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Distribution of Choline Acetyltransferase Immunoreactivity in the Brain of Anuran (*Rana perezi*, *Xenopus laevis*) and Urodele (*Pleurodeles waltl*) Amphibians

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ABSTRACT

Because our knowledge of cholinergic systems in the brains of amphibians is limited, the present study aimed to provide detailed information on the distribution of cholinergic cell bodies and fibers as revealed by immunohistochemistry with antibodies directed against the enzyme choline acetyltransferase (ChAT). To determine general and derived features of the cholinergic systems within the class of Amphibia, both anuran (*Rana perezi*, *Xenopus laevis*) and urodele (*Pleurodeles waltl*) amphibians were studied. Distinct groups of ChAT-immunoreactive cell bodies were observed in the basal telencephalon, hypothalamus, habenula, isthmic nucleus, isthmic reticular formation, cranial nerve motor nuclei, and spinal cord. Prominent plexuses of cholinergic fibers were found in the olfactory bulb, pallium, basal telencephalon, ventral thalamus, tectum, and nucleus interpeduncularis. Comparison of these results with those obtained in other vertebrates, including a segmental approach to correlate cell populations, reveals that the cholinergic systems in amphibians share many features with amniotes. Thus, cholinergic pedunculopontine and laterodorsal tegmental nuclei could be identified in the amphibian brain. The finding of weakly immunoreactive cells in the striatum of *Rana*, which is in contrast with the condition found in *Xenopus*, *Pleurodeles*, and other anamniotes studied so far, has revived the notion that basal ganglia organization is more preserved during evolution than previously thought. J. Comp. Neurol. 382:499–534, 1997.

Indexing terms: acetylcholine; striatum; basal forebrain; pontomesencephalic reticular formation; motor nuclei

Although acetylcholine was the first molecule shown to act as a neurotransmitter, it was not until the early 1980s that insight was gained into the organization of cholinergic systems in the central nervous system (CNS) of vertebrates. The morphological visualization of cholinergic systems in the CNS was hampered by the lack of a specific technique for unambiguous identification of cholinergic neurons and fiber tracts. In short, acetylcholine is synthetized from choline by the enzyme choline acetyltransferase (ChAT), whereas another enzyme, i.e., acetylcholinesterase (AChE), is involved in its degradation. The development of histochemical techniques to demonstrate AChE has led to a vast number of articles dealing with putative cholinergic cell bodies and fibers in the CNS of vertebrates (for reviews, see Kása, 1986; Woolf, 1991), including amphibians (Northcutt, 1974; Ciani et al., 1988). However, it became clear that AChE also occurred in noncholinergic cells, and, consequently, its demonstration could not be considered a reliable marker of cholinergic cells and fibers. On the contrary, ChAT appeared to be closely related with the distribution of acetylcholine. The development of anti-

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bodies against ChAT has, therefore, given new impetus to the study of cholinergic systems in vertebrates. Thus, on the basis of immunohistochemistry for ChAT, the cholinergic systems have been described in several mammalian species (cats: Kimura et al., 1981; Vincent and Reiner, 1987; rats: Houser et al., 1983; Tago et al., 1989; dogs: St-Jacques et al., 1996; primates: Mesulam et al., 1984; Satoh and Fibiger, 1985a; guinea pigs: Maley et al., 1988), birds (chickens: Sorenson et al., 1989; pigeons: Medina and Reiner, 1994), reptiles (crocodiles: Brauth et al., 1985; turtles: Mufson et al., 1984; Powers and Reiner, 1993; lizards: Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993), and several teleosts (Ekström, 1987; Brantley and Bass, 1988; Zottoli et al., 1988; Molist et al., 1993). These studies have shown many common features of the cholinergic systems among vertebrates. For example, in all vertebrates studied, cholinergic neurons are present in the basal telencephalon, habenula, isthmic tegmentum, and cranial nerve motor nuclei, and cholinergic fiber systems innervate the optic tectum and the interpeduncular nucleus. In addition, the striatum and the nucleus accumbens of amniotes contain intrinsic cholinergic neurons that exert influence on the activity of basal ganglia projection neurons (Kása, 1986; Alheid and Heimer, 1988). Such neurons are apparently lacking in the brain of teleosts (Ekström, 1987; Brantley and Bass, 1988), and the existence of basal ganglia interneurons therefore may be a derived feature of amniotic brains (Medina and Reiner, 1995). Unfortunately, data on the cholinergic systems in the CNS of amphibians are limited. Only the cholinergic isthmotectal pathway (Desan et al., 1987) and the efferent cells of the octavolateralis system (González et al., 1993a) have been studied by means of ChAT immunohistochemistry. So far, no attempts have been made to

Abbreviations

A	anterior thalamic nucleus
ac	anterior commissure
Acc	nucleus accumbens
Ad	nucleus anterodorsalis tegmenti
aob	accessory olfactory bulb
al	adenohypophysis
ap	anterior parencephalon
Apl	amygdala, pars lateralis
Apm	amygdala, pars medialis
C	central thalamic nucleus
Cb	cerebellum
cc	central canal
cStr	caudal striatum
DB	diagonal band of Broca
DCN	dorsal column nucleus
Dp	dorsal pallium
dStr	dorsal striatum
Ea	anterior entopeduncular nucleus
em	eminencia mediana
epl	external plexiform layer
EW	Edinger-Wesphal nucleus
flm	fasciculus longitudinalis medialis
fr	fasciculus retroflexus
gl	glomerular layer of the olfactory bulb
GT	griseum tectale
Hd	dorsal habenula
igl	internal granular layer
il	intermediate lobe of the hypophysis
inf	infundibulum
Ip	nucleus interpeduncularis
Is	nucleus isthmi
L	nucleus lentiformis
La	lateral thalamic nucleus, anterior division
Lc	locus coeruleus
LDT	laterodorsal tegmental nucleus
lfb	lateral forebrain bundle
Lp	lateral pallium
Lpd	lateral thalamic nucleus, posterodorsal division
Ls	lateral septum
m	mesencephalon
MC	Mauthner cell
MCa	Mauthner cell axon
ml	mitral cell layer of the olfactory bulb
Mp	medial pallium
Ms	medial septum
nl	neural lobe of the hypophysis
nPT	nucleus pretectalis
NPv	nucleus of the hypothalamic periventricular organ
nri	nucleus reticularis isthmi
Nsol	solitary tract nucleus
nsp 2	2nd spinal nerve
nII	nervus opticus
nIII	nervus oculomotorius
nIV	nervus trochlearis
nV	nervus trigeminus
nVI	nervus abducens
nVII	nervus facialis
nVIII	nervus octavus
nIX	nervus glossopharyngeus
nXII	nervus hypoglossus
oc	optic chiasm
Pb	parabrachial nucleus
pc	posterior commissure
PGPS	preganglionic parasympathetic cells
pp	posterior parencephalon
PPN	pedunculopontine tegmental nucleus
POa	preoptic area
Ra	raphe nuclei
rh1–8	rhombomeres 1–8
Ri	nucleus reticularis inferior
Rm	nucleus reticularis medius
Rs	nucleus reticularis superior
rStr	rostral striatum
s	synencephalon
SC	nucleus suprachiasmaticus
sol	solitary tract
sp.mot	spinal cord motoneurons
spta	striato-pallial transition area
Str	striatum
tect	tectum mesencephali
Tl	laminar nucleus of the torus semicircularis
Tp	principal nucleus of the torus semicircularis
TPdm	tuberculum posterius, dorsomedial part
TPvl	tuberculum posterius, ventrolateral part
v	ventricle
Ve	vestibular nuclei
VH	ventral hypothalamic nucleus
VL	ventrolateral thalamic nucleus
VM	ventromedial thalamic nucleus
vStr	ventral striatum
VT	ventral thalamus
zav	zona anteroventralis
ZIp	periventricular nucleus of the zona incerta
zpd	zona posterodorsalis
III	nucleus nervi oculomotorii
IV	nucleus nervi trochlearis
Vd	tractus descendens nervi trigemini
Vm	nucleus motorius nervi trigemini
VIa	accessory abducens nucleus
VIm	main abducens nucleus
VIIm	nucleus motorius nervi facialis
VIIIc	nucleus caudalis nervi octavi
VIIIe	efferent cells of the nucleus nervi octavi
VIIIv	nucleus ventralis nervi octavi
IXm	nucleus motorius nervi glossopharyngei
Xm	nucleus motorius dorsalis nervi vagi
XI	nucleus of the accessory nerve
XI-sp.mot	nucleus of the accessory nerve and spinal cord motoneurons
XII l	nucleus motorius lateralis nervi hypoglossi
XIIm	nucleus motorius medialis nervi hypoglossi

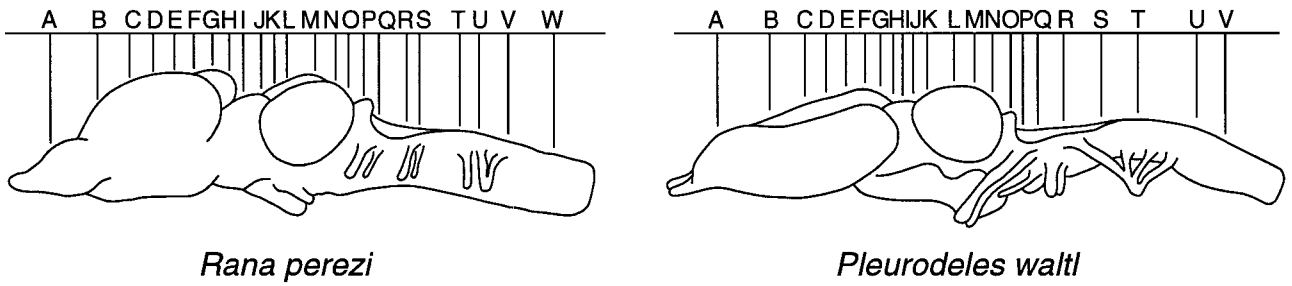

Fig. 1. Lateral view of the brains of the frog *Rana perezi* and the newt *Pleurodeles waltl*. The letters on the top refer to the levels of transverse sections shown in Figures 2 and 9, respectively.

study immunohistochemically the distribution of cholinergic perykarya and fibers throughout the entire brain of amphibians, although some histochemical and biochemical studies have determined ChAT activity in several areas of the brain and spinal cord of frogs (Ciani et al., 1988; Wallace et al., 1990).

The main goal of this study was to provide detailed information on the localization of cholinergic neuronal elements in anuran (*Rana perezi*, *Xenopus laevis*) and urodele (*Pleurodeles waltl*) amphibians. Because two different orders of amphibians were involved, we thought we could gain insight into primitive or derived conditions of the cholinergic system in the class of Amphibia. Furthermore, by comparing the cholinergic systems of amphibians with those of amniotes, a better understanding of the evolution of the cholinergic systems of vertebrates could be expected. In particular, the possibility of dopamine–acetylcholine interactions in the striatum and the basal ganglia projections to the cholinergic pedunculopontine tegmental nucleus seemed to be of interest.

MATERIALS AND METHODS

For the present study, 12 adult Iberian green frogs (*Rana perezi*), 10 South African clawed frogs (*Xenopus laevis*), and 10 Iberian ribbed newts (*Pleurodeles waltl*) were used. The animals were obtained from the laboratory stock of the Department of Cell Biology, University Complutense of Madrid. All animals were anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz; pH 7.3) and perfused transcardially with saline followed by 150–200 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer (PB; pH 7.4). The brain and spinal cord were removed and kept in the same fixative for 2–3 hours. Subsequently, they were immersed in a solution of 30% sucrose in PB for 3–5 hours at 4°C until they sank, embedded in a solution of 15% gelatin with 30% sucrose in PB, and then stored for 5 hours in a 4% formaldehyde solution at 4°C. The brains were cut on a freezing microtome at 40 µm in the frontal, sagittal, or horizontal plane and collected in PB. They were then rinsed twice in PB, treated with 1% H_2O_2 in PB saline (PBS; pH 7.4) for 15 minutes to reduce endogenous peroxidase activity, and rinsed again three times in PBS. Sections were then processed for ChAT immunohistochemistry by the peroxidase antiperoxidase (PAP) method (Sternberger, 1979). This method included a first incubation of the sections in a goat anti-ChAT serum (Chemicon), diluted 1:100 in PBS containing 0.5% Triton X-100 (PBS-T), 15% normal rabbit serum (NRS), and 2% bovine serum albumin (BSA) for 40 hours at 4°C. Subsequently, the sections were rinsed three times in PBS for 10 minutes and incubated for 1 hour in rabbit anti-goat serum (1:50, Chemicon). After rinsing again three times for 10 minutes, the sections were incubated for 90 minutes in goat PAP (1:600, Chemicon). Secondary antiserum and PAP complex were diluted in PBS-T, NRS, and BSA in the same concentrations as those used for the primary antiserum. Finally, the sections were rinsed three times for 10 minutes in PBS and twice in 0.05 M Tris-HCl buffer (TB; pH 7.6) and subsequently stained in 0.5 mg/ml 3,3′-diaminobenzidine (DAB) with 0.01% H_2O_2 in TB for 5–15 minutes. A series of sections was stained according to the glucose oxidase method (Shu et al., 1988), which specifically enhances the staining of nerve fibers and terminals. Briefly, after rinsing in PBS, the sections were rinsed in 0.1 M acetate buffer (AB; pH 6.0) for 10 minutes and then incubated in a medium containing 0.5 mg/ml DAB, 0.027 mg/ml glucose oxidase (Sigma, Madrid, Spain, type VII), 25 mg/ml nickel ammonium sulfate (Merck, Darmstadt, Germany), 2 mg/ml D-glucose (Merck), and 0.4 mg/ml ammonium chloride (Merck) in AB for 5–10 minutes. The sections were rinsed twice in AB and another three times in TB. The sections were then mounted (mounting medium: 0.25% gelatin in TB) and, after drying overnight, coverslipped. Some sections were counterstained with cresyl violet to facilitate the analysis of the results.

The specifity of the antisera has been previously tested (Shiromani et al., 1987; Medina and Reiner, 1994; Grosman et al., 1995). As a further control, the primary antiserum was omitted from some sections in each experiment, which resulted in no specific labeling of somata or fibers. For the description and mapping of ChAT-immunoreactive (ChATi) cell bodies and fibers in anurans, *Rana perezi* was chosen as the core species. If not stated otherwise, the description also holds for *Xenopus laevis*. The distribution of ChATi cell bodies and fibers in the brains of *Rana perezi* and *Pleurodeles waltl* was charted in representative transverse sections (levels indicated in Fig. 1) by a camera lucida. The nomenclature in the present study is essentially the same as that used in previous studies (González and Smeets, 1991; González et al., 1993b; Marín et al., 1997a). In addition, the nomenclature of Puelles et al. (1996) is largely followed for the anuran diencephalon, and that of Potter (1969) and Roth et al. (1990) are followed for the tectum of anurans and urodeles, respectively.

RESULTS

The antibodies against ChAT used in the present study revealed patterns of immunostaining that, for each of the

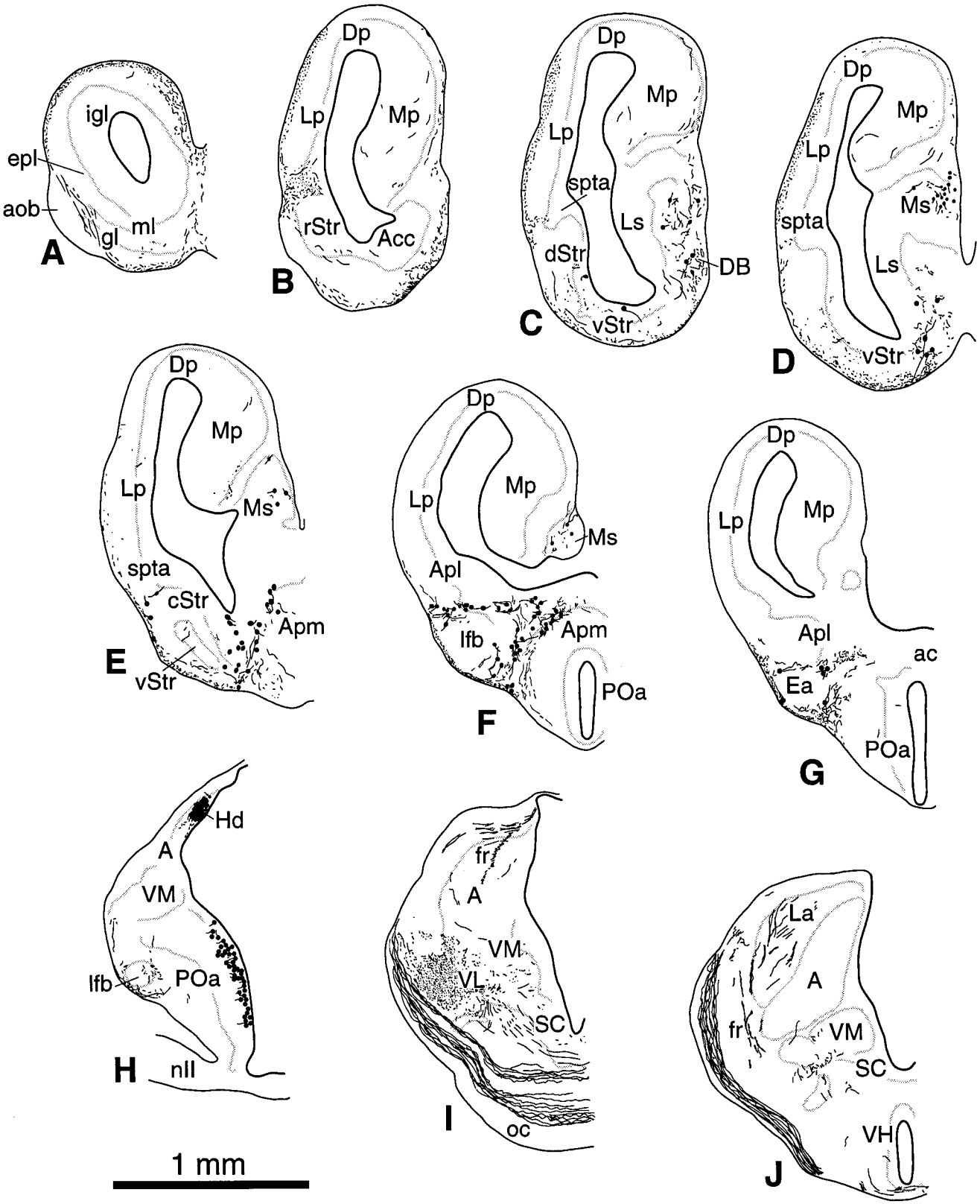

Fig. 2. **A–W:** Diagrams of transverse sections through the brain of the frog *Rana perezi* (levels indicated in Fig. 1) showing the distribution of immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines) in the left half of each section.

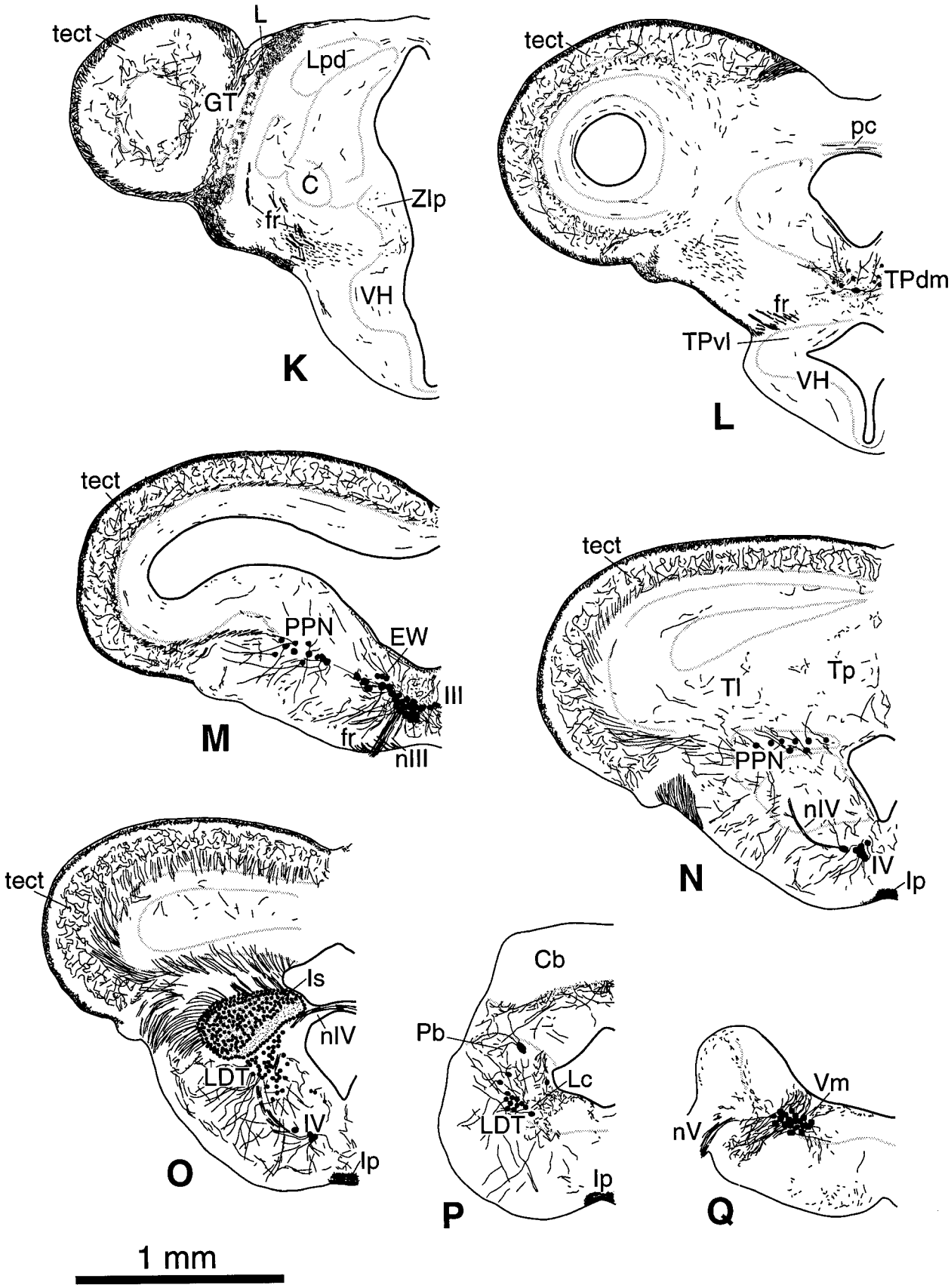

Figure 2 (Continued.)

