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Distribution of Choline Acetyltransferase Immunoreactivity in the Brain of an Elasmobranch, the Lesser Spotted Dogfish (*Scyliorhinus canicula*)

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ABSTRACT

Although the distribution of cholinergic cells is remarkably similar across the vertebrate species, no data are available on more primitive species, such as cartilaginous fishes. To extend the evolutionary analysis of the cholinergic systems, we studied the distribution of cholinergic neurons in the brain and rostral spinal cord of *Scyliorhinus canicula* by immunocytochemistry using an antibody against the enzyme choline acetyltransferase (ChAT). Western blot analysis of brain extracts of dogfish, sturgeon, trout, and rat showed that this antibody recognized similar bands in the four species. Putative cholinergic neurons were observed in most brain regions, including the telencephalon, diencephalon, cerebellum, and brainstem. In the retrobulbar region and superficial dorsal pallium of the telencephalon, numerous small pallial cells were ChAT-like immunoreactive. In addition, tufted cells of the olfactory bulb and some cells in the lateral pallium showed faint immunoreactivity. In the preoptic-hypothalamic region, ChAT-immunoreactive (ChAT-ir) cells were found in the preoptic nucleus, the vascular organ of the terminal lamina, and a small population in the caudal tuber. In the epithalamus, the pineal photoreceptors were intensely positive. Many cells of the habenula were faintly ChAT-ir, but the neuropil of the interpeduncular nucleus showed intense ChAT immunoreactivity. In the pretectal region, ChAT-ir cells were observed only in the superficial pretectal nucleus. In the brainstem, the somatomotor and branchiomotor nuclei, the octavolateral efferent nucleus, and a cell group just rostral to the Edinger-Westphal (EW) nucleus contained ChAT-ir neurons. In addition, the trigeminal mesencephalic nucleus, the nucleus G of the isthmus, some locus coeruleus cells, and some cell populations of the vestibular nuclei and of the electroreceptive nucleus of the octavolateral region exhibited ChAT immunoreactivity. In the reticular areas of the brainstem, the nucleus of the medial longitudinal fascicle, many reticular neurons of the rhombencephalon, and cells

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of the nucleus of the lateral funiculus were immunoreactive to this antibody. In the cerebellum, Golgi cells of the granule cell layer and some cells of the cerebellar nucleus were also ChAT-ir. In the rostral spinal cord, ChAT immunoreactivity was observed in cells of the motor column, the dorsal horn, the marginal nucleus (a putative stretch-receptor organ), and in interstitial cells of the ventral funiculus. These results demonstrate for the first time that cholinergic neurons are distributed widely in the central nervous system of elasmobranchs and that their cholinergic systems have evolved several characteristics that are unique to this group. J. Comp. Neurol. 420:139–170, 2000.

Indexing terms: acetylcholine; brain; immunohistochemistry; immunoblotting; dogfish; elasmobranchs

Knowledge of the distribution of cholinergic systems in the brain has relied classically on histochemical studies of the activities of acetylcholinesterase (AChE) and/or choline acetyltransferase (ChAT), the acetylcholine degradation and synthesizing enzymes, respectively (see Hoover et al., 1978; Butcher and Woolf, 1984). The early development of AChE histochemical techniques in the 1950s led to a large number of studies dealing with putative cholinergic cell bodies and fibers in the central nervous system (CNS) of vertebrates (for reviews, see Butcher and Woolf, 1984; Kása, 1986; Woolf, 1991). The introduction in the early 1980s of antibodies to ChAT (Kimura et al., 1980, 1981; Eckenstein and Thoenen, 1982; Levey and Wainer, 1982; Ishida et al., 1983; Levey et al., 1983) has given new

Abbreviations

AMV	anterior medullary velum
BSA	basal superficial area
btf	basal telencephalic fascicle
CC	cerebellar crest
CDP	central dorsal pallium
CER	cerebellum
CN	cerebellar nucleus
COM	commissural vagal nucleus
CS	central canal of the spinal cord
df	dorsal funiculus
DH	dorsal horn
DON	dorsal octavolateral nucleus
DT	dorsal thalamus
ENT	entopeduncular nucleus
EW	Edinger-Westphal nucleus
fs	solitary tract
G	nucleus G
GLOM	glomerular layer of the olfactory bulb
GR	granular layer (eminences) of the cerebellum
H	habenula
HY	hypothalamus
IAC	interstitial nucleus of the anterior commissure
ICs	interstitial cells of the ventral funiculus
IHL	inferior hypothalamic lobe
III	oculomotor nucleus
IO	inferior olive
IP	interpeduncular nucleus
IPN	caudal neuropil/tract of the interpeduncular nucleus
IS	isthmus
IV	trochlear nucleus
LAL	lower auricular leaf
LC	locus coeruleus
lf	lateral funiculus
LFN	lateral funiculus nucleus
LG	lateral geniculate nucleus
LP	lateral pallium
ME	median eminence
MES	mesencephalon
MESV	mesencephalic trigeminal nucleus
MG	marginal nucleus
mlf	medial longitudinal fascicle
MOL	molecular layer
MON	medial octavolateral nucleus
MP	medial pallium
N	nucleus N
NCT	nucleus of the transverse commissure
NHY	neurohypophysis
NMH	medial hypothalamic nucleus
Nmlf	nucleus of the medial longitudinal fascicle
OB	olfactory bulb
OC	optic chiasma
OEN	octavolateral efferent nucleus
ON	optic nerve
OT	optic tectum
OV	olfactory ventricle
OVH	organon vasculosum hypothalami
OVL	organon vasculosum lamina terminalis
P	pineal organ
P1–P4	prosomeres 1–4
PC	posterior commissure
PCN	periventricular region of the cerebellar nucleus
PEW	pre-Edinger-Westphal nucleus
POC	postchiasmatic hypothalamus
PON	preoptic nucleus
PRN	posterior recess nucleus
PT	posterior tubercle
RB	retrobulbar region
RI	inferior reticular nucleus
RM	intermediate reticular nucleus
RN	recessus neuroporicus
RS	superior reticular nucleus
SDP	superficial dorsal pallium
SF	Stieda's median fascicle
SN	substantia nigra
SP	nucleus of the spinooccipital nerves
SR	septal region
ST	striatum
SV	saccus vasculosus
TEL	telencephalon
UAL	upper auricular leaf
VD	trigeminal descending root
vf	ventral funiculus
VH	ventral horn
VI	abducens nucleus
VIIIa	anterior octaval nucleus
VIIId	descending octaval nucleus
VIIIm	magnocellular octaval nucleus
VIM	visceromotor column
VIS	viscerosensory column
VM	trigeminal motor nucleus
VS	trigeminal sensory nucleus
VT	ventral thalamus
VTA	ventral tegmental area

impetus to the study of cholinergic systems in vertebrates. The use of these antibodies has revealed a number of neuronal populations that are AChE-positive but not ChAT-immunoreactive (ChAT-ir; Eckenstein and Sofroniew, 1983; Butcher and Woolf, 1984). It has become clear that AChE also occurs in noncholinergic cells and, thus, that this activity is not a reliable marker of cholinergic cells. In contrast, the distribution of ChAT is related closely to that of acetylcholine.

The cholinergic systems of several mammals have been described using anti-ChAT antibodies (rats: Kimura et al., 1980; Houser et al., 1983; Tago et al., 1987, 1989; mice: Mufson and Cunningham, 1988; guinea pigs: Maley et al., 1988; cats: Kimura et al., 1981; Vincent and Reiner, 1987; dogs: St-Jacques et al., 1996; primates: Mesulam et al., 1984; Satoh and Fibiger, 1985). The majority of the cholinergic neurons in the CNS of mammals are distributed in five large groups of cells (medial basal forebrain, neostriatal, parabrachial, cranial motor nuclei, and reticular), and this distribution appears to be remarkably stable across species (see Tago et al., 1989). The use of anti-ChAT antibodies also has allowed the construction of detailed maps of cholinergic neurons in 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), amphibians (Marín et al., 1997), and several teleosts (Ekström, 1987; Zottoli et al., 1987, 1988; Brantley and Bass, 1988; Molist et al., 1993a). Some of the cholinergic groups found in mammals also are present in most these species, although there are noticeable differences as regards other neuronal groups.

The electric organ of the electric rays (*Torpedo*) is one of the best characterized vertebrate cholinergic systems. The electric organ contains a very large number of cholinergic synapses and is innervated by the electric lobe of the medulla oblongata, probably the largest cholinergic nucleus observed in any vertebrate (Anadón et al., 1995b; Herreros et al., 1995). Previous studies have accumulated extensive knowledge of this system. However, with the exception of a histochemical study of AChE activity in sharks (Kusunoki et al., 1973), there have been no studies of the distribution of other cholinergic markers in the brain of elasmobranchs. The aim of the current study was to provide a detailed description of the putative cholinergic neurons in the brain of a galeomorph elasmobranch, *Scyliorhinus canicula*. This dogfish is used widely in anatomic and physiologic studies, and a detailed atlas of its brain is available currently (Smeets et al., 1983). The results of this study may contribute to our knowledge of the evolution of cholinergic systems in vertebrates.

MATERIALS AND METHODS

Immunohistochemistry

Seven dogfish (*Scyliorhinus canicula* L.) that were caught in the Ría of Vigo (Spain) were used immediately after capture. All animals were anesthetized deeply with 0.05% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in sea water and perfused through the conus arteriosus with elasmobranch Ringer's solution containing 0.1% procaine following by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. All experiments were conducted in accordance with European Community guidelines on animal care and experimentation. After perfusion, the brains were removed and postfixed in the same fixative for 5–6 hours and then immersed in a cold solution of 30% sucrose in PB until they sank. Subsequently, three brains were embedded in a solution of 15% gelatin with 30% sucrose in PB and then stored overnight in 4% formaldehyde solution at 4°C. The brains were cut at a thickness of 40 μm on a freezing microtome in the transverse or sagittal planes and collected in 0.1 M PB. From each brain, five parallel series were obtained.

Four brains were embedded in optimum cutting temperature (OCT) compound (Tissue Tek, Torrance, CA), frozen with liquid-nitrogen-cooled isopentane, and cut on a cryostat at 18 μm thickness. Then, six parallel series of sections were mounted on chrome alum-gelatin coated slides.

Both free-floating and slide-mounted sections were treated with 1% H_2O_2 in phosphate-buffered saline (PBS), pH 7.4, for 15 minutes to reduce endogenous peroxidase activity. After three rinses in PBS, the sections were incubated by following the peroxidase-antiperoxidase method, as described by Marín et al. (1997). Briefly, this method involves the sequential incubation of sections in 1) purified goat anti-ChAT serum (anti-human placental enzyme; code AB144P; Chemicon, Temecula, CA) diluted 1:100 for 2 days at 4°C, 2) rabbit anti-goat serum (Chemicon) diluted 1:50 for 2 hours, 3) goat peroxidase-antiperoxidase (PAP) complex (Chemicon) diluted 1:600 for 2 hours, and 4) the complex was developed with 3,3′diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (Tris, pH 7.6) for 5–15 minutes. The antibodies and the PAP complex were diluted with 0.5% Triton X-100 (PBS-T), 15% normal rabbit serum (NRS), and 2% bovine serum albumin (BSA). Moreover, the sections were rinsed three times in PBS for 10 minutes before each new step. In some series of free-floating sections, visualization of the immunostaining was improved by processing the sections with nickel-enhanced DAB (0.05% DAB, 0.01% H_2O_2, 0.04% nickel-ammonium sulfate in PB). Another series of free-floating sections was stained by using the glucose oxidase method (Shu et al., 1988), which specifically enhances the staining of nerve fibers and terminals. Briefly, after rinsing in PBS, the sections were rinsed in 0.1 M acetate buffer (AB), pH 6.0, for 10 minutes and subsequently incubated in a medium containing 0.5 mg/ml DAB, 0.027 mg/ml glucose oxidase (type VII; Sigma), 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 Tris. The sections were then mounted (mounting medium: 0.25% gelatin in Tris) and, after drying overnight, coverslipped. Some sections were counterstained with cresyl violet to facilitate analysis of the results. As a control, the primary antiserum was omitted from a series of sections of each specimen in each experiment: This resulted in no immunostaining at all.

Photomicrographs were recorded with an Olympus photomicroscope (Olympus, Tokyo, Japan) by using Kodak T-Max100 professional black-and-white film (Eastman-Kodak, Rochester, NY), and the enlarged photomicrographs were printed on Brovira-Speed photographic paper (Agfa-Gevaert, Montsel, Belgium) in the darkroom. Measurement of neurons was performed directly on the screen

of a Visopan (Reichert, Austria) projection microscope. For description, the ChAT-ir neurons of the dogfish were divided into four size categories, roughly following Smeets et al. (1983): small (8–15 μm in minor diameter), medium-sized (15–30 μm), large (30-45 μm), and very large (>45 μm).

Western blot analysis

The antibodies used have been well characterized (Shiromani et al., 1987; Medina and Reiner, 1994; Grosman et al., 1995) and have been used before in a wide range of vertebrate species. To further assess the specificity of the antibody in the dogfish, it was checked by Western-blot analysis of brain extracts. For comparative purposes, brain extracts of rat, trout (*Salmo gairdneri*), and sturgeon (*Acipenser baeri*) were subjected to identical analyses.

Brains of two adult dogfish were homogenized mechanically in a six-fold volume of cold 0.05 M Tris-saline buffer, pH 7.6, containing 0.05 M ethylenediamine tetraacetic acid (Sigma), and the protease inhibitors phenylmethylsulfonyl fluoride (2 mM; Sigma) and N-ethylmethylmaleimide (10 mM; Sigma). Samples were centrifuged at ×20,000 g at 4°C for 20 minutes, then cold methanol was added to the supernatant, and the samples were kept overnight at −20°C. After a brief centrifugation, the methanol was eliminated, and the protein concentration of the samples was determined by using Bradford's method (1976). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide 80 × 70 × 0.75 mm slab gels at a constant 150 V for 1 hour (Mini-Protean II PAGE System; Bio-Rad, Richmond, CA). Samples of 50 μg of protein in 30 μl of loading buffer containing 10% β-mercaptoethanol (Sigma), pH 6.8, were applied to each lane, and low-range, prestained SDS-PAGE molecular weight standards (Bio-Rad) were run in additional lanes. The separated proteins were then electroblotted at 30 V overnight at 4°C onto a 0.45 μm pore size nitrocellulose membrane (Bio-Rad) by using a Mini-TransBlot system (Bio-Rad). Nonspecific binding sites on the membrane were blocked by incubating for 2 hours in 5% milk powder dissolved in 0.01 M Tris-saline buffer, pH 8.0, containing 0.5% Tween-20 (TBST). The blots were then incubated overnight in agitation at room temperature in anti-ChAT diluted at 1:200 in TBST containing 15% normal rabbit serum (Sigma). After repeated washings in TBST, the blots were incubated for 1 hour in rabbit anti-goat immunoglobulin G (IgG) horseradish peroxidase-conjugated antibody (Chemicon; 1:5,000 dilution). Staining was visualized with a rapid electrochemoluminiscent (ECL) detection system (ECL Western blotting system; Amersham, Buckinghamshire, United Kingdom) and exposed on to Hyperfilm-ECL (Amersham). To assess the specificity of the immunostaining, some blots were treated with the primary antibody preincubated for 24 hours at 4°C at working dilution with 50 μg/ml or 100 μg/ml of human placental ChAT (Sigma). Preabsorption completely abolished the staining of blots.

RESULTS

Western blot analysis

Western blot analysis of brain protein extracts with the polyclonal antiserum against human placental ChAT (hpChAT) in the four species studied showed the presence of bands of ≈68–72 kDa (Fig. 1) in addition to further bands of lower molecular weight (degradation fragments). Similar degradation fragments of ChAT were obtained in previous biochemical studies that characterized antibodies to this enzyme from crude preparations (Muñoz-Maines et al., 1988; Poethke et al., 1997). In the dogfish lane, the major band of ≈68 kDa was stained weakly and had a molecular weight similar to that of the rat but lower than the major bands of sturgeon and trout. In the four species studied, there was a degradation band that showed a similar weight (≈55 kDa; Fig. 1), and a few minor bands also were observed. All bands disappeared in parallel experiments in which the primary antibody was preincubated with hpChAT.

