neuronal components of the caudal part of the medullary alar plate in the ribbed newt, *Pleurodeles waltl*. It can be expected that within this ill-defined part of the brain a DCN and an LCN with appropriate fiber connections are present. The present study includes: 1) a discussion of the cytoarchitectonics of the caudal part of the medullary alar plate; 2) data on the chemical neuroanatomy of this ill-defined area, and 3) an analysis of its fiber connections.

MATERIALS AND METHODS

The data presented are based on a total of 77 adult speciments of *Pleurodeles waltl* obtained from laboratory stock of the Department of Cell Biology, University Complutense of Madrid or donated by Dr. Gerhard Roth from the University of Bremen (Germany). For a cytoarchitectonic analysis of the obex region, Nissl (cresylecht violet)-stained series were available, cut either transversally, horizontally or sagittally at a thickness of 20 µm. The histochemical, immunohistochemical and tract-tracing techniques used in this study are discussed below. The nomenclature used is based on studies by Opdam and Nieuwenhuys (1976) on the brain stem.

NADPH-diaphorase histochemistry

Eight animals were anesthetized in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz), and subsequently perfused transcardially with a 0.9% saline solution followed by a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were taken out and further fixed in the same fixative for six to eight hours at room temperature. They were subsequently immersed in a 30% phosphate buffer solution at 4°C, embedded in a 15% gelatin and 30% sucrose solution, and stored for five hours in a 4% formaldehyde solution at room temperature. On a freezing microtome, 30 or 40 µm frontal sections were cut and collected in phosphate buffer. Free-floating sections were incubated in a medium containing 1mM ß-NADPH, 0.8mM nitroblue tetrazolium and 0.06% Triton X-100 in 0.1M phosphate buffer (pH 7.6) at 37°C for one to two hours. After incubation, the sections were thoroughly rinsed in PB. In three cases after rinsing, the sections were also processed for tyrosine hydroxylase immunohistochemistry as described below.

Immunohistochemical procedures

For the immunohistochemical procedures used, animals were anesthetized with an overdose of MS222, and transcardially perfused with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were removed, postfixed for 4-7 hours in the same fixative and embedded in 15% gelatin with 30% sucrose. Brains were cut frontally on a freezing microtome or on a vibratome at 40 µm, and the sections were collected in Tris-saline (TBS) buffer (0.05M, pH 7.6). All antibodies were diluted in 0.1% normal serum of the species in which the secondary antibody was raised in TBS with 0.1% Triton X-100 (Sigma). The sections were preincubated for 1-2h in TBS containing 3% normal serum and 0.1% Triton X-100 and subsequently incubated in the primary antibody-containing solution for 12-60h at 4°C. Controls for the immunohistochemistry experiments included: 1) staining some selected sections with pre-immune mouse serum (1:1,000 for tyrosine hydroxylase, and calbindin D-28k immunohistochemistry), or with rabbit serum (1:1,000 for neuropeptide Y and serotonin; 1:2,000 for substance P and Leu-enkephalin immunohistochemistry) instead of the primary antibody, and 2) controls in which either the primary antibody, secondary antibody or the peroxidase-antiperoxidase complex (PAP) was omitted. As an additional control for the specificity of the labeling of calcium-binding proteins, some sections were stained using antibodies that had been pre-absorbed in an excess of calbindin (Sigma). The sections were processed according to the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979) in a series of incubations with the following antisera:

1) *tyrosine hydroxylase (TH) immunohistochemistry (8 cases):* a) mouse anti-TH (Incstar), diluted 1:1,000, for 24-72 hours; b) goat anti-mouse (Nordic), diluted 1:100, for three to five hours, and c) rat peroxidase-antiperoxidase (PAP) complex (Incstar), diluted 1:500 for two hours.

2) *Nitric oxide synthase (NOS): (5 cases)* a) sheep anti-NOS (gift from Dr. Emson), diluted 1:20,000, for 36-60 hours, b) biotinylated rabbit anti-sheep diluted 1:200 for two hours at room temperatute and c) ABC Elite Kit (Vector) for 1.5h at room temperature. For fluorescent immunolabeling a rhodamine-coupled donkey anti-sheep (Chemicon) or a fluorescein-coupled rabbit anti-sheep (Vector) were used as second antibody diluted 1:100 for two hours.

For double TH/NOS immunofluorescence labeling (3 cases) the section were simultaneusly incubated in a solution containing the same first antibodies as for single labeling (TH and NOS). After rinsing, sections were incubated first in a solution containing biotynilated horse anti-mouse (Vector) diluted 1:100 for one hour at room temperature, and then, after rinsing in a mixture containing a Texas red-coupled streptavidin complex (Vector) diluted 1:100 and a rabbit anti-sheep Fluoresceine-coupled (Vector) diluted 1:75 for two hours at room temperature.

3) *calbindin D-28k immunohistochemistry (5 cases):* a mouse anti-calbindin D-28k (Sigma), diluted 1:1,000 for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for three to five hours, and c) rat PAP complex (Incstar), diluted 1:500, for two hours.

4) *Substance P (SP) (5 cases) and Leu-enkephalin (L-Enk) immunohistochemistry (4 cases):* a) rabbit anti-SP or rabbit anti-L-Enk (CRB), diluted 1:2,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for two hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for two hours;

5) *Neuropeptide Y (NPY) immunohistochemistry (4 cases):* a) rabbit anti-NPY serum (gift from Dr. J.D. Mikkelsen), diluted 1:1,000, for 36 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for one hour, and c) rabbit PAP complex (Dakopatts), diluted 1:800, for one hour;

6) *serotonin (5-HT) immunohistochemistry (5 cases):* a) rabbit anti 5-HT (gift from Dr. H.W.M. Steinbusch), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for two hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for two hours.

In all cases, after rinsing, the sections were incubated with 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in phosphate buffer, for 10-15 minutes. After another rinsing, the sections were mounted on glass slides, dried overnight, and coverslipped. In most cases, visualization of the immunostaining was improved by processing the sections with nickel-enhanced DAB (0.05% DAB, 0.01% H_2O_2, 0.04% ammonium nickel sulphate in phosphate buffer).

Tract tracing experiments

For all the surgical procedures the animals were anesthetized by inmersion in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz) in tap water. For the tract-tracing experiments the bidirectionally transported tracer biotinylated dextran amine (BDA 10kD, Molecular Probes) was either iontophoretically (5-8 μA, 7 sec. on/7 sec. off) injected (a 10% solution in 0,1 phosphate buffer, pH 7.4) through a glass micropipette (outer tip 20-30 μm) or applied recrystallized at the tip of fine tungsten needles or glass micropipettes, to the dorsal part of the cervical spinal cord (7 cases), to the ventral thalamus (5 cases) and to the torus semicircularis (5 cases). The animals were allowed to survive for 7-10 days. They were then reanesthetized with an overdose of MS222 and perfused transcardially with saline followed by 200ml of 4% paraformaldehyde in PB. In all cases the brain and spinal cord were removed and further fixed for 3-7h, cryoprotected in 30% sucrose in PB for 3-5h at 4°C and embedded in a medium of 15% gelatin with 30% sucrose in PB. The blocks were fixed for 7h in a 2% formaldehyde, 30% sucrose. Sections were cut transversally at 40 μm on a freezing microtome and collected in PB. For visualizing BDA, an avidine biotin complex (Vectastain ABC Standard Kit, Vector Laboratories) was used. Histochemistry for HRP followed the heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product according to Adams (1981).

Selected sections from tract-tracing, histochemical and immunohistochemical experiments were counterstained with 1% cresyl violet or toluidine blue in distilled water. After rinsing all the sections were mounted on glass slides (mounting medium 0.25% gelatin in Tris buffer), dried, and coverslipped with Entellan (Sigma) or with Vectashiel (Vector) for experiments using fluorescence microscopy .

RESULTS

Delineation and (immuno)histochemical characterization of cell groups in the urodele caudal rhombencephalic alar plate.

Cytoarchitecture

In the alar plate of the caudal medulla of *Pleurodeles waltl*, at obex levels, evidence for different

cell components such as a dorsal column nucleus (DCN), a lateral cervical nucleus (LCN), a nucleus of the solitary tract and a nucleus of the descending tract of the trigeminal nerve can be observed in Nissl-stained material (Fig. 1).

The neurons in the alar plate in *Pleurodeles waltl* form a curved, continuous zone of periventricular gray in which individual cell groups are difficult to recognize. However, in the caudal brain stem and upper spinal cord levels a distinct cellular band (outer cell layer), oriented dorsomedially-to-ventrolaterally, separated from the main periventricular layer (inner cell layer) by a thin layer of white substance, can be distinuished (Figure 1B,C). In the upper cervical cord just caudal to the obex, the inner cell layer is rather thick and compact, whereas in its outer part a thinner cell layer (outer layer) is present although a clear boundary between the two layers can not be observed (Figure 1D,E). The outer cell layer is more evident dorsomedially where the cells form what will be tentatively considered as the DCN since they are closely related to the site of termination of the ascending dorsal funicular spinal fibers (Figure 1D,E). At the ventrolateral aspect of the outer cell layer, neurons are more sparsely organized and related to the fibers passing via the dorsolateral funiculus. Some of them are located even within the dorsolateral funiculus. This ventrolateral cluster of neurons forms what will be termed the LCN (Figure 1B,C). Here, a thin lamina of white matter separates the inner and the outer cell layers. Rostral to the obex, this separation is more evident and due to the presence of the solitary tract (Figure 1A). The DCN is found dorsal to the solitary tract and extends as far rostrally as the motor nucleus of the Xth nerve, where its cells become intermingled with cells of the caudal part of the lateral line area. The DCN consists of small (8-10 μm) and medium-sized (15 μm) multipolar neurons. No medial ('gracile') and lateral ('cuneate') compartments can be distinguished.

The ventrolateral aspect of the DCN extends into areas where its cells are difficult to distinguish from those of the LCN or even the nucleus of the descending trigeminal tract. The latter nucleus and the nucleus of the solitary tract are located within the dense inner cell layer. They are rather poorly segregated from each other or from the reticular elements located ventromedially. In general, neurons of the nucleus of the solitary tract are located dorsomedial to the trigeminal neurons. The nucleus of the descending trigeminal nerve can be characterized by the presence of trigeminal afferents in tract-tracing experiments.

Chemoarchitecture

NADPHd histochemistry

In the caudal part of the rhombencephalic alar plate of *Pleurodeles waltl*, just rostral to the obex, NADPHd-positive neurons were observed in the outer half of the inner cell layer (Fig. 2). This cell population forms part of the nucleus of the descending trigeminal tract and extends caudally up to the cervical spinal cord. It contains medium-sized, round-to-oval neurons with long NADPHd-positive processes directed dorsolaterally towards the descending tract of the trigeminal nerve. Additionally, NADPH-positive cells were found in the nucleus of the solitary tract intermingled with catecholaminergic neurons, in experiments in which NADPHd-staining was combined with immunohistochemistry against tyrosine hydroxylase. However, NADPHd-positive neurons in the nucleus of the solitary tract were more numerous rostrally. At the obex level only a few positive cells were observed in the outer half of the inner cell layer.

No NADPH-positive cells were found in the area of the DCN. NADPHd-positive fibers were found predominantly in two bundles, i.e. the descending trigeminal tract and the rostral portion of the solitary tract. Weakly labeled fibers were also present in the dorsal and dorsolateral funiculi close to the obex region. Heavier staining within these funiculi is present in the spinal cord.

Immunohistochemical data

The distribution of nitric oxide synthase (NOS)-immunopositive neurons in the caudal part of the rhombencephalic alar plate is shown in Figure 3, the presence of tyrosine hydroxylase-positive neurons in Figure 4, and the distribution of the calcium-binding protein calbindin D-28k in Figure 5.

Nitric Oxide Synthase (NOS): The distribution of NOS-immunopositive neurons in the medullary alar plate of *Pleurodeles waltl* is comparable to that observed after NADPHd-staining. Weak labeling was observed in the nucleus of the descending trigeminal tract. Neurons with long processes directed dorsolaterally towards the descending trigeminal tract were observed. Additionally, some, more dorsomedially located, neurons were observed that may belong to either the nucleus of the solitary tract or the DCN.

Tyrosine hydroxylase (TH): TH immunohistochemistry (Figure 4) revealed, in line with previous studies (González and Smeets, 1991, 1995), the presence of catecholaminergic neurons in the alar plate of the caudal medulla. This neuronal population extends from the level of the IXth motor nucleus to the transition with the spinal cord, and they are mainly located ventromedial to the solitary tract. At the obex level, TH-positive cells are located in the outer aspect of the inner cell layer as a band oriented from dorsomedial to ventrolateral. TH-positive neurons have long processes directed horizontally or ventrolaterally to the dorsolateral funiculus. In double labeling (TH/NADPHd) experiments it was observed that the most ventrolaterally located neurons overlap with the NADPHd-positive neurons of the nucleus of the descending trigeminal tract. TH-positive processes of neurons of the nucleus of the solitary tract are also directed towards the dorsolateral funiculus and intermingle with the NADPHd-positive, dorsolaterally oriented processes of trigeminal neurons. Double TH/NOS-immunofluorescence experiments revealed results in line with that described for TH/NADPHd-staining.