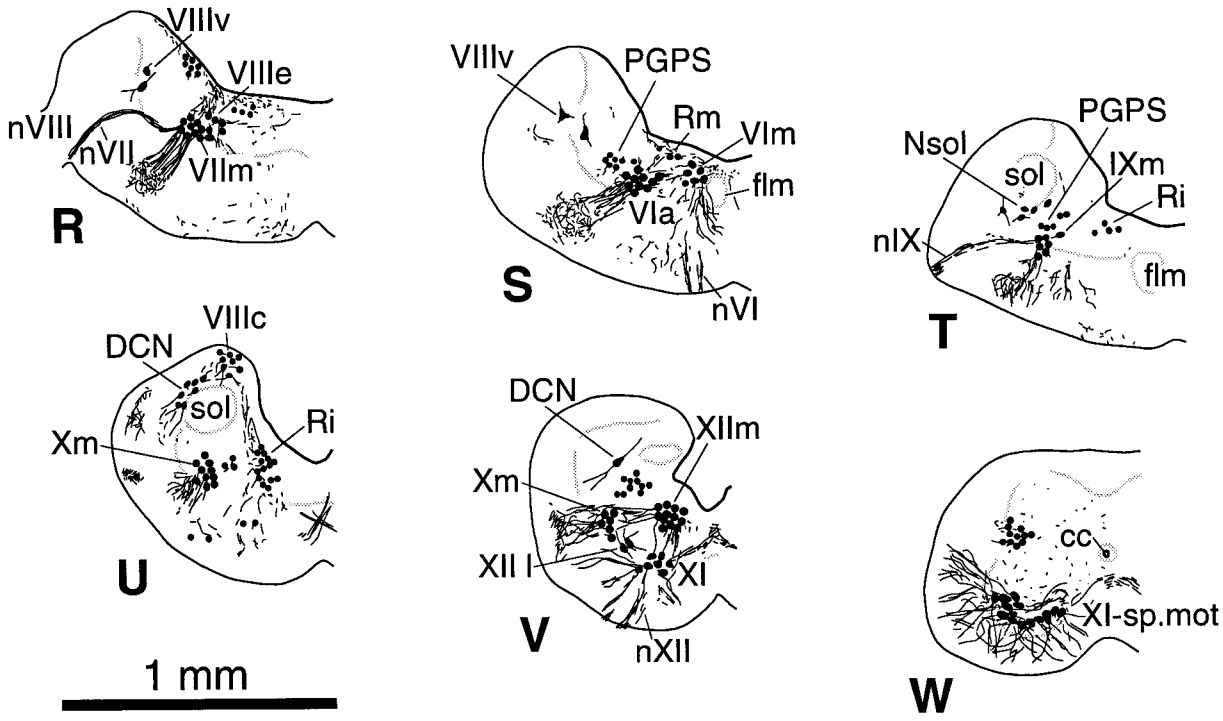

Figure 2 (Continued.)

three species examined, were constant from animal to animal. The distribution of cholinergic cells and fibers was studied in series processed with or without nickel intensification. The results obtained by the different procedures were essentially similar, but the use of the glucose oxidase-DAB-nickel technique appeared to be especially beneficial for visualizing nerve fibers and terminals.

ChATi cell bodies

Anurans

Telencephalon. The most rostrally located ChATi cell bodies were found in the striatum (Fig. 2C). The striatal ChATi cells, which were few in number and only weakly stained, are medium sized and possess a main process oriented ventrally (Fig. 3a). They are scattered throughout the striatal cell plate at intermediate hemispheric levels. From the intermediate hemispheric levels and farther caudally, numerous ChATi cell bodies were found in the medial wall of the hemisphere in the diagonal band of Broca (Fig. 2C–D). These cells are large and possess long processes. Dorsally, the cells of the diagonal band are continuous with another, rather compact group of ChATi cells in the dorsomedial part of the medial septal nucleus (Figs. 2D–F, 3b). The latter cells are less darkly stained and smaller in size than those in the diagonal band. More caudally in the telencephalon, ChATi cells lie intermingled with the fibers of the medial forebrain bundle (Fig. 2D). In addition, some ChATi cells were found in the medial amygdala, and others occurred in the ventrolateral telencephalic wall immediately lateral to the lateral forebrain bundle (Fig. 2D). At the level of the anterior preoptic area, numerous ChATi cell bodies surround the lateral forebrain bundle (Figs. 2E–G, 3c). In addition, strongly immunoreactive cells were observed within the lateral forebrain bundle and the medial amygdala, whereas weakly immunoreactive cells occurred in the superficial aspect of the caudal striatum (Fig. 2E–F). Occasionally, weakly ChATi cells were found in the lateral amygdala. The ChATi cells scattered throughout the basal telencephalon seem to constitute a more or less continuous field of immunoreactive neurons. In *Xenopus*, a similar distribution of ChATi cell bodies was observed, except for the striatum, which appeared to be devoid of immunoreactive cells.

Diencephalon. In the rostral hypothalamus, weakly stained ChATi cell bodies were observed in the ventral portion of the suprachiasmatic nucleus and among the magnocellular neurons in the caudal pole of the preoptic nucleus in both anuran species (Fig. 2H). Only in *Xenopus* were additional ChATi cell bodies seen, in large numbers, in the infundibular hypothalamus throughout its rostrocaudal extent. These cells line the infundibular ventricle and possess a single, long process, which is directed laterally or ventrolaterally. In spite of their position close to the ventricle, no evidence was found to indicate that these cells contact the cerebrospinal fluid (CSF). In the caudal hypothalamus of both species, small ChATi neurons were located in the retromammillary region and in the dorsomedial part of the posterior tubercle. In the epithalamus, a distinct population of ChATi cells was found in the habenula. Densely packed, small ChATi cells occurred in the asymmetric dorsal habenular nuclei but were absent in the ventral habenular nuclei (Fig. 3d). The axons of these cells form the fasciculus retroflexus, which courses ventrocaudalward through the thalamus as separate bundles of immunoreactive fibers.

Mesencephalon. In the mesencephalic tegmentum, the oculomotor motoneurons were strongly ChATi (Figs. 2M, 4a–c). In horizontal sections, the cells constitute two parallel columns of cells that remain separate in the rostral part of the nucleus but merge together in its caudal part, thus forming a single cell group along the midline (Fig. 4c). The cells have dendrites that extend dorsally and

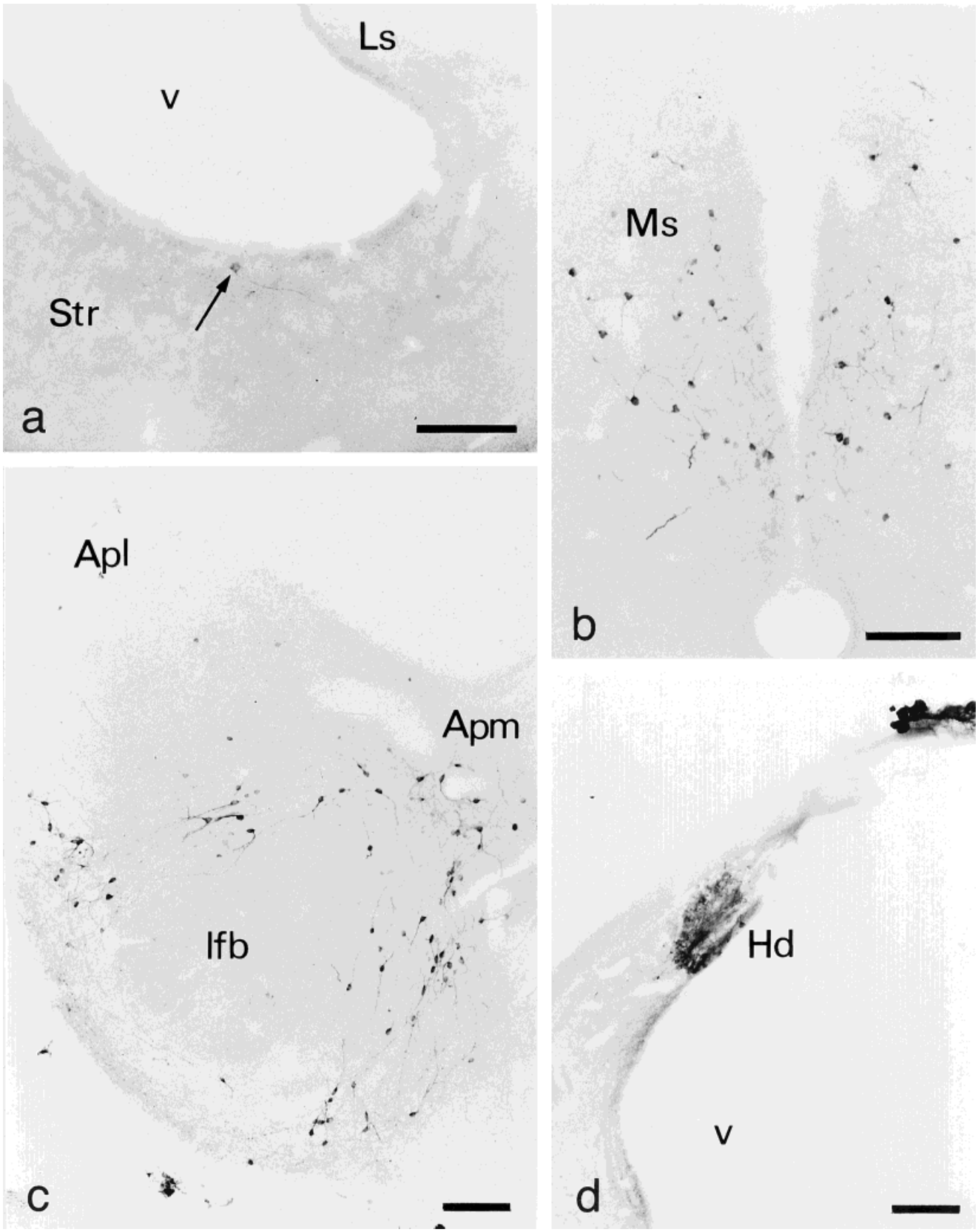

Fig. 3. Photomicrographs of transverse sections through the brain of *Rana perezi* showing choline acetyltransferase-immunoreactive (ChATi) cell bodies in the ventral striatum (**a**), the medial septum (**b**), the latero-caudal telencephalic regions (**c**), and the dorsal habenula (**d**). Scale bars = 200 µm in a, 50 µm in b, 100 µm in c,d.

laterally into the tegmentum, but their axons course ventrally to exit the brain as the oculomotor nerve (Fig. 4a). Some axons arising from the oculomotor nucleus on one side cross the midline and leave the brain via the contralateral nerve root. Along the caudal two-thirds of the oculomotor nucleus, a few small cells were found above the main motor nuclear complex. The latter cells are also ChATi, but less strongly immunoreactive than the large cells of the oculomotor nucleus (Fig. 4a). On the basis of their position and morphology, these cells may belong to the parasympathetic nucleus of Edinger-Westphal. Such a cell group was not recognized in the oculomotor nuclear complex of *Xenopus laevis*. However, exclusively in *Xenopus*, a group of strongly immunoreactive cells was observed in the midline. These cells, which were labeled nucleus interoculomotorius by Nikundiwe and Nieuwenhuys (1983), do not differ in size, morphology, and ChAT immunoreactivity from the other cells in the oculomotor nuclear complex (Fig. 4b). The medial tegmental region of both anuran species also contains small ChATi neurons that seem to constitute a caudal continuation of those present in the retromammillary and posterior tubercular regions.

Isthmus. Within the basal plate of the isthmus, strongly immunoreactive cell bodies were found in the trochlear nucleus (Figs. 2N–O, 4c,d). In the rostral pole of the nucleus, the cells form a compact group medial to the medial longitudinal fascicle. The number of motoneurons decreases caudally and the cells shift to a dorsomedial position in respect of the fascicle. The dendritic processes of the large neurons arborize profusely in the ventrolateral tegmentum, but their axons arch laterally and dorsally toward the dorsal surface of the brain, where they leave the brain in multiple rootlets. In the alar plate, a large group of ChATi neurons was found throughout the tegmentum, including the superficial aspect of the anterodorsal tegmental nucleus, the posterodorsal tegmental nucleus, and the region below the torus semicircularis (Fig. 2M–N). These neurons have long processes directed toward the lateral surface of the brain, where some of them appear to course into the tectum. In conventional transverse sections, this group occupies a progressively more dorsal position, going from rostral to caudal. This group of ChATi neurons appears to represent a single entity that strongly resembles the cholinergic cells of the pedunculopontine tegmental nucleus of amniotes and therefore has been labeled by us accordingly (Figs. 2M–N, 4d, 5a–c).

Nearly all the cell bodies in the nucleus isthmi are ChATi (Figs. 2O, 5a,d). The cells are tightly clustered in both the lateral aspect of the medulla and in the rim cortex of the nucleus (terminology according to Grobstein and Comer, 1983) but sparsely distributed in the medial aspect of the medulla. Axons arising from these neurons course rostrodorsally through tectal layer 7 and ramify within the superficial tectal layers. Medial to the isthmic nucleus, a group of strongly immunoreactive neurons was observed in the reticular formation (Figs. 2O–P, 5a,c,d), dorsal to the noradrenergic neurons of the locus coeruleus, although some overlap with the latter was obvious. The group consists of multipolar ChATi neurons with long dendrites that are directed ventrally and laterally toward the surface of the brain. We have labeled this the cholinergic cell group, which extends caudally to the rostral pole of the trigeminal nucleus, the laterodorsal tegmental nucleus. Some additional ChATi neurons were observed medial to this group.

Rhombencephalon. The most conspicuous ChATi cell groups were located in the hindbrain, where strongly immunoreactive cell bodies were present in all somato- and branchiomotor nuclei of the rhombencephalon (Figs. 2Q–V, 6–8). Moreover, at several levels, additional ChATi cell groups occurred outside the confines of the motor nuclei.

Immediately caudal to the cerebellum, the motor nucleus of the trigeminal nerve (Vm), located within the lateral zone of the rhombencephalic basal plate, contains ChATi cells (Figs. 2Q, 6, 7a). This nucleus extends caudally up to the level of the entrance of the octaval nerve, and the axons of all trigeminal motoneurons form a discrete fascicle that exits the brain in the ventral aspect of the thick trigeminal root (Figs. 2Q, 7a). Trigeminal motoneurons have abundant dendritic processes that arborize profusely within the ventrolateral rhombencephalon, whereas other processes, which are generally shorter and thinner, extend dorsally and medially toward the ventricle. At the rostral pole of the Vm nucleus, a few ChATi cells occur in the cerebellar peduncle, within the region of the parabrachial nucleus (Fig. 2P). Also in the ventrolateral gray, but more caudally, the facial motor nucleus (VIIm) consists of closely packed ChATi cells (Figs. 2R, 6, 7b). Like the Vm cell bodies, those of the VIIm have numerous dendritic processes that are directed ventrolaterally, whereas others, fewer in number, course dorsomedially. The axons of the facial motoneurons collect at the lateral margin of the gray and arch dorsally and then ventrolaterally to leave the brain in the ventralmost aspect of the octaval nerve roots. Slightly medial and dorsal to the VIIm, a few large immunoreactive cells are present, which most likely represent efferent cells of the octavolateral system (González et al., 1993a). At the same levels, but also farther caudally, scattered ChATi cells were found in the ventral octaval nucleus and the nucleus reticularis medius (Figs. 2R–S, 7b). Some of the ChATi cells in the alar plate are large neurons of the ventral vestibular nucleus (homologous to Deiters's nucleus; see Matesz, 1979; Fig. 7b). Additional small and weakly immunoreactive cells were observed medial to the latter cells, close to the ventricle.

The main abducens nucleus (VIm) is located dorsolateral to the medial longitudinal fascicle and is formed by a few, loosely arranged motoneurons that are strongly immunoreactive (Figs. 2S, 6, 7c). Their dendritic trees branch out in almost every direction, but their axons form several distinct rootlets that exit the brain ventrally. At the same levels as the VIm, but also more caudally, a conspicuous group of large ChATi neurons was observed in the accessory abducens nucleus (VIa, as distinguished by Matesz and Székely, 1977). This nucleus lies in the ventrolateral basal plate and its cells have a morphology similar to that of the facial motoneurons (Figs. 2S, 6, 7c). However, their axons course first medially toward the VIm and then ventrally to join the abducens rootlets. Dorsolateral to the VIa, another group of small ChATi cells was found, which on the basis of its position and cholinergic nature may represent the parasympathetic nucleus salivatorius superior (see Matesz and Székely, 1978; Székely and Matesz, 1993; Fig. 2S–T).

The rostral pole of the glossopharyngeal-vagal motor complex consists of a few large ChATi neurons (Fig. 6). Because the axons of these cells leave the brain via the

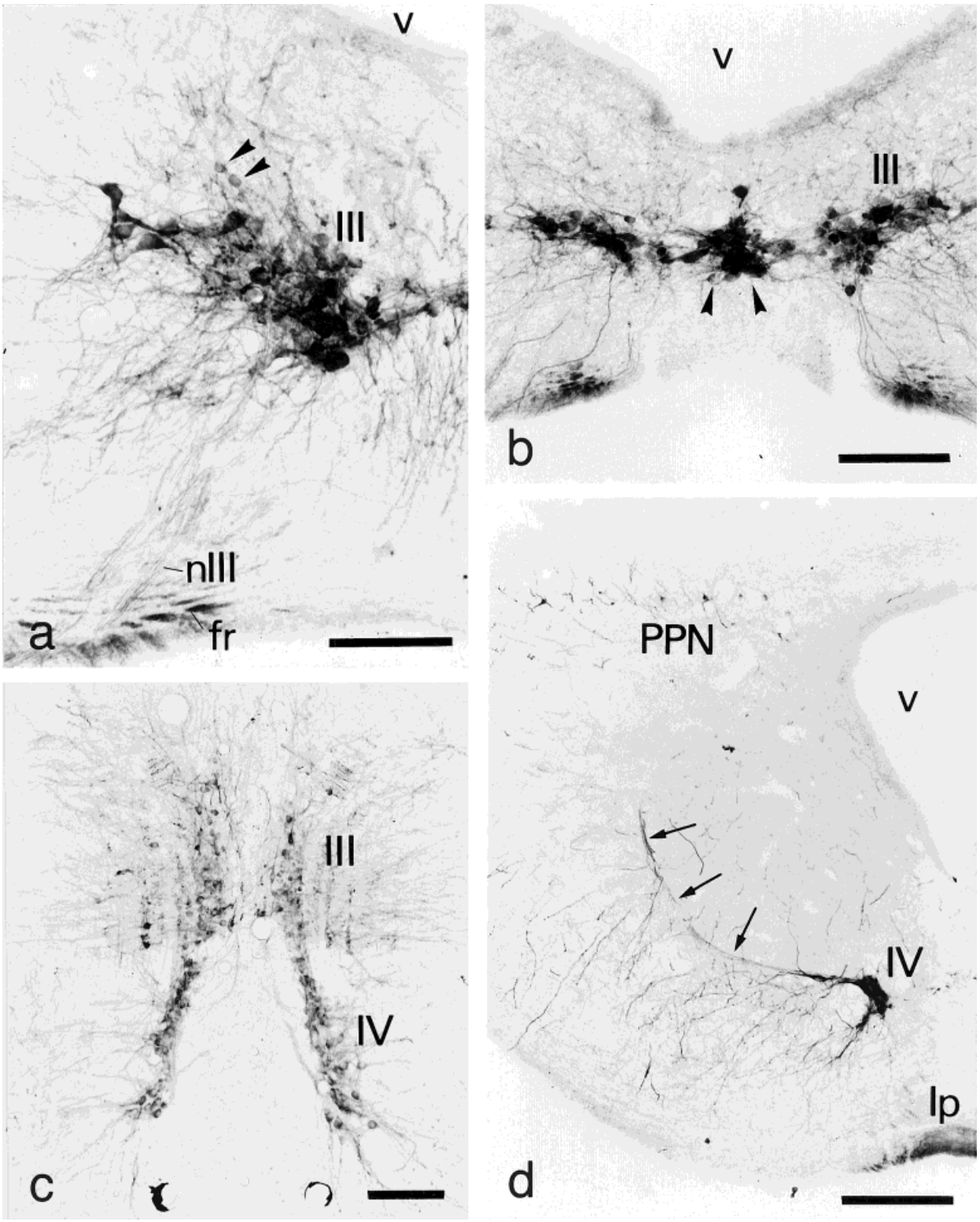

Fig. 4. Photomicrographs showing the ChATi cells in the anuran oculomotor and throchlear nuclei. **a:** Transverse section through the oculomotor nucleus of *Rana perezi* (arrowheads point to weakly labeled cells at the dorsal aspect of the nucleus). **b:** Transverse section through the oculomotor nuclei of *Xenopus laevis* (arrowheads point to the ChATi cells of the nucleus interoculomotorius). **c:** Horizontal section through the brain of *Rana perezi* showing the rostrocaudal continuation of the oculomotor and trochlear nuclei. **d:** Transverse section at the level of the trochlear nucleus of *Rana perezi* (arrows point to the trochlear axons). Scale bars = 100 µm in a,c, 200 µm in b,d.