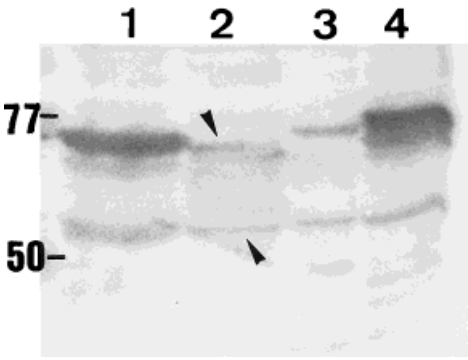


Fig. 1. Western blot analysis of protein extracts of brains of rat (lane 1), dogfish (lane 2), sturgeon (lane 3), and trout (lane 4) immunostained with anti-human placental choline acetyltransferase (anti-hpChAT) showing the presence of similar bands of ≈68–72 kDa and 55 kDa in the four species. The arrowheads indicate the bands of the dogfish. Molecular weight standards are indicated on the left.

Immunohistochemistry

The immunohistochemical methods used demonstrated the presence of immunostaining with the anti-ChAT antiserum in neurons of various brain regions and in peripheral ganglia as well as in a number of fiber systems. The pattern of labeling observed in the brain with immunohistochemistry (staining of the motor nuclei and other nuclei known to be cholinergic in other vertebrates) was similar in many respects to that reported in other vertebrates. Together with Western blot results, these findings strongly indicate that the substance recognized by the antibody in neural tissue of the dogfish was ChAT. Accordingly, the dogfish neurons that were positive to this antibody are described here as ChAT-like immunoreactive (ChAT-ir).

The general distribution of ChAT-ir neurons and fibers in the brain of the dogfish is presented summarily in Figures 2 and 3. For the description and mapping of ChAT-ir cell bodies and fibers in the dogfish, the nomenclature used in the current study is essentially the same as that used by Smeets et al. (1983).

The intensity of staining varied with the different protocols: The best staining was obtained with the procedure employing glucose oxidase during development of the immunoreaction. Differences in staining intensity also were observed between neuron groups and between fibers with the same protocol, probably due to the presence of different amounts of the enzyme.

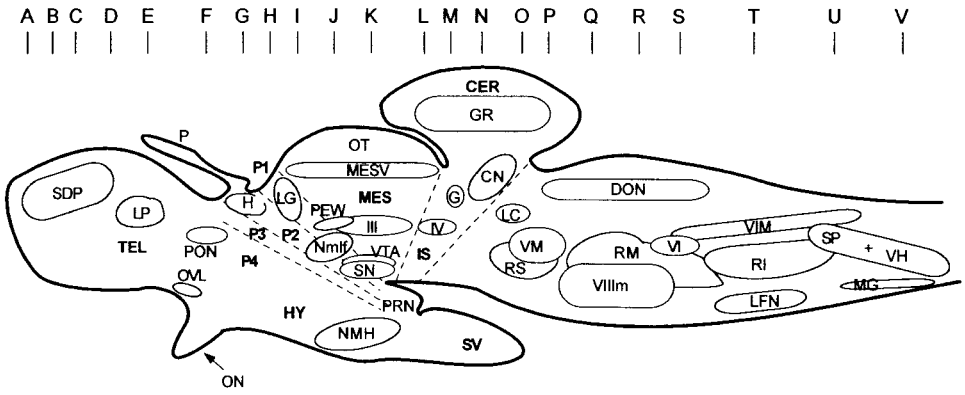


Fig. 2. Schematic representation of the distribution of ChAT-immunoreactive (ChAT-ir) neuronal groups in the brain of the dogfish. The vertical bars indicate the approximate levels of sections of Figure 3A–V, respectively. Rostral toward the left. For abbreviations, see list.

Telencephalon

A number of ChAT-ir neurons were observed in the telencephalon, both in the olfactory bulbs and in the telencephalic hemispheres (Figs. 2, 3A–E). Groups of small, ChAT-ir neurons were observed among the olfactory glomerules in the olfactory bulbs (Fig. 4A). These bipolar-shaped cells were stained weakly and exhibited long dendritic processes extending toward the olfactory glomerules. In view of their shape and glomerular relationships, these cells probably correspond to tufted cells. Although the individual axonal processes were extremely difficult to follow, an accumulation of ChAT-ir fibers at the junction with the telencephalic lobes was appreciable clearly in the series.

In the rostral telencephalic hemispheres, numerous small, strongly ChAT-ir neurons were observed scattered in the anterior, deep lateral, and deep medial regions of the superficial dorsal pallium, extending from the olfactory bulb peduncles (retrobulbar region) in the hemispheres and also in the deep medial region of the dorsal pallium near the anterior neuroporic recess and the septal nuclei (Figs. 3A–C, 4B,C). A few of these cells also were observed in the granule cell layer of the olfactory bulb. Such cells were absent in the central (caudal) regions of the dorsal pallium and in the middorsal region at intermediate levels. The size and appearance of these cells were rather homogeneous: They were bipolar, tripolar, or multipolar cells with a globular perikaryon and several thin, branched dendrites. Although, in our preparations, the axons of these cells could not be followed more than a few microns from the cell body, a number of bouton-like structures were observed in the dorsal pallium (Figs. 3A–C, 4C), suggesting that these cells are intrinsic pallial neurons.

In the caudal hemispheres, in the region of the lateral pallium (i.e., the olfactory tubercle), there was a group of faintly ChAT-ir neurons that accompanied the lateral olfactory tract (Figs. 2, 3C–E, 4D). These small cells clearly were larger and paler than the ChAT-ir dorsal pallial cells.

Abundant ChAT-ir fibers appeared intermingled with the ChAT-ir neurons of the pallial groups and also coursed in the septal area. The largest accumulation of ChAT-ir fibers and terminals was found in dorsal areas of the medial pallium forming part of a thick commissural region referred to as the dorsal pallial decussation (Fig. 3C). In the region of the striatum, caudal to the olfactory bulbs, there was a field of ChAT-ir terminals that appeared to outline the perikarya and dendrites of some striatal neurons (Figs. 3B,C). The forebrain peduncle also contained a few intensely stained, beaded fibers that ran near the midline from diencephalic regions to the septum, reaching the region of the neuroporic recess. However, in the basal superficial area (a characteristic neuronal field of the basal telencephalon), neither ChAT-ir neurons nor ChAT-ir fibers appeared to be present.

Preoptic region

In the preoptic region, ChAT-ir neurons were found in two different locations. At the level of the optic chiasma, the preoptic nucleus contained weakly ChAT-ir cell bodies (Figs. 2, 3F). These cells formed a population of small to medium-sized cells that were located dorsolaterally near the ventricular surface. The position of these ChAT-ir cells corresponded to that of the magnocellular preoptic nucleus (Mazzi, 1952; Scharrer, 1952; Mellinger, 1963; Meurling et al., 1996), which was located in the region referred to as the thalamic eminence by Smeets et al. (1983). The vascular organ of the lamina terminalis also contained a population of small ChAT-ir neurons in the ventral wall of the preoptic recess, just rostral to the optic chiasma (Figs. 2, 3F, 4E). These cells exhibited moderate ChAT immunoreactivity. A rich plexus of ChAT-ir fibers was observed in the preoptic recess organ. Numerous ChAT-ir fibers were found innervating the ventral preoptic region and extending in the region of the suprachiasmatic nucleus and the median nucleus of the hypothalamus (Figs. 3G–I, 4F), two of the most richly ChAT-ir innervated regions of the diencephalon.

Diencephalon

Hypothalamus. The dogfish hypothalamus contained very small numbers of ChAT-ir neurons. At the ventrolateral transition between the posterior recess organ (mammillary recess organ) and the neurohypophysis and saccus vasculosus, there was a small but distinct group of small, ChAT-ir bipolar cells of the cerebrospinal fluid-contacting type (Fig. 3D). In the postchiasmatic hypothalamus, some

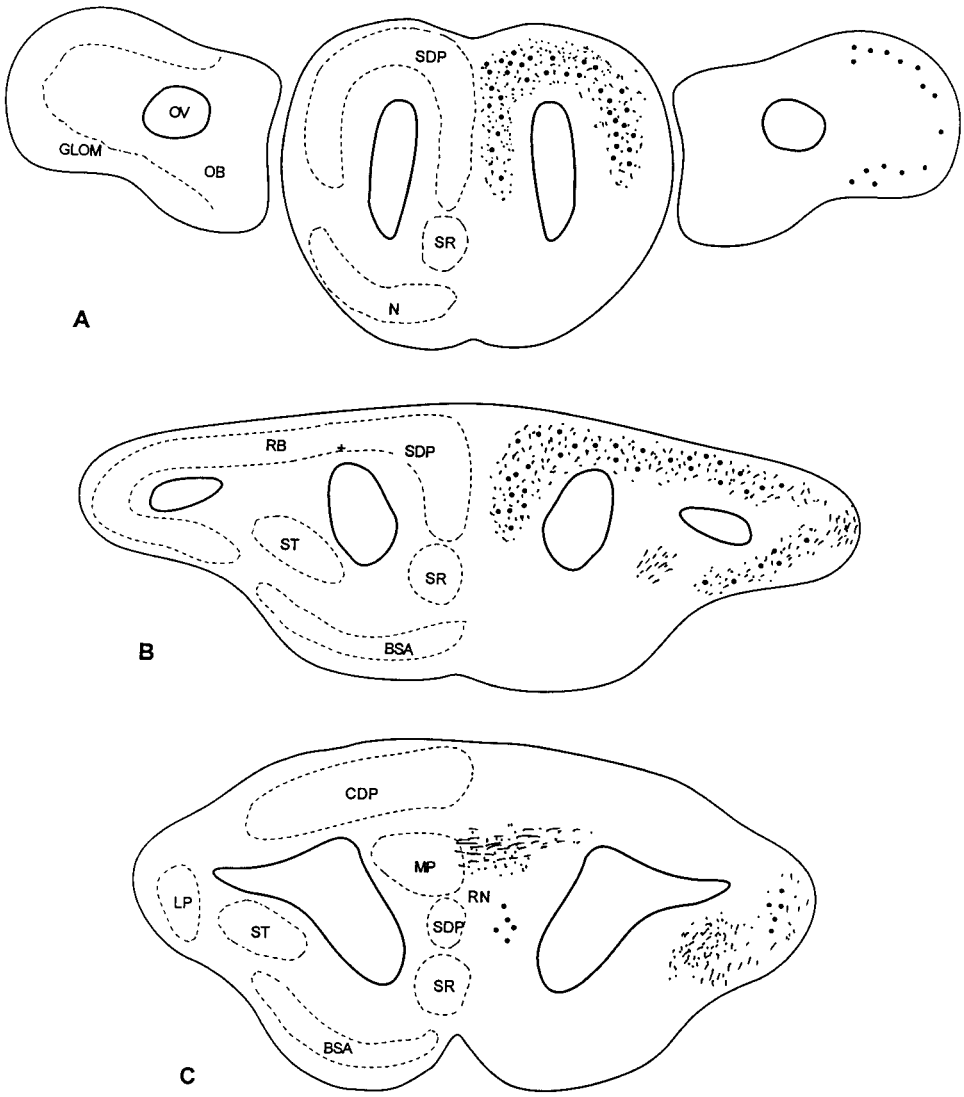


Fig. 3. **A–V:** Schematic drawings of transverse sections through the brain showing at the right of the figures the distribution of ChAT-ir neurons (solid circles), fibers (dashes), and main fields of ChAT-ir terminals (dotted areas). Large circles represent large neurons, and small circles represent small or medium-sized cells. For further explanation, see text. For abbreviations, see list.

faintly ChAT-ir neurons were seen contacting the third ventricle in the lateral tuberal nucleus (located periventricularly in the paired ventromedial eminences of the walls of the infundibulum). In the floor of the hypothalamus, some ChAT-ir fibers coursed along the median eminence and the hypophyseal stalk to the neurointermediate lobe. The hypothalamic vascular organ did not contain any ChAT-ir neurons but was innervated by ChAT-ir fibers (Fig. 3I). Likewise, the walls of the infundibulum and lateral and posterior hypothalamic recesses were innervated by ChAT-ir fibers (Fig. 3K,L). The tract of the saccus vasculosus contained faint ChAT-ir fibers that coursed to the nucleus of the saccus vasculosus located in the posterior tubercle. However, whether the epithelium of the saccus vasculosus contained ChAT-ir cells could not be determined. A number of intensely ChAT-ir, beaded fibers that apparently originated from groups of cells caudal to the diencephalon were observed in the posterior tubercle.

Thalamus. The thalamus did not contain ChAT-ir neurons. The medial thalamic region, however, received considerable numbers of intensely stained ChAT-ir beaded fibers from the region of the posterior tubercle that ascended through the periventricular region to the dorsal and ventral thalami (Fig. 3H,I).

Epithalamus. The epithalamus contained two ChAT-ir neuronal systems: the pineal organ and the habenula. The pineal organ of the dogfish, a blind sac that extends in the dorsal meninges from the posterior commissure, contained large numbers of intensely ChAT-ir bipolar cells that covered most of the inner surface of the pineal organ (Figs. 3F–H, 5A,B). These very small cells showed dendrite-like, ChAT-ir apical processes that protruded in the pineal recess. The basal region of the cell appeared to give rise to a process, although it was practically impossible to follow. The basal region of the pineal neuroepithelium contained large numbers of ChAT-ir

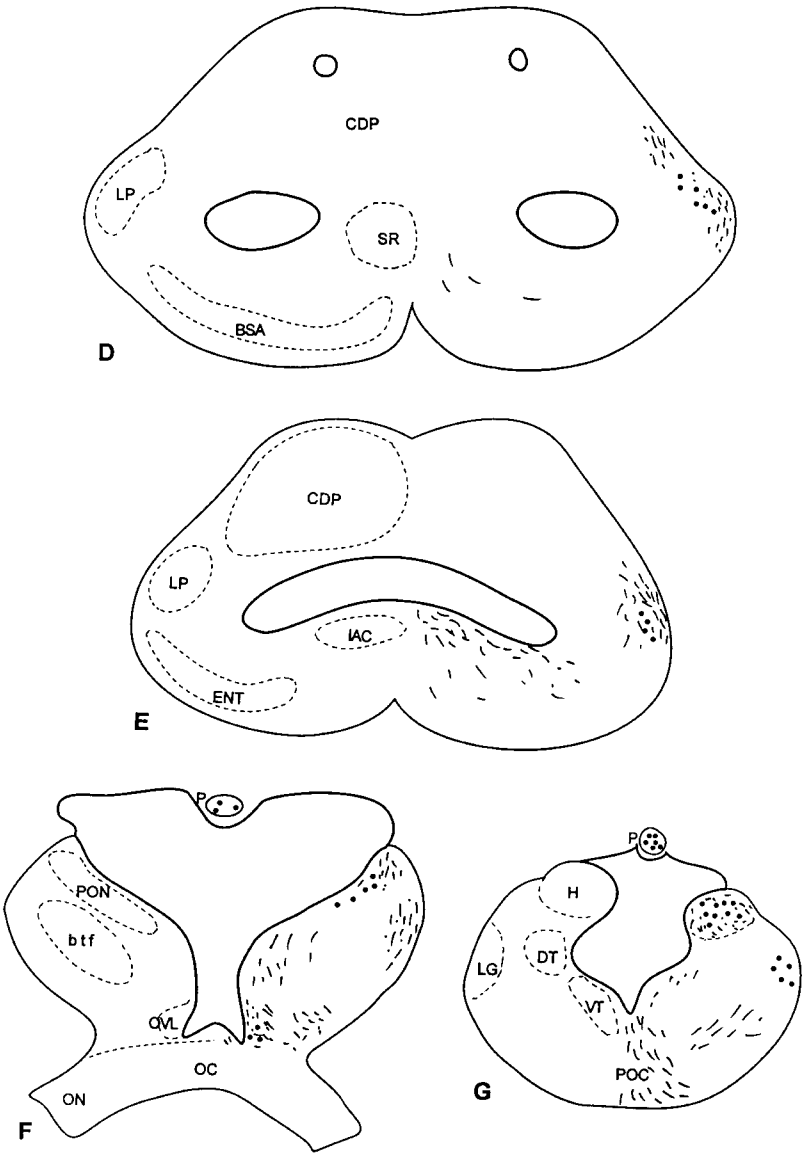


Figure 3 (Continued)

dots, presumably boutons of these cells. These cells had the appearance of photoreceptor neurons. They were observed up to the insertion of the pineal stalk in the posterior commissure, but whether ChAT-ir fibers ran from the pineal organ in the brain could not be ascertained.