Calbindin D-28K (Calb): In the caudal rhombencephalic alar plate two Calb-positive neuron populations can be distinguished (Figure 5). The first coincides with the nucleus of the solitary tract and it contains a band of neurons with processes towards the dorsolateral funiculus. The second is located in the dorsolateral gray and forms part of the nucleus of the descending trigeminal tract. Its cells have positive processes extending to the descending trigeminal tract. The highest density of both cell populations were observed at the obex level where the labeled processes of each population cross each other in their course to the dorsolateral funiculus and the descending trigeminal tract, respectively. Calb-positive neurons were not found in the DCN. In the dorsal horn of the spinal cord, however, an abundant Calb-positive cell population was observed that at cervical levels includes those cells of the caudal extent of the nucleus of the descending trigeminal tract.

Serotonergic (5-HT) and peptidergic labeling: Immunohistochemical labeling showed

that substance P-immunoreactive fibers are present in the dorsal aspect of the dorsolateral funiculus, more sparsely in the dorsal funiculus and almost no positive fibers are present in the descending trigeminal tract (Figure 7C,D). Some fibers leave the dorsolateral funiculus and may include primary afferents of the the tract of Lissauer and other non-primary projections, and innervate mainly the LCN area. Additionally, a few substance P-positive varicose fibers are present in the DCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract. A similar pattern of Leu-enkephalin-immunoreactive terminal structures was found in the dorsal grey at obex levels in both the inner and the outer cell layers, including the DCN, LCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract. These enkephalinergic fibers penetrate the gray from the dorsal funiculus, descending trigeminal tract and mainly from the dorsolateral funiculus. Neuropeptide Y-immunoreactive fibers enter the dorsolateral gray from the dorsal aspect of the lateral funiculus and form varicose fibers and terminal boutons. Serotonin-immunoreactive fibers innervate the DCN and LCN, and the adjacent structures of the inner cell layer including the nucleus of the solitary tract and nucleus of the descending trigeminal tract.

Tract-tracing experiments

In the previous sections evidence was provided for the presence of a DCN, an LCN, a nucleus of the descending trigeminal tract and a nucleus of the solitary tract within the ill-defined caudal rhombencephalic alar plate. In order to further characterize the DCN and the LCN of *Pleurodeles waltl*, the afferent and efferent connections of these structures were analyzed. To delineate the nucleus of the descending trigeminal tract, also the trigeminal innervation of the caudal rhombencephalon was studied.

Afferent connections

The primary afferent projections to the caudal rhombencephalic alar plate have been studied by applying BDA to cervical and lumbar spinal dorsal roots as well as to the trigeminal nerve root. Non-primary ascending projections from the spinal cord were studied by applying BDA to the dorsal horn of the cervical spinal cord.

Ascending dorsal root afferents.

Following the application of BDA to either brachial or lumbar dorsal roots, the labeled afferent fibers upon entering the spinal cord bifurcate into ascending and descending tracts. Two components can be distinguished in the spinal white matter: a medial bundle of thick fibers that enters the dorsal funiculus, and a more laterally located group of thin fibers within the dorsal portion of the dorsolateral funiculus, i.e. the tract of Lissauer. Both fiber systems project to widespread spinal and supraspinal regions in a manner that largely resembles that described for anurans (Antal et al., 1980; Székely et al., 1980; Nikundiwe et al., 1982a; Muñoz et al., 1996b). In the present study only the distribution of axons that innervate the caudal part of the rhombencephalic alar plate will be discussed. Ascending fibers in Lissauer's tract could be traced rostrally for only a few spinal segments giving off collateral branches that arborize and terminate in a ventral neuropil located at the lateral aspect of the deep dorsal and intermediate spinal grey. In the lumbar BDA experiments, ascending fibers in Lissauer's tract fade at thoracic levels and do not reach the obex region (Figure 6A). BDA applications to the second spinal dorsal root

labeled fibers in Lissauer's tract that ascend in a superficial position within the dorsolateral funiculus and reach the rostral pole of the IX-Xth cranial nerve root complex (Figure 6B). At upper cervical spinal levels and in the obex region a few branches leave the tract and turn medially to innervate the LCN in the outer cell layer and, to a lesser extent, the outer aspect of the inner cell layer where the caudal aspect of the nucleus of the descending trigeminal tract is located. More rostrally, Lissauer's tract courses ventrolaterally and some fibers leave the tract to arborize within the white matter adjacent to the reticular formation. The medial component is formed by ascending and descending dorsal funicular fibers that innervate the spinal gray of adjacent levels rostral and caudal to the entrance of the different roots. The rostrocaudal extent of this spinal projection exceeds that of Lissauer's tract. Most of the fibers arborize and form a terminal field in the dorsal aspect of the spinal gray, although some fibers continue ventrally to terminate within the central spinal gray.

Spinal primary afferents ascend to the obex region, somatotopically organized, within the dorsal funiculus. Fibers from lumbar dorsal roots occupy positions medial to those of cervical origin (Figure 6). These ascending spinal projections outline the DCN at the obex level (Figure A,B). The terminal fields in this area largely resemble the organization of the fibers in the dorsal funiculus. Thus, medially situated axons from lumbar dorsal root ganglion cells terminate on medial cells in the DCN, whereas laterally located fibers arising from cervical dorsal root ganglion cells end on lateral cells, with a certain degree of overlap in the projection. Most of the primary afferents terminate dorsal to the cells of the DCN. However, some varicose fibers extend more ventrolaterally and reach the area where the cells of the LCN are located. Rostral to the obex level, tightly packed dorsal funicular fibers gradually turn ventrolaterally and ascend throughout the medulla in a position dorsal to the descending trigeminal tract. Lumbar primary afferents hardly extend beyond the rostral limits of the DCN. Brachial primary afferents reach the cerebellum where they arborize profusely within the granule layer. Throughout the rhombencephalon, varicose fibers leave the tract and arborize within the white matter where dendrites of the adjacent periventricular cells of the reticular formation, nucleus of the solitary tract, nucleus of the descending trigeminal tract and the octavolateral area may be contacted.

Trigeminal afferents

After application of BDA to the proximal stump of a severed trigeminal nerve root, the primary trigeminal afferents were labeled (Figure 8). At the obex level, primary trigeminal fibers leave the descending trigeminal tract ventromedially and give off terminal branches to the outer and inner cell layers. These primary trigeminal branches are directed ventromedially, whereas dendrites of trigeminal neurons are directed dorsolaterally towards the descending trigeminal tract and they intermingle profusely (Figure 8C,D). Additionally, sparse trigeminal fibers reach the DCN, the nucleus of the solitary tract and the LCN.

Non-primary afferents

Spinal second-order projections also ascend via the dorsal funiculus and the dorsolateral funiculus. Tracer application to the cervical spinal cord revealed thin fibers coursing rostralwards throughout the rhombencephalon up to the cerebellum and the

subcerebellar region. These data are discussed in a separate paper (A. Muñoz et al., 1996b).

Efferent projections

Retrograde BDA tracing was used to further characterize the DCN and LCN with respect to their efferent projections. Experiments with tracer application to the ventral thalamus and to the torus semicircularis were done to label the cell populations in the caudal medulla and upper spinal cord that project to these targets of somatosensory projections.

Thalamic applications

In this group of experiments, BDA was applied to the thalamus. Although the tracer was applied mainly to the ventral thalamus, in some cases the dorsal thalamus was also affected. A distinct population of retrogradely labeled cells was observed, in the alar plate at the obex level composed of irregular and large cells together with round, small cells (Figure 9). Most of the cells were found contralateral to the injection site although a minor component of ipsilateral cells was also present. At the obex level, dorsomedially located neurons with processes directed dorsally into the dorsal funiculus, where spinal primary afferents ascend, were identified as the DCN. This neuronal population is formed by round, bipolar, irregular and oval shaped neurons that extend from medullary levels slightly rostral to the obex into the upper cervical spinal segment where they are tightly packed in the dorsalmost part of the gray. Additionally, ventrolaterally located neurons with dendritic processes directed ventrolaterally towards the dorsolateral funiculus were identified as the LCN. The LCN extends from the obex level rostrally to the cervical spinal cord, but extends more caudally than the DCN. LCN neurons are round, bipolar, oval shaped or irregular, and some of them are segregated from the gray within the dorsolateral funiculus proper. Although there is some degree of segregation between the DCN and the LCN, some labeled neurons were observed with processes directed both dorsally and ventrolaterally. The axons of both the DCN and LCN neurons could be traced into the contralateral medial lemniscus.

Toral applications

In five cases BDA was applied to the torus semicircularis. In some cases spread of the tracer to the mesencephalic dorsal tegmentum could not be avoided. In all cases neurons were retrogradely labeled within both the DCN and the LCN, but in higher numbers than in the cases of thalamic applications. They were more abundant on the contralateral side although a small ipsilateral component was also present. Dorsomedially located DCN cells possess several processes extending into the dorsal funiculus. Their axons mainly course ventromedially, cross the midline and form part of the contralateral medial lemniscus. LCN labeled cells are located in the lateral marginal zone of the dorsal grey or within the dorsolateral funiculus itself and extend more caudally than the DCN. LCN cells are large, bipolar or irregular cells with long processes directed mainly into the dorsolateral funiculus. Their axons could also be traced into the medial lemniscus.

Additionally, in both thalamic and toral BDA experiments retrogradely labeled neurons were observed in the dorsolateral part of the outer cell layer, thought to be part of the nucleus of the descending trigeminal nerve, since here trigeminal primary afferents arborize.

DISCUSSION

In the present study the organization, immunohistochemical characterization and the fiber connections of some of the neuronal components of the ill-defined alar gray at the spinomedullary transition level were investigated in the ribbed new, *Pleurodeles waltl*. More in particular, a DCN, an LCN, the nucleus of the solitary tract and the nucleus of the descending trigeminal tract which are hard to distinguish in Nissl-stained material, were (immuno)histochemically characterized. Additionally, the distribution of the spinal primary afferents from fore- and hindlimb-innervating spinal nerves, non-primary cervical spinal projections, the pattern of termination at the obex region of trigeminal primary afferents, and the organization of DCN and LCN neurons projecting to the thalamus and mesencephalon were studied with the BDA tract-tracing technique. In general, in *Pleurodeles waltl*, a similar pattern of organization of the various cell components of the alar plate at the spinomedullary transition level was found as in anurans (A. Muñoz et al., 1995, 1996a).

In cytoarchitectonic studies of the urodele brain stem no distinct DCN could be distinguished from the adjacent gray (Herrick, 1930, 1944, 1948; Kreht, 1940; Opdam and Nieuwenhuys, 1976). Herrick (1944) described a nucleus of the funiculus dorsalis in the alar plate of the obex region. Its boundaries with the nucleus commissuralis, i.e. the caudal continuation of the nucleus of the solitary tract, are not clear though its neurons are larger than those of the commissural nucleus. As far as we know, no LCN was described at the obex level or in the cervical spinal cord, and even controversial data are found in the literature concerning the existence of a cytoarchitectonically dictinct nucleus of the descending trigeminal tract in different species of urodeles (Herrick, 1930; Woodburne, 1936; Opdam and Nieuwenhuys, 1976; González and Muñoz, 1988). Herrick (1948) already noted the existence of the nucleus of the solitary tract in urodeles, and in a later study the nucleus of the solitary tract was described as a group of cells that surround the solitary tract throughout the medulla, although it could not be clearly delimited from the adjacent gray (Opdam and Nieuwenhuys, 1976).

The DCN has been considered as the site of termination of dorsal funicular fibers in the caudal brain stem, but not as a cytoarchitectonic entity (Nieuwenhuys and Cornelisz, 1971; Roth and Wake, 1985). Nevertheless, Nissl-stained sections of the brain stem at the obex level and of upper cervical levels allow the delineation of a DCN and even an LCN. Moreover, BDA labeling of spinal primary afferent projections passing via the dorsal funiculus to the obex level, labeling of non-primary ascending spinal projections at the dorsolateral funiculus (A. Muñoz et al., 1996b), and retrograde labeling from the thalamus and the torus semicircularis, clearly delineate the DCN and LCN in urodeles. Histochemical and immunohistochemical data are of great help in characterizing the nucleus of the solitary tract and nucleus of the descending trigeminal tract.

The NADPH-diaphorase (NADPHd) histochemical technique, known to stain specific neurons (Thomas and Pearse, 1964), can selectively stain particular populations of neurons in a Golgi-like manner (Scherer-Singler et al., 1983; Alonso et al., 1995; M. Muñoz et al., 1996). Throughout the brain, NADPHd and NOS localizations are identical (Bredt and Snyder, 1992). The distribution of NADPHd staining and neuronal NOS immunolabeling in the present study was also found identical. Therefore,

NADPHd can be used as a marker for NOS. Nitric oxide most likely plays a major role as a neuronal messenger (Bredt and Snyder, 1992; Meller and Gebhart, 1993; Schuman and Madison, 1994). The presence of NADPHd-positive cells and fibers in the mammalian spinal cord (Valtschanoff et al., 1992) suggests that nitric oxide may be involved in spinal sensory processing. In the rat DCN, Valtschanoff et al. (1993) found that most NOS-positive neurons are also immunoreactive for GABA, but not for the excitatory transmitters glutamate and aspartate. Moreover, since NOS-positive neurons could not be labeled retrogradely from the thalamus or spinal cord, they probably are local circuit neurons (Valtschanoff et al., 1993). In anurans, the presence of NADPHd-positive neurons in the alar plate at the obex level was recently described (A. Muñoz et al., 1995; M. Muñoz et al., 1996). NADPHd-positive neurons were found in the DCN, but more abundantly in the adjacent nucleus of the solitary tract and the nucleus of the tract of the trigeminal nerve in line with data in mammals (e.g., Leight et al., 1990; Vincent and Kimura, 1992; Dohrn et al., 1994; Takemura et al., 1994). In the present study in *Pleurodeles*, however, at the obex level labeling was restricted to neurons of the nucleus of the descending trigeminal tract. Only very few lightly stained cells were found more dorsally in the cell area that includes the DCN and the nucleus of the solitary tract. Weakly stained neurons were also found more rostrally in the nucleus of the solitary tract.