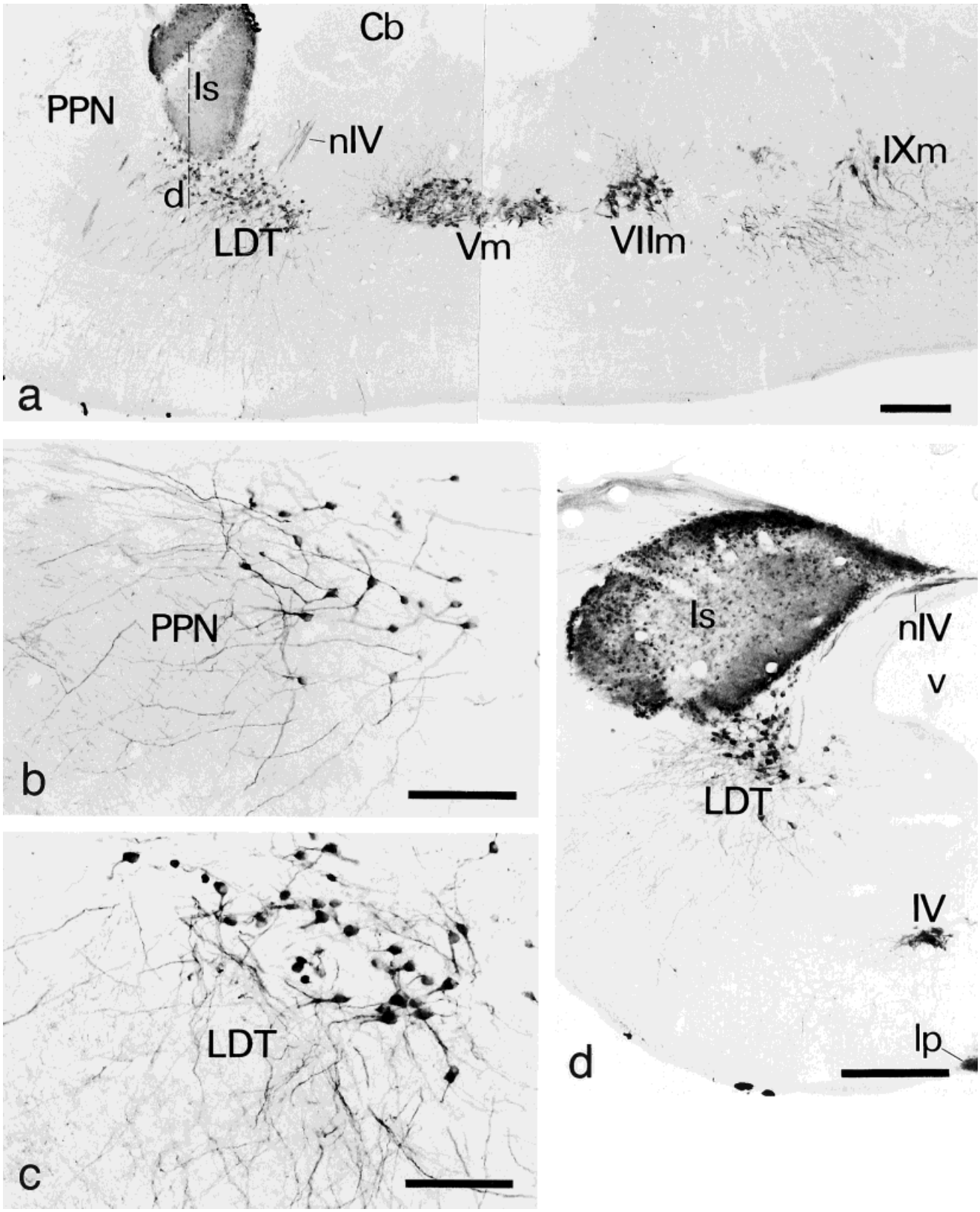

Fig. 5. Photomicrographs of ChATi cells and fibers in the brainstem of *Rana perezi*. **a:** Sagittal section at a mid-lateral level showing the isthmic and rostral rhombencephalic ChATi cell groups. **b,c:** Transverse sections illustrating the morphology of the ChATi neurons in the pedunculopontine and laterodorsal tegmental nuclei, respectively. **d:** Transverse section at the level indicated by the dashed line in a. Scale bars = 200 µm in a,d, 100 µm in b,c.

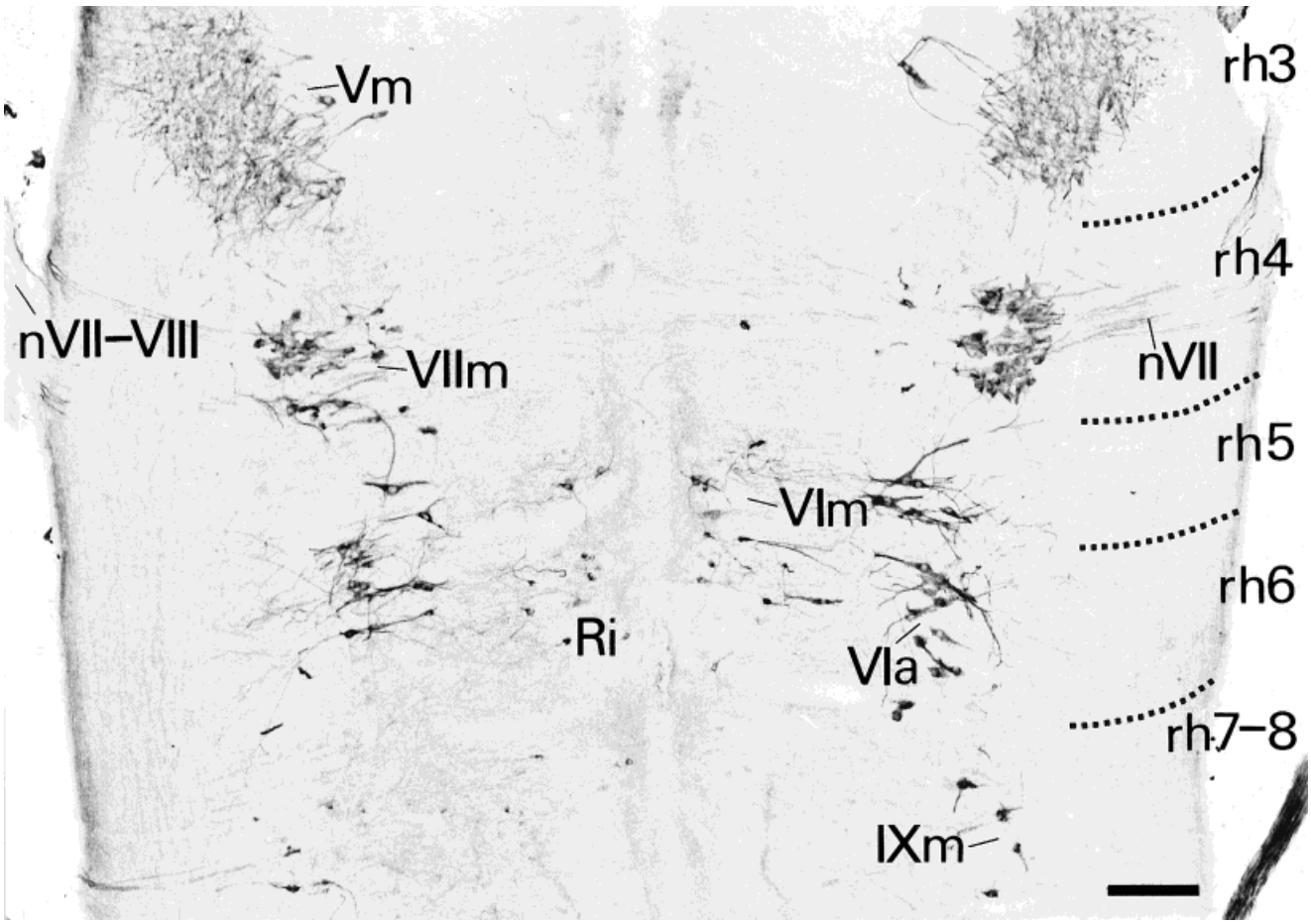

Fig. 6. Photomicrograph of a horizontal section through the rhombencephalon of *Xenopus laevis* at the level of the branchiomotor nuclei. The approximate boundaries of the rhombomeres are indicated on the right side. Scale bar = 100 μm.

IXth cranial nerve, they are considered glossopharyngeal motoneurons (IXm; Fig. 2T). In *Rana*, they are separated from the vagal part by a cell-free gap, but in *Xenopus*, the glossopharyngeal motoneurons are continuous with those of the vagal nerve. More dorsally, a population of small, weakly immunoreactive cells scattered within the nucleus reticularis inferior extended medially to the ventricle. At this level, large multipolar ChATi neurons lie ventral to the solitary tract. The ChATi cells of the vagal motor nucleus (Xm) are large, numerous, and located within the lateralmost zone of the basal plate and extend as far as the obex (Fig. 2U–V). In addition, small ChATi cells are present dorsal and medial to the Xm and are particularly abundant in the nucleus reticularis inferior (Fig. 2V). In the dorsal alar plate, strongly immunoreactive cell bodies occur in the caudal pole of the caudal octaval nucleus and in the dorsal column nuclei, both located dorsal to the solitary tract (Fig. 7d).

At the obex level, ChATi cell bodies were observed not only in the caudal extent of the Xm but also in several other nuclei (Figs. 2V, 8a). The most conspicuous cell group lies within the confines of the medial hypoglossal nucleus (XIIm). This group consists of large neurons with long dendritic processes that ramify laterally, almost horizontally, while their axons course ventrally to exit the brain as the ventral root of the second spinal nerve. A few ChATi cells located more ventrolaterally may represent the lateral hypoglossal nucleus (XIIl; see Stuesse et al., 1983). At these levels, a few, large elongated cells were observed in the ventral aspect of the gray. They are almost horizontally oriented and caudally continuous with the somatic motor column of the spinal cord. These cells most likely represent the efferent cells of the accessory nerve, which extend caudally as far as the second spinal segment (see Székely and Matesz , 1993).

The description of the organization of the ChATi cell populations in the rhombencephalon of *Rana perezi* holds essentially for *Xenopus laevis*. A few peculiarities, however, deserve some comment. First, all branchiomotor nuclei (Vm, VIIm, IXm, Xm) migrate farther away from the ventricle in *Xenopus* than in *Rana*. Moreover, *Xenopus* lacks small ChATi cells associated with the large immunoreactive motoneurons of IXm and Xm. The most striking difference, however, is the absence of a ChATi medial hypoglossal cell group in *Xenopus*, probably reflecting the lack of a tongue in this species (Fig. 8b). The apparent lack of the XIIm, which innervates the intrinsic tongue muscles, contrasts with the presence of ChATi cells in *Xenopus* in a position comparable to that of the lateral hypoglossal cell group in *Rana*. This observation suggests that the lateral

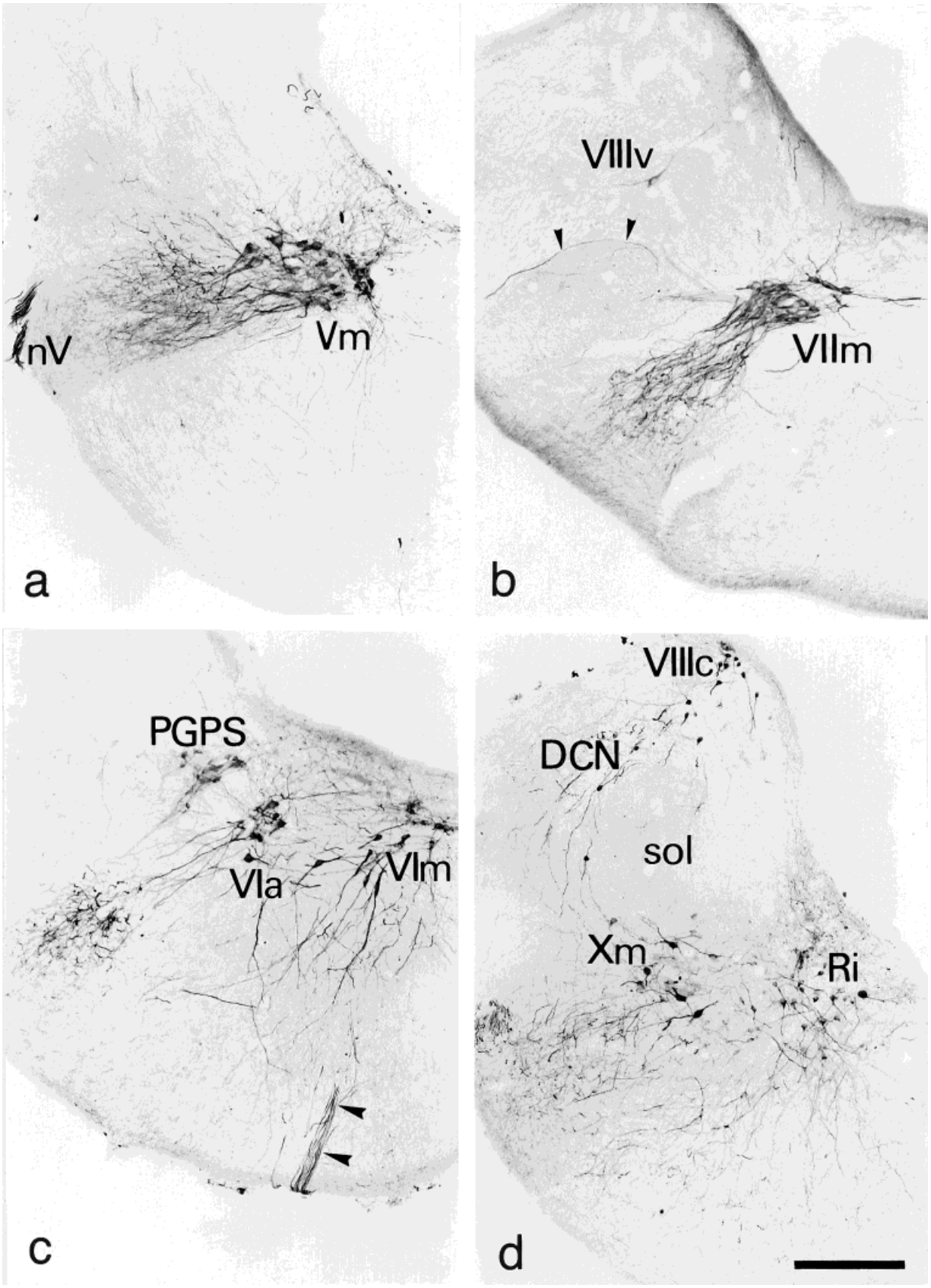

Fig. 7. Photomicrographs of transverse sections through the rhombencephalon of *Rana perezi* at the levels of the trigeminal motor nucleus (**a**), the facial motor nucleus (**b**), the abducens nuclei (**c**), and the vagal motor nucleus (**d**). Arrowheads point to facial axons in b and to abducens axons in c. Scale bar = 200 µm.

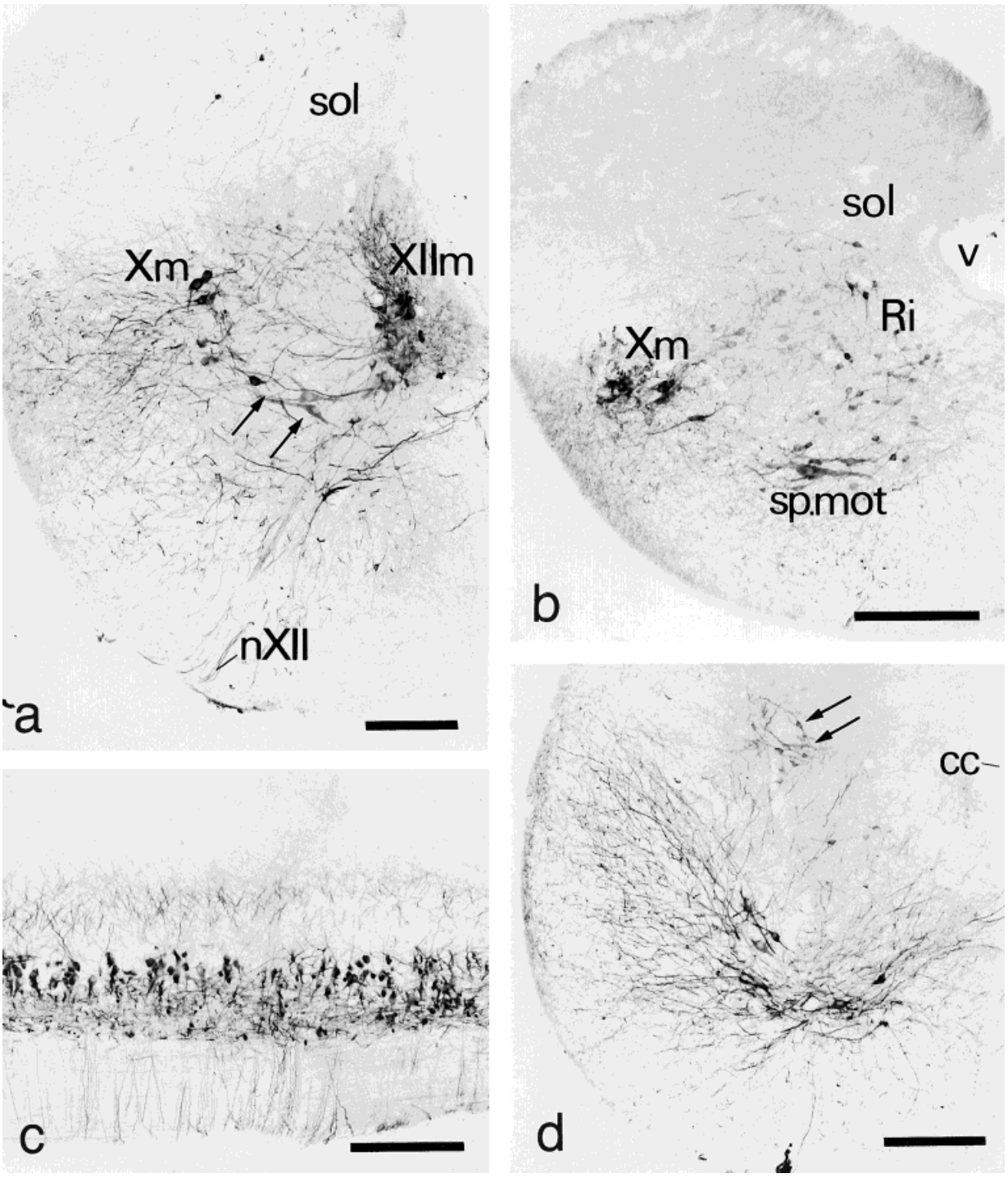

Fig. 8. Photomicrographs of ChATi cells and fibers in the caudal rhombencephalon and upper spinal cord of anurans. **a:** Transverse section through the hypoglossal nucleus of *Rana perezi* (arrows point to lateral hypoglossal labeled neurons). **b:** Transverse section of *Xenopus laevis* at a level comparable to that shown in a showing the lack of a hypoglossal nucleus. **c:** Sagittal section of the upper spinal cord at lateral levels showing the ChATi motor neurons. **d:** Transverse section through the spinal cord of *Rana perezi*, where, in addition to the ventral motoneurons, small and weakly labeled cells are present in the intermediate zone (arrows). Scale bars = 200 μm.

hypoglossal nucleus, which innervates the sternohyoid muscle in ranid frogs (Stuesse et al., 1983), is also present in *Xenopus*.