Numerous small, ChAT-ir neurons were observed in the habenula, where they generally appeared as faintly immunoreactive cells (Figs. 3G,H, 5C). They were denser and smaller in the left (small) habenula and larger and more scattered in the right (large) habenula. The lateral and ventral parts of this nucleus contained moderate numbers of ChAT-ir fibers. ChAT-ir fibers also were observed coursing along the fasciculus retroflexus, although the ChAT immunoreactivity of this fascicle was very faint over most of its trajectory. This fascicle could be followed to the interpeduncular nucleus in the isthmus and the rostral medulla (see below).

Pretectum and pretectal tegmentum

The dogfish dorsal pretectal region contained a single ChAT-ir nucleus, the superficial pretectal nucleus, or lateral geniculate nucleus, which was located near the outer surface of the brain (Figs. 3G–I, 5D). The small to medium-sized, ChAT-ir neurons of this nucleus were scattered among the bundles of the optic tract. In addition to neurons, this nucleus exhibited a well-developed ChAT-ir neuropil of reticular appearance that extended around the optic tract bundles, mainly caudal to the neurons and in part intermingled with them.

The pretectal tegmentum of the dogfish contained medium-sized to large, multipolar, ChAT-ir neurons of the nucleus of the medial longitudinal fascicle (Nflm; Figs. 2, 3J, 5E). These cells were found over a considerable rostrocaudal distance, extending rostrally to the posterior tuber-

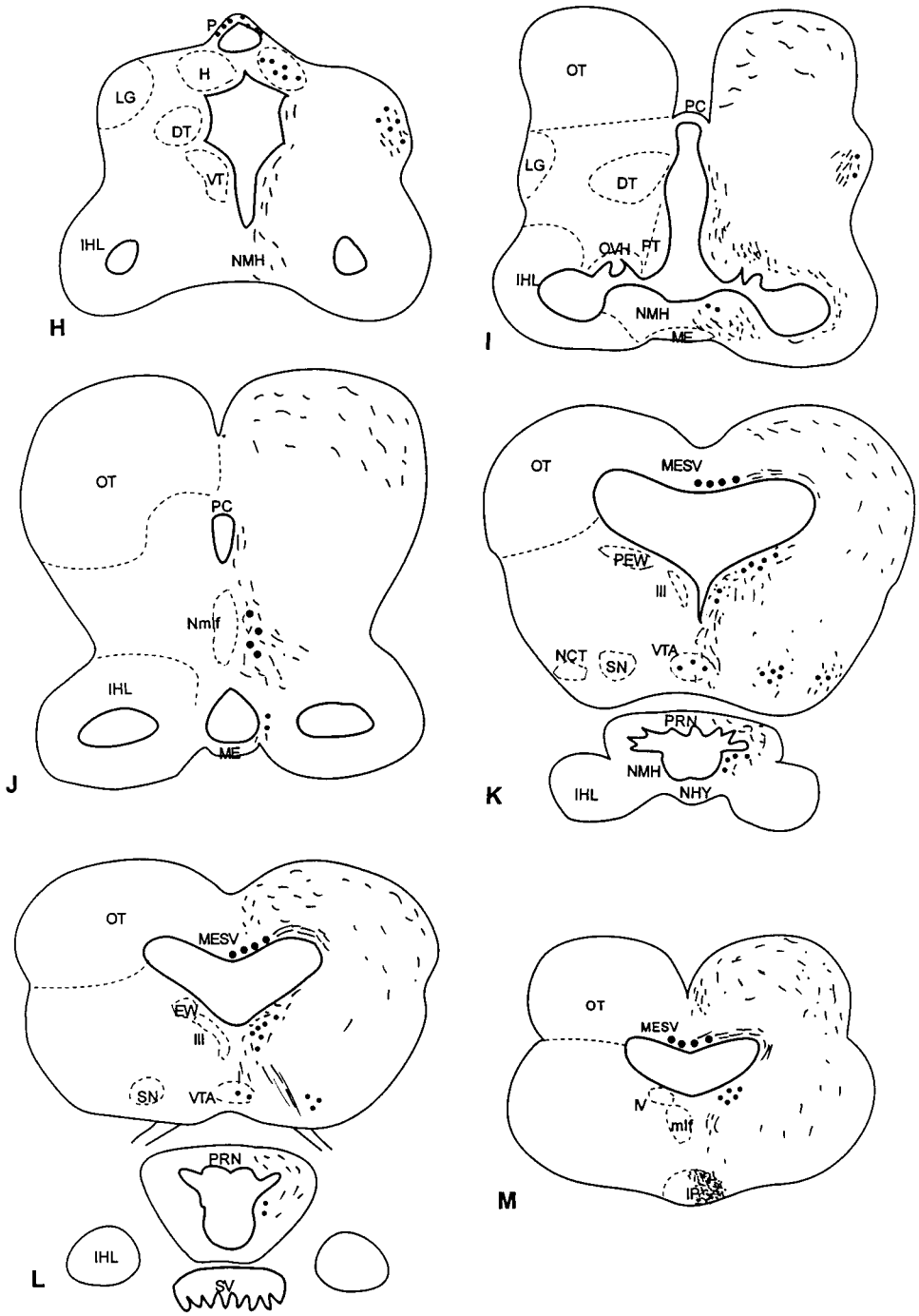


Figure 3 (Continued)

cle and thalamus, rostral to the fasciculus retroflexus, and caudally to the rostral region of the oculomotor nucleus. However, the boundary with the mesencephalic tegmentum was unclear, and similar cells also were found scattered more caudally at the level of the oculomotor nucleus.

Mesencephalon

In the mesencephalon, ChAT-ir neurons were observed in both the optic tectum and the tegmentum (Figs. 2, 3K–M). The mesencephalon also contained a number of ChAT-ir fibers.

Optic tectum. The only ChAT-ir neurons observed in the optic tectum were the large to very large neurons of the trigeminal mesencephalic nucleus, which showed intense immunoreactivity, as well as the fibers (unite processes) of the trigeminal mesencephalic root (Figs. 3K–M, 5F). These ChAT-ir cells were numerous and occupied a periventricular location (internal cellular layer) along

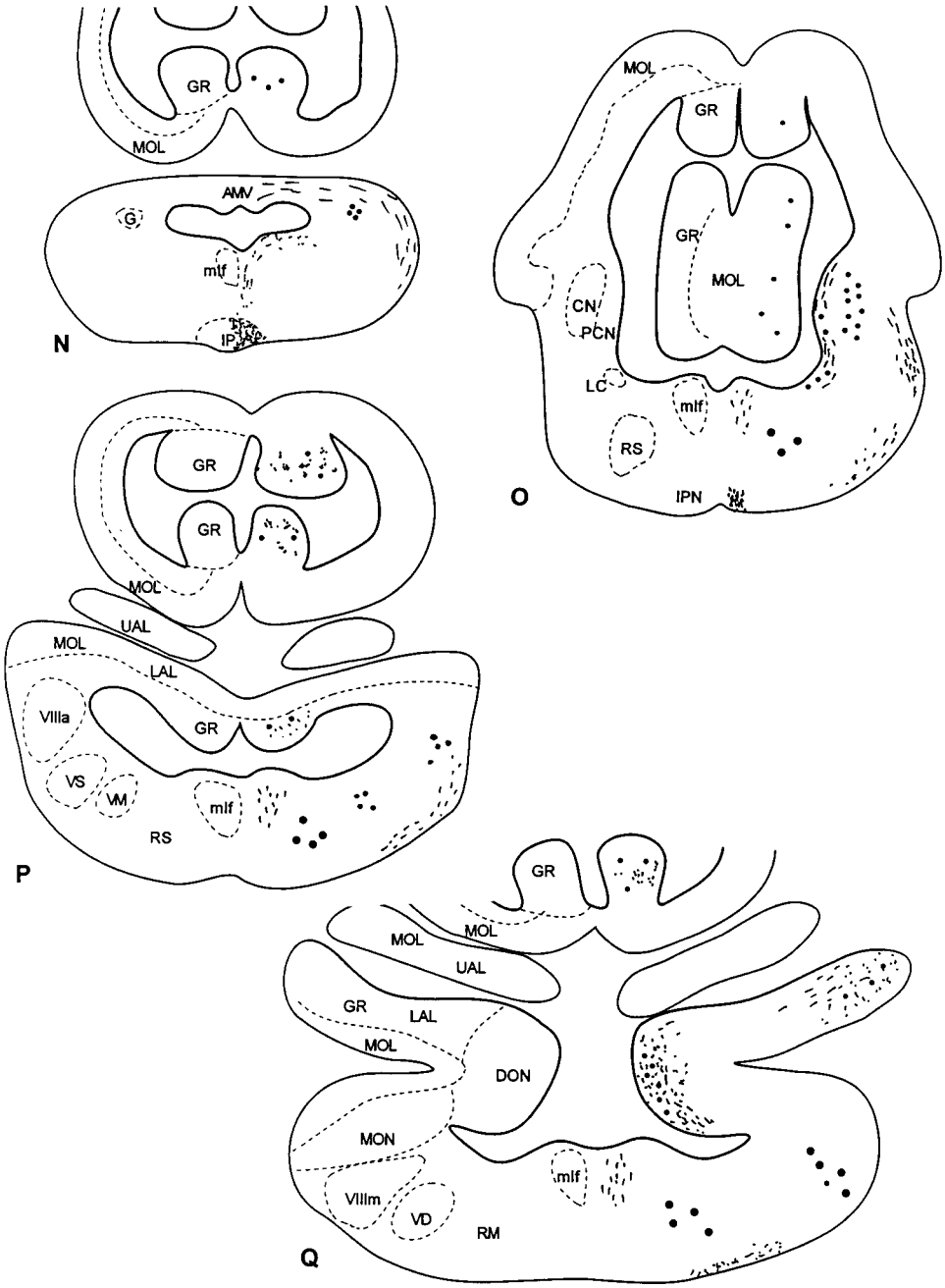
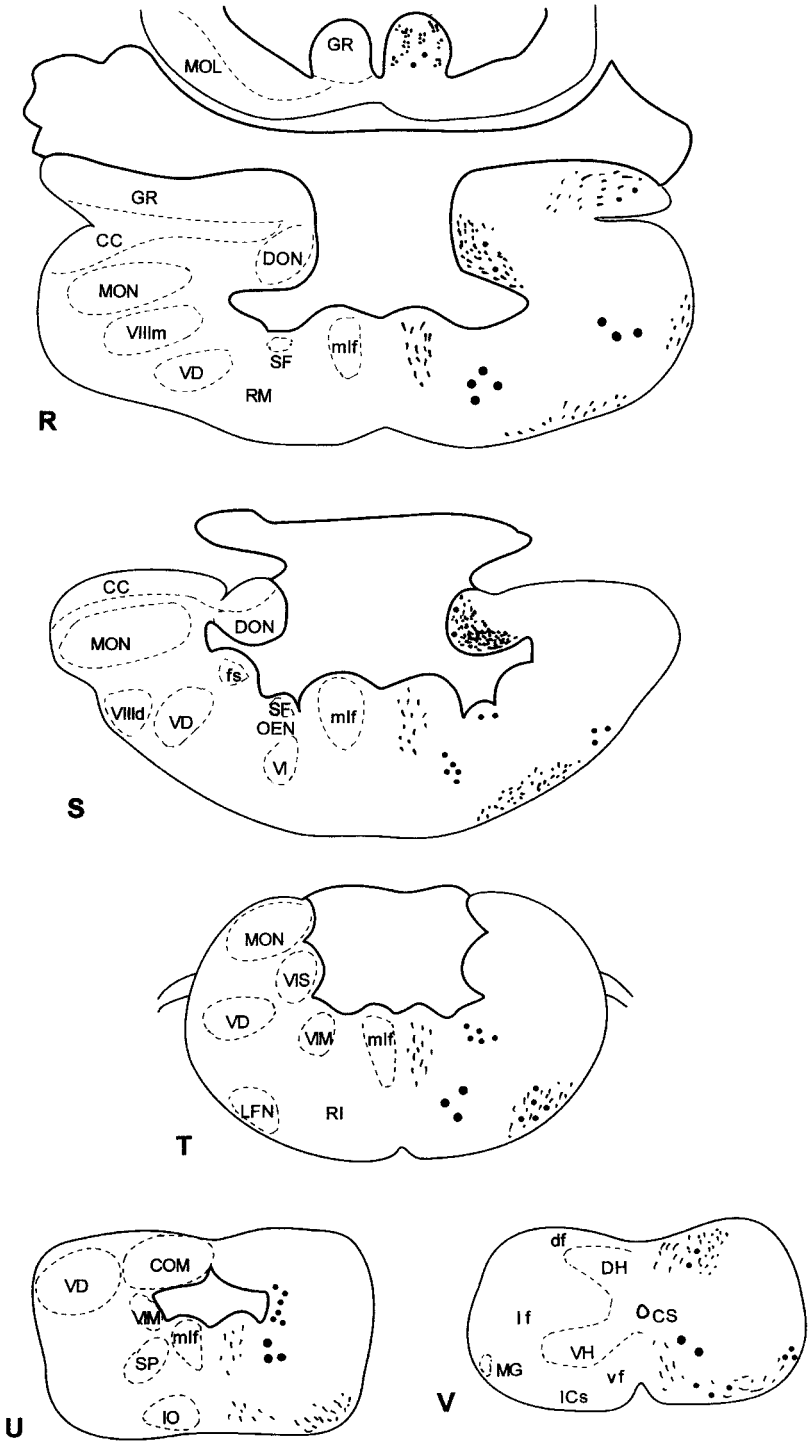


Figure 3 (Continued)

most of the length of the tectum, except its most rostral region. A few of these cells also were observed in the granular layer of the cerebellar auricles. The very thick, myelinated unite processes of these cells were moderately ChAT-ir and coursed caudally in the deep region of the internal medullary layer of the optic tectum.

The optic tectum received a number of intensely stained, ChAT-ir fibers (Fig. 3I–M). These fibers were thin and regularly beaded at short or very short intervals, and they were distributed throughout all layers along the entire length of the optic tectum. They were more abundant in the external cellular layer zona externa and were sparser in the internal cellular layer and the periventricular fibrous layer. Comparison of transverse and sagittal sections indicated that, in the medial and lateral optic bundles, ChAT-ir fibers tended to course longitudinally, and a portion of them arose from fibers ascending laterally in the medulla and running through the region of the anterior medullary velum. However, other fibers entered the tectum following other routes.