In mammals, calcium-binding proteins such as calbindin and parvalbumin show a preferential distribution for somatosensory structures including the DCN (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menétrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994). Parvalbumin appears to be abundant in the pathway for epicritic sensibility, i.e. the dorsal column-medial lemniscal system, calbindin D-28k (Calb) occurs in the whole taste pathway of rats (Celio, 1990). In rats, Calb-positive neurons are found in certain laminae of the dorsal horn (see Antal et al., 1990; Ren and Ruda, 1994) including the cells of origin of ascending spinal projections (Menétrey et al., 1992b), in the sensory trigeminal nuclei as well as in the gracile and cuneate nuclei (Celio, 1990). Also in rats, Menétrey et al. (1992a) showed that Calb-positive neurons form a major part of the solitary and trigeminal projection systems. In monkeys, both proteins are differentially expressed in trigeminothalamic projections (Rausell and Jones, 1991a,b) and in spinothalamic projections (Rausell et al., 1992).

The presence of calbindin D-28k and parvalbumin in the alar plate of the spinomedullary transition area was recently demonstrated in the anuran brain (A. Muñoz et al., 1995). In line with these anuran data, in *Pleurodeles waltl*, Calb-positive neurons were found in the nucleus of the solitary tract and in the nucleus of the descending trigeminal tract, but not in the DCN or LCN. This pattern of distribution of Calb-positive neurons suggests that as in mammals, in amphibians, Calb positive cells are present in part of the somatosensory system including some neurons of the dorsal horn of the spinal cord and the nucleus of the trigeminal tract. In the anuran obex region, a distinct parvalbumin-positive cell population was found (A. Muñoz et al., 1995). Immunostaining for parvalbumin, rather clearly delineates the anuran DCN. However, with the same antibody and protocol no clear parvalbumin labeling, was found in the brain of *Pleurodeles*.

The caudal medullary and upper spinal alar plate in *Pleurodeles* is innervated by substance P-fibers

that course via the dorsal funiculus and mainly, the dorsolateral funiculus. Although all main regions, including the DCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract are innervated, the LCN located at the dorsolateral aspect of the gray, in close relation to the dorsolateral funiculus receives the weakest innervation. These projections include primary spinal afferents in the dorsal funiculus and the tract of Lissauer according to Taban and Cathieni (1983) who described substace P-positive neurons in the spinal dorsal root ganglia, but may include other non-primary projections coursing in the dorsal funiculus and dorsolateral funiculus. In anurans, in which also CGRP-immunoreactive fibers was described (A. Muñoz et al., 1995), a similar innervation pattern exists (see also Adli et al., 1988; Petkó and Sánta, 1992). Additionally, this region is innervated by Leu-enkephalin, neuropeptide Y and serotonin-immunoreactive fibers resembling the situation in anurans (Ueda et al., 1984; Merchenthaler et al., 1989; Lázár et al., 1990; A. Muñoz et al., 1995). A similar peptidergic and serotoninergic innervation of the DCN is found in mammals (e.g., Steinbusch, 1981; Westman et al., 1984; Halliday et al., 1988; Blomqvist and Broman, 1993).

Urodele DCN: Cytoarchitecture and afferent connections

The urodele DCN was first described by Herrick (1944) as the nucleus of the funiculus dorsalis, located dorsolaterally to the nucleus commissuralis of Cajal from which it could not be clearly separated. Although in the axolotl a DCN was not considered a separated cytoarchitectonic entity (Opdam and Nieuwenhuys, 1976), Nissl-stained material of *Pleurodeles* revealed, in line with Herrick's (1944) observations that the DCN extends from the caudal rhombencephalic midvagal level up to the first cervical spinal segment. The rostral part of the DCN is composed of large neurons located dorsal to the solitary tract and is partially intermingled with smaller neurons of the caudal part of the lateral line area. At the obex level, the DCN is segregated at the dorsal aspect of the outer cell layer by a thin white matter layer. More caudally it occupies the most dorsomedial aspect of the spinal dorsal horn and the boundaries with the proper spinal neurons are hardly distinguishable. The urodele DCN is the main site of termination of spinal primary afferent fibers (Herrick, 1944; Nieuwenhuys and Cornelisz, 1971; Roth and Wake, 1985; the present study). As already noted in the axolotl (Nieuwenhuys and Cornelisz, 1971), in *Pleurodeles*, the medial component of the dorsal funiculus arises from lumbar ganglia and innervates the medial part of the DCN, whereas ascending fibers from cervical ganglia ascend in the lateral part of the dorsal funiculus and innervate the lateral aspect of the DCN. This somatotopic arrangement of dorsal column projections is comparable to that found in anurans (Antal et al., 1980; Nikundiwe et al., 1982; M. Muñoz et al., 1991; A. Muñoz et al., 1995). However, in contrast to anurans, in *Pleurodeles*, medial, "gracile" and lateral, "cuneate" components of the DCN can not be recogniced, neither cytoarchitectonically nor by means of retrograde tracing from the thalamus or the torus semicircularis.

Dorsal funicular fibers in *Pleurodeles* continue rostrally to the obex level and innervate the nucleus of the solitary tract, reticular formation, the nucleus of the descending trigeminal tract as well as the vestibular nuclear complex, and reach the granule layer of the cerebellum. Apart from spinal primary afferents, the presence of non-primary spinal fibers to the DCN or postsynaptic dorsal column system

(PDCS) is suggested for urodeles (A. Muñoz et al., 1996b). The presence of a somatotopically arranged PDCS projection was demonstrated in anurans (ten Donkelaar and de Boer-van Huizen, 1991; A. Muñoz et al., 1995) and throughout other terrestrial vertebrates (e.g., Rustioni, 1973; Angaut-Petit, 1975a, b; Uddenberg, 1968; Rustioni and Kaufman, 1977; Bennett et al., 1984; Giesler et al., 1984; Funke, 1988; Pritz and Stritzel, 1994).

Some fibers of the descending trigeminal tract, including cutaneous fibers from the Vth, VIIth and IXth cranial nerves also reach the DCN in *Pleurodeles* (González and Muñoz, 1987; A. Muñoz et al., 1995). Additionally, dorsolateral funicular fibers, some of them substance-P-, Leu-Enk-, or NPY-positive, five off collateral branches, and innervate the LCN and the more dorsomedially located targets i.e. the nucleus of the descending trigeminal tract and the DCN.

Urodele LCN: Cytoarchitecture and afferent connections

The LCN in urodeles was not noticed in previous studies (Herrick, 1944, 1948; Opdam and Nieuwenhuys, 1976). In *Pleurodeles* the LCN, is not a clearly segregated cell mass in the ventrolateral aspect of the outer cell layer at the spinomedullary transition level, but it can be identified by means of immunohistochemical and hodological studies, as also shown for anurans (A. Muñoz et al., 1996a). Some of the LCN neurons are segregated into the dorsolateral funiculus. LCN afferents include spinal primary afferents of Lissauer's tract, at least from brachial dorsal roots, and non-primary spinal projections from the spinocervical tract. Some of the LCN afferents are immunopositive for substance-P, Leu-Enk, NPY and serotinin-positive. Additionally, the LCN cells probably receive a trigeminal primary afferent input (González and Muñoz, 1988; the present study). A similar immunohistochemical characterization of afferent terminals upon cells of the LCN was recently described for anurans (A. Muñoz et al., 1995).

Urodele DCN and LCN: Efferent connections

Herrick (1944, 1948; see also Herrick and Bishop, 1958) denied the existence of a medial lemniscus in *Amblystoma tigrinum*, and compared the nucleus of the funiculus dorsalis in urodeles with the external cuneate nucleus and not with the gracile and cuneate nuclei of mammals. Herrick suggested that proprioceptive arcuate fibers, that arise at the region of the calamus, including the nucleus of the funiculus dorsalis, join the dorsal component of the spinal lemniscus where they ascend, ventral to the descending trigeminal tract to innervate the medullary and midbrain reticular formation, the tectum and the dorsal (sensory) thalamus. Additionally, Herrick (1944, 1948; see also Herrick and Bishop, 1958) reported the existence of the general bulbar lemniscus as a crossed fiber system that arises from the nucleus of the funiculus dorsalis (exteroceptive fibers), the spinal nucleus of the trigeminal nerve and other medullary centers and ascend at the ventrolaterally white matter of the medulla to innervate the reticular formation, the tectum mesencephali and also the dorsal (sensory) thalamus, overlapping with those projections of the spinal lemniscus. However, the present study clearly demonstrates that the 'nucleus' identified in *Pleurodeles waltl* is comparable to the dorsal column nuclei of anurans and other vertebrates, which together with the LCN, give rise to the medial lemniscus. Herrick (1944, 1948) also described ipsilateral fibers from the nucleus of the funiculus dorsalis that would reach the

cerbellum through the tracts A and B of Kingsbury (1895), that would have different function from those fibers from the dorsal funicular nucleus that reach the cerebellum through the spinal lemniscus. Although in anurans no equivalents of the urodele tracts A and B have been described it is noteworthy that an ipsilateral cerebellar projection from the DCN ascending dorsal to the descending trigeminal tract together with the spinal primary afferents, was demonstrated in *Rana perezi* and in *Xenopus laevis* (González et al., 1984; A, Muñoz et al., 1995).

In the present study the existence in urodeles of medial lemniscal projections from the DCN and the LCN to the ventral thalamus and the torus semicircularis was demonstrated. Axons of DCN and LCN projection neurons cross the midline ventral to the caudalmost aspect of the fourth ventricle or the central canal to join the contralateral medial lemniscus where they ascend towards the torus semicircularis and the thalamus. In urodeles and in anurans a convergence of spinal (A. Muñoz et al., 1994a, 1996b) and medial lemniscal (DCN and LCN) inputs (Silvey et al., 1974; Neary and Wilczynski, 1977, 1979; Forehand and Farel, 1982; Urbán and Székely, 1982; A. Muñoz et al., 1994b, 1995, 1996a) in the torus semicircularis was demonstrated. A similar convergence appears in the ventral thalamus. The torus semicircularis of urodeles is not clearly distinguishable in silver or Nissl-stained sections (Herrick, 1942; Opdam and Nieuwenhuys, 1976), although it was proposed as a center for integration of lateral line and vestibular information (Herrick, 1948). However, in later studies the torus semicircularis was unequivocally delineated (Roth et al., 1990) based on its afferent connections (González and Muñoz, 1987; Manteuffel and Naujoks-Manteuffel, 1990), its descending medullary and spinal projections (Naujoks-Manteuffel and Manteuffel, 1988) and by registration of visual, vibration and auditory responses (Manteuffel and Naujoks-Manteuffel, 1990). In line with our data on the medial lemniscal (DCN and LCN) innervation of the torus semicircularis in *Pleurodeles*, Manteuffel and Naujoks-Manteuffel (1990) found retrogradely labeled neurons bilaterally in the dorsal periventricular gray of the most caudal part of the medulla oblongata. Although convergence of somatosensory spinal (A. Muñoz et al., 1996b) and medial lemniscal inputs including DCN/LCN (the present study) seems to exist in the urodele torus semicircularis, detailed physiological studies are needed in order to establish the somatotopic representation of the contralateral body surface as found in anurans (Comer and Grobstein, 1981).

Apart from the thalamus and the torus semicircularis, it seems likely (unpublished observations) that the medial lemniscus in urodeles includes fibers that reach the posterior tubercle, the mesencephalic optic tectum, mesencephalic tegmental nuclei, the isthmic region, the cerebellum, the area octavolateralis and the rhombencephalic reticular formation in line with data in anurans (A. Muñoz et al., 1995).

Various studies have dealt with somatosensory processing in the deep neuropil and the gray of the optic tectum of different urodele species (Grüsser-Cornehls and Himstedt, 1973; Gruberg and Solish, 1978; Gruberg and Harris, 1981; Harris, 1982, 1989; Stirling and Brändle, 1982). Roth et al. (1990) described the tectum as a polysensory integrative center of retinal and non-retinal afferents. Their cell types 1 and 2c are probably involved in the processing of somatosensory information. Gruberg and Solish (1978) observed a rostrocaudal and lateromedial somatosensory body representation in layer 3 of the tectum formed by

contralateral and deeper bilateral somatosensory proccessing units. In various studies a spinal innervation of the urodele tectum mesencephali was shown, although some controversies with regard to the cells of origin of this projection exist (Herrick, 1914, 1942, 1948; Nieuwenhuys and Cornelisz, 1971; Jakway and Riss, 1972; Gruberg, 1972; Finkenstädt et al., 1983; Rettig, 1988; A. Muñoz et al., 1996b). The urodele tectum may also receive somatosensory information from the DCN/LCN via the medial lemniscus as demonstrated in *Xenopus laevis* (A. Muñoz et al., 1995). Until now, the latter pathway has not been unequivocally demonstrated for urodeles in studies on the cells of origin of non-retinal tectal afferents (Rettig, 1988, Finkenstädt et al., 1983). After HRP injections in the tectum, Finkenstädt et al. (1983) labeled cells in the upper cervical spinal cord in positions corresponding to the DCN or LCN, as observed in the present study in *Pleurodeles*.