Spinal cord. In the present study, only the rostral part of the spinal cord has been analyzed. The most conspicuous ChATi cells lie in the ventral somatomotor column (Figs. 2W, 8c,d), where they form a fanlike dendritic arborization, which occupies almost the whole ventrolateral aspect of the spinal cord. The caudal components of the XI motor nucleus could not be distinguished from those

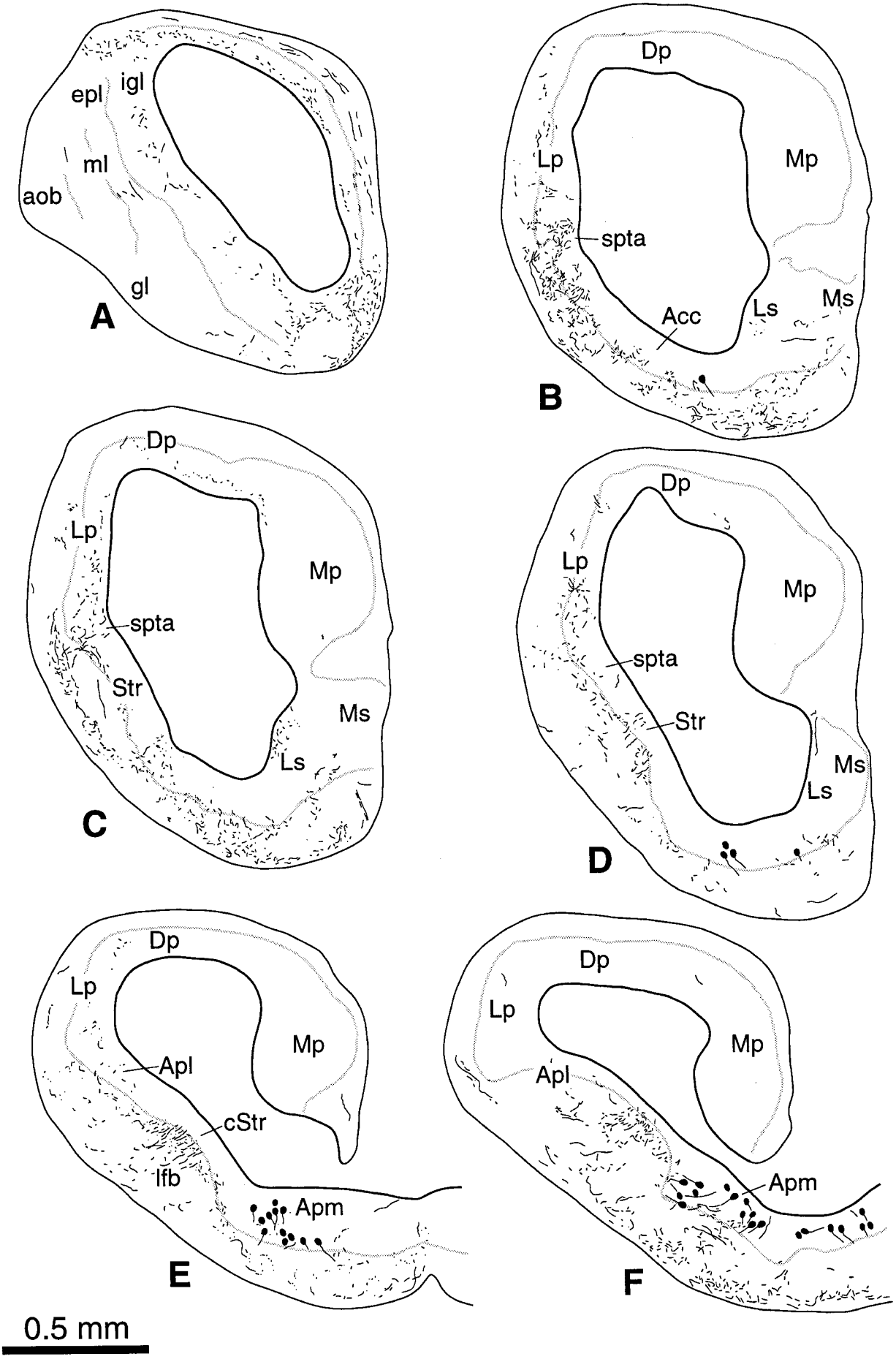

Fig. 9. **A–V:** Diagrams of transverse sections through the brain of the newt *Pleurodeles waltl* (levels as indicated in Fig. 1) showing the distribution of immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines) in the left half of each section.

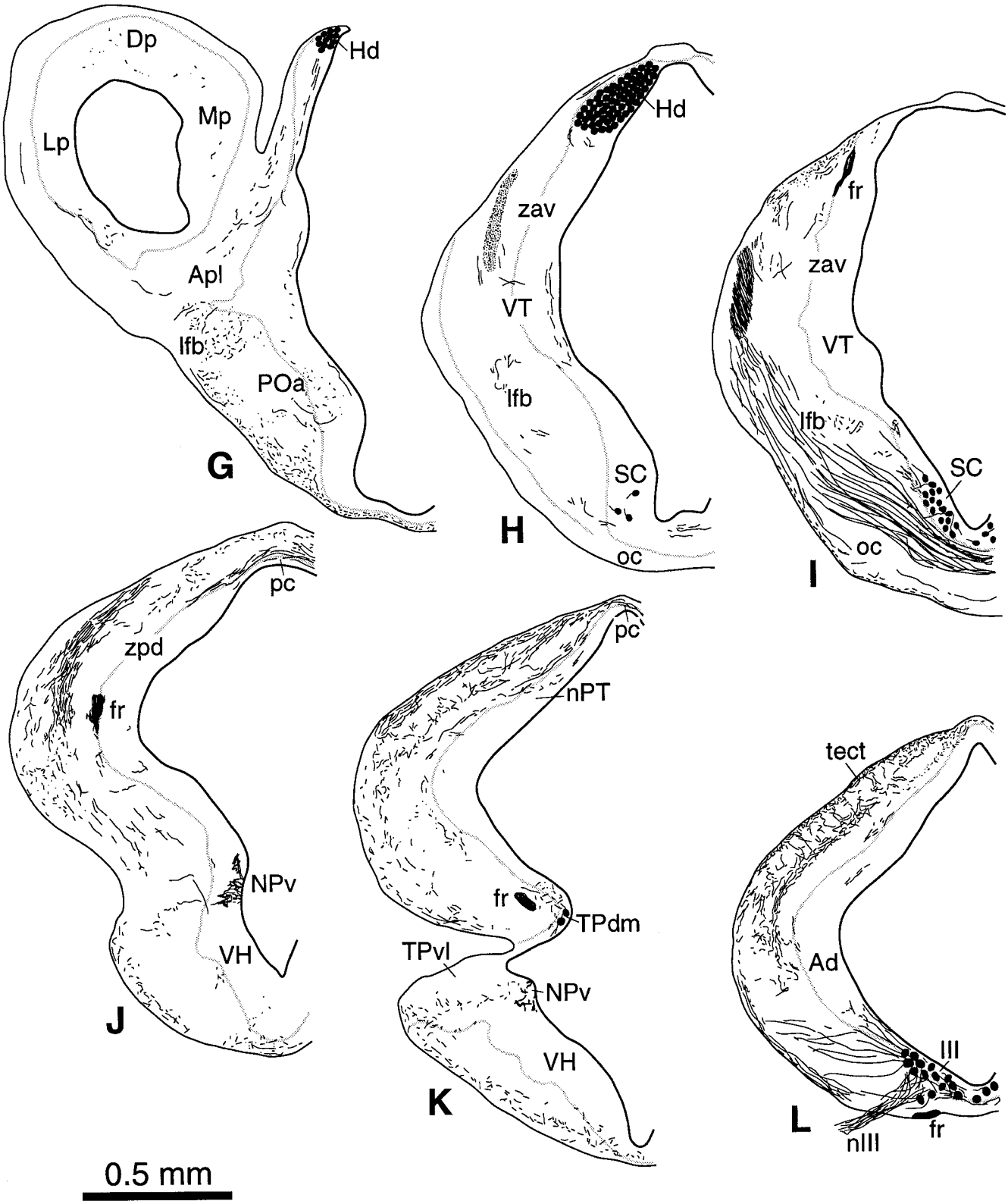

Figure 9 (Continued.)

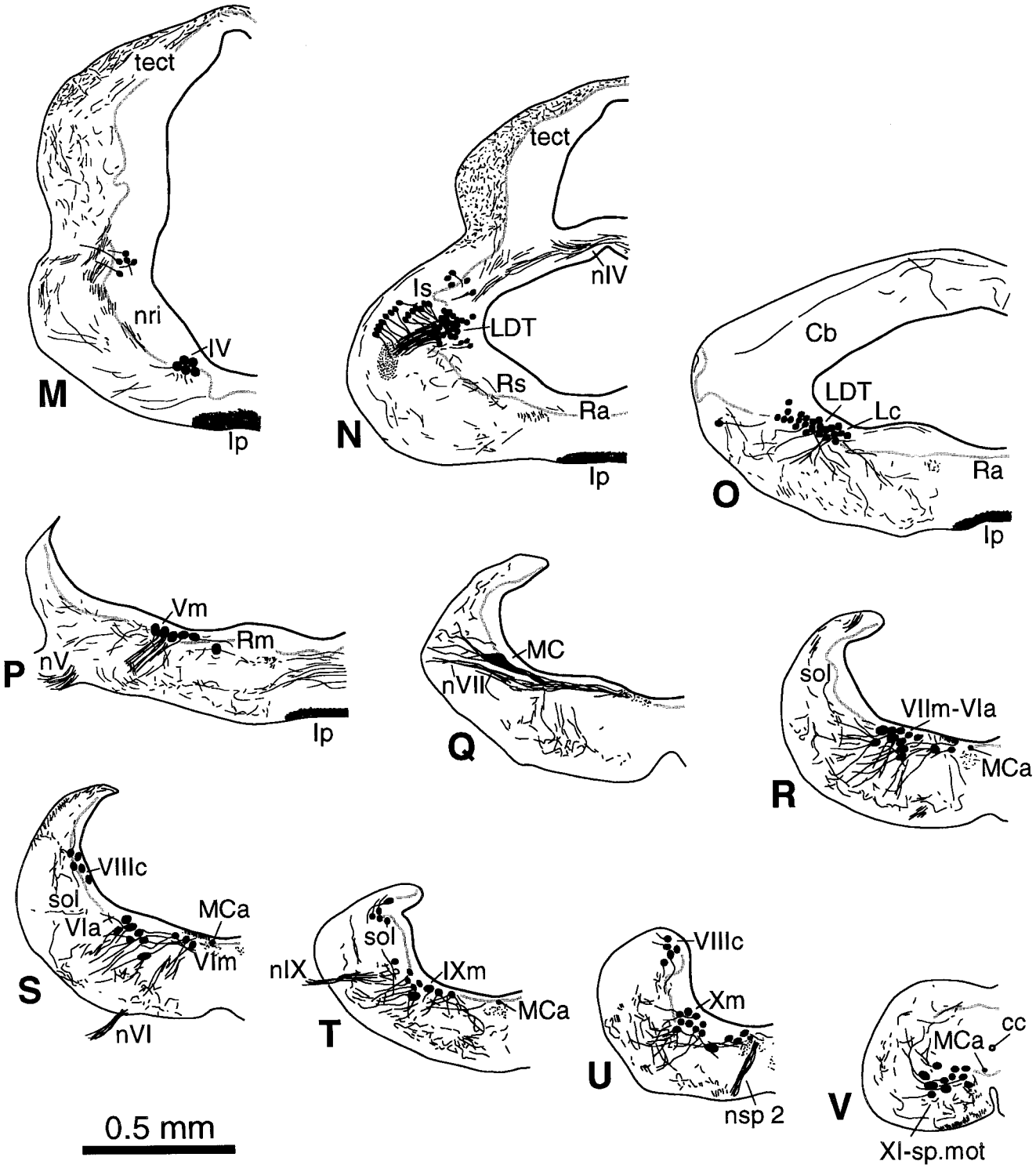

Figure 9 (Continued.)

of the spinal somatomotor column. Apart from the somatomotor neurons, small ChATi cells were found at the lateral border of the intermediate gray zone (Figs. 2W, 8d). These cells formed a small group that most likely represents the column of sympathetic neurons, which will continue caudally in the spinal cord. No ChATi cells were observed in the dorsal horn of the rostral spinal cord.

Urodeles

Telencephalon. At intermediate hemispheric levels, a few ChATi cell bodies were found medial to the nucleus

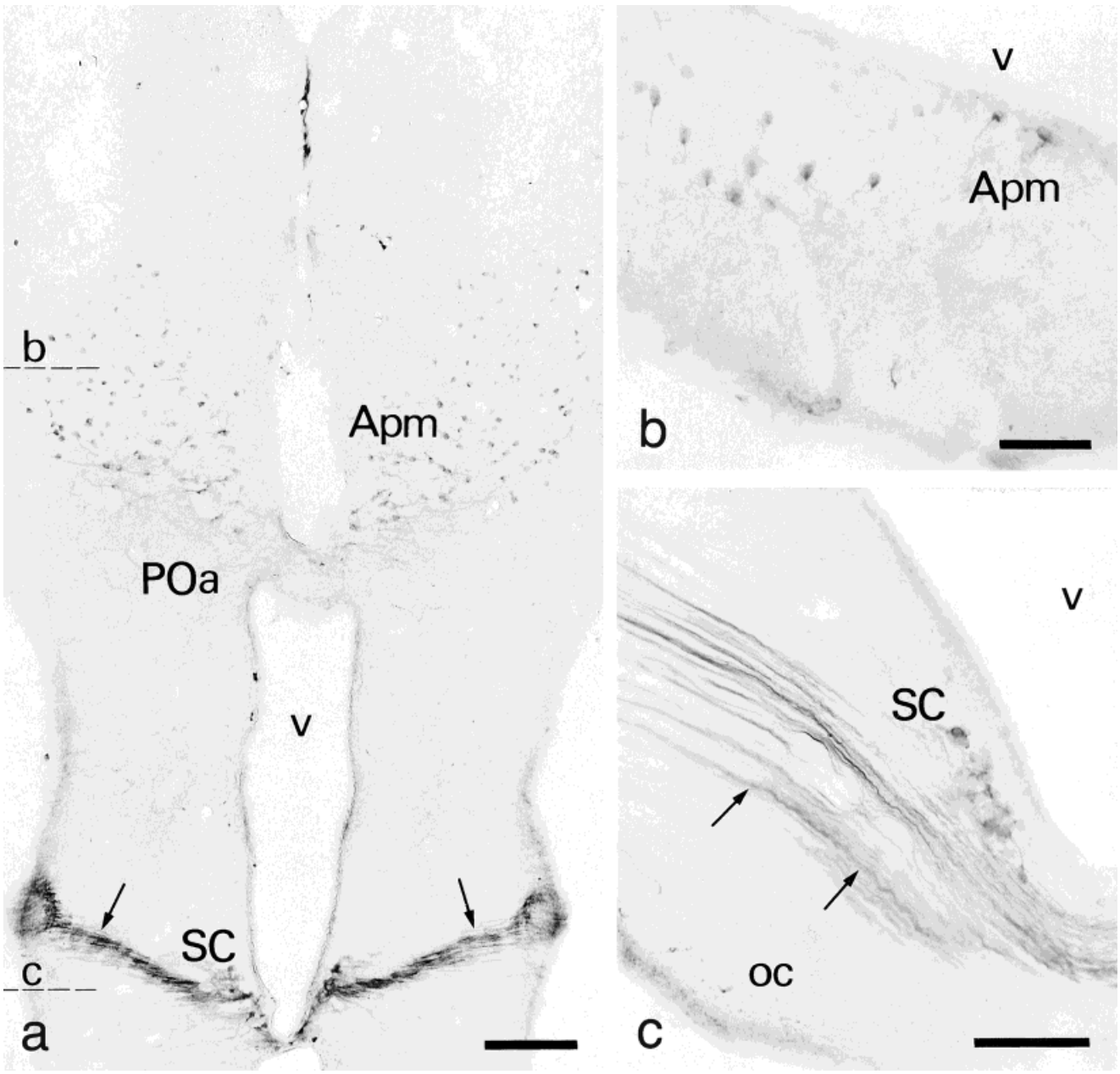

Fig. 10. Photomicrographs through the brain of *Pleurodeles waltl* showing ChATi cell bodies and fibers. **a:** Horizontal section through the basal forebrain. **b,c:** Transverse sections at levels indicated by dashed lines in a. Arrows in a and c point to ChATi fibers in the upper portion of the optic chiasm. Scale bars = 200 µm in a, 50 µm in b, 100 µm in c.

accumbens in the ventromedial aspect of the basal telencephalon (Fig. 9B–D). Caudally, these cells are continuous with a prominent ChATi cell group located in the medial amygdala (Fig. 10a,b). This group consists of medium-sized cells with a main process directed ventrally (Fig. 9E). At the level of the anterior preoptic area, the ChATi cells of the medial amygdala are organized in medial and lateral divisions (Fig. 9F).

Diencephalon. In the epithalamus, a group of small, closely packed ChATi cells was found in the habenula (Fig. 9G,H). Their axons course ventrally, constituting the fasciculus retroflexus. A second group of diencephalic ChATi cell bodies was observed in the suprachiasmatic nucleus (Figs. 9H,I, 10c). These cells are small and stain less darkly than those in the telencephalon and the habenula. Caudally, a few small ChATi cells were identified in the dorsomedial aspect of the posterior tubercle (Fig. 9K). The cells are located close to the ventricle but do not have processes that contact the CSF.

Mesencephalon. The oculomotor nucleus is located in the ventral mesencephalic tegmentum, where it occupies most of the gray surrounding the ventral tip of the mesencephalic ventricle (Figs. 9L, 11a,b). This conspicuous ChATi cell group can be subdivided into a compact

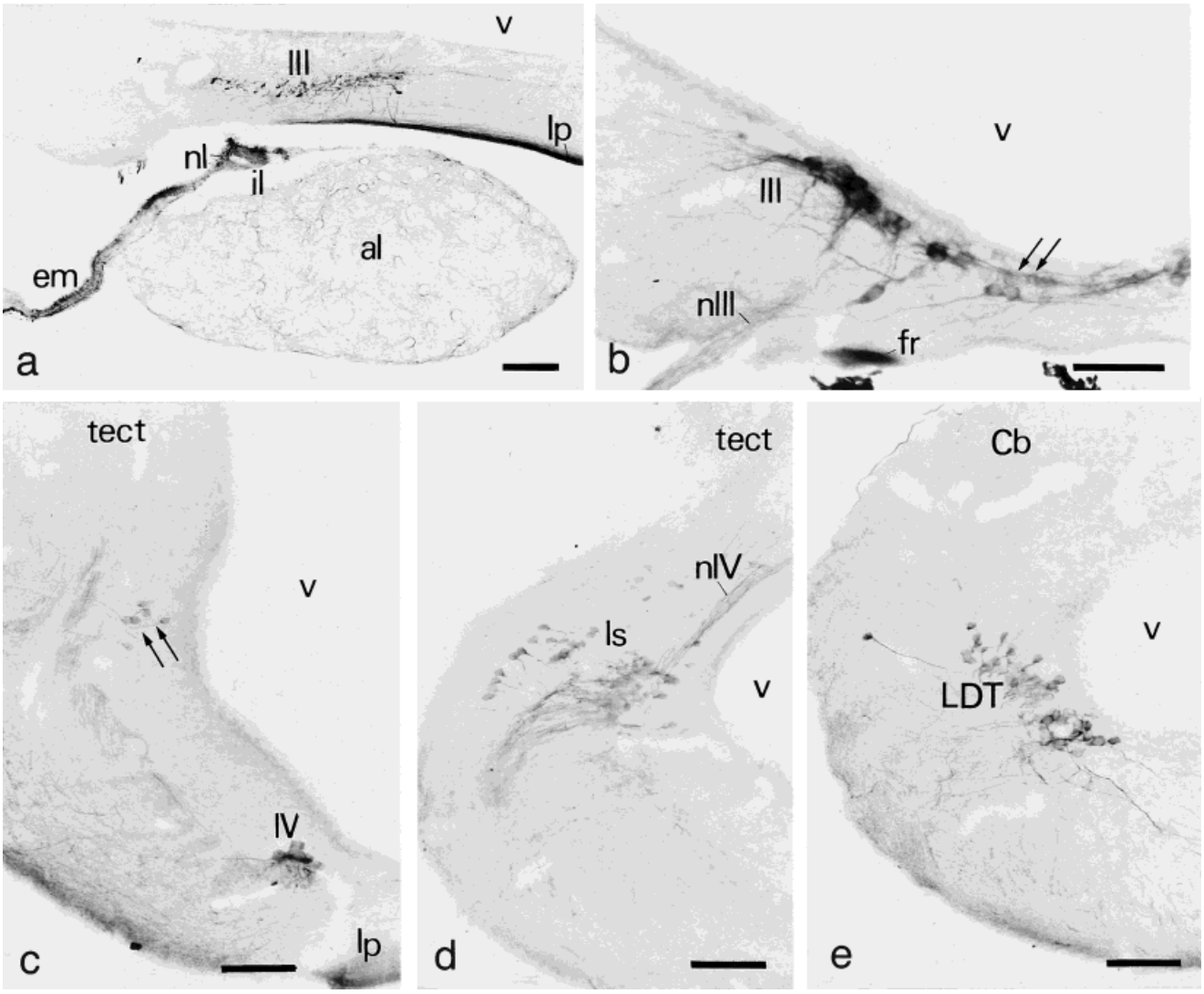

Fig. 11. Photomicrographs through the brain of *Pleurodeles waltl* showing ChATi cell bodies and fibers. **a:** Sagittal section through the oculomotor nucleus showing also the hypophysis. **b:** Transverse section at the level of the oculomotor nucleus (arrows point to medially located cells). **c:** Transverse section at the level of the trochlear nucleus (arrows point to dorsally located ChATi cells in the isthmic tegmentum). **d:** Transverse section at the level of the isthmic nucleus. **e:** Transverse section through the caudal isthmic region, just rostral to the trigeminal motor nucleus. Scale bars = 200 µm in a, 100 µm in b–e.

group of large cells located within the intermediate and lateral aspects of the oculomotor nucleus and a more loosely organized group of smaller cells close to the midline (Fig. 11b). The axons of the smaller cells course to the contralateral side and therefore correpond to the neurons that innervate the contralateral musculus rectus superior (Naujoks-Manteuffel et al., 1986). The axons of all oculomotor neurons collect at the ventrolateral aspect of the nucleus and leave the brain ventrally in the oculomotor nerve. The ventral half of the mesencephalon is almost entirely filled with dendritic arborizations of the oculomotor motoneurons. Unlike anurans, a nucleus of Edinger-Westphal is not distinguishable in urodeles, although cells that, at some levels, are located in the dorsal aspect of the oculomotor nucleus and more independently arranged may represent a primordium of such a nucleus (see Naujoks-Manteuffel et al., 1986).