Tegmentum. In the mesencephalic tegmentum, there were several groups of ChAT-ir cells. The oculomotor nucleus contained moderately ChAT-ir motoneurons that



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were located mostly dorsal and dorsolateral to the medial longitudinal fascicle (Figs. 3K,L, 6A). It was possible to distinguish large and medium-sized ChAT-ir neurons in this oculomotor nucleus, the latter outnumbering the former. These cells sent dendrites ventral and ventrolateral, crossing the bundles of the medial longitudinal fascicle. Oculomotor cells gave rise to faintly to moderately ChAT-ir, thick axons that formed the roots of the oculomotor nerve. Coextensive with the caudal part of the Nflm and the rostral part of the oculomotor nucleus and dorsal

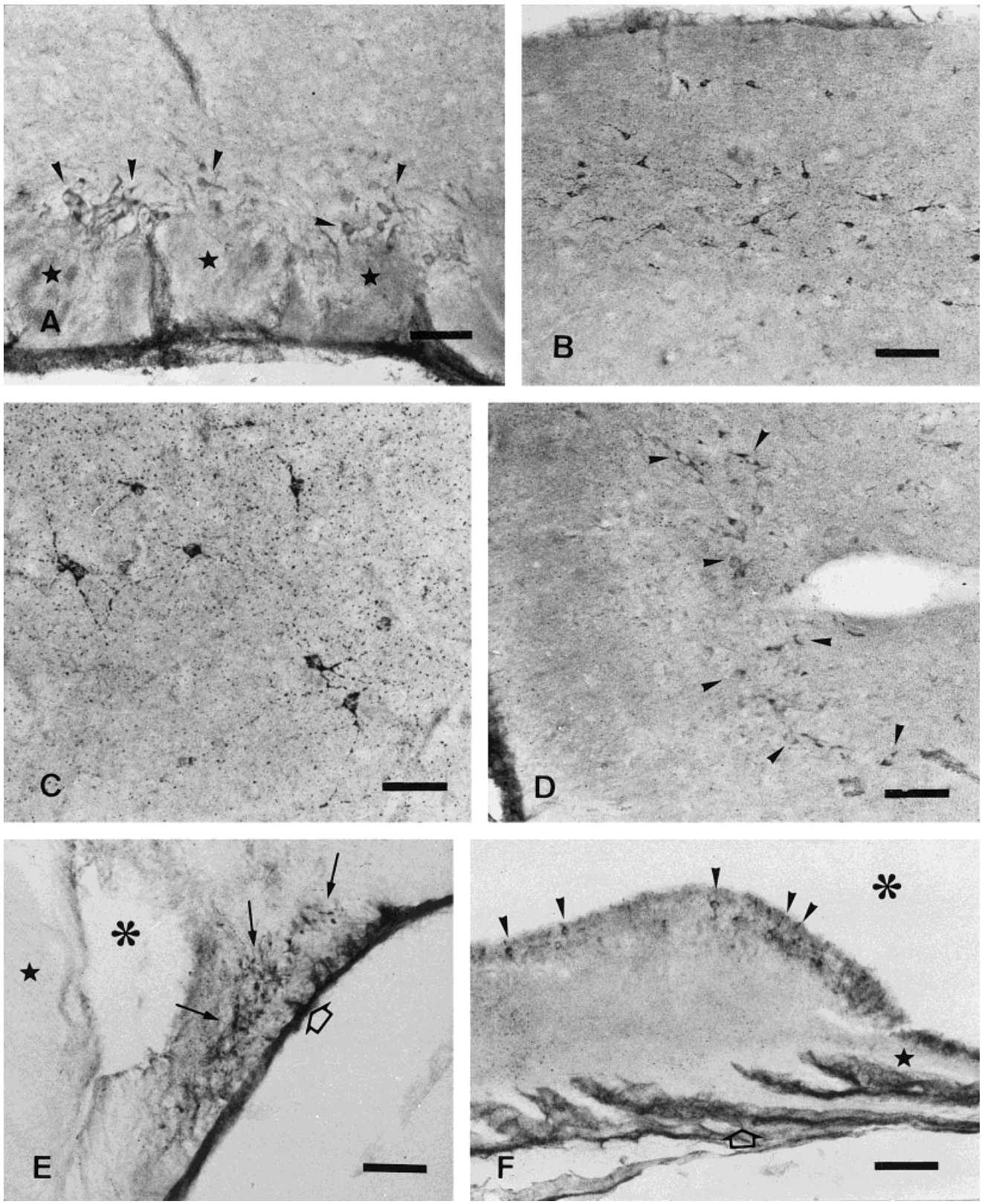

Fig. 4. Photomicrographs from transverse sections through the telencephalon (A–D) and longitudinal sections through the preoptic region and medial hypothalamus (E,F). **A:** Groups of ChAT-ir tufted cells (arrowheads) in the ventral region of the olfactory bulb. Stars indicate olfactory glomerules. **B:** Section through the superficial dorsal pallium showing large numbers of small, ChAT-ir neurons. **C:** Detail of ChAT-ir pallial neurons showing thin, beaded processes and the large number of boutons. **D:** ChAT-ir neurons (arrowheads) of the lateral pallium. **E:** ChAT-ir neurons (thin arrows) in the vascular organ of the lamina terminalis. Rostral is toward the right. An asterisk indicates the optic recess, the thick arrow indicates nonspecific staining of the meninges, and a star indicates the optic chiasma. **F:** Cerebrospinal fluid-contacting, ChAT-ir cells (arrowheads) in the floor of the infundibulum (asterisk). Rostral is toward the left. A star indicates the neurohypophysial stalk, and the arrow indicates nonspecific staining of the meningeal structures of the median eminence. Scale bars = 100 μm in A,B,D–F; 50 μm in C.

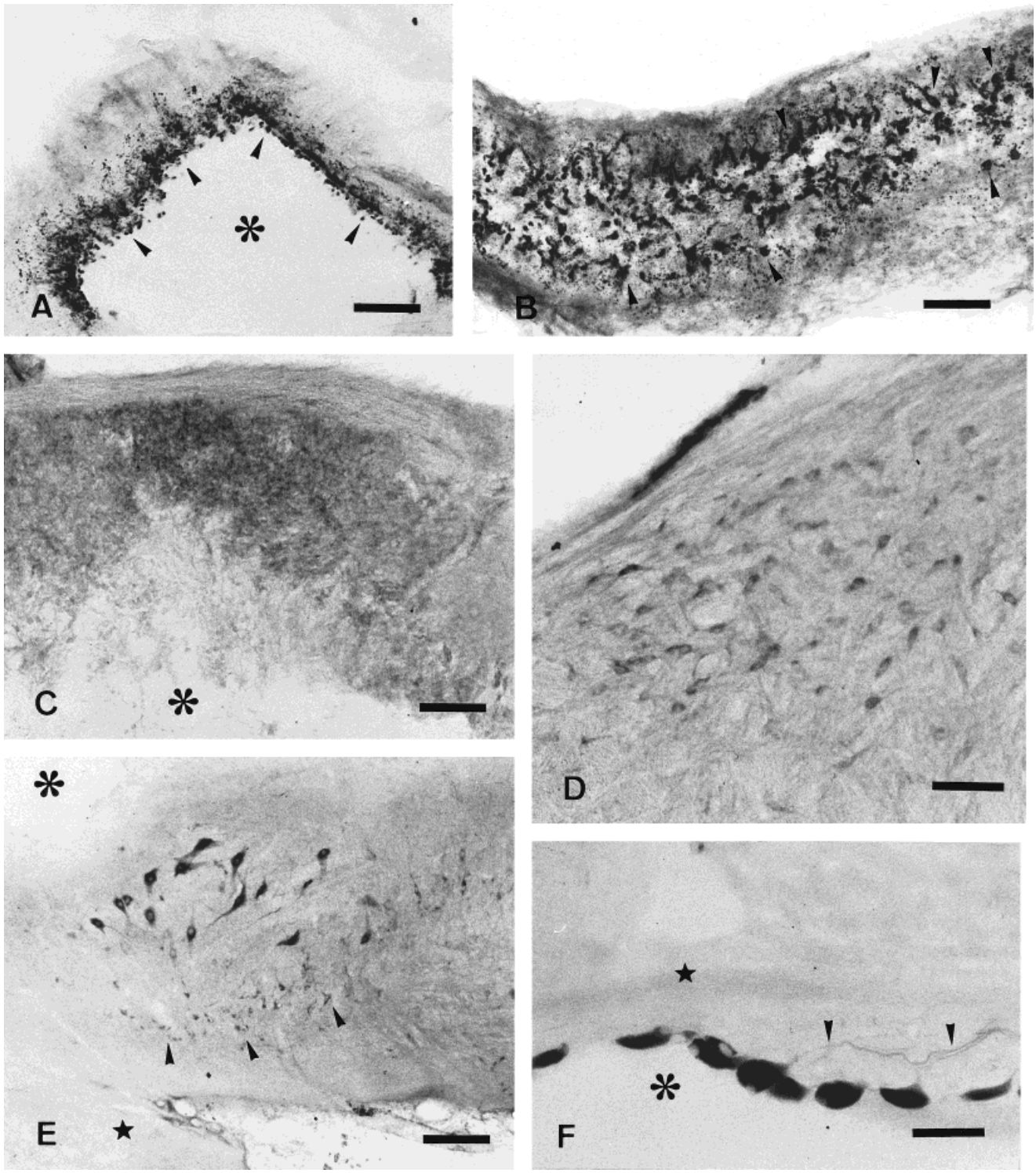

Fig. 5. Photomicrographs from transverse sections through the epithalamus (A–C), sagittal sections through the pretectal segment (D,E), and transverse section through the periventricular region of the optic tectum (F). **A:** Numerous ChAT-ir bipolar cells (arrowheads) at the junction between the pineal organ and the roof of the third ventricle (asterisk). **B:** ChAT-ir bipolar cells (arrowheads) in the pineal organ. **C:** Left habenula showing numerous, faint, ChAT-ir small cells and neuropil. An asterisk indicates the third ventricle. **D:** ChAT-ir neurons of the lateral geniculate nucleus. Rostral is toward the left. **E:** Sagittal section showing large cells of the nucleus of the medial longitudinal fascicle. Rostral is toward the left. Arrowheads indicate small cells of the ventral tegmental area, an asterisk indicates the third ventricle, and a star indicates the hypothalamic lobes. **F:** Large neurons of the trigeminal mesencephalic nucleus showing faint immunoreactive fibers (arrowheads). An asterisk indicates the mesencephalic ventricle, and a star indicates the intertectal commissure. Scale bars = 100 μm in A,C–F; 50 μm in B.

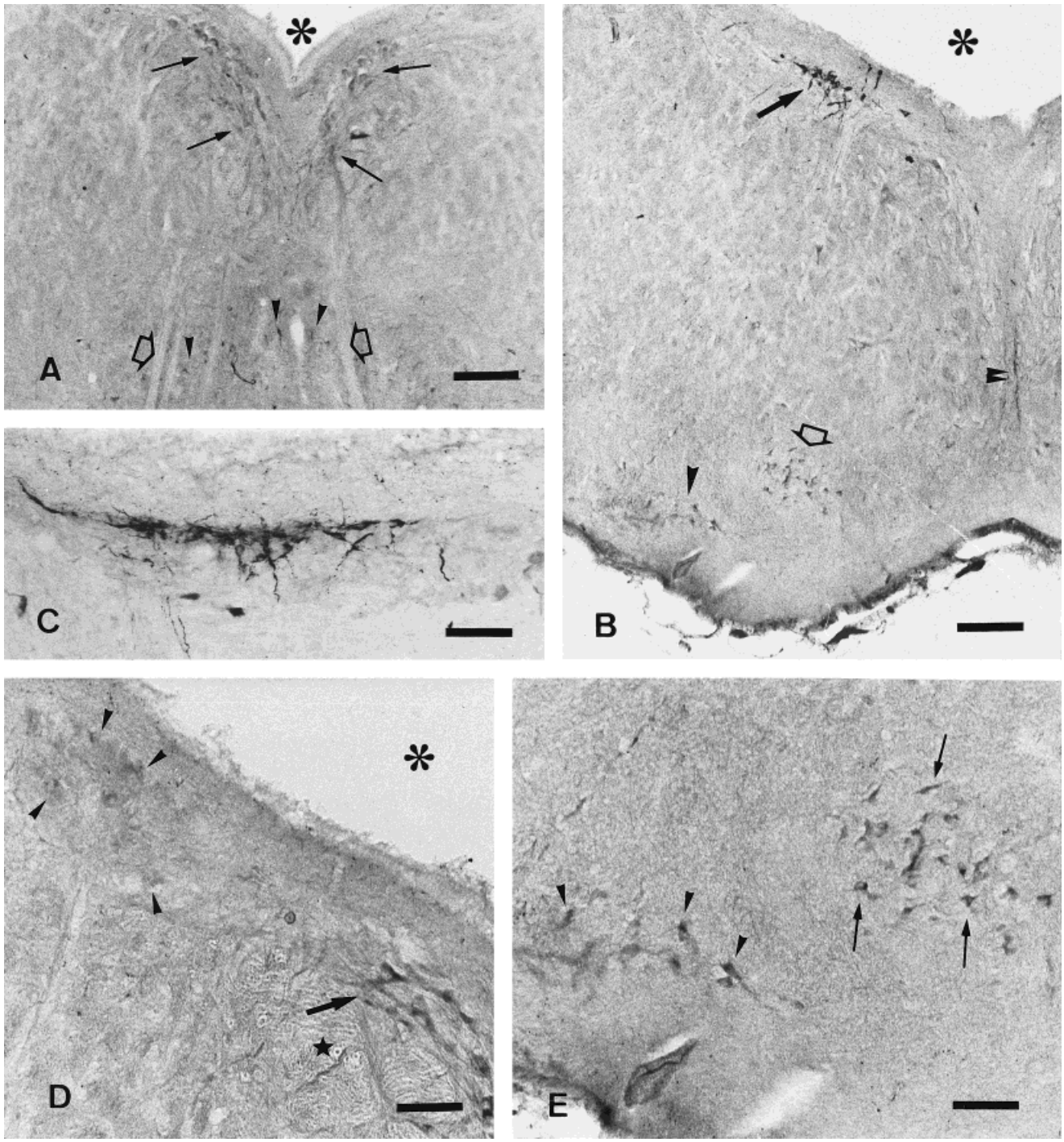

Fig. 6. Photomicrographs from transverse (A,B,D,E) and sagittal (C) sections through the mesencephalic tegmentum. **A:** Section showing ChAT-ir neurons in the oculomotor nucleus (thin arrows) and the ventral tegmental area (arrowheads). An asterisk indicates the mesencephalic ventricle, and open arrows indicate the third nerve roots. **B:** Group of intensely ChAT-ir neurons (thin arrow) of the rostral tegmentum. The thick arrow and the single arrowhead indicate two lateral groups of faintly ChAT-ir cells; the double arrowheads indicate ChAT-ir fibers ascending in the raphe. An asterisk indicates the mesencephalic ventricle. **C:** Detail of the intensely immunoreactive group of cells. Rostral is toward the left. **D:** Section through the Edinger-Westphal nucleus (arrowheads) showing the faint immunoreactivity of its cells. An arrow indicates the oculomotor nucleus, an asterisk indicates the mesencephalic ventricle, and a star indicates the medial longitudinal fascicle. **E:** Detail of the ChAT-ir cells of the putative substantia nigra (arrows) and the ChAT-ir cells near the tract of the transverse commissure (arrowheads). Scale bars = 250 μm in A,B; 100 μm in C–E.

and lateral to these areas, there was a group of intensely ChAT-ir neurons (Figs. 2, 3K, 6B,C). Here, this group is referred to as the pre-EW nucleus (see Discussion, below). These small, ChAT-ir neurons were bipolar or triangular and had dendritic processes that coursed mostly parallel to the mesencephalic ventricle. Thin, beaded fibers that appeared to originate from these cells coursed mostly in the ventral direction, although they could not be followed to the third nerve exit. Just caudal to this group, there was a conspicuous nucleus that was elongated rostrocaudally and oval in section that has been considered to be the dogfish EW nucleus (Smeets et al., 1983; Rodríguez-Moldes et al., 1993). The cells of this group were pear-shaped, were larger than those of the rostral ChAT-ir group, and showed only very faint ChAT immunoreactivity (Figs. 3L, 6D).