CONCLUSIONS

In the caudal rhombencephalic alar plate of the ribbed newt, *Pleurodeles waltl*, a DCN and an LCN can be distinguished lateral to an inner periventricular zone composed of the nucleus of the solitary tract and the nucleus of the descending trigeminal tract. NADPH-diaphorase histochemistry and calbindin D-28k immunohistochemistry stain only neurons in the inner compact cell layer. Tracing experiments showed that the dorsal column is somatotopically arranged: lumbar primary afferents terminate on medial DCN neurons, whereas cervical primary afferents terminate on lateral DCN neurons. The LCN is densely innervated by the spinocervical tract present in the dorsolateral funiculus. Retrograde tracing showed extensive predominantly contralateral projections of both the DCN and LCN to the torus semicircularis and the ventral thalamus.

These data show that in urodeles a dorsal column-medial lemniscus system via the DCN as well as a spinocervicothalamic system via the LCN are present.

ACKNOWLEDGEMENTS

This research was supported by a Grant from the Spanish Government (DGICYT PB 93-0083) to A. González and by a NATO Collaborative Research Grant (930542) to H.J. ten Donkelaar. The authors would like to thank Dr. Gerhard Roth for providing some of the *Pleurodeles waltl* speciments and Ms. Inge Eijkhout for secretarial assistance.

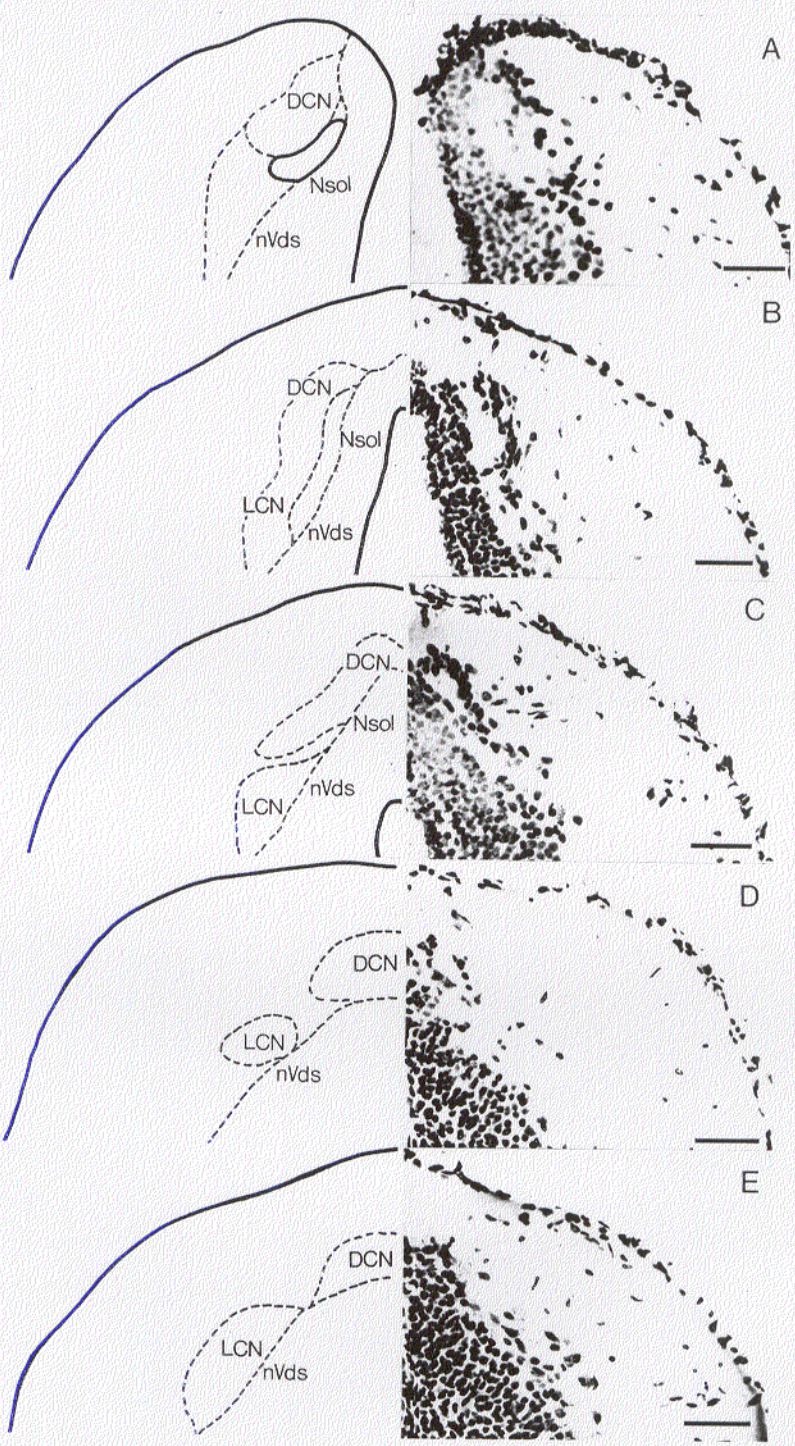

Figure 1: Photomicrographs of Nissl-stained transverse sections and schematic drawings of the caudal part of the rhombencephalic alar plate in *Pleurodeles waltl*. Scale bars indicate 100 µm.

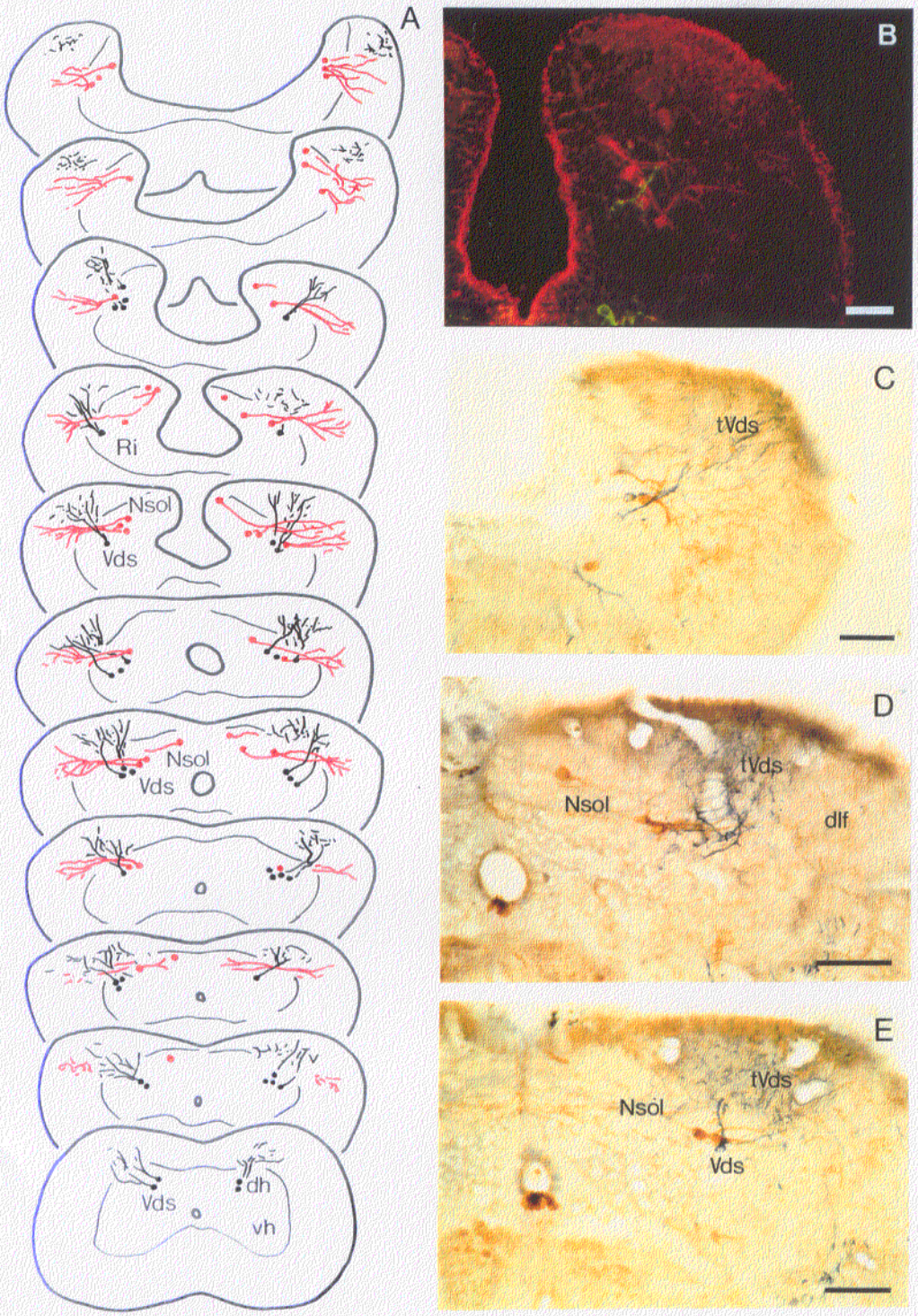

Figure 2: A, The distribution of NADPH diaphorase-positive (black) and TH (red) cells in the caudal part of the rhombencephalic alar plate of *Pleurodeles waltl.* C-E, Photomicrographs illustrating examples of NADPH diaphorase-positive (blue) and TH (brown) positive cells. B, an example of the NOS (green) and TH (red)-positive neurons in the alar plate at the obex region. Scale bars indicate 100 µm.

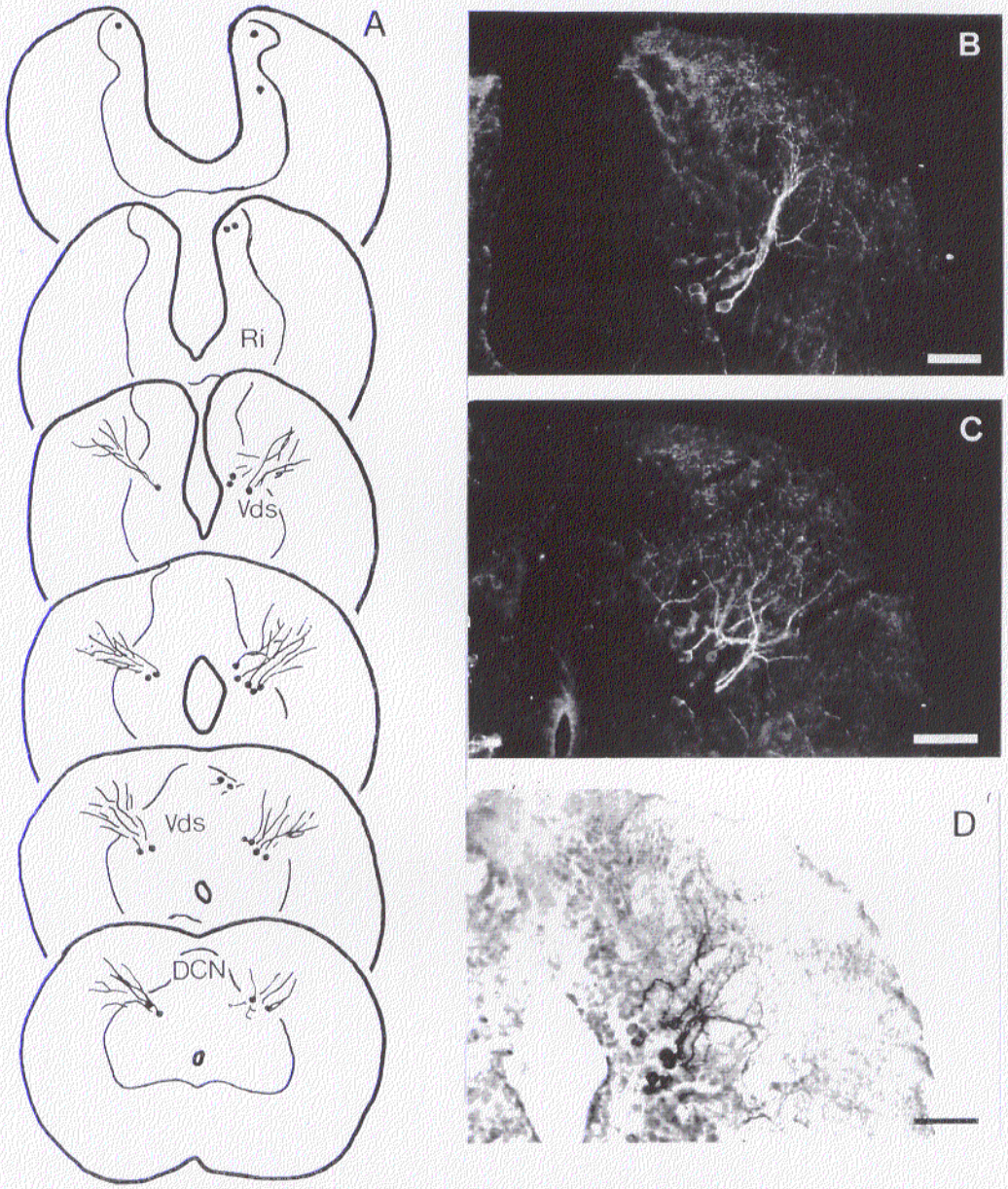

Figure 3: The distribution of NOS-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Pleurodeles waltl*. Photomicrographs show examples of the labeling observed in the nucleus of the descending trigeminal tract. Scale bars indicate 100 µm.