Isthmus. The ChATi cells of the trochlear nerve nucleus form a compact group of cells in the basal plate of the isthmus, caudal to the oculomotor nucleus (Figs. 9M, 11c). The nucleus is constituted by a few large neurons at the border of the periventricular gray, dorsomedial to the fasciculus longitudinalis medialis. The dendritic arborizations of the trochlear neurons are more restricted than those of the oculomotor neurons. Moreover, their axons collect into several fascicles, which course dorsocaudally in the tegmentum and leave the brain dorsally in the contralateral trochlear nerve, immediately rostral to the cerebellum (Figs. 9N, 11d). At this level, ChATi cells were found in the rostral aspect of the isthmic tegmentum (Fig. 9M). The medium-sized cells form a compact group located close to the ventricle and have a main process that courses dorsally into the optic tectum (Fig. 11c). More caudally, ChATi cells are found in the nucleus isthmi and are smaller in

size and located more laterally in the tegmentum (Figs. 9N, 11d). These cells have long processes that course first ventrally and then turn 90° in a rostral direction to enter the tectum. Another conspicuous group of ChATi cells was found in the tegmentum, medial to the caudal pole of the nucleus isthmi and extending caudally up to the level of the trigeminal motor nucleus (Figs. 9N,O, 11e). The cells of this group are located mainly dorsal to the noradrenergic cells of the locus coeruleus, although some overlap with the latter occurs. As in anurans, we have named this group of ChATi cells the laterodorsal tegmental group.

Rhombencephalon. As in anurans, the rhombencephalon contains a very large population of ChATi cells, which are confined mainly to the motor nuclei of the cranial nerves (Figs. 12–14). The ChATi cells of the branchiomotor nuclei form an almost continuous cell column, with only a few gaps (Fig. 12). Moreover, extensive overlap of motoneurons belonging to different cranial nerves occurs at caudal rhombenephalic levels (see Roth et al., 1988).

In the rostral rhombencephalon, the trigeminal motor nucleus is located in the ventrolateral periventricular gray (Figs. 9P, 12, 13a). The nucleus is constituted by a band of cells that lies close to and parallel to the ventricular surface between the level of the entrances of the trigeminal and octaval nerves. The cells are pear shaped, with their main dendritic trunks directed ventrolaterally. The axons of the trigeminal motoneurons course in a ventrolateral direction and exit the brainstem in the ventral one-third of the trigeminal root.

Caudal to the trigeminal motor nucleus, there is a gap in the branchiomotor column, which is marked by the presence of a thick, tranversely coursing fascicle of facial nerve fibers (Figs. 9Q, 13b), leaving the brain ventral to the octaval nerve. At this level, on each side of the brain, a single giant ChATi soma of the Mauthner cell is present just ventral to the octaval nuclei, at the lateral margin of the reticular formation (Figs. 9Q, 12, 13b). The thick axons of the two Mauthner cells cross immediately caudal to the cell bodies and descend along the rhombencephalon and spinal cord, keeping a position in the dorsal aspect of the fasciculus longitudinalis medialis (Figs. 12, 13c,d).

Caudal to the level of the Mauthner cells, numerous ChATi cells are present in the branchiomotor column extending caudally beyond the obex (Fig. 12). On the basis of the course of the immunoreactive axons in the facial nerve, the rostral part of this column is, at least in part, constituted by facial motoneurons (Fig. 12). The morphology of these cells resembles that of the trigeminal motoneurons, but their axons follow a peculiar course. They course initially medially and are arranged as a thick fascicle dorsal to the fasciculus longitudinalis medialis; the fascicle then turns 90° rostralward and continues to the level of the facial nerve roots. There, the fibers seem to genuflect (genu facialis) and course laterally to exit the brain (Fig. 13b). In horizontal sections, the rostrocaudal extent of the facial motor nucleus is very long and on the basis of the ChAT immunostaining impossible to distinguish between facial and abducens motoneurons (Naujoks-Manteuffel et al., 1986; Roth et al., 1988). In fact, the abducens motoneurons are largely overlapping with the facial motor nucleus. Nevertheless, as in anurans, a main and accessory subdivision are recognized in the abducens nucleus (Fig. 13c). The main nucleus is easily identified by its medial position and the course of the axons of its constituent cells (Fig. 9S). The rostral pole of the main abducens nucleus lies just caudal to the facial knee and its large, spindle-shaped neurons have axons that collect as several vertical-running fascicles, leaving the brain ventrally as separate abducens rootlets before joining together as the abducens nerve. In contrast, the motoneurons of the accessory abducens nucleus intermingle with those of the facial motor nucleus,

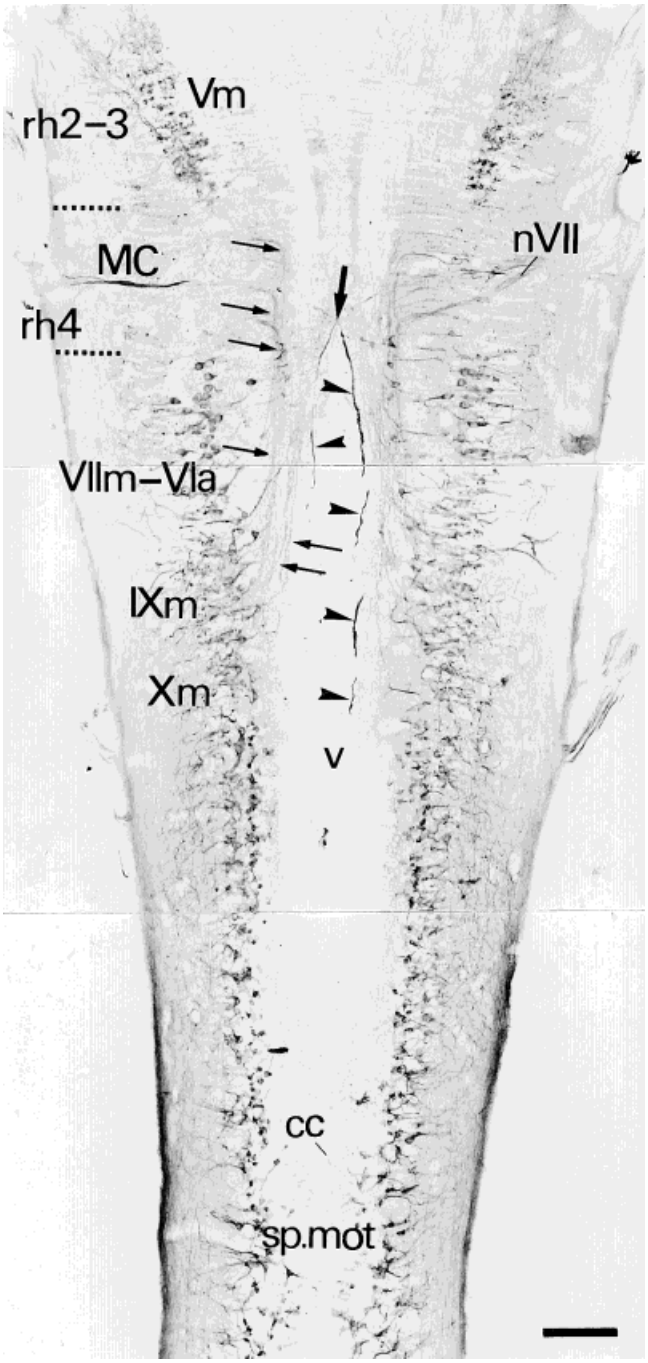

Fig. 12. Photomicrograph of a horizontal section through the rhombencephalon of *Pleurodeles waltl*. Thick arrow points to the decussation of the Mauthner cell axons (arrowheads), and small arrows indicate the facial motoneuron axons. The rostral rhombomeres are indicated on the left side. Conventions as in Figure 6. Scale bar = 200 µm.

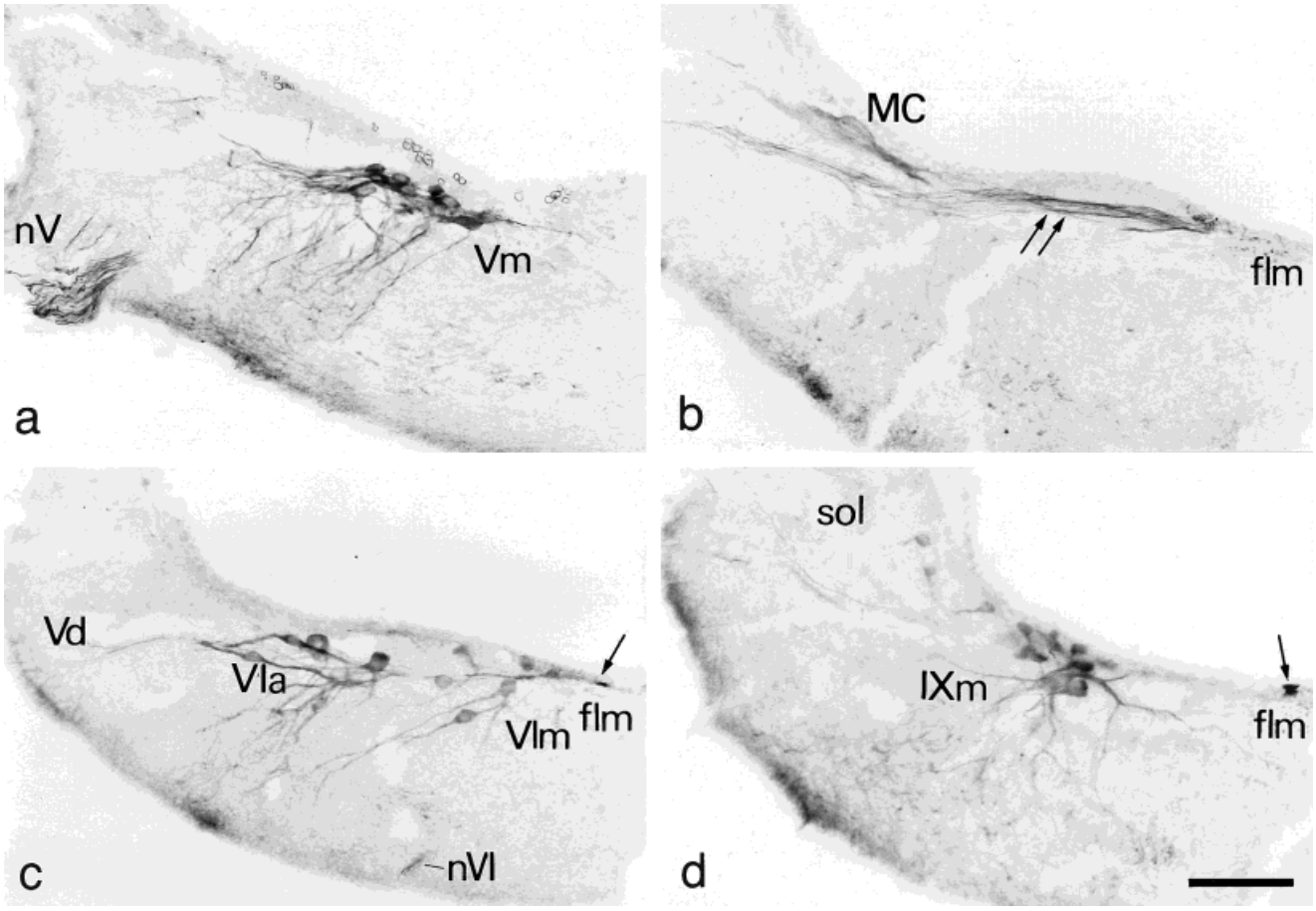

Fig. 13. Photomicrographs of transverse sections through the rhombencephalon of *Pleurodeles waltl* at the levels of the trigeminal motor nucleus (**a**), the Mauthner cell body and facial transverse bundle (arrows) (**b**), the abducens nuclei (**c**), and the glosopharyngeal motor nucleus (**d**). Arrows in c and d indicate the Mauthner cell axons. Scale bar = 100 μm.

and their morphology is similar. Both the accessory abducens and facial motoneuron axons course medially to the main abducens nucleus, but whereas the accessory abducens fibers turn ventrally to exit the brain, those of the facial nerve turn rostrally into the facial fascicle above the fasciculus longitudinalis medialis. Apart from the motoneurons, large ChATi cells are present among them, which may represent either dispersed abducens neurons (Naujoks-Manteuffel et al., 1986) or octavolateral efferent cells (González et al., 1993a). From intermediate to caudal rhombencephalic levels, smaller ChATi cells occur in the caudal octaval nucleus, which at levels close to the obex join a ChATi cell group in a position comparable to the dorsal column nucleus (A. Muñoz et al., 1996; Fig. 14a).

From the level of the first root of the IXth–Xth nerve complex and farther caudalward, strongly immunoreactive neurons form a continuous column in the ventrolateral gray (Figs. 9T,U, 14a). The rostral part of this column, containing the glossopharyngeal motoneurons, lies at the same level as the caudal pole of the facial motor nucleus (Fig. 13d). Caudalward, the column is constituted by the motoneurons of the vagus, the accessory nerve, and the first two spinal nerves. Close to the obex level, a medially located group of ChATi cells is present, which may represent the hypoglossal nucleus. The axons of this cell group course ventrally and leave the brain in the second spinal nerve (Fig. 9U). In fact, the homologue of the XIIth cranial nerve in urodeles is considered to be composed of the ventral roots of the first two spinal nerves, of which the cells of origin form dorsomedial and lateral groups in the caudal rhombencephalon (Wake et al., 1988). Neurons in the lateral group are intermingled with other spinal motoneurons, from which they cannot be distinguished on the basis of ChAT immunoreactivity.

Spinal cord. In the upper spinal cord, ChATi motoneurons lie within the ventrolateral margin of the gray matter (Figs. 9V, 14b,c). They possess profuse dendritic ramifications that fill almost entirely the ventrolateral white matter and even reach the dorsal half of the spinal cord. At these levels, the motoneurons in the caudal extent of the branchiomotor column intermingle with the spinal motoneurons. A tiny group of small ChATi cells lies within the intermediate zone of the gray and therefore may represent components of the sympathetic spinal system (Fig. 14c).

ChATi fibers

Anurans

Telencephalon. In the olfactory bulb, ChATi fibers and terminals are present in the external plexiform layer,

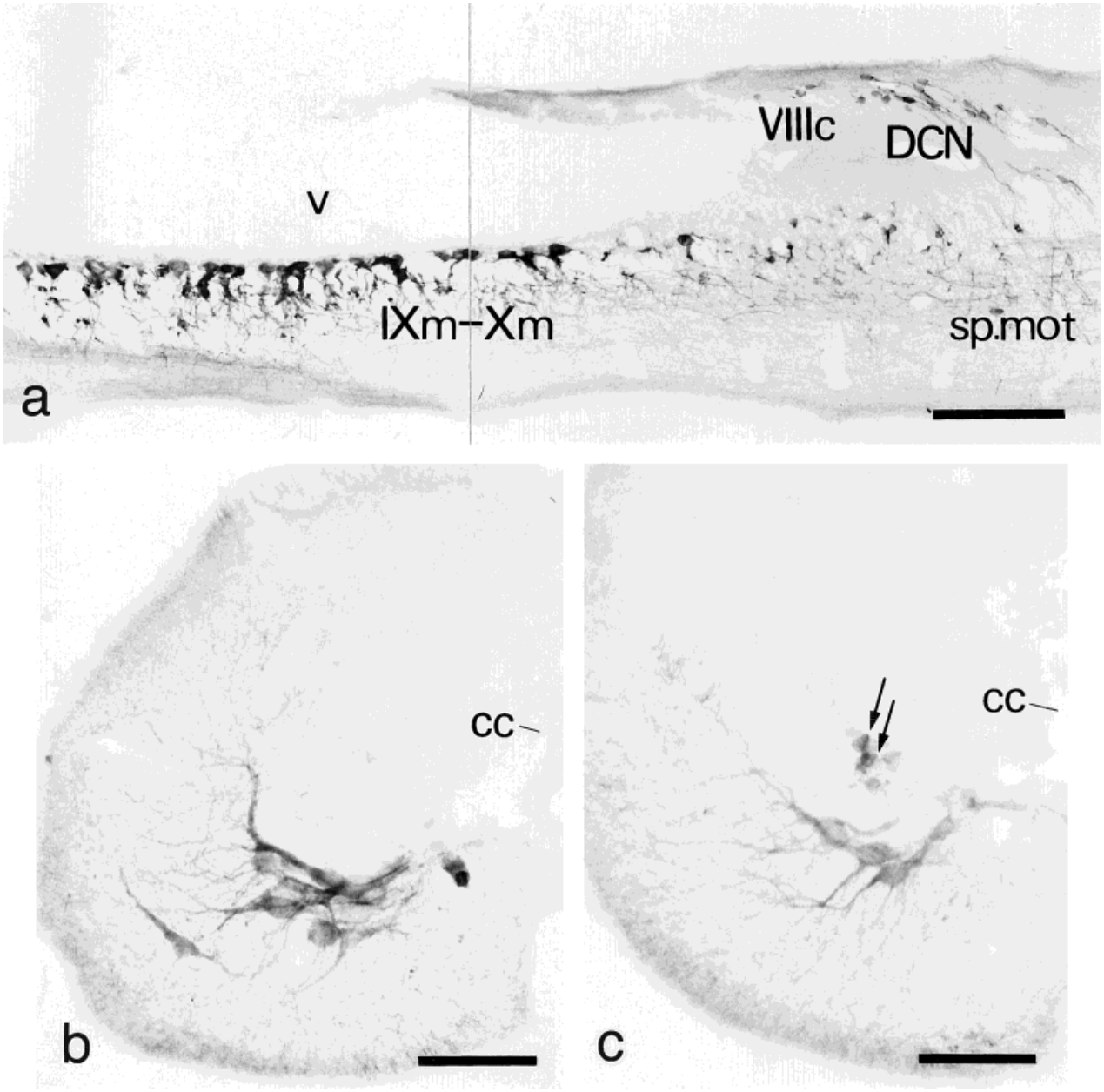

Fig. 14. Photomicrographs through the caudal rhombencephalon and spinal cord of *Pleurodeles waltl*. **a:** Mid-lateral sagittal section through the caudal medulla and upper spinal cord. **b,c:** Transverse sections through the upper spinal cord. Arrows in c point to a group of ChATi cells dorsal to the large motoneurons. Scale bars = 200 µm in a, 100 µm in b,c.

where they constitute a prominent plexus in its dorsal portion. ChATi fibers also occur, but are less numerous, in the mitral cell layer and the glomerular layer. At caudal levels of the main olfactory bulb, numerous ChATi fibers were found widely distributed in all layers of the main olfactory bulb but preferentially in its medial part close to the surface of the bulb. These fibers can be traced caudally within the medial olfactory tract. In contrast, the accessory olfactory bulb does not contain ChATi fibers (Fig. 2A).