Ventral and lateroventral in the tegmentum, three groups of ChAT-ir neurons were observed (Figs. 2, 3K,L, 6A,D,E). The most characteristic group was a long and narrow column of cells located just lateral to the brachium conjunctivum and extending from the region of the posterior tubercle to the caudal end of the mesencephalon, i.e., the level of exit of the third nerve. These small, ChAT-ir cells were bipolar or triangular and formed a rather compact group. In view of its position, this group may correspond either to the red nucleus of Smeets et al. (1983) or, in part, to the substantia nigra of the dogfish (Meredith and Smeets, 1987; Molist et al., 1995). In the midline ventral region, there were numerous small, ChAT-ir neurons that extended below the oculomotor nucleus from the caudal part of the Nflm to the region between the two third nerves near the ventral midline. The location of these scattered neurons corresponded to that of the ventral tegmental area (Meredith and Smeets, 1987; Molist et al., 1995). The third ChAT-ir nucleus was a group of elongated cells located in the ventrolateral corner of the rostral tegmentum near the transverse commissure of Smeets et al. (1983). These cells were close to a ChAT-ir reticular neuropil associated with a pretectotegmental tract, which apparently originated from the superficial pretectal nucleus. In addition to these three ventral tegmental groups, there were some medium-sized to large, ChAT-ir neurons scattered in the intermediate reticular region ventrolateral to the oculomotor nucleus.

The mesencephalic tegmentum showed dense ChAT-ir innervation of the central gray and the raphe midline (Fig. 3K). In other tegmental regions, ChAT-ir innervation was sparse.

Isthmus

At the level of the isthmus, the medium-sized, ChAT-ir, trochlear neurons formed an oval-shaped group with processes directed toward the center of the nucleus rather than toward the medial longitudinal fascicle (Fig. 3M). The faintly ChAT-ir axons of these neurons extended dorsally, crossing in the anterior medullary velum to exit in the contralateral trochlear nerve.

A small group of moderately stained, ChAT-ir neurons was located rostroventral to the cerebellar nucleus within the cerebellar peduncle. The cells of this nucleus were rather medium-sized and pear-shaped or multipolar. This ChAT-ir group had a rounded appearance in both sagittal sections and transverse sections (Figs. 2, 3N, 7A,B) and probably corresponds to nucleus G of Smeets and Timerick (1981).

Caudally in the cerebellar peduncle, a small group of intensely stained, ChAT-ir neurons was observed periependymally close to the sulcus limitans, just at pretrigeminal levels (Figs. 2, 3O, 7C). The location of this group of medium-sized multipolar cells corresponded to that of the locus coeruleus.

The isthmus appeared to be a passage region for numerous ChAT-ir fibers. Most of them were observed coursing in periventricular and marginal regions, ascending either to the cerebellum or to the optic tectum. Although ChAT-ir fibers were abundant near the outer surface of the brain in the region of the nucleus isthmi of Smeets et al. (1983) and frequently showed a beaded appearance, ChAT-ir cells were not observed in this region. Some ChAT-ir fibers crossed the midline in the anterior medullary velum.

Just caudal to the third nerve exit, the habenulointerpeduncular projection became intensely ChAT-ir and formed two differentiated areas of neuropil: one in the interpeduncular nucleus (Fig. 3M–O) and other in thin but conspicuous caudal neuropil/fascicle that extended in the ventral midline of the medulla to trigeminal levels (Fig. 3O). The most rostral neuropil (interpeduncular nucleus) was the largest (Figs. 3M,N, 7D), and most of the ChAT-ir fibers in this neuropil coursed transversely, giving it a characteristic striated appearance.

Cerebellum and cerebellar nucleus

In the granule cell layer of the cerebellum, in both the corpus cerebelli and the auricles, numerous medium-sized, ChAT-ir cells were observed, the appearance and distribution of which suggested that they were Golgi cells (Figs. 2, 3N–R, 7E,F). In addition to perikarya, ChAT-ir terminals and fibers were abundant in the cerebellum. Small, ChAT-ir dots were observed in association with the cerebellar glomerules in the granule cell layer (Figs. 3N–R, 7E). Rather thick fibers also entered the cerebellum, mainly in the auricles, coursing to the granule cell layer. In the medial region of the lower (caudal) auricular leaf, where a number of these fibers enter, there were characteristic large, ChAT-ir boutons in the granule cell layer (Fig. 7F) that were not observed in other cerebellar regions. This region covers the cerebellar crest of the dorsal octavolateral nucleus and gives rise to their parallel fibers.

Numerous small to medium-sized, moderately ChAT-ir neurons were observed in the main portion of the large cerebellar nucleus of the dogfish, which was located in the cerebellar peduncle (Figs. 2, 3O, 7A, 8A). In addition, smaller but more intensely ChAT-ir, spindle-shaped cells were observed in the periventricular region of this nucleus (see Alvarez-Otero et al., 1996), mostly in its rostral region (Fig. 8B). The periventricular region also was traversed by intensely ChAT-ir fibers (Fig. 8C).

Medulla oblongata

Several ChAT-ir cell populations, including the motor nuclei, neurons of the octavolateral nuclei, efferent neurons of the octavolateral system, the nucleus of the lateral funiculus, and different reticular nuclei (Figs. 2, 3O–U), were observed in the medulla oblongata.

Motor nuclei. All motoneurons of the rhombencephalon were ChAT-ir, although the intensity of staining varied considerably among motor nuclei. The medium-sized motoneurons of the facial, glossopharyngeal, and vagal nerves, which formed a continuous column between the

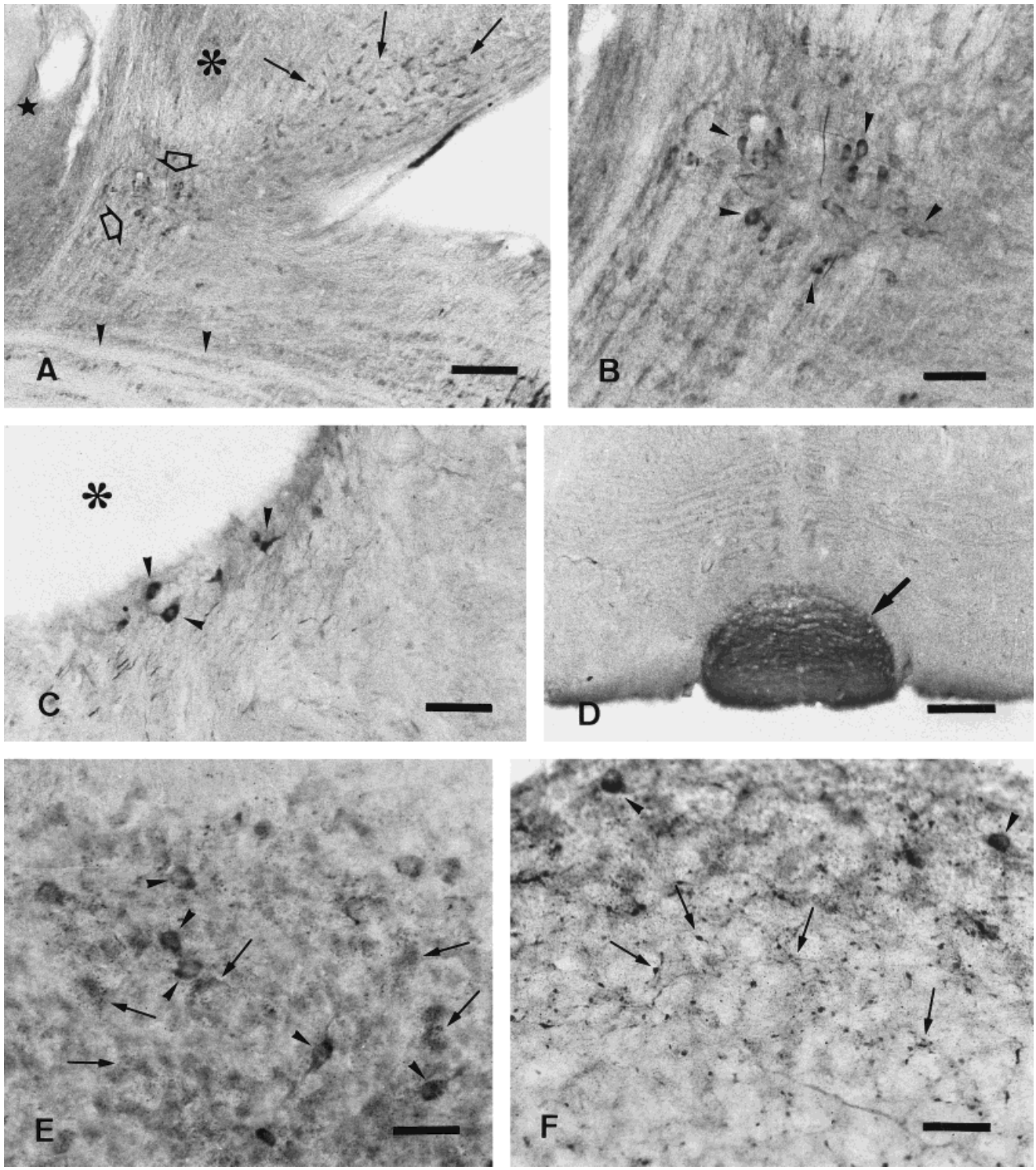

Fig. 7. Photomicrographs from sagittal sections (A,B,F) and transverse sections (C,D,E) through the isthmus (A–D) and the cerebellum (E,F). **A:** Section through the cerebellar peduncle (asterisk) showing ChAT immunoreactivity in the nucleus G (thick arrows) and the cerebellar nucleus (thin arrows). Rostral is toward the left. Arrowheads indicate longitudinal white tracts, and a star indicates the optic tectum. **B:** Detail of the ChAT-ir cells of the nucleus G (arrowheads). **C:** ChAT-ir cells of the locus coeruleus (arrowheads). Note also the abundance of ChAT-ir fibers. An asterisk indicates the fourth ventricle. **D:** Section showing intense ChAT immunoreactivity in the neuropil of the interpeduncular nucleus (arrow). **E:** Section through the granular layer of the corpus cerebelli showing Golgi-like ChAT-ir cells (arrowheads) and areas of small terminals (arrows) around the cerebellar glomerules. **F:** Section through the granular layer of the lower auricular leaf showing large, ChAT-ir boutons (arrows) that are characteristic of this cerebellar region. Arrowheads indicate Golgi-like cells. Scale bars = 250 μm in A,D; 100 μm in B,C; 50 μm in E,F.

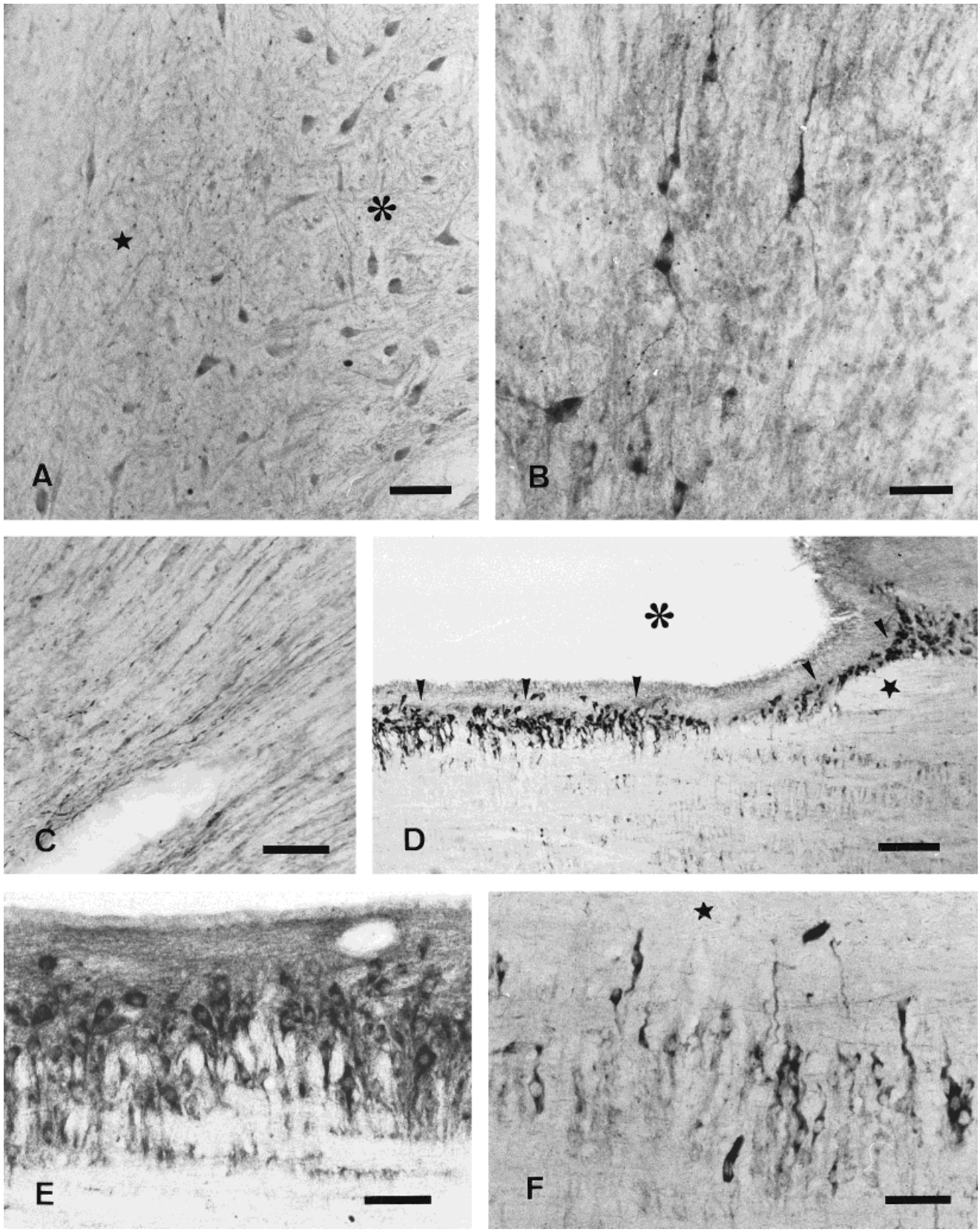

Fig. 8. Photomicrographs from transverse sections (A) and sagittal sections (B–F) through the cerebellar peduncle (A–C) and the medulla oblongata (D–F). **A:** Section through the cerebellar nucleus showing ChAT-ir cells of the main region (asterisk) and numerous fibers in its lateral portion (star). **B:** Section parallel to the ventricle showing the characteristic intensely ChAT-ir cells of the periventricular region of the cerebellar nucleus. **C:** ChAT-ir fibers coursing among fiber tracts of the cerebellar peduncles. **D:** Section passing through the glossopharyngeal-vagal motor column (arrowheads). Rostral is toward the left. An asterisk indicates the fourth ventricle rostral to the calamus scriptorius, and a star indicates Stieda's median fascicle at the level of the obex. **E:** Detail of neurons of the facial-vagal visceromotor column. **F:** Sagittal section showing the motoneurons of the abducens nucleus. A star indicates the region of dorsal arcuate fibers. Scale bars = 100 μm in A,C,E,F; 50 μm in B; 250 μm in D.