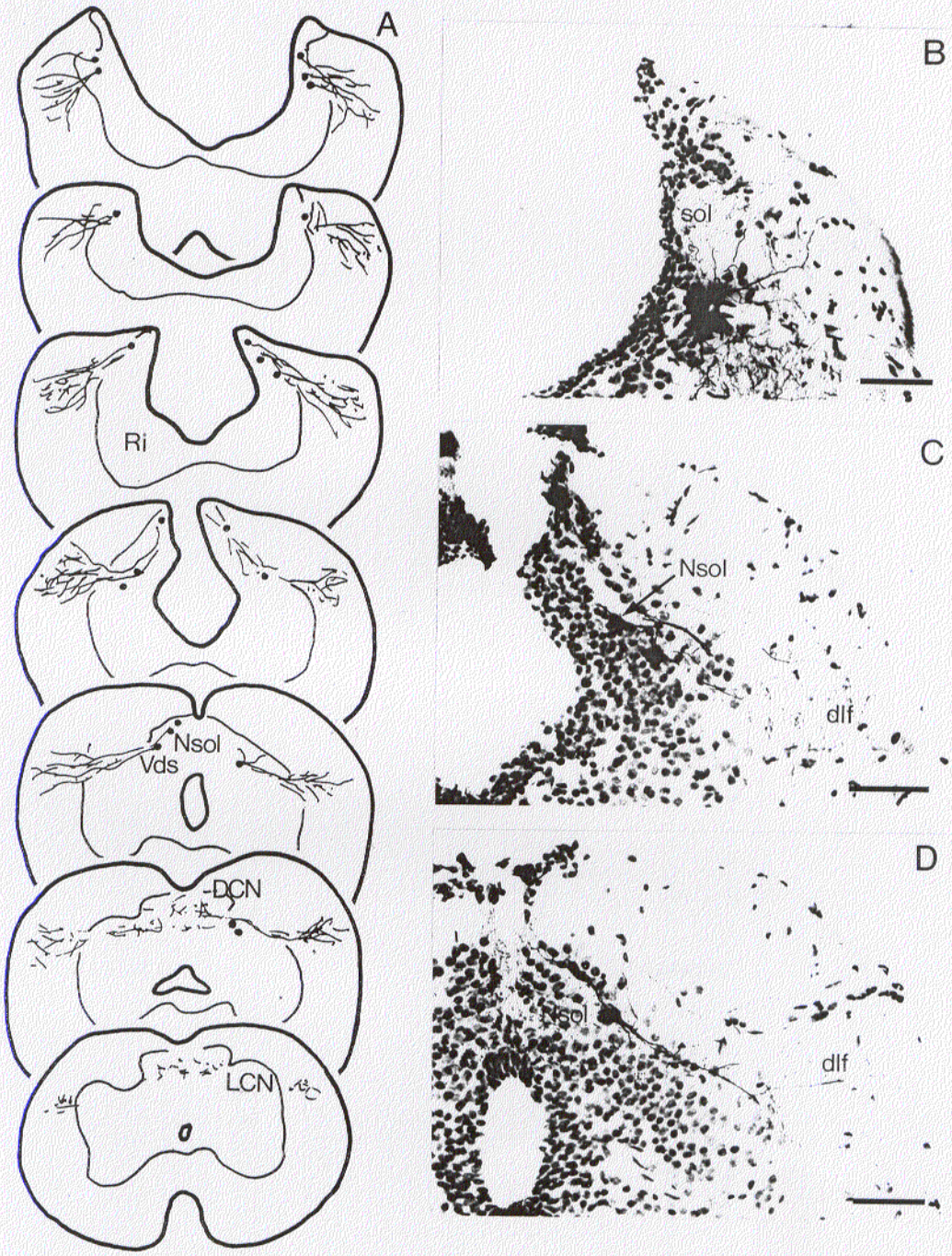

Figure 4: A, The distribution of TH-positive neurons in the caudal part of the rhombencephalon and the most rostral part of the spinal cord of *Pleurodeles waltl.* B-D, Photomicrographs of examples of TH labeling in the nucleus of the solitary tract. Scale bars indicate 100 µm.

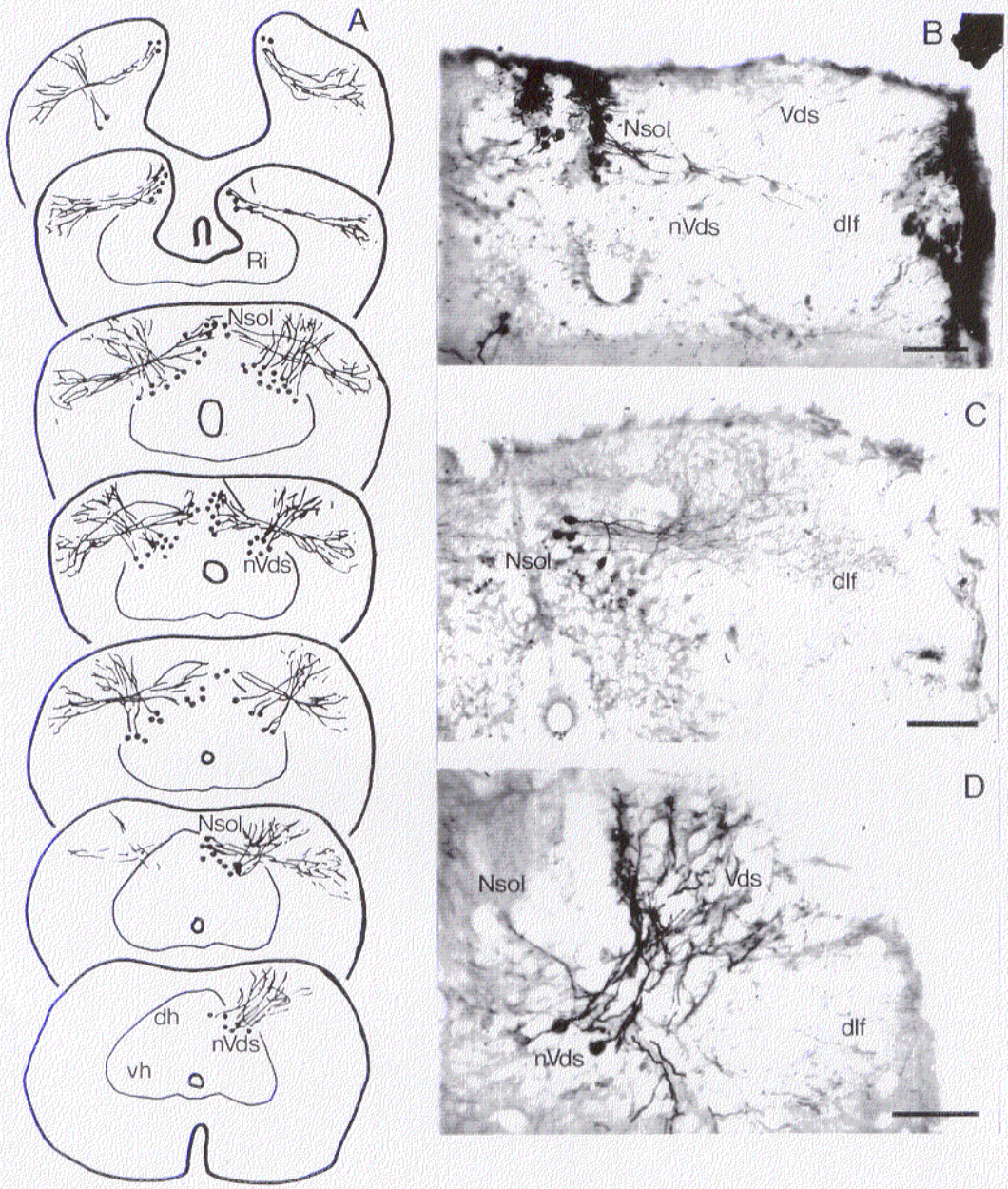

Figure 5: The distribution of calbindin D-28k-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Pleurodeles waltl*. Photomicrographs show examples of the labeling observed in the nucleus of the solitary tract and in the nucleus of the descending trigeminal tract. Scale bars indicate 100 µm.

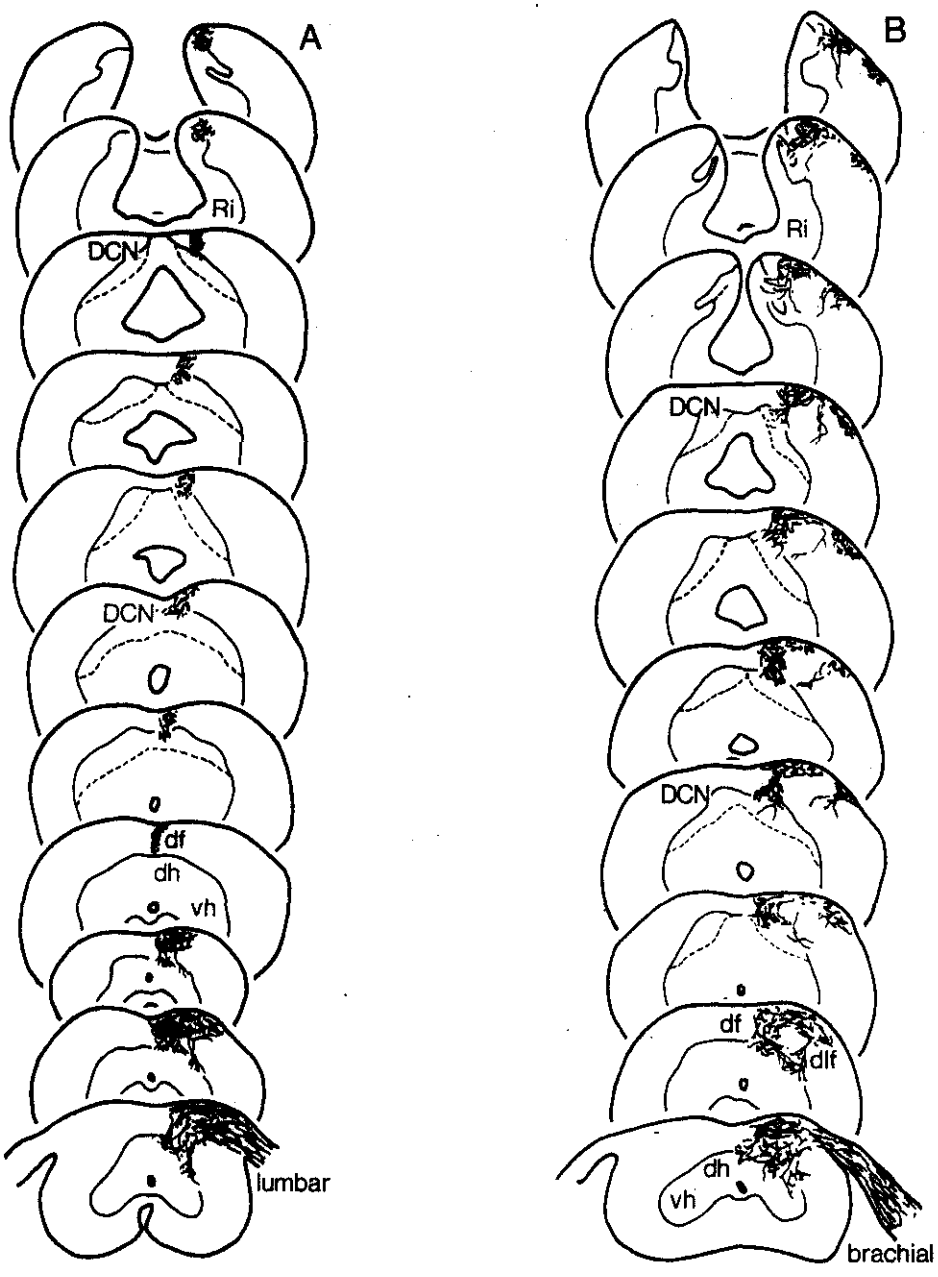

Figure 6: Schematic drawings of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* showing the distribution of lumbar (A) and brachial (B) dorsal root afferents.