In the telencephalon proper, dense plexuses of ChATi fibers and varicosities are present in the neuropil of the lateral pallium and the striatopallial transition area throughout the entire hemisphere (Fig. 2B–E). The medial pallium contains a moderate number of fine varicose fibers, whereas the dorsal pallium contains only a few ChATi fibers. Within the basal forebrain, some varicose immunoreactive fibers were observed in the nucleus accumbens. In the striatum, a modest number of ChATi fibers was found in its rostral and dorsal parts, but a substantially larger number was seen in the neuropil of the ventral striatum and the lateral forebrain bundle (Fig. 2C–E). Moderate plexuses of ChATi fibers intermingled with ChATi neurons were found in the medial septum, the medial amygdala, and the medial forebrain bundle. In

addition, the caudal portion of the striatum contained a moderate number of ChATi fibers, whereas numerous immunoreactive fibers were observed lateral to the lateral forebrain bundle, close to the brain surface (Fig. 2E–F). Finally, moderate numbers of ChATi fibers were located in the lateral amygdala and dorsal to the anterior commissure. Some fibers crossed within the latter commissure.

Preoptic area and hypothalamus. The preoptic area contains some ChATi fibers, which are located mainly lateral to the preoptic recess (Fig. 2F–H). Caudally, fine varicose ChATi fibers are located within the caudal aspect of the suprachiasmatic nucleus, and numerous nonvaricose immunoreactive fibers course immediately lateral to the nucleus. A large number of ChATi fibers decussates in the supraoptic commissure and then courses dorsally, forming two separate bundles that run parallel to the optic tract (Fig. 2J). These fibers can be traced caudally as far as the isthmic region, where they seem to have their origin. A moderate number of ChATi fibers was observed in the infundibular hypothalamus and the neural lobe of the hypophysis.

Thalamus, epithalamus, and pretectum. The prominent bundles of immunoreactive fibers, which have their origin in the isthmic region, issue numerous collaterals to the ventral thalamus, the dorsal thalamus, and the pretectal region (Figs. 2I–K, 15a). In the ventral thalamus, a moderate-to-dense plexus of ChATi varicose fibers is found within the neuropil of the ventrolateral thalamic nucleus and the ventral geniculate nucleus. In addition, fine varicose fibers are located within the confines of the ventrolateral and ventromedial thalamic nuclei (Fig. 2I–J). Caudally, ChATi fibers are present in the periventricular nucleus of the zona incerta (Fig. 2K). The dorsal thalamus is less well innervated by ChATi fibers. Small numbers of immunoreactive fibers occur in the dorsal geniculate neuropil, the anterior thalamic nucleus, the anterior division of the lateral thalamic nucleus, and the central thalamic nucleus (Fig. 2I–K).

Fine ChATi fibers reach the habenula via the stria medullaris, where they form a moderate plexus around the ChATi cell bodies. In both anuran species, the fasciculus retroflexus consists of several distinct bundles of ChATi fibers (Figs. 2H–I, 15a), which arise from the cells in the dorsal habenular nucleus. The course of the fasciculus retroflexus, however, differs between the two species. In *Xenopus*, the bundles course along the meningeal surface of the diencephalon; in *Rana*, they traverse the anterior division of the lateral thalamic nucleus and then course laterally to the ventral thalamic nuclei (Fig. 2J).

In the pretectum, moderate plexuses of ChATi fibers are present in the precommissural neuropile and the nucleus lentiformis (Figs. 2K, 15a). A weak immunoreactive innervation was found in the lateral posterodorsal thalamic nucleus and the nucleus of the posterior commissure. Some ChATi fibers decussate in the latter commissure (Fig. 2L).

Basal diencephalon. Numerous varicose ChATi fibers intermingle with the ChATi neurons of the retromammillary and posterior tubercular regions (Fig. 2K–L). Horizontal sections revealed that the posterior tubercular regions are invaded by numerous immunoreactive processes of oculomotor motoneurons. A moderate plexus of ChATi fibers was found in the nucleus of the basal optic root.

Mesencephalon. A dense plexus of ChATi fibers is located in the griseum tectale, which, following Puelles et al. (1996), has to be considered a midbrain structure. In addition, the optic tectum contains numerous ChATi fibers, which show a laminar organization (Figs. 2K–O, 15b,c), but the ChATi fibers are restricted predominantly to the superficial tectal layers. A very dense plexus of ChATi terminals is present in the superficial layer A (nomenclature according to Potter, 1969), whereas numerous nonvaricose and varicose fibers are distributed sparsely through tectal layers C–G. In addition, some ChATi fibers are visible in deep tectal layers 2–6. In the torus semicircularis, the rostral portion of the nucleus laminaris receives a moderate number of ChATi fibers. Caudally, a moderate-to-dense plexus of fine varicose ChATi fibers is present in the lateral aspect of the toral complex, including the laminar and the magnocelular nuclei. Only a few, scattered fibers occur in the principal nucleus (Fig. 2N).

In the basal plate, fine varicose ChATi fibers are present within the medial portion of the tegmental region, where they intermingle with the cholinergic nerurons of the oculomotor nucleus. Some immunoreactive fibers were observed in the lateral aspect of the midbrain tegmentum. In addition, proccesses of the cholinergic neurons of the pedunculopontine nucleus extend laterally and enter the tectum (Fig. 2M–N).

Isthmus and cerebellum. Varicose ChATi fibers are distributed homogeneously in the anteroventral, anterodorsal, posteroventral, and posterodorsal tegmental nuclei. In addition, a rather dense plexus of varicosities is located close to the ventricle. Numerous nonvaricose ChATi fibers course within the lateral aspect of the tegmentum, and fine varicose fibers are present in the superficial reticular isthmic nucleus. A dense plexus of immunoreactive nerve terminals is located in the interpeduncular nucleus (Figs. 2N–P, 4d).

Smooth ChATi fibers course dorsally and rostrally from the isthmic nucleus to the tectum (Figs. 2O, 15d). In addition, numerous fine varicose immunoreactive fibers are present through the lateral surface of the tegmentum, where they intermingle with the labeled processes of the laterodorsal tegmental neurons. A dense plexus of ChATi fibers is located in the periventricular neuropil deep to the laterodorsal tegmental nucleus and the locus coeruleus (Fig. 2P). In the cerebellum, very thick immunoreactive fibers are present (Fig. 15e). These fibers have numerous clusters of thick varicosities with a mossy appearance. They occur throughout the granule cell layer but are observed most frequently close to the Purkinje cell layer (Fig. 2P).

Rhombencephalon. The rhombencephalon contains abundant ChATi fiber tracts, which are primarily the immunoreactive axons of the neurons in the motor nuclei of the Vth, VIth, VIIth, IXth, Xth, XIth, and XIIth cranial nerves. In addition, an extensive network is formed by the dendritic processes of these cells. Apart from the ChATi fibers related to motoneurons, distinct plexuses of immunoreactive fibers are present in the dorsal, ventral, and caudal octaval nuclei. Fine ChATi thin fibers and terminals occur also close to the motor nuclei and in the reticular nuclei and in the dorsal column nucleus. In the ventral aspect of the rhombencephalon, ChATi fibers course in rostrocaudal direction and extend into the spinal cord.

Spinal cord. In the upper spinal cord, segments of the spinal cord the longitudinal fiber tracts already noted for the rhombencephalon proceed in the ventral and ventrolateral funiculi. ChATi varicose fibers were observed to

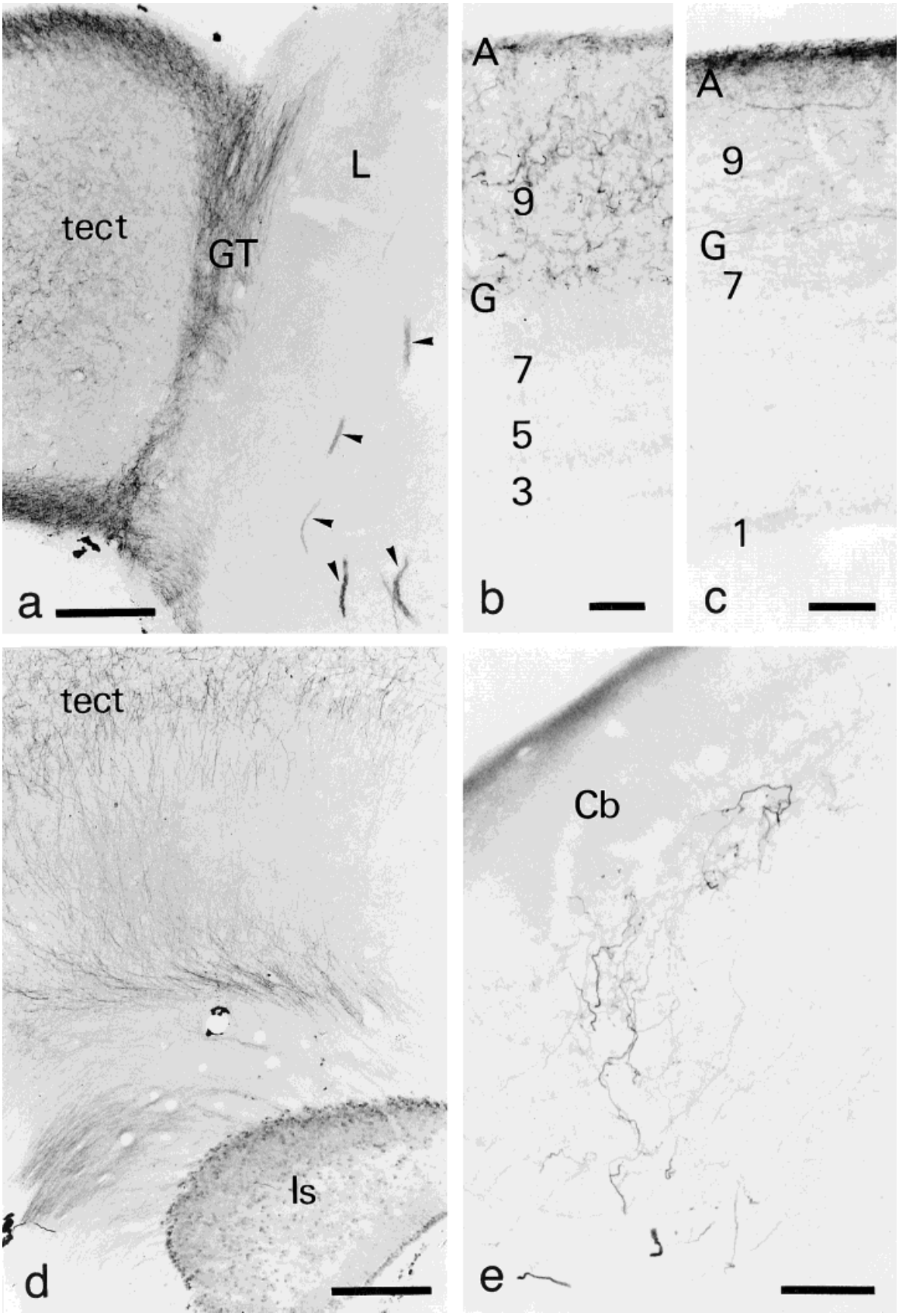

Fig. 15. Photomicrographs of transverse sections through the brain of anurans illustrating ChATi fibers in the pretectal region (**a**), the optic tectum of *Rana perezi* (**b**) and *Xenopus laevis* (**c**), the caudal tectum and the istmus (**d**), and the cerebellum (**e**). Numbers in b and c indicate some of the tectal layers. Arrowheads in a point to fiber bundles of the fasciculus retroflexus. Scale bars = 200 µm in a,d, 50 µm in b,c,e.

surround the spinal motor neurons. Occasionally, thin ChATi fibers were seen in the dorsal aspect of the spinal cord in areas corresponding to the caudal extent of the descending trigeminal tract and the substantia gelatinosa.

Urodeles

Telencephalon. In the olfactory bulb, numerous ChATi fibers are present in the internal granular layer, whereas only a few occur in the external plexiform layer (Fig. 9A). As in anurans, the accessory olfactory bulbs are devoid of fibers. Cholinergic fibers and varicosities are widely, but not uniformly, distributed throughout the telencephalon proper. In the pallium, ChATi fibers are almost exclusively restricted to rostral telencephalic levels, where the dorsal pallium shows the strongest cholinergic input, although the medial and lateral pallia also show a substantial innervation (Fig. 9A). At intermediate and caudal hemispheric levels, the ChATi fibers are mainly confined to the lateral pallium, with only a few fibers dorsally (Fig. 9C–D).

Within the subpallium, abundant ChATi fibers are located throughout the rostrocaudal extent of the striatopallial transition area (Fig. 9B–D). ChATi varicose fibers occur in the neuropil of nucleus accumbens and the striatum (Figs. 9B–D, 16b), whereas in the septal region, fine varicose ChATi fibers are present in the lateral septum (Fig. 9C). At the level of the medial amygdala, numerous ChATi fibers are located in the lateral forebrain bundle, the neuropil of the caudal division of the striatum, and the basal telencephalon (Fig. 9E–F).

Preoptic area and hypothalamus. In the preoptic area, moderate-to-dense plexuses of ChATi fibers are located laterally to the cell plate, whereas a smaller number of fibers are present within the cell plate in the dorsal aspect of the preoptic area (Fig. 9G). Caudally, ChATi fibers were found in the suprachiasmatic nucleus (Fig. 9H–I). Distinct bundles of ChATi fibers decussate in the commissura supraoptica and course parallel to the optic tract (Figs. 9I, 10a,c, 16a). A number of thin varicose ChATi fibers courses to the infundibular region and, further caudally, to the neural lobe of the hypophysis (Figs. 9J,K, 11a). Numerous, coarse varicose ChATi fibers are located around the unstained cell bodies of the periventricular organ (Fig. 9J,K). In addition, fine varicose fibers are present lateral to this organ.

Epithalamus, thalamus, and pretectum. The dorsal habenula contains a moderate plexus of ChATi fibers, which surround the cholinergic neurons (Fig. 9G,H). The axons of the cholinergic cells are densely packed within the fasciculus retroflexus (Fig. 9I–L). In sagittal sections (Fig. 16a), these fibers course caudally to the level of the thalamic-pretectal boundary, then turn ventrally to reach the basal plate, and continue caudally to terminate in the interpeduncular nucleus (Fig. 11c). A dense plexus of ChATi fibers is located lateral to the ventral thalamus and anteroventral thalamic zone (Fig. 9H), which most likely constitutes the terminal site of the cholinergic cells at isthmic levels. Caudally, other dense plexuses of immunoreactive fibers occur lateral to the posterodorsal thalamic zone (Fig. 9J), the posterior commissure (Fig. 9J), and the nucleus pretectalis (Fig. 9K). Apart from these distinct fiber bundles and plexuses, small numbers of fibers were also found within the cell plate of the ventral thalamus (Fig. 9H).

Mesencephalon. A laminar organization of ChATi fibers is easily recognized in the optic tectum of *Pleurodeles* (Fig. 16c). Dense plexuses of varicose ChATi fibers are present in the stratum opticum and tectal layer 3 and are separated from each other by a weakly innervated layer 2 (Fig. 9L–N). Layers 4 and 5 contain a modest number of ChATi fibers (Fig. 9L–M), whereas layer 7 only occasionally contained such fibers (Fig. 9L).

A moderate number of fibers is present immediately lateral to the region, where the presumptive urodele homologue of the torus semicircularis is located (Manteuffel and Naujoks-Manteuffel, 1990; Fig. 9M). In the basal plate, varicose fibers are present in the medial tegmental area, where they intermingle with the ChATi neurons of the oculomotor nucleus.

Isthmus and cerebellum. As in anurans, many ChATi axons were found to arise from the the nucleus isthmi and course to the tectum (Figs. 9M,N, 16a). Other ChATi fibers arise from the laterodorsal tegmental nucleus and form a bundle that can be traced rostrally (Fig. 9N,O). Fine varicose ChATi fibers are present in the nucleus reticularis superior and in a region medial to it, which includes the raphe nucleus (Fig. 9N,O). A very dense plexus of ChATi fibers is located in the interpeduncular nucleus (Fig. 9M–P). In the cerebellum, long coarse ChATi fibers, which give rise to numerous varicosities, are located in the granule cell layer.

Rhombencephalon. As in anurans, the rhombencephalon of urodeles contains extensive arborizations of dendrites that belong to the cranial nerve motoneurons (Fig. 9P–V). The course of their axons has been described above, and because most of the motoneurons lie far from the level where they leave the brain, these axons form distinct pathways within the rhombencephalon. In addition, moderate plexuses of ChATi fibers are present in the octavolateral area and the nucleus reticularis medius and inferior. Longitudinally coursing fiber tracts were observed in the ventral and ventrolateral aspects of the white matter and in the medial longitudinal fascicle, which includes the conspicuous axons of the Mauthner cells (Figs. 9R–V, 12).

Spinal cord. ChATi elements are restricted to the ventral half of the spinal cord in urodeles. Strongly stained axons accompany the thick Mauthner axons in the dorsal aspect of the ventral funiculus, and others more diffusely organized course within the ventral and ventrolateral funiculi (Fig. 9V). At several places in the upper spinal cord, collaterals of the Mauthner axons branch off and enter the zone of the ventral motoneurons. Among the motoneurons in the ventral gray, thin ChATi fibers were also observed. Occasionally, ChATi fibers were observed in the dorsal aspect of the spinal cord.

DISCUSSION

The aim of the present study was to provide a detailed description of the organization of the cholinergic cell bodies and fibers in the brains of amphibians. Comparison with previous reports about the localization of cholinergic systems in amphibians is limited because only two studies are available for anurans, and in those two the scope was limited to the isthmotectal pathway (Desan et al., 1987) and the efferent cells of the octavolateralis system (González et al., 1993a). However, some biochemical, physiological, or pharmacological aspects of the cholinergic systems of anuran amphibians have been studied (Ricciuti and Gruberg, 1985; Fite and Wang, 1986; Ciani et al., 1988; Sargent et al., 1989; Wallace et al., 1990; Jardon and Bonaventure, 1992a,b). In the following sections, the gen-

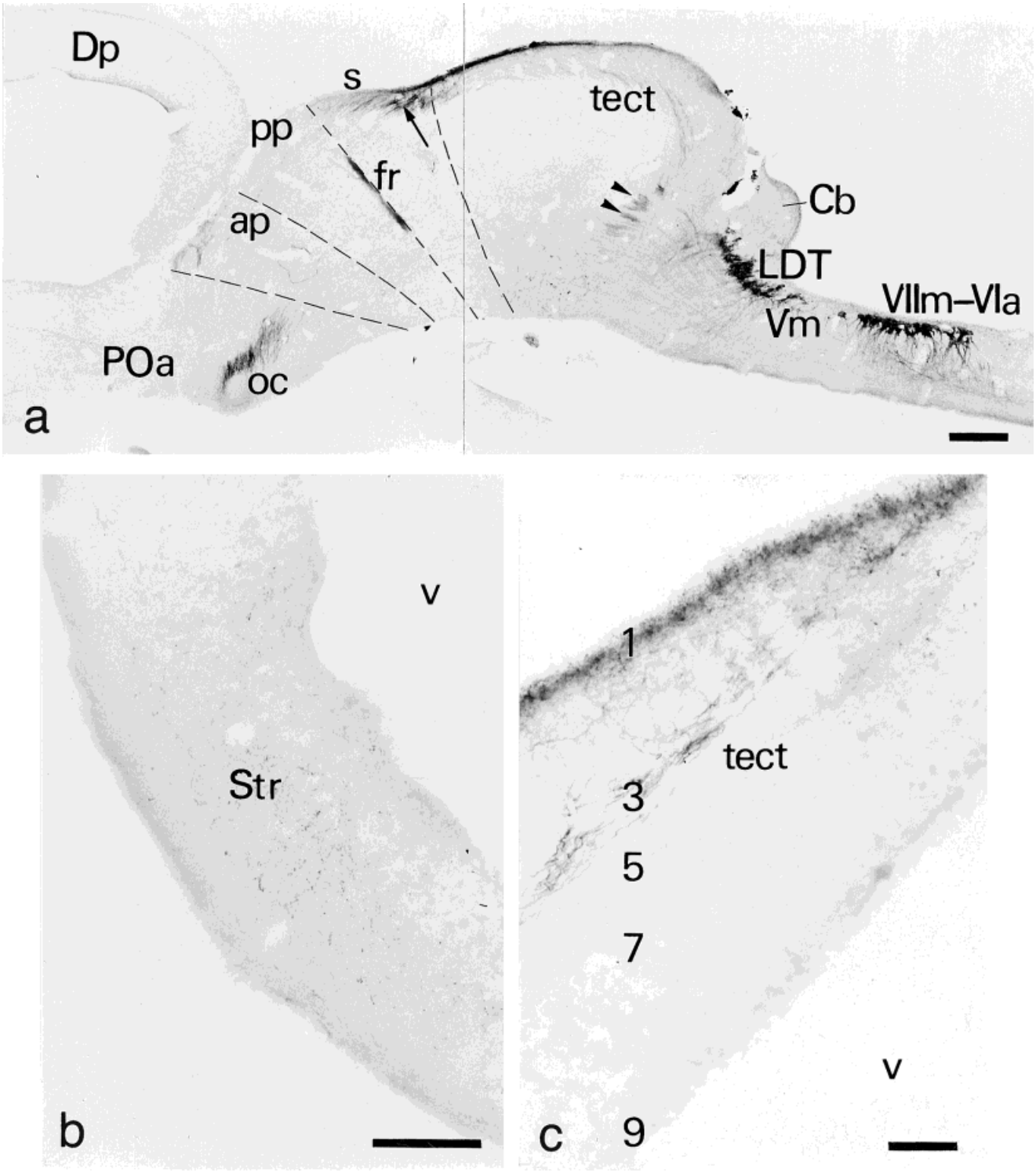

Fig. 16. Photomicrographs illustrating the distribution of ChATi fibers in the brain of *Pleurodeles waltl*. **a:** Mid-lateral sagittal section, in which the diencephalic neuromeres are indicated (arrowheads point to the isthmotectal radiation, and the arrow indicates the pretectal region). **b:** The fine innervation of the striatum. **c:** ChATi fibers in the superficial tectal layers. Scale bars = 200 μm in a, 50 μm in b,c.

eral organization and variations of the cholinergic systems in amphibians are discussed, based primarily on the results of the present study. Attention is also paid to putative interactions with other neurotransmitter systems. Subsequently, the cholinergic systems of amphibians are compared with those of other vertebrates to define

primitive (general) and derived features. Finally, the topography of the cholinergic cell groups is discussed following a segmental approach (Puelles, 1995).