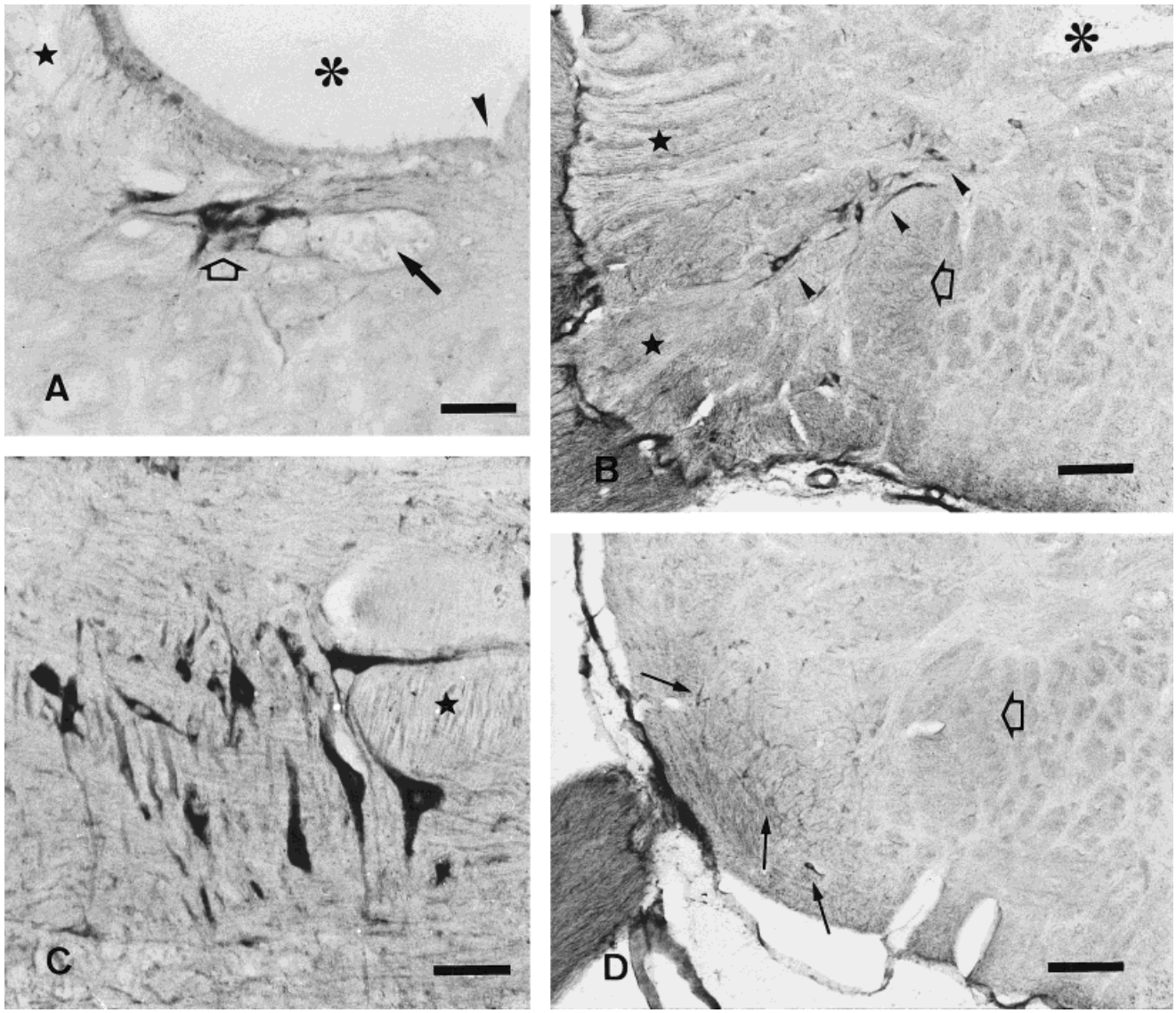

Fig. 9. Photomicrograph from transverse sections (A,B,D) and sagittal sections (C) through the medulla oblongata. **A:** ChAT-ir octavolateral efferent neurons (thick arrow) are located close to the Stieda's median fascicle (thin arrow). An arrowhead indicates sulcus limitans, an asterisk indicates the fourth ventricle, and a star indicates the median longitudinal fascicle. **B:** Section showing ChAT-ir cells (arrowheads) of the magnocellular octaval nucleus at the entrance of the octaval nerve. An asterisk indicates the fourth ventricle, an arrow indicates the trigeminal descending root, and stars indicate the octaval nerve roots. **C:** Detail of magnocellular octaval cells. Rostral is toward the left. A star indicates the facial nerve root. **D:** Small, ChAT-ir cells (thin arrows) of the descending octaval nucleus. A thick arrow indicates the trigeminal descending root. Scale bars = 100 μm in A,C; 250 μm in B,D.

level of the glossopharyngeal nerve entrance to the rostral spinal cord, generally exhibited intense ChAT immunoreactivity (Figs. 2, 3T,U, 8D,E). Unlike the motoneurons of these branchiomeric nerves, the cells of the trigeminal motor nucleus generally showed faint ChAT immunoreactivity. These trigeminal cells occupied an intermediate ventrolateral location in the wall of the rhombencephalon (Fig. 3P). The abducens motoneurons exhibited faint-to-moderate ChAT immunoreactivity (Figs. 3S, 8F). These medium-sized, ChAT-ir multipolar neurons exhibited long dendrites extending in the reticular area and occupied an intermediate medial location in the rhombencephalon. In the caudal medulla, medium-sized to large, ChAT-ir neurons of the spinooccipital nerves (i.e., the hypoglossal nerve homologues) formed a continuous column with the ChAT-ir motoneurons of the ventral horn of the spinal cord, from which they were indistinguishable in appearance (Fig. 2). A few ChAT-ir roots originating from this column exited from the ventral medulla at vagal levels and from the rostralmost spinal cord.

The efferent cells of the octavolateral system were moderately to intensely ChAT-ir (Fig. 9A). These medium-sized to large, spindle-shaped or multipolar cells characteristically were located near the fourth ventricle associated with Stieda's median fascicle, as was found in a previous experimental study of this species (Meredith and Roberts, 1986).

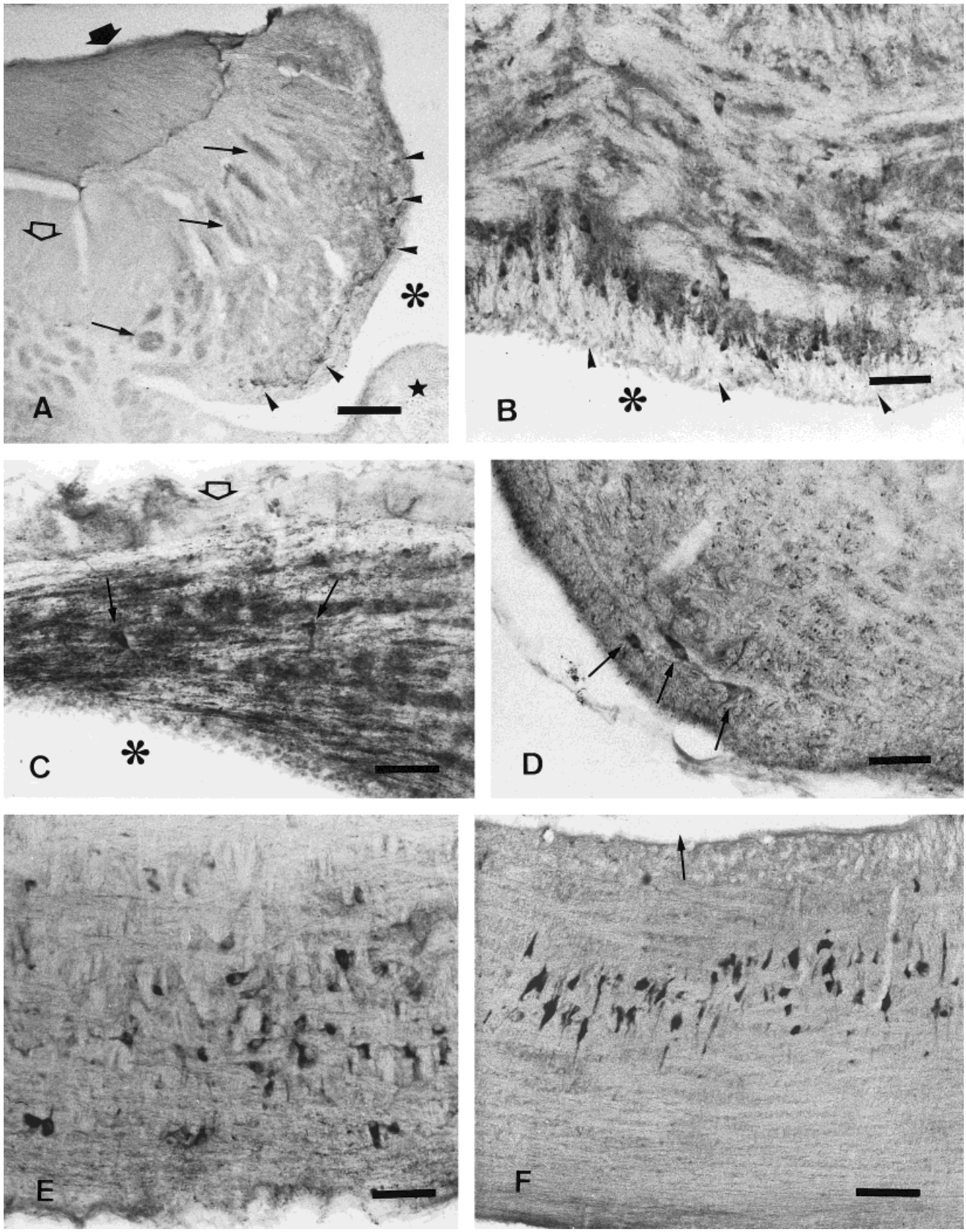

Figure 10

In the octavolateral region of the dogfish rhombencephalon, ChAT-ir cells were observed mainly in two regions: the octaval area (ventral octavolateral nucleus) and the electrosensory lobe (dorsal octavolateral nucleus). In contrast, the medial octavolateral nucleus (mechanoreceptive lateral-line area) did not show ChAT-ir neurons.

In the octaval area, ChAT-ir neurons were observed in the anterior, magnocellular, and descending octaval nuclei (Figs. 3P–S, 9B–D). The anterior octaval nucleus possessed a fairly abundant population of medium-sized, ChAT-ir cells extending caudally from the region caudal to the cerebellar nucleus to the magnocellular nucleus. The large to very large neurons of the magnocellular octaval nucleus, which is located at the level of entrance of the octaval nerve (Fig. 9B), showed fairly intense ChAT immunoreactivity (Fig. 9C). These triangular cells gave rise to stout dendritic processes. Caudal to the magnocellular octaval nucleus, the descending octaval nucleus extended into a field triangular in transverse section that was characterized by the presence of numerous thick, moderately ChAT-ir fibers (probably primary vestibular fibers). The ChAT-ir neurons of the descending octaval nucleus were multipolar and medium-sized (Fig. 9D).

The dorsal octavolateral nucleus contained a number of ChAT-ir neurons and was one of the regions with the highest ChAT-ir fiber density (Fig. 3P–S). This nucleus consisted basically of an external molecular layer (the cerebellar crest) and an inner region of neuropil enmeshing numerous longitudinal bundles of fibers and containing at least two types of neuron: small cells located periventricularly and larger cells located among thick bundles of fiber. Numerous small to medium-sized neurons exhibiting intense ChAT immunoreactivity were located in the nucleus in a periventricular position (Figs. 3Q–S, 10A,B). Although these cells were found throughout the nucleus, they were numerous only rostral to the entrance of the dorsal root of the anterior lateral-line nerve. This dorsal root carries the electroreceptive lateral-line fibers. The neuropil showed numerous ChAT-ir boutons that became very numerous in the caudal region of the nucleus (Fig. 10C). Numerous beaded fibers also were observed coursing in the longitudinal fiber bundles, both beneath the cerebellar crest (Fig. 10A) and in more internal positions.

The ventrolateral region of the medulla oblongata, between the levels of entrance of the glossopharyngeal and vagal nerves, contained a large group of ChAT-ir cells (Figs. 2, 3T, 10D,E) that corresponded to the nucleus of the lateral funiculus of Smeets et al. (1983). These medium-sized cells were distributed among tracts of fibers near the meninges and lateral to the inferior olivary nucleus. This nucleus gave rise to numerous thick, ChAT-ir fibers that ran in the lateral funiculus, ascending to the isthmus and mesencephalon or descending to the spinal cord. In transverse sections, these ChAT-ir fibers ran scattered near the meninges.

In addition to these well-defined nuclei, ChAT-ir reticular neurons were observed ventrolateral to the medial longitudinal fascicle, forming a long column of cells scattered among longitudinal bundles of fibers. At pretrigeminal-trigeminal levels, the medium-sized to large, ChAT-ir reticular cells formed a rather compact group medial to the trigeminal motor nucleus (Fig. 2, 3P) in a position corresponding to the superior reticular rhombencephalic nucleus of Smeets et al. (1983). The greatest numbers of large, ChAT-ir reticular cells, however, were observed at the level of the octaval nuclei, where the cells were scattered over a much wider area (Figs. 2, 3Q,R, 10F). These neurons corresponded to the intermediate reticular rhombencephalic nucleus of Smeets et al. (1983). In glossopharyngeal-vagal regions, the reticular cells were medium-sized and tended to occupy progressively more ventral positions in the caudal medulla. The distribution of these ChAT-ir reticular cells corresponded closely to the inferior reticular rhombencephalic nucleus (Smeets et al., 1983). Thick, ChAT-ir fibers that probably originated from ChAT-ir reticular perikarya were numerous in the medial longitudinal fascicle.

Rostral spinal cord

In the rostral spinal cord, ChAT immunoreactivity was observed mainly in the cells of the motor column and the marginal nucleus (Figs. 2, 3V, 11A,B). The ChAT-ir motoneurons were located in the ventral horn and, in transverse sections, appeared as large multipolar cells (Fig. 2). Ventrolaterally in the spinal cord, there was a column of medium-sized neurons located near the meninges (Figs. 3V, 11B). These neurons exhibited intense ChAT immunoreactivity both in the perikaryon and in their thick dendrites (which coursed to a lateral glomerular neuropil). These cells formed the marginal nucleus of the spinal cord (Anadón et al., 1995a).

In addition to these well-defined groups, the dorsal horn of the spinal cord and the ventral funiculus contained scattered ChAT-ir cells (Fig. 3V). In the dorsal horn, small bipolar or triangular ChAT-ir cells were observed in the neuropil among small longitudinal bundles of fibers of the substantia gelatinosa. Numerous beaded, ChAT-ir fibers also were observed coursing longitudinally in this region. The neuropil of the dorsal horn contained numerous small, ChAT-ir boutons, sometimes at a density similar to that observed in the electrosensory lobe of the octavolat-

Fig. 10. Photomicrograph from transverse sections (A,D) and sagittal sections (B,C,E,F) through the medulla oblongata. **A:** Section through the dorsal octavolateral nucleus (lateral-line lobe) showing numerous ChAT-ir cells in periventricular regions (arrowheads) as well as fiber-rich areas (thin arrows). An asterisk indicates the fourth ventricle; a solid, thick arrow indicates the dorsal root of the anterior lateral-line nerve; an open, thick arrow indicates the cerebellar crest (molecular layer) of the medial octavolateral nucleus; and a star indicates the medial longitudinal fascicle. **B:** Sagittal section passing through the periventricular region of the dorsal octavolateral nucleus near the lateral-line nerve entrance showing numerous ChAT-ir neurons and areas of positive neuropil. **C:** Similar section through the caudal region of the dorsal octavolateral nucleus showing the intensely ChAT-ir regions of neuropil. Note also the scarcity of ChAT-ir neurons (thin arrows). An asterisk indicates the fourth ventricle, and a thick arrow indicates the cerebellar crest of the dorsal octavolateral nucleus. **D:** Ventrolateral region of the medulla oblongata at the level of the glossopharyngeal nerve showing ChAT-ir neurons of the nucleus of the lateral funiculus (arrows). Note also the abundance of ChAT-ir fibers in this region. **E:** Sagittal section at the ventrolateral border of the medulla passing through the nucleus of the lateral funiculus. **F:** Sagittal section showing large, ChAT-ir reticular cells of the intermediate reticular rhombencephalic nucleus. An arrow indicates the fourth ventricle. Scale bars = 250 μm in A,F; 100 μm in B–E.