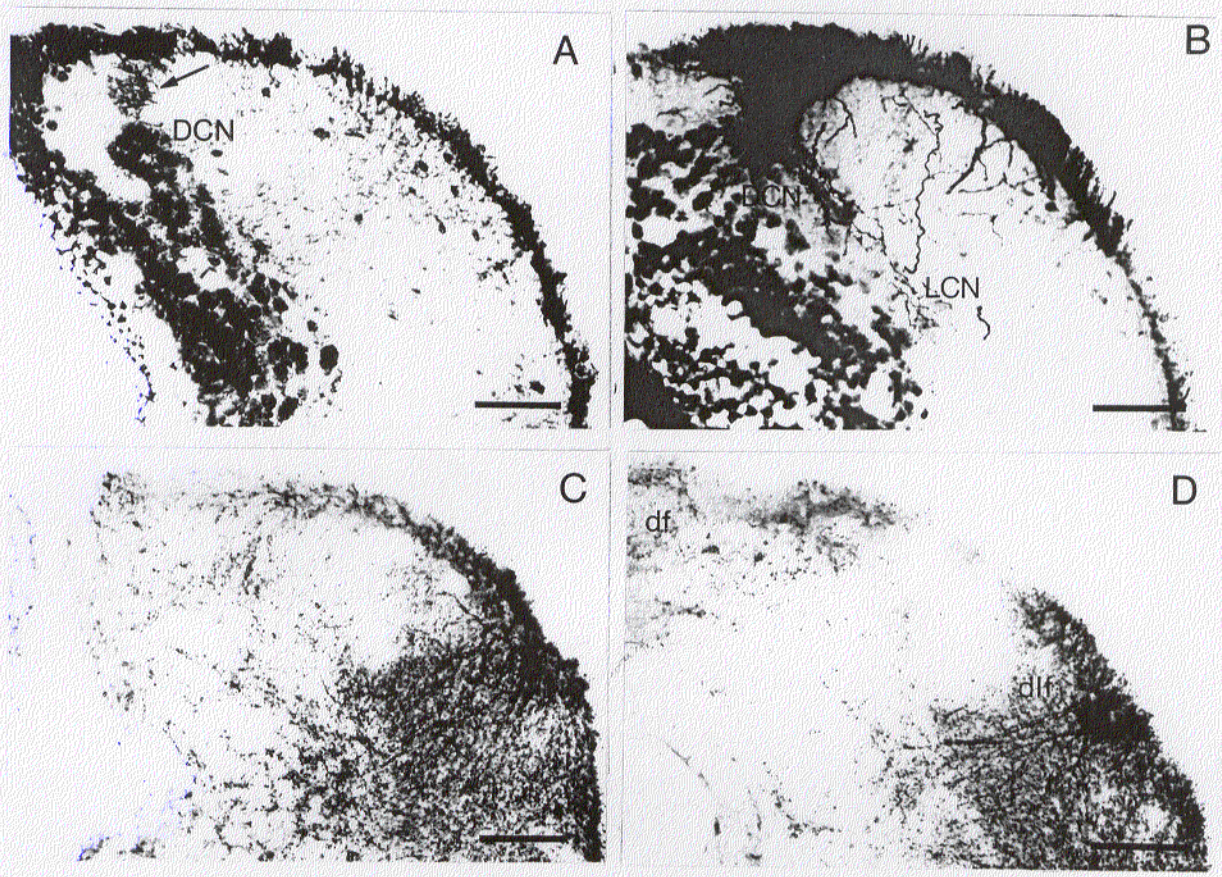

Figure 7: A, B, Photomicrograph showing the termination pattern of BDA labeled brachial and lumbar dorsal root afferents at the rostral DCN of *Pleurodeles waltl*. Scale bars indicate 100 µm. C,D, Photomicrographs showing the substance P-immunoreactive innervation of the caudal medullary (C) and rostral spinal (D) gray. Scale bars indicate 100 µm.

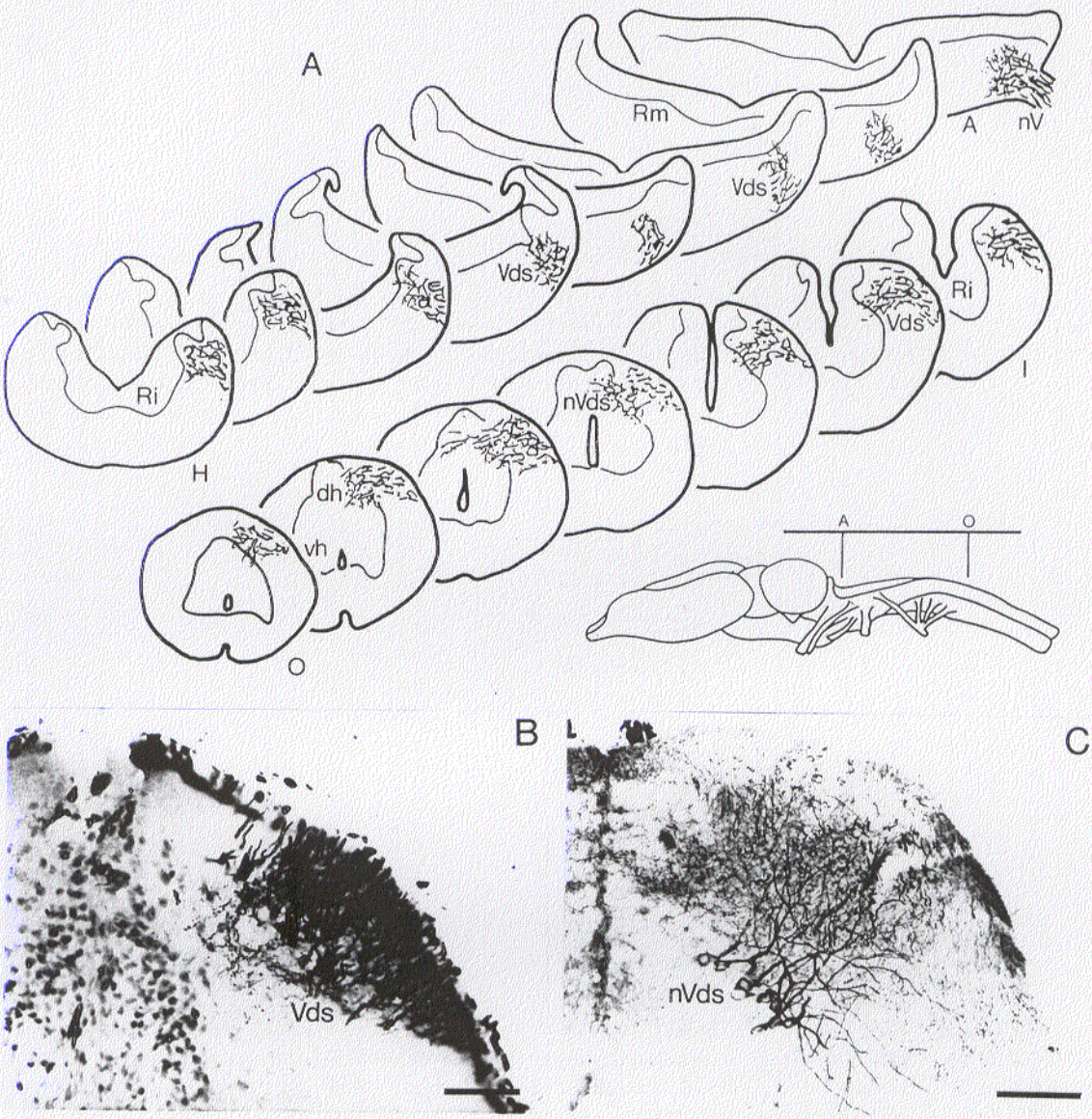

Figure 8: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodesle waltl* showing the distribution of trigeminal afferent fibers. B, Photomicrograph showing ipsilateral trigeminal afferents to the obex region. C, Photomicrograph showing NAHPHd positive neurons in the nucleus of the descending trigeminal tract with dendrites directed to the Vds.

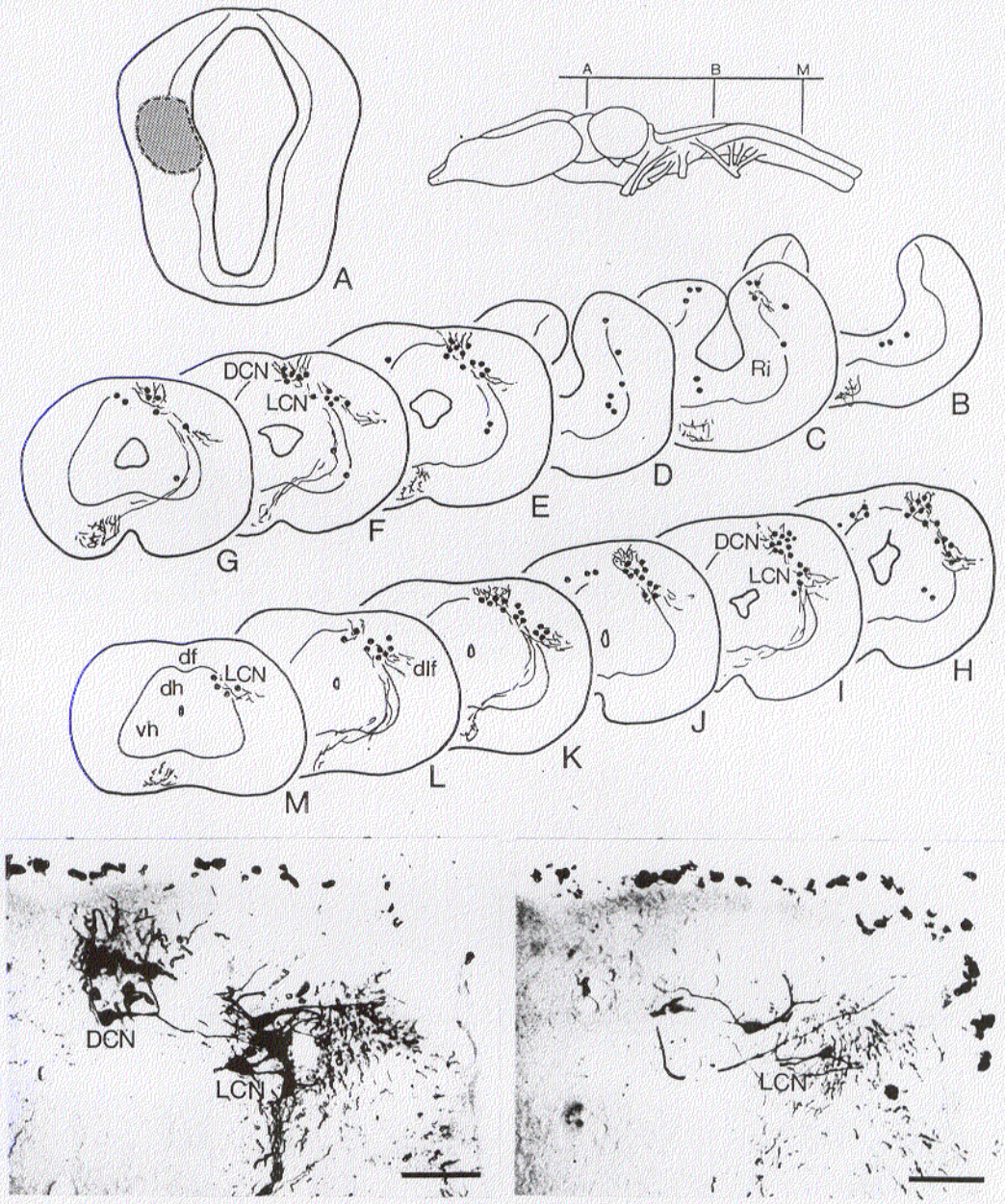

Figure 9: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* the distribution of retrogradely labeled neurons following a BDA application to the thalamus. Examples of labeling are shown in two photomicrographs. Scale bars indicate 100 µm.