Amphibian cholinergic systems

Olfactory bulb. In both anuran and urodele amphibians, cholinergic fibers are present within the main olfactory bulb but apparently absent in the accessory olfactory bulb. Whereas in anurans ChATi fibers occur primarily in the external plexiform layer, in urodeles the internal granular layer contains the highest number of immunoreactive fibers. Although these observations point to clear species differences, the number of species studied is too small to draw any conclusion. Because cholinergic neurons are lacking in the olfactory bulb of amphibians, the ChATi innervation observed in the bulb probably has an extrinsic origin, most likely from the diagonal band of Broca (Neary, 1990).

Pallium. The pallial regions of the amphibians studied have in common that they contain moderate plexuses of ChATi fibers. The majority of these fibers lie within the medial and lateral pallium and are more abundant at rostral than at caudal levels. In both amphibian orders, the pallium receives afferent projections from cells located in the diagonal band, the medial septum, the medial amygdala, and adjacent areas (Neary, 1990; Sassoè-Pogneto et al., 1991, 1995; Northcutt and Ronan, 1992). Therefore, these basal forebrain areas may contain the cells of origin of the cholinergic input to the amphibian pallium.

Basal telencephalon: Striatum. An interesting finding of the present study is the demonstration of ChATi cell bodies in the striatum of the frog, *Rana perezi*. However, such neurons are few in number, weakly immunoreactive, and apparently lacking in *Xenopus* and *Pleurodeles*. Because the distribution of other cholinergic cell groups in the brain of both anuran species is almost identical, this difference may be a true species difference. However, cells in the striatum of *Xenopus* and *Pleurodeles* may actually contain acetylcholine but below the immunodetection level. Consistent with this view is the notion that the cholinergic neurons in the striatum of *Rana* are always weakly immunoreactive with the method used in the present study.

Basal telencephalon: Diagonal band, septum, and amygdala. All three amphibian species studied have in common a large number of ChATi cell bodies in the basal telencephalon. In anurans, these cells were found within the medial septum, the diagonal band of Broca, the medial and lateral amygdala, and the caudal part of the striatum. In addition, numerous ChATi cells were located around the lateral forebrain bundle and intermingled with the fibers of the medial forebrain bundle. These cholinergic cells constitute a rather large field that extends over several cell groups and may correspond, as a whole, to the cholinergic basal telencephalic neurons found in urodeles. Apart from the olfactory bulb and the pallium, the cholinergic neurons of the basal telencephalon most likely contribute to the innervation of the striatum and the nucleus accumbens, a notion that is supported by the results of recent hodological studies of basal ganglia afferent connections in amphibians (Marín et al., 1997a). These neurons may also be the origin of projections outside the telencephalon, e.g., cholinergic fibers course within the stria medullaris to the habenula, and injections of retrograde tracers in the habenular complex retrogradely label cells in the septal region of frogs (Kemali et al., 1980) and the basal telencephalon of newts (Clairambault et al., 1986).

Hypothalamus. Common features of the hypothalamus of the three amphibian species studied are ChATi cells in the suprachiasmatic nucleus and a moderate number of immunoreactive fibers in the infundibulum and the neural lobe of the hypophysis. In previous studies with application of retrograde tracers to the neural lobe, retrogradely labeled cells were found in the suprachiasmatic hypothalamus (Pasquier et al., 1980; Tuinhof et al., 1994), suggesting that the suprachiasmatic ChATi cells might be the source of the cholinergic innervation of the neural lobe. In contrast to *Rana* and *Pleurodeles*, in *Xenopus* an additional group of ChATi cells was found in the infundibular hypothalamus, but the functional significance of this difference is unclear.

Epithalamus, thalamus, and pretectum. Numerous, closely packed, cholinergic neurons in the dorsal habenula are a common feature of anuran and urodele amphibians. Consistent with previous hodological studies, the axons of these cells constituted the fasciculus retroflexus, which terminated in the interpeduncular nucleus (Kemali et al., 1980; Kemali and Lázár, 1985; Clairambault et al., 1986). Neurons in the dorsal habenula have been reported to contain substance P, which is also found in the fasciculus retroflexus and in the interpeduncular nucleus (Inagaki et al., 1981; Taban and Cathieni, 1983; Kemali and Guglielmotti, 1984). Therefore, acetylcholine and substance P may colocalize within dorsal habenular neurons.

The present study shows a prominent cholinergic innervation of the retinorecipient thalamic and pretectal nuclei. Accordingly, moderate levels of ChAT activity were measured in the anuran thalamus (Ciani et al., 1988), whereas microinjections of cholinergic drugs in the pretectum revealed prominent effects on the activity of pretectal cells via both muscarinic and nicotinic receptors (Jardon and Bonaventure, 1992a). Analysis of the distribution of ChATi fibers that reach the thalamus and the pretectum suggests that the cholinergic innervation of these nuclei arises from the isthmic tegmentum. However, double-labeling studies combining retrograde tracing techniques and immunohistochemistry for ChAT are required to establish the origin of the cholinergic innervation of the amphibian thalamus and pretectum.

Mesencephalic tectum. As previously described in the frog *Rana pipiens* (Desan et al., 1987), the tectum of amphibians is densely innervated by cholinergic fibers, which originate primarily from the isthmic nucleus (Desan et al., 1987; present study). Consistent with these results, the anuran tectum contains the highest level of ChAT-specific activity in the brain (Ciani et al., 1988), as well as elevated levels of muscarinic and nicotinic receptors (Freeman and Norden, 1984; Sargent et al., 1989). Although the retina may be a source of cholinergic input to the tectum (Oswald et al., 1979), both lesion and immunohistochemical studies have failed to confirm that notion (Ricciuti and Gruberg, 1985; Desan et al., 1987; present study). The optic tectum contains nicotinic acetylcholine receptorlike molecules located within the terminals of retinofugal fibers, which are partly codistributed with ChATi fibers (Desan et al., 1987; Sargent et al., 1989; present study). Therefore, acetylcholine may influence retinal afferents by modulating synaptic function in the optic tectum (Sargent et al., 1989; King, 1990). Apart from an action on nicotinic

receptors, acetylcholine may influence visual information processing in the tectum via muscarinic receptors (Fite and Wang, 1986).

Isthmus. For anurans (Desan et al., 1987; present study) and for urodeles, the majority of the neurons in the nucleus isthmi have been proven to be cholinergic. The nucleus isthmi is known to project bilaterally to the tectum (Glasser and Ingle, 1978; Gruberg and Udin, 1978; Rettig, 1988; Wiggers and Roth, 1991). The contralateral isthmotectal fibers decussate in the dorsocaudal part of the optic chiasm (Gruberg and Udin, 1978; Rettig, 1988; Gruberg et al., 1989), which is in agreement with the present finding of cholinergic fibers abundantly crossing in the supraoptic commissure. Moreover, the isthmic nucleus may provide virtually the entire cholinergic input to the anuran tectum (Ricciuti and Gruberg, 1985; Desan et al., 1987; Wallace et al., 1990). Although our findings largely support this notion, results of a preliminary study, in which dextran amine as retrograde tracer was combined with ChAT immunohistochemistry, suggest that cells within the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus also contribute (Marín et al., unpublished observations). Finally, it is obvious that, within the class of amphibians, significant differences exist in the organization of the cholinergic cell groups at the isthmic level. Thus, in anurans, conspicuous ChATi cell groups form the pedunculopontine and the laterodorsal tegmental nuclei, whereas in urodeles, only the laterodorsal tegmental nuclues is well organized; if present, the pedunculopontine tegmental nucleus is formed only by a few ChATi cells located rostral to the nucleus isthmi.

Cerebellum. The present study has revealed the existence of cholinergic fibers in the amphibian cerebellum, which is in agreement with previously reported moderate levels of ChAT activity in another ranid frog (Ciani et al., 1988). A comparison of cell groups known to project to the cerebellum (González et al., 1984; Grover and Grüsser-Cornehls, 1984; A. Muñoz et al., 1995) with those containing ChATi cell bodies (present study) suggests that the nucleus reticularis inferior, the ventral and caudal octaval nuclei, and the dorsal column nucleus are likely sources of cholinergic input to the anuran cerebellum. Apart from a projection from the dorsal column nucleus to the cerebellum (A. Muñoz et al., 1996), nothing is known about cerebellar afferent connections in urodeles.

Motor nuclei. The arrangement of the somatomotor (III, IV, VI, XII) and branchiomotor (V, VII, IX–X) nuclei was clearly observed by ChAT immunohistochemistry in the species studied, and the results are largely consistent with those obtained in other species of anurans and urodeles on the basis of cytoarchitectonic criteria (Opdam and Nieuwenhuys, 1976; Opdam et al., 1976; Nikundiwe and Nieuwenhuys, 1983) or experimental tracing techniques (Matesz and Székely, 1977, 1978; Stuesse et al., 1983, 1984; Naujoks-Manteuffel et al., 1986; González and Muñoz, 1987, 1988; Oka et al., 1987b; Roth et al., 1988; Muñoz and González, 1995). An interesting finding, however, is the presence of additional ChATi cell groups related to the motor nuclei of the glossopharyngeal, vagus, accessory abducens, and, less clearly, oculomotor nerve. These groups most likely represent the preganglionic parasympathetic nuclei within the brainstem of amphibians, which have been noted in *Rana* by Matesz and Székely (1978) and in *Bufo* by Oka et al. (1987a) by using the retrograde cobalt tracing technique. With the horseradish perxodase technique, these cell groups were not distinctly observed (Stuesse et al., 1984). Whereas in *Rana perezi* a subgroup of ChATi cells in the dorsal part of the oculomotor complex, putatively homologous to the Edinger-Westphal nucleus of amniotes, could be recognized, such a cell group was difficult to distinguish in urodeles (Naujoks-Manteuffel et al., 1986; present study). Associated to the accessory abducens nucleus, a distinct ChATi cell group was found in both anuran species studied, whereas small ChATi cells associated to the IXm–Xm complex were observed only in *Rana*. These two cell populations can be readly compared to the superior and inferior salivatory nuclei of amniotes, and they innervate lacrimal glands and encapsulated salivary glands that appear first in amphibians and are related to the tongue and oral cavity. Because *Xenopus* lacks a tongue, the parasympathetic cells associated to the IXm–Xm complex are also missing (Simpson et al., 1986). Large lacrimal glands located medial to the eye (Harder's gland) may be innervated by the ChATi cells at the level of the accessory abducens nucleus in both anurans (Székely and Matesz, 1993). In the rhombencephalon of *Pleurodeles*, putative parasympathetic cells associated to the branchiomotor nuclei could be distinguished as separate entities, which is in line with results of retrograde tracer experiments in another urodele species (Roth et al., 1988). Most likely, the parasympathetic elements lie intermingled with the branchiomotor cells.

Other rhombencephalic cell groups. Previous retrograde tracer studies have revealed that the efferent cells of the VIIIth nerve are located in close relation to the VIIm, primarily at its medial aspect (Claas et al., 1981; Fritzsch, 1981; Strutz et al., 1982; Will, 1982). Their cholinergic nature has been proven by double labeling with tracing and ChAT immunohistochemistry (González et al., 1993a). In *Xenopus* and *Pleurodeles*, the lateral line system persists throughout adulthood. The efferent cells of the anterior and posterior lateral line nerves are also cholinergic and are found in association with the VIIm and IXm, respectively (Will, 1982; González et al., 1993a).

In *Pleurodeles*, the morphology and location of the giant Mauthner cells are in full agreement with the description given by Will (1991) on the basis of Nissl staining and retrograde tracing. In contrast, with ChAT immunohistochemistry, Mauthner cells are not identifiable in *Xenopus*, although it has been reported that such cells are retained in the adult after the metamorphosis (Will, 1986). A possible explanation could be that the neurotransmitter involved differs between species, but that seems unlikely. Because the function of the Mauthner cells is primarily related to movements in escape behavior, which changes during the transformation from tadpole to adult *Xenopus*, it would be interesting to know whether these cells are ChATi during larval stages.

Finally, ChATi cells were found in the solitary tract nucleus and the dorsal column nucleus. Both nuclei are chemically heterogeneous structures. Apart from acetylcholine, cells within the confines of the solitary tract nucleus contain, among others, the neurotransmitters dopamine, noradrenaline, adrenaline, and gamma-aminobutyric acid (GABA; A. Muñoz et al., 1995; González and Smeets, 1991, 1993, 1995; González et al., 1993b) and also calbindin D-28k and NADPH-diaphorase/nitric oxide synthase (A. Muñoz et al., 1995; González et al., 1996; M. Muñoz et al., 1996). In addition, the dorsal column nucleus consists of a heterogeneous population of cells containing, among oth-

ers, acetylcholine, GABA, glycine, NADPH-diaphorase/nitric oxide synthase, and the calcium-binding protein parvalbumin (A. Muñoz et al., 1995, 1996; González et al., 1996; M. Muñoz et al., 1996; present study). As yet, it is unknown whether neurons in the solitary tract nucleus and the dorsal column nucleus contain acetylcholine colocalized with any other of the chemical markers, such as NADPH-diaphorase or calcium-binding proteins, or whether they are projection neurons or interneurons.

Comparison with other vertebrates

Pallial/cortical regions. Comparison of the results obtained in amphibians with those in teleost fishes suggests that the absence of cholinergic neurons in the pallium of anamniotes is a common feature, and the same may hold for reptiles and birds. Except for the lizard *Gallotia galloti* (Medina et al., 1993), cholinergic neurons were never observed in crocodiles, turtles, or other lizards (Mufson et al., 1984; Brauth et al., 1985; Hoogland and Vermeulen-VanderZee, 1990; Reiner, 1991; Powers and Reiner, 1993). Cortical cholinergic neurons are almost entirely absent from cortical regions in birds (Medina and Reiner, 1994). Previously, it has been proposed that the evolution of the neocortex in mammals involve laminar differentiation with the addition of new cell types, including those containing acetylcholine (Reiner, 1991). However, a scrutiny of the available data reveals that even in mammals the presence of cholinergic neurons in the cortex is not a shared feature. For example, such neurons are present in the cortex of rats (Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1984; Ichikawa and Hirata, 1986; Parnavelas et al., 1986; Blaker et al., 1988; Mufson and Cunningham, 1988; Reiner, 1991) but not in guinea pigs (Maley et al., 1988), cats, or dogs (Kimura et al., 1981; Vincent and Reiner, 1987; St-Jacques et al., 1996). Moreover, in situ hybridization localization of ChAT mRNA in rat cortical neurons is still a matter of debate (Oh et al., 1992; Lauterborn et al., 1993). Furthermore, ChATi neurons have been identified in fetal monkey cerebral cortex (Hendry et al., 1987) but not in the cortex of adult primates, including human (Mesulam et al., 1984; Satoh and Fibiger, 1985a; Mesulam and Geula, 1988; Geula et al., 1993; Alonso and Amaral, 1995). Thus, the presence of cortical cholinergic neurons seems to be a feature that is acquired relatively late during the evolution of vertebrates, as has been suggested previously (Reiner, 1991; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994), but it is certainly not a feature generally shared by amniotes. As yet, it is unclear whether these species differences are due to variations of transmitter content reaching nonimmunodetectable levels or represent true species differences.

Striatum. In amniotes, the dorsal striatum (caudate-putamen), the nucleus accumbens, and the olfactory tubercle contain cholinergic neurons (Kimura et al., 1981; Armstrong et al., 1983; Hedren et al., 1983; Brauth et al., 1985; Phelps et al., 1985; Satoh and Fibiger, 1985; Vincent and Reiner, 1987; Maley et al., 1988; Mufson and Cunningham, 1988; Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993; Powers and Reiner, 1993; Henselmans and Wouterlood, 1994; Medina and Reiner, 1994; Holt et al., 1996). In mammals, these cholinergic cells are local circuit neurons that have a perikaryon that is larger than that of the projection neurons (Kása, 1986; Woolf, 1991; Alheid et al., 1995). Similar observations have been made in birds (Medina and Reiner, 1994) and reptiles (Medina et al., 1993; Henselmans and Wouterlood, 1994). In contrast, the cholinergic interneurons seem to be lacking in the telencephalon of teleost fishes (Ekström, 1987; Brantley and Bass, 1988). In amphibians, cholinergic neurons, although few in number and only weakly immunoreactive, have been found in the striatum of the frog *Rana perezi* but not in *Xenopus* and *Pleurodeles*. It is not known whether these neurons are local circuit neurons or projection neurons. Nevertheless, the demonstration of cholinergic neurons in at least some amphibians may have consequences for our understanding of the organization and evolution of the vertebrate basal ganglia, e.g., in anamniotes, dopamine–acetylcholine interactions may occur within the striatum. However, even though the presence of cholinergic neurons in the striatum and the nucleus accumbens is a common feature of all amniotes studied so far, substantial differences exist in the number, arrangement, and physiological properties of these cells among reptiles, birds, and mammals (Meredith et al., 1989; Henselmans and Stoof, 1991; Henselmans et al., 1991; Medina et al., 1993; Powers and Reiner, 1993; Henselmans and Wouterlood, 1994; Medina and Reiner, 1994). Therefore, the cholinergic neurons in the striatum of amphibians need further characterization before they can be compared with those of amniotes.

Other telencephalic groups. Under this heading, the cholinergic cell groups identified as the Ch1–Ch4 groups by Mesulam et al. (1983a,b, 1984) are discussed. In brief, four groups of cholinergic cells have been described within the basal telencephalon in mammals. The Ch1 cell group is represented by cholinergic cells in the medial septal nucleus. Cholinergic neurons in the vertical and horizontal limbs of the diagonal band of Broca constitute the Ch2 and Ch3 cell groups, respectively. In some mammalian species, no clear boundary exists between the Ch1 and Ch2 groups. The Ch4 cell group is comprised of neurons that extend over several nuclei, including the nucleus basalis of Meynert, the globus pallidus, the ventral pallidum, and some adjacent areas.

In anurans, the distribution of cholinergic neurons in the septal region (i.e., medial septal nucleus and nucleus of the diagonal band) resembles that found in mammals, and the same holds true for turtles and crocodiles (Mufson et al., 1984; Brauth et al., 1985; Powers and Reiner, 1993). In contrast, the cholinergic cells in the septal region of urodeles, lizards, and birds are restricted to the diagonal band nucleus and do not extend into the medial septal nucleus (Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993; Medina and Reiner, 1994; present study). However, part of the cholinergic cells of the globus pallidus, ventral pallidum, and lateral forebrain bundle found in reptiles and birds have been compared with the mammalian nucleus basalis of Meynert (Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994). The present account reveals that the basal telencephalon of amphibians contains a large population of cholinergic cells disseminated across the ventromedial telencephalic wall and in close relation with the fibers of the lateral forebrain bundle, the anterior commissure, and the medial forebrain bundle. Recent hodological studies have suggested that the amphibian basal telencephalon may contain pallidal structures because the striatum and the nucleus accumbens give raise to prominent intratelencephalic projections that innervate the caudal telencephalic regions (Marín et

al., 1997b). The present finding of cholinergic neurons around the lateral forebrain bundle and among the fibers of the medial forebrain bundle in amphibians supports the hypothesis that these basal telencephalic regions actually contain the amphibian counterparts of the amniote globus pallidus and ventral pallidum.

Hypothalamus. Cholinergic neurons have been described in the supraoptic hypothalamus and in the infundibular hypothalamus of several mammalian species (Mason et al., 1983; Tago et al., 1987). Similarly, cholinergic neurons have been described in the suprachiasmatic and infundibular hypothalamus of reptiles and birds (Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994). As in amniotes, cholinergic cells are present in comparable positions in the hypothalamus of amphibians and teleosts (Ekström, 1987; present study), suggesting that the existence of cholinergic hypothalamic neurons is a primitive feature of cholinergic systems in vertebrates. The existence of cholinergic CSF-contacting cells in the periventricular organ of teleosts, however, most probably is a derived feature of actinopterygians because such cells have not been found in amphibians or amniotes. Remarkably, the periventricular organ receives a prominent and characteristic cholinergic innervation in both birds and urodeles (Medina and Reiner, 1994; present study).