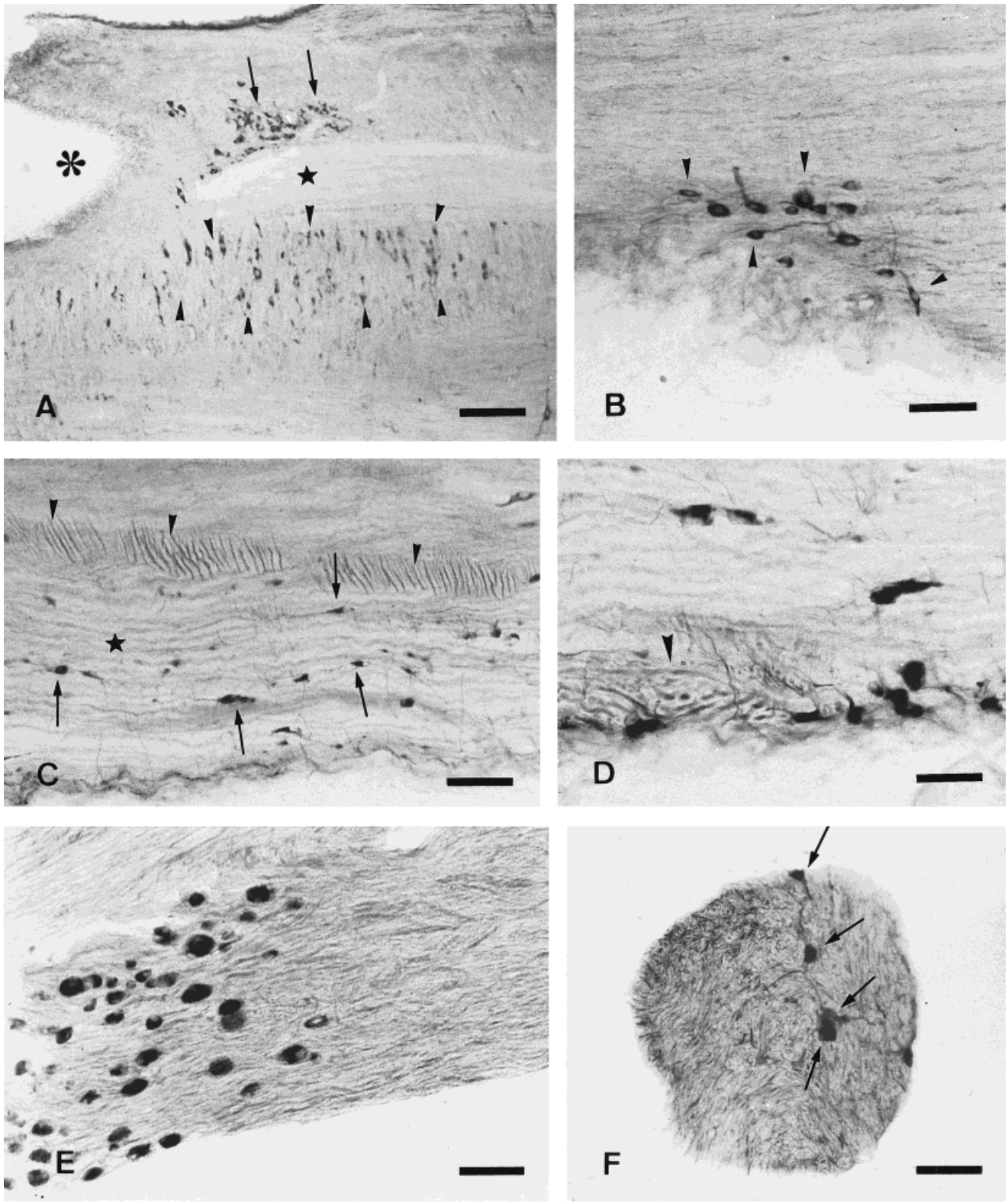

Fig. 11. Photomicrographs of sagittal sections through the rostral spinal cord (A–D) and through the octaval (E) and oculomotor (F) nerves. **A:** ChAT-ir motoneurons of the spinal motor column (arrowheads) and of the caudal part of the vagal visceromotor column (arrows). An asterisk indicates the fourth ventricle, and a star indicates Stieda's median fascicle. **B:** Submeningeal ChAT-ir neurons of the marginal nucleus of the spinal cord (arrowheads). **C:** Section through the ventral funiculus showing interstitial ChAT-ir cells (arrows). Arrowheads indicate the spinal motor roots. **D:** Detail of the interstitial ChAT-ir cells of the ventral funiculus. An arrowhead indicates the motor root. **E:** ChAT-ir ganglion cells of an octaval nerve ganglion. **F:** Transverse section through the oculomotor nerve showing ChAT-ir neurons of ganglion cell appearance (arrows). Scale bars = 250 μm in A; 100 μm in B,C,E,F; 50 μm in D.

eral region. The cholinergic population of the ventral funiculus consisted of scattered, small, fusiform, interstitial elements, often with eccentrically located nuclei, that gave rise to rather thick processes running in the white matter (Figs. 11C,D).

Peripheral ganglia

ChAT immunoreactivity was observed in the neurons of the ganglia of the trigeminal, octaval, and anterior lateral-line nerves that were included in the sections of the brain. These large to very large ganglion cells exhibited strong ChAT immunoreactivity (Fig. 11E). The thick sensory fibers of these nerves also were faintly ChAT-ir. The intracranial course of the oculomotor nerve of the dogfish contained medium-sized, ChAT-ir ganglion cells either singly or in small groups (Fig. 11F). The presence of ChAT-ir beaded fibers, such as those arising from the rostral mesencephalic group of cells, or of boutons contacting these ganglion cells was not observed in the third nerve.

DISCUSSION

The Western blot analysis of brain extracts from the dogfish and from two ray-finned fishes (sturgeon and trout) indicated the presence of a major protein band of ≈68–72 kDa together with minor bands of lower molecular weight. In general, the pattern of bands observed in extracts from these species was similar to that originated in similarly treated rat brain extracts. In the four species studied here, all the bands disappeared after preincubation of the antibody with hpChAT (which is believed to be identical to brain ChAT; Bruce et al., 1985), supporting the finding that the molecules revealed in the blots are closely related to it. Considerable controversy was raised by the significance of the different bands revealed in preparations of ChAT from brains of different mammalian species and whether there is one or more forms of the enzyme. Most authors consider than these bands originate by proteolysis of native ChAT (e.g., see Hersh et al., 1984; Bruce et al., 1985; Poethke et al., 1997). In *Drosophila*, ChAT bands of 75 kDa, 67 kDa, and 54 kDa have been detected in head extracts, the 75-kDa and 67-kDa forms of which are active enzymatically and the 54-kDa form of which was originated by proteolysis (Muñoz-Maines et al., 1988). The finding of a similar band of ≈55 kDa in the immunoblots of rat and fishes is notable, suggesting both that similar proteolysis occurred in our brain extracts and that relevant sites of proteolytic processing of ChAT are well conserved between species. The deduced sequence for ChAT in *Drosophila* contains unusually large numbers of dibasic amino acids, which may be sites for proteolytic processing (Muñoz-Maines et al., 1988).

The current study has revealed ChAT immunoreactivity in the motor nuclei of the brainstem and spinal cord of the dogfish as well as in other neuronal systems that are distributed widely in the brain and in peripheral ganglia. The similarity of our results of distribution in most brain regions with those obtained in other vertebrate groups and the results obtained by Western blot analysis support the finding that the molecule recognized by the primary antibody in the neural tissue was the enzyme ChAT. Comparison of intense staining of neurons obtained with the hpChAT antibody in the brains of rat and teleosts (unpublished observations) with the faint staining obtained in dogfish in the same conditions suggests a poor affinity of this antibody for ChAT in this species.

In general, most nuclei showed faint-to-moderate ChAT immunoreactivity, although some types of cells were stained intensely. Axonal processes were labeled faintly or were not stained in many regions, and, in general, it was not possible to provide a detailed description of ChAT-ir innervation except for some regions in which fibers were stained more intensely. The distribution of some nonmotor cholinergic groups in the dogfish differed markedly from the distributions reported in other vertebrates, although the general pattern of cholinergic systems basically was similar. The peculiar immunoreactivity observed in our study (such as in the pallial cells) must have been due to true species differences, which is not surprising, because the same has been reported for other neurotransmitter systems in elasmobranchs (for example, dopamine; Meredith and Smeets, 1987).

Distribution of ChAT-ir structures in the dogfish brain

Telencephalon. The olfactory bulbs contains two populations of ChAT-ir cells. The most rostral ChAT-ir neurons are groups of bipolar cells of the olfactory bulbs. In view of their bipolar shape, tufted dendrites, and close association with olfactory glomerules, this cell type probably corresponds to small mitral (tufted) cells similar to cells described in *Scyllium catulus* (Catois, 1901) and *Acanthias vulgaris* (Sterzi, 1909) and similar to those found in other vertebrates (Ramón y Cajal, 1911). The large mitral cells did not appear to be ChAT-ir, although they have been reported to be AChE -positive in sharks (Kusunoki et al., 1973). The presence of ChAT-ir fibers in the lateral olfactory tract suggests that these fibers may originate from the putative small mitral cells. However, experimental studies of their projections are lacking in elasmobranchs. The second ChAT-ir population corresponds to cells that are scattered in the granular layer and the retrobulbar region similar to those of the superficial dorsal pallium (see below).

ChAT-ir cells have not been described in the olfactory bulbs of most vertebrate species, although cholinergic cells associated with the olfactory pathways have been observed in the olfactory tubercle of reptiles (Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993), birds (Medina and Reiner, 1994), and mammals (Armstrong et al., 1983; Phelps and Vaughn, 1986; Gould et al., 1991; Ichikawa et al., 1997). Cholinergic projections to the olfactory bulbs have been proposed to arise from the basal forebrain (Medina and Reiner, 1994), from the diagonal band of Broca in anurans and urodeles (Marín et al., 1997), and from the ventral zone of area ventralis telencephali in teleosts (Brantley and Bass, 1988). In *Scyliorhinus*, the olfactory projections run in the medial and lateral olfactory tracts, which were traced to the contralateral olfactory bulb, several regions of the telencephalic hemisphere (basal superficial area, pallium), and a caudolateral region referred to as the olfactory tubercle or the lateral pallium (Smeets et al., 1983). The latter region contains a ChAT-ir population of small to medium-sized cells, but whether the cholinergic cells observed in the lateral pallium of the dogfish project to the olfactory bulb is not known. One possibility is that this population corresponds to the ChAT-ir group described in the ventrolat-

eral telencephalon of teleosts (Ekström, 1987), which also is related to lateral olfactory tracts.

The rostral, medial, and lateral regions of the superficial dorsal pallium contain large populations of small ChAT-ir cells that correspond to at least a portion of the small cells of bipolar, tripolar, and multipolar appearance described with Golgi methods in the pallium of this species (Manso and Anadón, 1993). This characteristic population also extends into the retrobulbar region and even reaches inner regions of the olfactory bulbs. In view of their small size and widespread distribution, these cells may be part of intrinsic pallial circuits, although other possibilities cannot be ruled out. The caudal regions of the dorsal pallium (central dorsal pallium), which appear to give rise to most pallial efferents, are practically devoid of these small, ChAT-ir cells. The abundance of ChAT-ir neurons is not the only striking feature noted in the dorsal pallium of elasmobranchs. In the pallium of skates and sharks, there are numerous small, catecholaminergic cells (Meredith and Smeets, 1987; Northcutt et al., 1988; Molist, 1990; Stuesse et al., 1990), and we have observed the same in the dogfish by using immunohistochemistry to tyrosine hydroxylase (TH; unpublished observations). The abundance of TH-ir neurons in the caudal pallium and periventricular locations of the dogfish (unpublished results), however, is quite different from that of ChAT-ir cells. Taken together, these observations indicate that the elasmobranch pallia have evolved certain chemical characteristics that are strikingly different from those of all other vertebrate groups. The absence of ChAT-ir tracts coursing away from the pallium and the presence of numerous boutons and short, beaded processes in the pallium suggest that the small, cholinergic cells participate in intrinsic pallial circuits.

The abundance of cholinergic neurons in the dorsal pallial regions of the dogfish is in contrast with the absence of cholinergic cells in the dorsal telencephalic area of teleosts (Ekström, 1987; Brantley and Bass, 1988) and in the pallium of amphibians (Marín et al., 1997) and most reptiles (Mufson et al., 1984; Brauth et al., 1985; Hoogland and Vermeulen-VanderZee, 1990; Powers and Reiner, 1993). Comparison of the results obtained in elasmobranchs with those in teleosts (Ekström, 1987; Brantley and Bass, 1988) and amphibians (Marín et al., 1997) suggests that an absence of cholinergic neurons in the pallium may be a "primitive" feature. Pallial cholinergic neurons have been reported only in a lizard (Medina et al., 1993) and are absent almost entirely from cortical regions in birds (Medina and Reiner, 1994). Moreover, examination of the available data reveals that the presence of cholinergic neurons in the cortex is not a shared feature in mammals. For example, small, ChAT-ir neurons are present in the cortex of rat and mouse (Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1984; Parnavelas et al., 1986; Blaker et al., 1988; Mufson and Cunningham, 1988; Reiner, 1991) but not in guinea pig (Maley et al., 1988). These results suggest that, in cortical/pallial areas, cholinergic neurons appeared several times during the evolution of vertebrates, which is in agreement with its putative late origin (Reiner, 1991; Medina et al., 1993; Powers and Reiner, 1993). This also raises the possibility that the ChAT-ir neurons found in the dogfish pallium do not correspond with any of those found in pallial/cortical areas of the rest of vertebrates, i.e., that they have different origins.

In most vertebrates, the presence of ChAT-ir cells is a recurrent feature of striatal and septal regions (mammals: Kimura et al., 1980, 1981, 1984; Armstrong et al., 1983; Hedreen et al., 1983; Houser et al., 1983; Satoh et al., 1983; Mesulam et al., 1984; Mufson et al., 1984; Brauth et al., 1985; Satoh and Fibiger, 1985; Phelps and Vaughn, 1986; Vincent and Reiner, 1987; Maley et al., 1988; Mufson and Cunningham, 1988; St-Jacques et al., 1996; Ichikawa et al., 1997; sauropsids: Brauth et al., 1985; Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993; Medina and Reiner, 1994; Helselmans and Wouterlood, 1994; Li and Sakaguchi, 1997; amphibians: Marín et al., 1997). A striking result of this study in the dogfish was the practical absence of ChAT-ir cells in the subpallial regions, although AChE histochemistry has revealed the presence of positive (cholinoceptive?) perikarya in the basal olfactory area and septum of sharks (Kusunoki et al., 1973). Also noteworthy was the scarcity of ChAT-ir fibers in these regions in contrast with their abundance in the basal telencephalon of birds and mammals.

Preoptic region and hypothalamus. Weakly ChAT-ir neurons were observed in the magnocellular preoptic nucleus of the dogfish. Cells of this nucleus are AChE-positive in other sharks (Kusunoki et al., 1973). ChAT immunoreactivity of cells of the preoptic nucleus has been observed in lampreys (Pombal et al., 1999a), teleosts (Ekström, 1987), and amphibians (Marín et al., 1997), although not in all of the neurons of this nucleus. There also have been reports of ChAT immunoreactivity in some cells related to the supraoptic nucleus of mammals (Mason et al., 1983; Ichikawa et al., 1997), and ChAT immunoreactivity is very conspicuous in the mammalian median eminence (Ichikawa et al., 1997). Studies with immunocytochemical methods indicate that cells of the elasmobranch preoptic nucleus project both to the hypophysis and to several extrahypophysial targets (Pérez et al., 1995; Meurling et al., 1996). It seems possible that the ChAT-ir preoptic neurons contribute to the cholinergic innervation observed in the dogfish median eminence and neurohypophysis. Calcitonin gene-related peptide (CGRP), a neuropeptide that is expressed in cholinergic motoneurons (Takami et al., 1985; Batten et al., 1989; Hietanen et al., 1990), also has been observed in neurons of the dogfish preoptic nucleus (Molist et al., 1995). Thus, this neuropeptide also may be colocalized with ChAT in preoptic neurons.