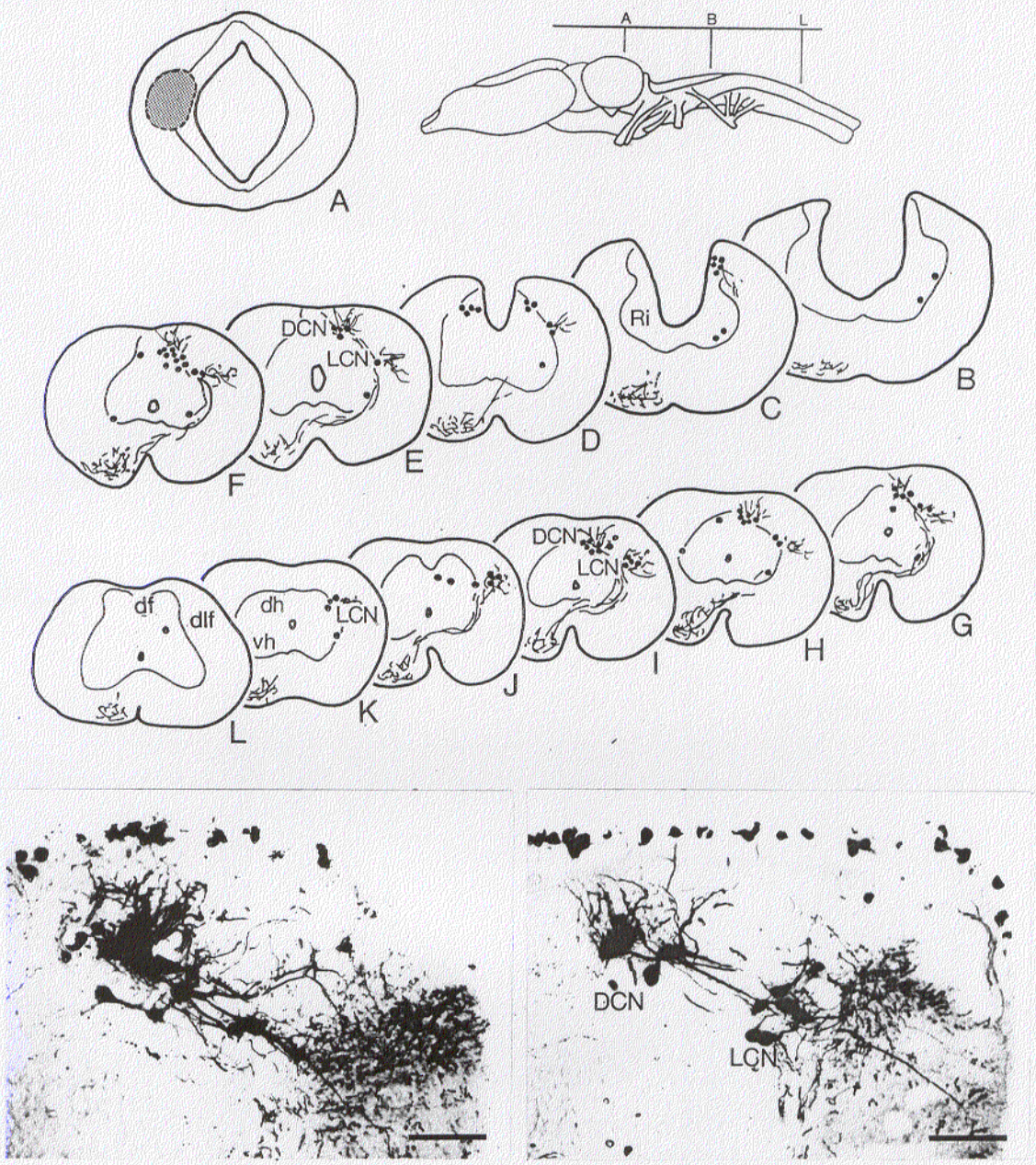

Figure 10: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* showing the distribution of retrogradely neurons following a BDA application to the torus semicircularis. Examples of labeling are shown in two photomicrographs. Scale bars indicate 100 µm.

ABBREVIATIONS

DCN	dorsal column nucleus
df	dorsal funiculus
dh	dorsal horn
dlf	dorsolateral funiculus
LCN	lateral cervical nucleus
Nsol	nucleus of the solitary tract
nV	trigeminal nerve
nVds	nucleus of the descending tract of the trigeminal nerve
Ri	nucleus reticularis inferior
Vds	descending tract of the trigeminal nerve
vh	ventral horn

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Organization of the caudal rhombencephalic alar plate of the ribbed newt Pleurodeles waltl: Evidence for the presence of dorsal column and lateral cervical nuclei

COMENTARIOS

6.2

El lemnisco medial, formado en parte por las proyecciones ascendentes procedentes de los núcleos de la columna dorsal y cervical lateral, constituye uno de los principales sistemas ascendentes de transmisión de información somatosensorial a centros supraespinales, en vertebrados terrestres. Los núcleos de la columna dorsal reciben aferencias espinales primarias y no primarias, a través del funículo dorsal, mientras que el núcleo cervical lateral se encuentra inervado por el tracto espinocervical que asciende por el funículo dorsolateral (Willis y Coggeshall, 1991). En anuros se ha demostrado recientemente la presencia del núcleo de la columna dorsal (DCN) y cervical lateral (LCN) en la placa alar de la región del óbex, así como del lemnisco medial (capítulo 5 de la presente memoria). Sin embargo, en urodelos no existen hasta el momento evidencias experimentales que demuestren la presencia de este sistema.

El sistema nervioso central de urodelos se ha considerado menos evolucionado que el de otros anfibios, debido a su escasa diferenciación citoarquitectónica, y emigración de las masas celulares, que, por ejemplo en el tronco cerebral (Opdam y Nieuwenhuys, 1976) se disponen, densamente agrupadas, en una zona continua de sustancia gris periventricular. Herrick (1948), sin embargo, sugirió que dentro de la simple disposición de neuronas y fibras, el cerebro de la salamandra *Ambystoma tigrinum* presenta una organización similar a la de otros vertebrados. Estudios posteriores han demostrado que al menos algunos sistemas del cerebro de urodelos presentan características anatómicas y funcionales similares a las de anuros, lo que conduce a un patrón de comportamiento comparable (Roth, 1987, Roth y cols., 1993), por lo que se ha sugerido una *simplificación secundaria* (Northcutt, 1987; Roth y cols., 1992, 1993), según la cual el cerebro de este grupo de vertebrados aparenta ser más primitivo de lo que cabría esperar, según su posición filogénica (Roth y cols., 1992).

Citoarquitectura

En el presente estudio se han caracterizado, en el urodelo *Pleurodeles waltl*, los distintos componentes neuronales relacionados con el procesamiento de información somática, presentes en la placa alar de la región del óbex, citoarquitectónica y quimioarquitectónicamente, así como su conectividad.

Debido a la escasa diferenciación en estudios citoarquitectónicos en urodelos (Herrick, 1930, 1944, 1948; Kreht, 1940; Opdam y Nieuwenhuys, 1976) no se han descrito el DCN ni el núcleo del tracto solitario (Nsol), como centros diferenciables de las masas celulares adyacentes, y existen datos controvertidos en cuanto a la presencia del núcleo del tracto descendente del nervio trigémino (nVds) como entidad citoarquitectónica (Herrick, 1930; Woodburne, 1936, Opdam y Nieuwenhuys, 1976; González y Muñoz, 1988). Herrick (1944), en *Ambystoma tigrinum*, en estudios con ténicas de Golgi sugirió la presencia del *nucleus funiculus dorsalis*, en la placa alar de la región del óbex, relacionado con fibras del funículo dorsal y en menor grado con fibras vestibulares, trigeminales y de la línea lateral; escasamente segregado del *nucleus comisuralis*, el cual representa la prolongación caudal del núcleo del tracto solitario y recibe aferencias viscerales del tracto solitario. La presencia del DCN ha sido considerada en estudios posteriores, en base al patrón de terminación de las aferencias primarias espinales en esta región (Nieuwenhuys y Cornelisz, 1971; Roth y Wake, 1985). Sin embargo, hasta el momento en uodelos no se ha sugerido la existencia del LCN en niveles de transición espinorombencefálicos.

En el presente trabajo se han identificado en *Pleurodeles waltl*, el DCN y el LCN en la placa alar a nivel del óbex mediante técnicas de Nissl. En dicha región hemos observado, además de una *banda celular interna* periventricular, gruesa y con células densamente empaquetadas, una *banda celular externa*, delgada y separada de la anterior por una estrecha lámina de sustancia blanca. El DCN consiste en una agrupación celular localizada en la región dorsomedial de la banda celular externa, en relación con la sustancia blanca del funículo dorsal. Dicho núcleo se extiende desde los primeros segmentos espinales rostrales hasta niveles del núcleo motor del nervio vago, y en él no se distingue una segregación mediolateral de los componentes *gracilis* y *cuneatus*. El LCN, situado ventrolateralmente en la banda celular externa, está formado por neuronas, agrupadas menos densamente que en el caso del DCN, que se entremezclan con la sustancia blanca del funículo dorsolateral, y se extiende desde el óbex hasta los primeros segmentos espinales. Esta organización citoarquitectónica ha sido corroborada con experimentos inmunohistoquímicos y mediante el estudio de las conexiones aferentes y eferentes de ambos núcleos (ver más adelante).

El Nsol y el nVds, identificados en base a resultados hodológicos e inmunohistoquímicos, se localizan en la banda celular interna en posiciones dorsomedial y ventrolateral respectivamente, si bien no están citoarquitectónicamente segregados entre si, ni de otras celulas ventromediales a ellos, por lo que no pudieron ser identificados mediante tinciones de Nissl.

Quimioarquitectura

En mamíferos la sintasa del óxido nítrico (NOS), el cual probablemente desempeña un papel importante como mensajero neuronal (Bredt y Snyder, 1992; Meller y Gebhart, 1993; Schuman y Madison, 1994), se expresa en neuronas de núcleos relacionados con el procesamiento de información somática, como los núcleos de la columna dorsal, el nVds y el Nsol, presentes en la placa alar de la región del óbex (Leight y cols., 1990; Vincent y Kimura, 1992; Valtschanoff y cols., 1993; Dohrn y cols., 1994; Takemura y cols., 1994). Debido a que la NOS tiene actividad NADPH-d (Dawson et al., 1991; Hope et al., 1991), la distribución de ambas son idénticas (Bredt y Snyder, 1992), por lo que la NADPHd puede usarse como un marcador para NOS.

En nuestros experimentos en *Pleurodeles waltl*, con tinciones tanto histoquímicas frente a NADPHd como inmunohistoquímicos frente a NOS, hemos observado una población muy patente de neuronas positivas en la región del óbex, localizadas en la mitad externa y ventrolateralmente dentro de la banda celular interna. Las dendritas de estas células se dirigen dorsolateralmente e invaden la sustancia blanca dorsolateral, correspondiente al tracto descendente del nervio trigémino (Vds), por lo que dicha población neuronal se ha considerado como una parte del nVds, coincidiendo con los datos descritos en mamíferos (Leight y cols., el 1990; Vincent y Kimura, 1992; Dohrn y cols., 1994; Takemura y cols., 1994).

En *Pleurodeles waltl* únicamente hemos observado un escaso número de neuronas positivas para NADPHd o NOS en el DCN o el Nsol a nivel de óbex, contrastando con los datos descritos en anuros (A. Muñoz y cols., 1995: M. Muñoz y cols., 1996) y en mamíferos (Leight y cols., 1990; Vincent y Kimura, 1992; Valtschanoff y cols., 1993; Dohrn y cols, 1994; Takemura y cols., 1994). La mayor concentración de neuronas positivas para NADPHd o NOS en el Nsol está presente en los niveles rombencefálicos más

rostrales. En experimentos en los que se combinaron las tinciones para NADPHd o NOS con la inmunodetección de la tirosina hydroxilasa (TH), se comprobó que la población neuronal positiva para NADPHd-NOS en estos niveles, se dispone ventralmente al tracto solitario y está entremezclada con neuronas catecolaminérgicas (positivas para TH) del Nsol, por lo que consideramos que pertenece a dicho núcleo. En la región del óbex las neuronas catecolaminérgicas del Nsol se disponen en la zona dorsomedial de la banda celular interna, y sus prolongaciones se dirigen lateral y ventrolateralmente hasta el funículo dorsolateral. En su recorrido dichas prolongaciones se cruzan con las dendritas de las neuronas del nVds, positivas para NOS-NADPHd y dirigidas hacia el Vds.

En mamíferos la proteína ligante de calcio, calbindina D28k (Calb), se expresa en algunas estructuras somatosensoriales (Celio, 1990; Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a,b; Maslany y cols., 1992; Ren y Ruda, 1994). En la rata, existen neuronas positivas para Calb en determinadas láminas del asta dorsal de la médula espinal (Antal y cols., 1990; Menétrey y cols., 1992b; Ren y Ruda, 1994), en los núcleos sensitivos trigeminales y en menor número en los núcleos *gracilis* y *cuneatus* (Celio, 1990; Maslany y cols., 1992). En distintas especies de mamíferos se ha demostrado que las neuronas positivas para Calb, constituyen una parte importante de los sistemas de proyección trigeminales, del núcleo del tracto solitario y de las proyecciones espinales ascendentes (Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a).

Nuestros resultados en *Pleurodeles waltl*, de acuerdo con las observaciones realizadas en anuros (A. Muñoz y cols., 1995), demuestran la existencia de dos poblaciones neuronales positivas para Calb en la placa alar de la región del óbex. Se localizan dorsomedial y ventrolateralmente en la banda celular interna y pertenecen al Nsol y nVds respectivamente. Las prolongaciones de las neuronas positivas para Calb del Nsol y nVds se entremezclan en sus recorridos hacia el DLF y el Vds respectivamente.

En experimentos inmunohistoquímicos hemos podido comprobar la presencia de fibras inmunoreactivas para sustancia P y Leu-encefalina, en el DLF y en menor grado en el DF y el Vds. Igualmente se observó que existen terminales positivos de sustancia P y Leu-encefalina, así como para serotonina, que alcanzan el DCN, LCN, Nsol y nVds. El patrón de marcaje para neuropéptido Y en esta región es más restringido y se limita a fibras positivas que abandonan el DLF, para inervar la región lateral de la placa alar. Esta inervación peptidérgica y serotoninérgica está de acuerdo con los datos obtenidos en anuros (capítulo 5 de la presente memoria; Ueda y cols., 1984; Adli y cols., 1988; Merchenthaler y cols., 1989; Lázár y cols., 1990; Petkó y Sánta, 1992; A. Muñoz y cols., 1995) y con resultados descritos en mamíferos (Steinbusch, 1981; Westman y cols., 1984; Halliday y cols., 1988; Ibuki y cols., 1989; Tamatani y cols., 1989; Conti y cols., 1990; Fabri y Conti, 1990; Blomqvist y Broman, 1993).