Although numerous data exist about the distribution of cholinergic neurons in the hypothalamus in vertebrates, the function of the hypothalamic cholinergic system is not clear. In mammals, cholinergic stimulation influences anterior pituitary function through several hypothalamic centers (Bioulac et al., 1978; García et al., 1992a,b). In addition, specific acetylcholinesterase has been demonstrated in nonneurosecretory nerve fibers in the neural lobe of the hypophysis, suggesting a cholinergic innervation of this lobe (Barron and Hoover, 1983; Alexandrova et al., 1987). Furthermore, acetylcholine has a strong stimulatory effect on the release of vasopressin and oxytocin at places where the neural lobe is in connection with hypothalamic tissue (Bridges et al., 1976; Gregg, 1985). The presence of ChATi fibers in the neural lobe of amphibians, which is densely innervated by vasotocin and mesotocin immunoreactive fibers (González and Smeets, 1992a,b), suggests that acetylcholine could play a similar role in the regulation of hormone release in the amphibian hypophysis.

Epithalamus, thalamus, and pretectum. In amniotes, cholinergic neurons are present in the medial habenula and project through the fasciculus retroflexus to the interpeduncular nucleus (Houser et al., 1983; Mesulam et al., 1984; Satoh and Fibiger, 1985a; Vincent and Reiner, 1987; Maley et al., 1988; Sorenson et al., 1989; Tago et al., 1989; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994). Thus, the present finding of cholinergic neurons in the amphibian dorsal habenula supports its correspondence with the amniote medial habenular nucleus. In contrast, although lesion studies have suggested the existence of a cholinergic habenulointerpeduncular projection in teleosts (Villani et al., 1987), available immunohistochemical data do not unequivocally support this notion (Ekström, 1987; Brantley and Bass, 1988; Villani et al., 1994). If so, the existence of cholinergic cells within the habenular complex may be considered as a common feature of the cholinergic systems in tetrapods, which appeared initially in early amphibians.

The pretectum of amphibians, reptiles, and mammals does not contain cholinergic neurons (Kása, 1986; Woolf, 1991; Medina et al., 1993; Powers and Reiner, 1993; present study), in contrast to the situation found in birds and teleost fishes (Ekström, 1987; Sorenson et al., 1989; Medina and Reiner, 1994). Numerous retinorecipient areas in the thalamus and pretectum of amphibians contain cholinergic fibers, as has been described in amniotes (Medina and Smeets, 1992; Medina et al., 1993; Medina and Reiner, 1994; Woolf, 1991), and, therefore, seems to be a primitive feature of cholinergic systems in vertebrates.

Mesencephalic tectum. As in amphibians, the tecta of reptiles and most mammalian species do not contain cholinergic neurons (Hedren et al., 1983; Mesulam et al., 1984; Brauth et al., 1985; Satoh and Fibiger, 1985a; Mizukawa et al., 1986; Maley et al., 1988; Mufson and Cunningham, 1988; Medina and Smeets, 1992; Medina et al., 1993; Powers and Reiner, 1993; present study). In contrast, tectal cholinergic neurons have been described in teleost fishes, birds, and some mammals. In teleosts, cholinergic neurons are distributed in the stratum periventriculare (Tumosa et al., 1986; Ekström, 1987; Zottoli et al., 1987; Molist et al., 1993), resembling type XIV cells, which may contribute to the efferent projections of the tectum (Meek and Schellart, 1978; Molist et al., 1993). Accordingly, in birds, tectal cholinergic neurons are present in layer 10, which contains the perikarya of neurons projecting outside the tectum (Sorenson et al., 1989; Bagnoli et al., 1992; Medina and Reiner, 1994). In rats, a few weakly stained cells have been described in deep tectal layers (Tago et al., 1989), whereas in cats these neurons are distributed in superficial tectal layers (Vincent and Reiner, 1987). The absence of cholinergic neurons in the tectum of a wide variety of vertebrates, on the one hand, and their presence in the tectum of teleost fishes, birds, and some mammals, on the other, make it difficult to determine what the primitive condition was in the brain of vertebrates.

Isthmus. In all classes of nonmammalian vertebrates, where a nucleus isthmi is present, the cholinergic nature of the majority of its cells has been demonstrated (teleosts: Ekström, 1987; Brantley and Bass, 1988; Zottoli et al., 1988; amphibians: Desan et al., 1987; present study; reptiles: Brauth et al., 1985; Medina and Smeets, 1992; Medina et al., 1993; Powers and Reiner, 1993; birds: Sorenson et al., 1989; Bagnoli et al., 1992; Medina and Reiner, 1994). Hodologically, the nucleus isthmi is reciprocally connected with the tectum (Hunt et al., 1977; Künzle and Schnyder, 1984; Ricciuti and Gruber, 1985; Desan et al., 1987; Zottoli et al., 1988). The parabigeminal nucleus of mammals is considered homologous to the nucleus isthmi and contains cholinergic neurons (Ch8 cell group; Mufson et al., 1986; Vincent and Reiner, 1987; Tago et al., 1989; Woolf, 1991) that project to the superior colliculus (Beninato and Spencer, 1986; Mufson et al., 1986). Therefore, the existence of a cholinergic isthmic/parabigeminal nucleus seems to be a primitive feature in the brain of vertebrates.

Abundant cholinergic cells have been present in the isthmic reticular formation of all vertebrates studied so far. In mammals and birds, two distinct groups of cholinergic neurons have been recognized, largely included within the boundaries of the pedunculopontine tegmental nucleus (Ch5 cell group) and the laterodorsal tegmental nucleus (Ch6 cell group) (Kimura et al., 1981; Armstrong et al.,

1983; Mesulam et al., 1983b, 1984; Satoh and Fibiger, 1985a; Mizukawa et al., 1986; Jones and Beaudet, 1987; Rye et al., 1987; Vincent and Reiner, 1987; Mufson and Cunningham, 1988; Tago et al., 1989; Lavoie and Parent, 1994). Similarly, two comparable cholinergic cell populations have been described in lizards and anurans (Medina et al., 1993; present study). In contrast, a single cholinergic cell group seems to be present in turtles, urodeles, and teleosts (Powers and Reiner, 1993; Ekström, 1987; Brantley and Bass, 1988; Zottoli et al., 1988; Molist et al., 1993; present study). The notion that the cholinergic cell groups found in amphibians are homologous to the pedunculopontine/laterodorsal tegmental cholinergic cell groups of amniotes is supported by the following findings: (1) in both amphibians and amniotes, these cell groups are located in similar areas of the brain and contain large cholinergic neurons that give rise to long dendrites that extend into adjacent fiber systems (Rye et al., 1987; Jones, 1990; Honda and Semba, 1995; present study); (2) they have similar projections, namely to the midbrain tectum, the retinocipient thalamic nuclei, and the pretectal nuclei (Sofroniew et al., 1985; Beninato and Spencer, 1986; Woolf and Butcher, 1986; Hall et al., 1989; Woolf, 1991; Medina et al., 1993; Medina and Reiner, 1994; present study); (3) basal ganglian efferents reach the pedunculopontine tegmental nucleus in mammals (for a recent review, see Parent and Hazrati, 1995), as seems to happen in amphibians (Marín et al., 1997b); and (4) NADPH-diaphorase is expressed in virtually all the cholinergic neurons in the mesopontine tegmentum in mammals (Vincent et al., 1983; Geula et al., 1993). Similarly, NADPH-diaphorase cells are present in the presumed homologues of the mammalian pedunculopontine and the laterodorsal tegmental nuclei in amphibians (M. Muñoz et al., 1996; González et al., 1996) and in birds (Brüning, 1993; Panzica et al., 1994), reptiles (Luebke et al., 1992; Panzica et al., 1994; Smeets et al., 1997), and fish (Brüning et al., 1995). Double-labeling studies are still needed, however, to prove the existence of colocalization in nonmammalian species.

Motoneurons of the cranial nerve nuclei and spinal cord. The motoneurons of the cranial nerve nuclei and the spinal cord are cholinergic in all vertebrates (teleosts: Rhodes et al., 1986; Ekström, 1987; Brantley and Bass, 1988; Molist et al., 1993; amphibians: González et al., 1993a; present study; reptiles: Medina et al., 1993; Powers and Reiner, 1993; birds: Sorenson et al., 1989; Reiner et al., 1991; Medina and Reiner, 1994; mammals: see e.g., Woolf, 1991).

Other rhombencephalic cell groups. The rhombencephalic reticular formation in amniotes and teleost fishes, as in amphibians, contains cholinergic neurons (Kimura et al., 1981; Mizukawa et al., 1986; Ekström, 1987; Hendry et al., 1987; Vincent and Reiner, 1987; Brantley and Bass, 1988; Maley et al., 1988; Mufson and Cunningham, 1988; Ruggiero et al., 1990; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994). Among the reticular ChATi cells, the Mauthner cells in *Pleurodeles waltl* are consistently labeled. From a comparative point of view, this result is surprising because in fish (teleost) the Mauthner cells are not immunoreactive for ChAT (Rhodes et al., 1986; Ekström, 1987).

Cholinergic neurons are present in various medullary sensory nuclei, including the gracile, cuneate, and external cuneate nuclei in mammals (Tago et al., 1989; Lan et al., 1995a,b). Similarly, a population of cholinergic cells is present in the amphibian dorsal column nucleus, although, consistent with previous (immuno)histochemical studies (A. Muñoz et al., 1995), no separation between medial and lateral cell populations is obvious. In contrast, the existence of cholinergic neurons in the gracile and cuneate nuclei in birds and reptiles has never been reported (Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994), although a few cholinergic cells are located in the external cuneate nucleus in the pigeon (Medina and Reiner, 1994). Finally, as in amphibians, the solitary tract nucleus contains a subpopulation of cholinergic neurons in mammals and birds (Armstrong et al., 1988; Tago et al., 1989; Ruggiero et al., 1990; Lan et al., 1995b), whereas the reptilian homologue nucleus does not seem to contain cholinergic neurons (Medina et al., 1993; Powers and Reiner, 1993).

Segmental organization of cholinergic cell groups

A segmental approach appears to be a framework well suited for presenting anatomical data of the vertebrate brain because the number and organization of the brain segments (neuromeres) are constant features in all vertebrates (Vaage 1969; Puelles et al., 1987; Lumsden and Keynes, 1989; Puelles, 1995). A schematic representation of the topography of the cholinergic cell groups in relation to the segmental domains in the amphibian brain is presented in Figure 17. A similar approach was used in the studies of the cholinergic systems in the brain of reptiles and birds (Medina et al., 1993; Medina and Reiner, 1994). The presentation of our results in the same manner facilitates a direct comparison between amniotes and amphibians (anamniotes) and contributes to a better understanding of the evolution of this neurotransmitter system. Briefly, in early stages of development, the hindbrain consists of seven to eight rhombomeres (rh1–rh8) and the mesencephalon of one mesomere (Puelles et al., 1987; Lumsden and Keynes, 1989; Noden, 1991). The diencephalon and the secondary prosencephalon has been proposed to consist of three prosomeres each (p1–p3 and p4–p6, respectively; see Puelles, 1995). Because neuromeric-derived domains are also evident in the adult brain, a segmental analysis of brain organization has been successfully applied in several tetrapods.

A detailed demonstration of the interneuromeric boundaries is not available for the mesencephalic and rhombencephalic cytoarchitecture in adult amphibians. However, the boundaries of the different segments in the midbrain and hindbrain of adult vertebrates can be inferred by using the cranial nerves and the cranial nerve motor nuclei as landmarks (Lumsden and Keynes, 1989; Noden, 1991; Medina et al., 1993; Medina and Reiner, 1994; Puelles and Medina, 1994). For the analysis of the diencephalon, we have followed a recent segmental analysis of the anuran diencephalon (Puelles et al., 1996). The schematic drawings presented in Figure 17 correspond to the brain of *Rana perezi*, and the main features of the distribution of the cholinergic cell groups of the two anurans studied are considered. A segmental approach in the adult brain of urodeles is more difficult to make because abundant cell migration occurs in the hindbrain. For example, at the level of the roots of nerves IX and X (Fig. 13), there is an intermingling of four brainstem motor nuclei (VI, VII, IX, X) and, in addition, the rostral extent of the spinal motor column (Roth et al., 1988; present study).

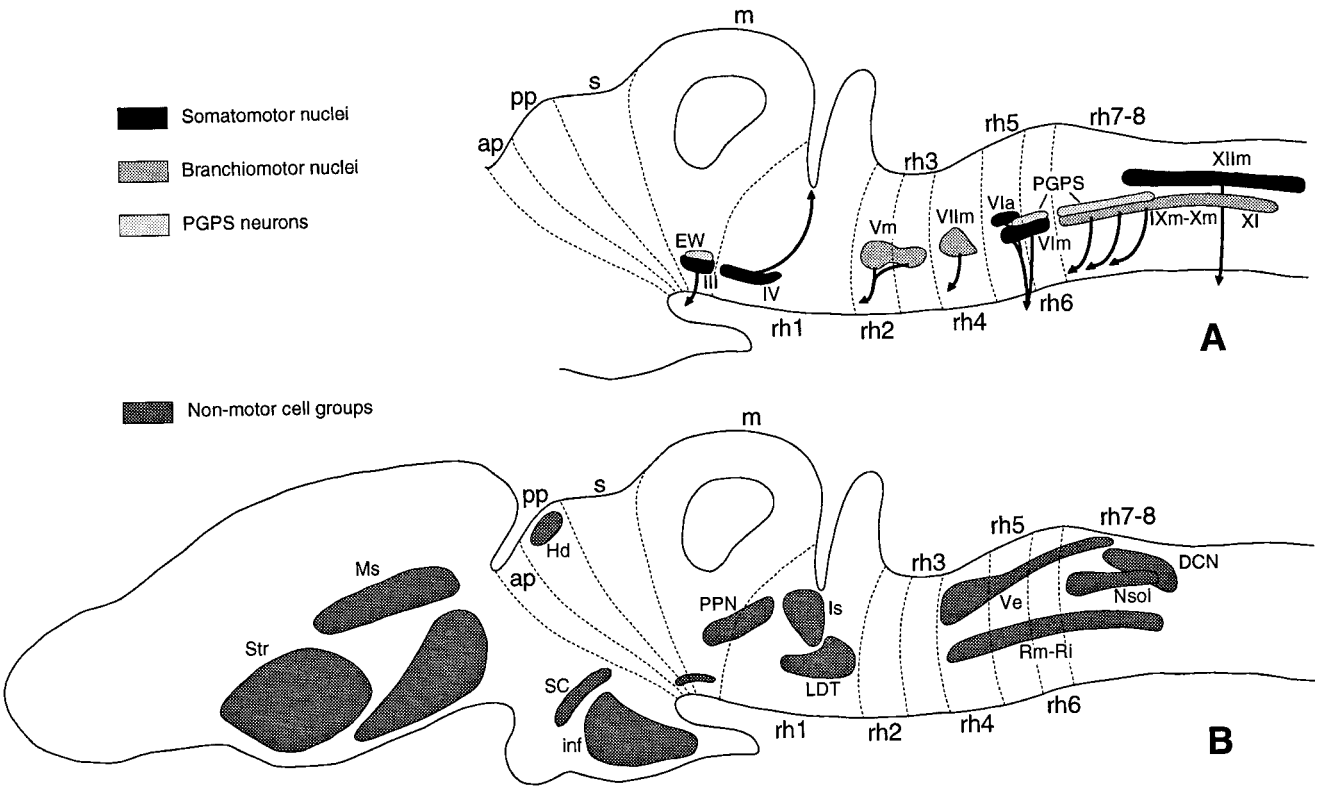

Fig. 17. Schematic drawings of sagittal sections through the brain of anurans showing the topography of the ChATi cell groups in relation to the segmental domains. **A:** ChATi motor nuclei of the cranial nerves. **B:** Non-motor cell groups.

The diencephalon has been subdivided in three transverse neuromeres: synencephalon, posterior parencephalon, and anterior parencephalon. This subdivision was proposed mainly on the basis of acetylcholinesterase staining in the brain of the frog (Puelles et al., 1996). In our material, these subdivisions can be recognized by the different density of cholinergic fibers observed in sagittal sections through the diencephalon in the three species studied. The presence of cholinergic fibers coursing adjacent to the limits supports the identification of diencephalic neuromeres. For example, the fasciculus retroflexus, which is clearly observed as a compact bundle in *Pleurodeles*, marks the limit between the synencephalon and the posterior parencephalon (Fig. 16). Similar findings have been reported for the diencephalon in reptiles and birds (Medina et al., 1993; Medina and Reiner, 1994). However, no definitive evidence exists of transverse neuromeres within the secondary prosencephalon in amphibians, which therefore includes the telencephalon, preoptic region, and hypothalamus (Puelles et al., 1996). Thus, the cholinergic cell groups observed in these regions are represented within a single segment domain in Figure 17.

Segmental analysis of the cholinergic neurons in the pedunculopontine tegmental nucleus reveals that a large portion of these cells is located within the isthmic tegmentum (Fig. 17), as has been described for amniotes (Medina et al., 1993; Medina and Reiner, 1994). It is also evident, however, that the rostral portion of the anuran pedunculopontine tegmental nucleus extends into the mesencephalic tegmentum, in contrast to what has been suggested for reptiles and birds (Medina et al., 1993; Medina and Reiner, 1994). Such a difference is also evident in conventional transverse sections, where the anuran pedunculopontine tegmental nucleus is located in a slightly more dorsal position than the corresponding cell group in reptiles and birds (Medina et al., 1993; Medina and Reiner, 1994; present study). As observed in sagittal sections, the cholinergic neurons of the pedunculopontine tegmental nucleus in mammals are restricted mainly within the isthmic neuromere, although it is not clear whether the rostral limit of the group is also located within the mesencephalic tegmentum (see Fig. 2F–H in Tago et al., 1989).

The hindbrain is one of the most conservative regions of the vertebrate brain. To explain the segmental arrangement of the cholinergic cell masses, the obvious match between segments (rhombomeres) and the adjacent branchial nerves offers helpful criteria. Actually, the cell bodies of individual cranial nerves have a precise relationship to specific rhombomeres in all vertebrates (Lumsden and Keynes, 1989; Noden, 1991; Medina et al., 1993; Medina and Reiner, 1994).

The somatomotor nuclei occupy the ventromedial portion of several segments. The oculomotor nucleus, which lies ventrally in the mesencephalic neuromere, is continuous caudally with the neurons of the trochlear nucleus, located in rh1 (sometimes called the isthmic neuromere). The motoneurons of the VIth nerve span rh5 and rh6, whereas the adjacent branchial motor nuclei of the VIIth and IXth nerves lie in rh4 and rh7, respectively. Finally, the motor nucleus of nerve XII is located in rh8.

The branchiomotor nuclei constitute a cell column, which extends throughout the brainstem, that is interrupted at some levels by more or less distinct gaps. The Vth nerve motoneurons form a single nucleus located within rh2 and rh3, with the trigeminal root in rh2. The organization and topographical relationships of the facial motor nucleus is one of the most striking differences between anurans and urodeles. In *Pleurodeles*, rh4 contains the giant Mauthner cells and the fascicles of the facial axons arise from more caudally located motoneurons and the genu facialis, whereas in anurans both the motoneurons and the fascicles lie within rh4 and a genu is lacking (Matesz and Székely, 1978; Stuesse and Cruce, 1986; Oka et al., 1987b). Interestingly, the two conditions observed in amphibians are also found in the brains of adult amniotes (Székely and Matesz, 1993). Thus, in birds, the facial nerve motor nucleus lies in rh4 (but extends into rh5), rostral to the abducens nucleus (Medina and Reiner, 1994), as in anurans. In contrast, in the brain of adult reptiles and mammals, the facial motor nucleus migrates caudally into rh6 during development and, as in urodeles, a prominent genu is formed (Medina et al., 1993). In anurans, the glossopharyngeal motor nucleus begins at the caudal pole of the nucleus abducens and proceeds along rh7 and rh8, forming a single column with the vagal motor cells (*Xenopus*), or with a small gap between the two entities (ranid frogs). Caudally, this column overlaps with the accessory nerve nucleus.

ChATi cells, which are topographically associated to the III, VIa, and IXm–Xm nuclei of amphibians (PGPS, Fig. 17), most likely represent the preganglionic parasympathetic column reported in amniotes (e.g., Contreras et al., 1980). A mesencephalic component comparable to the Edinger-Westphal nucleus and a rhombencephalic component extending throughout rh5–rh8 were observed. The rhombencephalic component could be further divided into rostral (rh5–rh6) and caudal (rh7–rh8) portions. Apart from the cranial motor nuclei and the preganglionic parasympathetic cell column, cholinergic cell groups in the rhombencephalon are present in the vestibular area (rh4–rh8), the reticular formation (rh4–rh8), and, more caudally, the solitary tract nucleus and the dorsal column nucleus (rh7–rh8).

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