Conectividad

Además de los criterios citoarquitectónicos e inmunohistoquímicos, en el presente trabajo se han caracterizado las distintas poblaciones neuronales de la placa alar de la región del óbex, mediante el estudio de algunas de sus conexiones tanto aferentes como eferentes.

Aplicaciones del trazador en las raíces dorsales espinales.

En experimentos con aplicaciones de dextrano amina combinada con biotina (BDA) en raíces espinales cervicales y lumbares en *Pleurodeles waltl*, hemos observado que las aferencias primarias espinales, al entrar en la médula espinal, se dividen en un componente medial y otro lateral (tracto de Lissauer) cuyas fibras ascienden y descienden en el DF y DLF respectivamente. En niveles espinales ambos componentes inervan distintas regiones, si bien la atención del presente trabajo se centró, fundamentalmente, en el estudio de los componentes ascendentes que alcanzan la región del óbex.

Fibras del tracto de Lissauer correspondientes a la segunda raíz espinal, ascienden en el DLF hasta el polo rostral del complejo motor de los núcleos de los nervios craneales VII, IX y X, atravesando por lo tanto región del óbex. En su recorrido, algunas fibras alcanzan la banda celular externa donde se localiza el LCN, y en menor medida la banda celular interna, donde se sitúa el nVds. Igualmente observamos que las fibras del componente medial ascienden en el DF, organizadas somatotópicamente, confirmando datos previos aislados, basados en tinciones argénticas (Herrick, 1914; 1930; 1944), y de degeneración anterógrada (Nieuwenhuys y Cornelisz, 1971), y coincidiendo con el patrón de organización presente en anuros (ver capítulo 5 de la presente memoria). Las aferencias primarias procedentes de segmentos lumbares ascienden en la parte medial del DF, mientras que las aferencias braquiales lo hacen en posición más lateral. De la misma manera terminan en la región del óbex, principalmente en el DCN situado en la banda celular extrena, si bien un pequeño componente de fibras varicosas podría alcanzar al Nsol, nVds y LCN. Rostralmente al óbex, el componente medial de las aferencias braquiales asciende a través del rombencéfalo, hasta la capa granular del cerebelo.

Aplicaciones del trazador en el nervio trigémino

En experimentos con aplicaciones de BDA o de peroxidasa de rábano (HRP) en la raíz del nervio trigemino, se observó el recorrido descendente de algunas de sus aferencias primarias a través del Vds. En la placa alar de la región del óbex algunas fibras varicosas abandonan dicho tracto ventromedialmente, para dar terminales en las bandas celulares externa e interna, mayoritariamente en el nVds y en menor número en las zonas del DCN, Nsol y LCN.

Aplicaciones del trazador en el asta dorsal cervical

En nuestros experimentos con aplicaciones de BDA en niveles espinales cervicales, se marcaron fibras que discurren en el DF, DLF y Vds.

En el DF se observaron dos componentes: 1) El localizado en su porción medial, un tracto de fibras gruesas y agrupadas de manera muy compacta, que atraviesa la región del óbex sin dar ramas terminales, y termina en el lóbulo de la línea lateral. Dicho componente corresponde al tracto A de Kingsbury (1895), formado principalmente por aferencias primarias de la segunda raíz del complejo de los nervios IX-X (Roth y Wake, 1985), que inervan el sistema de la línea lateral (Kreht, 1930) y descienden hasta la médula espinal, donde se incorporan al DF (Kreht, 1930; Herrick, 1944; 1948; Roth y Wake, 1985). Herrick (1944; 1948) sugirió que el DCN podría recibir información del tracto A de Kingsbury, sin embargo, según nuestros resultados, esto parece poco probable, debido a que a nivel del óbex sus fibras, muy gruesas,

se disponen muy empaquetadas y no emiten colaterales. Además, en algunas especies de anuros, como *Xenopus laevis*, que retienen el sistema de la línea lateral en estadios adultos, sus aferencias no terminan en la región del DCN (Lowe y Russell, 1982; Altmany Dawes, 1983; Fritzsch y cols., 1984; Will y cols., 1985a). 2) El segundo componente ocupa una posición lateral en el DF y está formado por un sistema ascendente de fibras que terminan fundamentalmente en el DCN, si bien un menor número continúa rostralmente hasta la capa granular del cerebelo. Dicho sistema está constituído por aferencias primarias espinales, ya que las características de sus fibras coinciden con las que presentan las fibras marcadas en nuestros experimentos con aplicaciones en las raíces primarias espinales; aunque además podría incluir proyecciones no primarias, como las del sistema postsináptico de la columna dorsal, cuya presencia, aunque no se ha descrito hasta el momento en urodelos, ha sido demostrada en otros vertebrados, incluyendo los anuros (Rustioni, 1973; Angaut-Petit, 1975a,b; Uddenberg, 1968; Rustioni y Kaufman, 1977; Bennett y cols., 1984; Giesler y cols., 1974; Kamogawa y Bennett, 1986; Funke, 1988; ten Donkelaar y de Boer-van Huizen, 1991; Pritz y Stritzel, 1994; A. Muñoz y cols., 1995).

Las fibras ascendentes marcadas en el funículo dorsolateral, abandonan medialmente el tracto en niveles cervicales superiores y organizan un campo de terminales que alcanza el LCN. Dichas fibras corresponden a aferencias espinales primarias del tracto de Lissauer así como a proyecciones no primarias, que podrían incluir el tracto espinocervical, presente en anuros (ver capítulos 3 y 5 de la presente memoria).

Lemnisco medial

Existen muy pocos datos en la bibliografía sobre las conexiones eferentes del DCN en urodelos, y están basados únicamente en estudios realizados mediante tinciones argénticas. Herrick (1944; 1948) y Herrick y Bishop (1958) negaron la existencia del lemnisco medial en *Ambystoma tigrinum*, y compararon el DCN de urodelos con el núcleo cuneado externo, y no con los núcleos *gracilis* y *cuneatus* de mamíferos. Los citados autores decribieron proyecciones que se originan en la región del óbex y ascienden a través de los lemniscos espinal y general bulbar, a la formación reticular, cerebelo, techo mesencefálico y al tálamo dorsal; así como proyecciones ipsilaterales que alcanzan el cerebelo mezcladas con fibras de los tractos A y B de Kingsbury. Sin embargo, no existen trabajos experimentales, basados en técnicas de degeneración o de trazado neuronal, que confirmen las sugerencias de Herrick (1944, 1948). Únicamente en un estudio con aplicaciones de HRP en el torus semicircularis de *Salamandra salamandra* se describieron neuronas retógradamente marcadas en la región rombencefálica más caudal (Manteuffel y Naujoks-Manteuffel; 1990). Por el contrario en algunos trabajos se ha sugerido la ausencia en urodelos del lemnisco medial (Ebbesson y cols., 1972; Naujoks-Manteuffel y Manteuffel, 1986; Wicht y Himstdet, 1988).

Aplicaciones en el tálamo ventral y en el torus semicircularis.

En el presente estudio se han realizado aplicaciones de BDA en el tálamo ventral y en el torus semicircularis, con objeto de comprobar la posible existencia del lemnisco medial en *Pleurodeles waltl*, e identificar las neuronas que lo originan. En ambos casos se han observado en la placa alar de la región del óbex, neuronas retrógradamente marcadas,

mayoritariamente en el lado contralateral, en dos poblaciones neuronales diferenciables. La primera corresponde al DCN y se localiza en posición dorsomedial en la capa celular externa, a nivel del óbex y los primeros segmentos espinales. Dicha población está formada por neuronas de diferentes morfologías con dendritas orientadas hacia el funículo dorsal, a través del cual, presumiblemente, reciben información espinal ascendente. En los citados experimentos no se observó una división evidente entre componentes *gracilis* (medial) y *cuneatus* (lateral). La segunda población corresponde al LCN y se sitúa en posición ventrolateral en la capa celular externa, y se extiende desde el óbex hasta niveles ligeramente más caudales que el DCN. Sus neuronas tienen dendritas dirigidas lateralmente hacia el DLF, y en ocasiones algunas de sus neuronas se encuentran segregadas dentro de la propia sustancia blanca del DLF. En algunos casos se observaron células retrógradamente marcadas en posiciones intermedias entre ambas poblaciones, en el lugar donde se arborizan las aferencias trigeminales por lo que podrían corresponder al nVds. Los axones de la neuronas del DCN y LCN cruzan la línea media ventralmente al canal central, para inorporarse al funículo ventrolateral contralateral, y ascender a través del lemnisco medial hasta el torus semicircularis y el tálamo.

Además del tálamo y del torus semicircularis, el lemnisco medial de urodelos podría inervar otras regiones cerebrales como ocurre en otros vertebrados (ver capítulo 5 de la presente memoria). Algunos trabajos han descrito, por ejemplo, el procesamiento de información somatosensorial en el neuropilo profundo del techo óptico en diversas especies de urodelos (Grüsser-Cornehls y Himstedt, 1973; Gruberg y Solish, 1978; Gruberg y Harris, 1981; Harris, 1982, 1989; Stirling y Brandle, 1982; Roth y cols., 1990), que además de mediante las aferencias espinales (Naujoks Manteuffel y Manteuffel, 1988; Herrick, 1914, 1942, 1948; Nieuwenhuys y Cornelisz, 1971; Jakway y Riss, 1972; Gruberg, 1973; Gruberg y Solish, 1978; Finkenstadt y cols., 1983; Rettig, 1988, 1989; A. Muñoz y cols. capítulo 3 de la presente memoria) podrían recibirla a través de las aferencias somatosensoriales procedentes de la región del óbex, como en anuros (capítulo 5 de la presente memoria). Cabe resaltar que Finkenstädt y cols. (1983), en experimentos con aplicaciones de HRP en el techo óptico de *Salamandra salamandra*, observaron neuronas retrógradamente marcadas en los segmentos espinales más rostrales, en una localización similar a la descrita en este estudio para el DCN.

Así pues en el presente trabajo se ha demostrado la existencia del lemnisco medial en urodelos, mediante el estudio de sus proyecciones al tálamo ventral y al torus semicircularis. Sin embargo, resulta necesaria la aplicación de trazadores anterógrados en la región del DCN y del LCN, para conocer la anatomía detallada del lemnisco medial en urodelos así como la totalidad de los centros a los que pueda proyectar.

CAPÍTULO 7

1- Se ha demostrado la **viabilidad de preparaciones *in vitro*** en las que el sistema nervioso central de anfibios, completo y aislado del cuerpo del animal, puede mantenerse vivo durante varios días en unas condiciones que permiten la realización de experimentos anatómicos y electrofisiológicos, cuyos **resultados son comparables** a los obtenidos con preparaciones *in vivo*.

2- Mediante trazado neuronal así como técnicas histoquímicas e inmunohistoquímicas, se han caracterizado en anuros y urodelos, los **núcleos de la columna dorsal, cervical lateral, descendente del nervio trigémino y del tracto solitario.**

3- En anuros y en urodelos las proyecciones somatosensoriales espinales ascendentes se organizan, al igual que en vertebrados amniotas, en **tres componentes: funículo dorsal**, funículo **dorsolateral** y **cuadrante ventral** (funículos dorsal y dorsolateral).

4- El **funículo dorsal** de anuros y urodelos posee **fibras primarias** procedentes de las neuronas ganglionares espinales, en anuros incluye además fibras **no primarias** del **sistema postsináptico de la columna dorsal.** Todos los componentes presentan una **somatotopía mediolateral** y terminan mayoritariamente en el **núcleo de la columna dorsal.**

5- **El funículo dorsolateral** de los anfibios estudiados está formado por fibras **primarias** del **tracto de Lissauer** y fibras ascendentes **no primarias**. En las últimas se incluyen el **tracto espinocervical,** que termina en el **núcleo cervical lateral** así como otras fibras que rostralmente alcanzan el cerebelo y distintos centros del tronco cerebral como la **formación reticular lateral** y el **área parabraquial.**

6- Tanto en anuros como en urodelos se ha demostrado la existencia, en el **cuadrante ventral,** de **proyecciones espinotalámicas** directas que alcanzan diversos núcleos del tálamo dorsal y ventral. Así como proyecciones que terminan en distintos núcleos del **torus semicircularis** que, junto con las del lemnisco medial, constituyen el sustrato anatómico por el que la información somatosensorial alcanza dicho centro, en el que se ha descrito un mapa somatotópico de representación de la superficie corporal.

7- Los **núcleos de la columna dorsal** y **cervical lateral** proyectan, en anuros y urodelos, a través del **lemnisco medial,** al **torus semicircularis** y al **tálamo.**

8- En *Xenopus laevis* el **desarrollo** de las aferencias al núcleo de la columna dorsal precede al de sus eferencias al mesencéfalo y al tálamo, por lo que se puede considerar que existe un **patrón de determinación periférico-central** en el sistema columna dorsal-lemnisco medial.

9- Los datos hodológicos e inmunohistoquímicos obtenidos demuestran la existencia, tanto en anuros como en urodelos, de los sistemas **columna dorsal-lemnisco medial** y **espino-cervico-talámico, comparables** a los descritos en vertebrados amniotas.