The organon vasculosum laminae terminalis of the dogfish contains an abundant ChAT-ir neuronal population. The close vascular relations of this nucleus have been noted with electron microscopy in mammals and in some teleosts (see Gómez-Segade et al., 1991), but similar studies in elasmobranchs are lacking. In teleosts, neurons located in the region of this organ give rise to hypophyseal projections (Holmqvist and Ekström, 1995), and it is probable that the same occurs in dogfish, contributing to the ChAT immunoreactivity observed in the neurohypophysis.

The hypothalamus in dogfish contains small numbers of ChAT-ir neurons, a feature shared by most vertebrates. Those observed in the dogfish were located in the region of the infundibulum in the lateral tuberal nucleus and the caudoventral part of the posterior recess, which are putative hypophysiotrophic regions (Meurling and Rodríguez, 1990; Molist et al., 1993b). This is consistent with the fact that most ChAT-ir hypothalamic neurons described to

date in amphibians (Marín et al., 1997), reptiles (Medina et al., 1993; Powers and Reiner, 1993), birds (Medina and Reiner, 1994), and mammals (Tago et al., 1987; Ichikawa et al., 1997) are located in the ventral hypothalamus/arcuatus region.

Epithalamus. A notable result of this study was the finding of a large population of bipolar ChAT-ir cells in the pineal organ of the dogfish. These very small cells have the characteristic apical inner segment of pineal photoreceptors, and they practically cover the inner surface of the pineal vesicle up to levels close to the posterior commissure. The presence of ChAT-ir photoreceptors in the pineal and parapineal organs of lampreys has been reported recently (Pombal et al., 1999b; Yáñez et al., 1999). The current results expand the available information on fishes that, together, suggests that the presence of cholinergic pineal photoreceptors may be a primitive feature of vertebrates. In this regard, pinealocytes (a photoreceptor-derived cell line) also express ChAT and produce acetylcholine in the rat (Wessler et al., 1997). In the pineal organ of a teleost, Ekström (1987) observed ChAT immunoreactivity in some neurons but not in photoreceptors.

The presence of cholinergic neurons in the medial habenular nucleus has been reported in mammals (Houser et al., 1983; Mesulam et al., 1984; Contestabile et al., 1987; Vincent and Reiner, 1987; Mufson and Cunningham, 1988; Ichikawa et al., 1997). In other vertebrates, some habenular cells and the interpeduncular neuropil were immunoreactive to ChAT (teleosts: Villani et al., 1994; amphibians: Marín et al., 1997; reptiles: Medina et al., 1993; birds: Sorenson et al., 1989; Medina and Reiner, 1994). The current results indicate that a subset of habenular neurons are cholinergic in the dogfish and that the interpeduncular neuropil shows strong ChAT immunoreactivity, in line with results in other vertebrates. Cells of the habenula also have been found to be AChE-positive in other sharks (Kusunoki et al., 1973). In view of its connections, the entire habenula of lampreys and teleosts has been suggested to be homologous to the mammalian medial habenular nucleus (Yáñez and Anadón, 1994, 1996). The distribution of ChAT in the dogfish habenula suggests that this also is the case in elasmobranchs. Our results in the dogfish indicated the presence of a ChAT-ir ventromedial tract/neuropil, which very probably originated from the habenula, extending caudally from the interpeduncular nucleus in the rhombencephalon to levels of the trigeminal motor nucleus. The presence of a ventromedial rhombencephalic projection of the fasciculus retroflexus has been demonstrated experimentally in lampreys (Yáñez and Anadón, 1994) and in a lizard (Díaz and Puelles, 1992). These results suggest that the habenular projections of elasmobranchs are very similar to those of other nonmammalian vertebrates. However, it is worth noting that substance P (SP), although it is present in the medial habenula of mammals (Hamill and Jacobowitz, 1984; Contestabile et al., 1987), has not been detected in the habenulointerpeduncular system of the dogfish (Rodríguez-Moldes et al., 1993).

Thalamus-pretectum. There have been scarce reports of the presence of ChAT-ir neurons in the thalamic-pretectal region of vertebrates. Cholinergic cells have been described in the superficial pretectum of teleosts (Ekström, 1987; Wullimann and Roth, 1992) and birds (Sorenson et al., 1989; Medina and Reiner, 1994) but not in the pretectum of amphibians, reptiles, or mammals (Kása, 1986; Woolf, 1991; Medina et al., 1993; Powers and Reiner, 1993; Marín et al., 1997). A ChAT-ir thalamic group has been reported in a recent study of a monkey (Rico and Cavada, 1998). The current results in the dogfish indicated that the superficial pretectal nucleus also contains numerous ChAT-ir cells. The presence of pretectal cholinergic cells associated with the visual pathways in two separate groups of fish may suggest an evolutionary correlation between these nuclei. In the dogfish, the superficial pretectum receives retinal fibers (Smeets, 1981; Repérant et al., 1986) and projects to the optic tectum (Smeets, 1982), suggesting the presence of a second-order cholinergic visuotectal circuit. Application of horseradish peroxidase (HRP) to the dogfish optic tectum also labeled neurons in other nuclei that contain ChAT-ir neurons, such as the cerebellar nucleus, the dorsal octavolateral nucleus, and the nucleus of the lateral funiculus (Smeets, 1982). All of these nuclei may give rise to ChAT-ir fibers of the optic tectum.

In the tegmentum of the pretectal region and the posterior parencephalon (rostral to the oculomotor nucleus), we observed large, ChAT-ir neurons of the Nflm. This nucleus extends in the basal plate rostral and caudal to the fasciculus retroflexus. In the dogfish, Smeets and Timerick (1981) have reported that neurons of the Nflm were labeled retrogradely after HRP injection into the spinal cord, and similar results have been obtained in other elasmobranchs (Cruce et al., 1999). The presence of ChAT-ir fibers in the medial longitudinal fascicle, together with results of tract-tracing studies, suggests that Nflm cholinergic neurons project to the brainstem and spinal cord. This nucleus does not appear to be ChAT-ir in other vertebrates.

Mesencephalon. ChAT-ir perikarya were not observed in the optic tectum of the dogfish, with the exception of the large neurons of the trigeminal mesencephalic nucleus (see below). These results are in contrast with the large numbers of ChAT-ir cells found in the optic tectum of teleosts (Ekström, 1987; Zottoli et al., 1987; Brantley and Bass, 1988; Molist et al., 1993a) and birds (Sorenson et al., 1989; 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, such cells are observed in superficial tectal layers (Vincent and Reiner, 1987). ChAT-ir cells have not been observed in the optic tectum of amphibians (Marín et al., 1997) or reptiles (Brauth et al., 1985; Medina et al., 1993; Powers and Reiner, 1993). Together, these results suggest that tectal cholinergic cells appeared separately in teleost and bird lines and that the lack of these cells is a primitive feature in vertebrates. Despite the absence of ChAT-ir cells in most vertebrate groups, their optic tecta are innervated richly by ChAT-ir fibers, although the origin of these fibers may differ between groups. Several ChAT-ir nuclei (the superficial pretectal nucleus, cerebellar nucleus, dorsal octavolateral nucleus, and lateral funiculus nucleus), as indicated above, may constitute the origin of the cholinergic innervation observed in the dogfish optic tectum.

In the rostral mesencephalic tegmentum of the dogfish, ChAT-ir cells are abundant in the region corresponding to the dopaminergic nuclei referred to as the ventral tegmental area and substantia nigra in elasmobranchs (Meredith and Smeets, 1987; Stuesse et al., 1990, 1991a). In the dogfish, these two nuclei have been shown to contain CGRP-ir neurons (Molist et al., 1995). Because ChAT and

CGRP coexist in other cholinergic neurons of the dogfish, it is possible that the CGRP-ir cells and the ChAT-ir cells of this region constitute the same population. Although TH-ir (dopaminergic) neurons appear to have a distribution similar to that of ChAT-ir cells of this region in the dogfish (Molist, 1990; unpublished observations), the possibility that TH and ChAT are expressed in the same cell requires further investigation. In mammals, the substantia nigra also contains some cholinergic neurons (Gould and Butcher, 1986). ChAT-ir cells were observed in the dorsomedial posterior tubercle and the interoculomotor nucleus of amphibians (Marín et al., 1997), and a ventral tegmental group has been reported in birds (Medina and Reiner, 1994). The possible evolutionary correlations of these structures are not clear, because similar cholinergic nuclei have not been reported in either teleosts or reptiles.

The mesencephalic trigeminal nucleus (MesV) is very conspicuous in elasmobranchs: It is characterized by its numerous, large, primary sensory neurons occupying the periventricular regions of the tectum (Witkovsky and Roberts, 1975). The mesencephalic trigeminal root also is unmistakable in view of its very coarse axons and characteristic course. In the dogfish, both the MesV and its root showed intense ChAT immunoreactivity. This finding is noticeable, because these cells are the only primary sensory neurons reported within the dogfish CNS. Recently, immunoreactivity to ChAT and to vesicular acetylcholine transporter has been found in the MesV of the rat (Schäfer et al., 1998). Thus, the MesV appears to be cholinergic in both rats and dogfish, but determination of whether this feature is primitive or derived will require studies in other vertebrate groups.

The intensely ChAT-ir cells that were observed in the central gray of the dogfish rostral mesencephalon deserve special attention. In the dogfish, a group of faintly ChAT-ir cells appears just lateral to the oculomotor nucleus in the position previously indicated for the EW nucleus (Smeets et al., 1983; Rodríguez-Moldes et al., 1993). The latter authors described the EW nucleus as SP-ir. In two elasmobranch species, the EW nucleus has been shown to give rise to spinal projections (Cruce et al., 1999). The position of the conspicuous ChAT-ir group suggests that it does not correspond to the SP-ir EW nucleus. The presence of two different populations in this area, the putative EW nucleus and the intensely ChAT-ir cells (pre-EW nucleus), is reminiscent of the fact that, in mammals, intensely ChAT-ir neurons were found near (but not in) the EW nucleus (Strassman et al., 1987). The latter authors also showed that the cells that project to the ciliary ganglion are the intensely ChAT-ir cells. In pigeon, EW neurons have been reported to be intensely ChAT-ir (Medina and Reiner, 1994). Because the central neurons that project to the ciliary ganglion in elasmobranchs have not yet been identified, experimental studies are necessary to clarify the nature of this nucleus.

Isthmus. A differential characteristic of the cholinergic systems of the dogfish appears to be the small number of ChAT-ir neurons in the isthmus. In mammals and other vertebrates, several ChAT-ir nuclei have been described in the isthmus region. These nuclei include the isthmic nucleus, the secondary gustatory nucleus, and the superior reticular nucleus in teleosts (Ekström, 1987; Brantley and Bass, 1988; Molist et al., 1993a) and the isthmus nuclei, pedunculopontine nucleus, locus coeruleus cells, laterodorsal tegmental nucleus, and reticular nuclei in tetrapods (Kimura et al., 1981, 1984; Armstrong et al., 1983; Satoh et al., 1983; Mesulam et al., 1984; Jones and Beaudet, 1987; Mufson and Cunningham, 1988; Sorenson et al., 1989; Tago et al., 1989; Medina et al., 1993; Powers and Reiner, 1993; Lavoie and Parent, 1994a,b; Medina and Reiner, 1994). In the dogfish, we observed small numbers of ChAT-ir cells in the periventricular lateroventral region of the fourth ventricle at levels just rostral to the trigeminal nerve entrance. This periventricular region, which also contains catecholaminergic neurons in other elasmobranchs, has been considered as a locus coeruleus (Stuesse et al., 1990, Stuesse et al., Stuesse et al., 1991a). Like what was found in the ventral tegmental area and the substantia nigra, catecholaminergic and cholinergic neurons appear to have a similar distribution in this putative locus coeruleus, although they probably represent separate populations. A recent study of two elasmobranch species reported that the locus coeruleus has spinal projections (Cruce et al., 1999) like the mammalian locus coeruleus (see Aston-Jones et al., 1995). In addition to the locus coeruleus, the cells of nucleus G of Smeets and Timerick (1981), as well as many neurons of the cerebellar nucleus, also were ChAT-ir in the dogfish. Smeets et al. (1983) previously pointed out the difficulty of defining a nucleus isthmi in the dogfish on the basis of its connections, and our results with ChAT immunocytochemistry point in the same direction. Thus, the dogfish nucleus referred to as the nucleus isthmi (Smeets et al., 1983) does not project to the optic tectum (Smeets, 1982) and is not ChAT-ir (current results). The possibility that the ChAT-ir nucleus G, which, like the isthmus nuclei, is located in the rostral region of the cerebellar peduncle, may be a nucleus isthmi homologue is not supported by its lack of tectal projections: This nucleus projects to the spinal cord (Smeets and Timerick, 1981). It is particularly puzzling that the only ChAT-ir nucleus of this region that projects to the optic tectum is the cerebellar nucleus (see below).

Cerebellum. The granule cell layer of the cerebellum of the dogfish showed ChAT-ir neurons that appeared to correspond in both size and distribution to the Golgi cells described in this species (Alvarez-Otero and Anadón, 1992; Alvarez-Otero et al., 1995). This result is similar to that reported in a teleost, *Porichthys notatus* (Brantley and Bass, 1988). Putative Golgi cells and some granule cells also exhibited ChAT immunoreactivity in the cat cerebellum (Ikeda et al., 1991). However, the presence of cholinergic cells in the cerebellar cortex has not been noted in the rat (with the exception of a transient ChAT immunoreactivity found in Purkinje cells during development; Gould and Butcher, 1987) or in sauropsids and amphibians. The results with ChAT immunocytochemistry in Golgi cells of the dogfish and *Porichthys* suggest that fish Golgi cells use acetylcholine as a neurotransmitter. Golgi cells also show immunoreactivity to γ-aminobutyric acid (GABA) in the dogfish (Alvarez-Otero et al., 1995), suggesting that GABA and ChAT are colocalized in these cells. Colocalization of GABA and ChAT has been observed in some neuronal groups of other vertebrates (Davidoff and Schulze, 1988; Kosaka et al., 1988). Whatever is the case, the dogfish cerebellum appears to have an intrinsic cholinergic innervation in addition to cholinergic fibers that also run in the cerebellum from octavolateral centers or other brain regions. ChAT-ir, mossy-like fibers have been reported in cerebella of other

vertebrates (mammals: Ikeda et al., 1991; Barmack et al., 1992; birds: Medina and Reiner, 1994), which is in agreement with our results in the dogfish. The finding of ChAT-ir structures in the dogfish cerebellum is in agreement with the abundance of muscarinic acetylcholine receptors found in a previous elasmobranch cerebellum study (Nicholson et al., 1994).

One finding of this study in the dogfish was the presence of ChAT-ir neurons in the cerebellar nucleus. In elasmobranchs, this nucleus receives fibers from the cerebellar cortex (Paul and Roberts, 1984; Alvarez-Otero et al., 1996), and it has been reported to project to the optic tectum (Smeets, 1982) and the corpus cerebelli (Fiebig, 1988). To our knowledge, the existence of cholinergic neurons in the cerebellar nuclei has been reported only in the cat (Ikeda et al., 1991), in which ≈10% of the neurons were ChAT-ir. The large neurons of the dogfish cerebellar nucleus appear to be both glutamate-ir (Alvarez-Otero et al., 1996) and ChAT-ir (current results). The colocalization of immunoreactivity to these two substances has been reported previously in neurons of the mesopontine tegmentum of the squirrel monkey (Lavoie and Parent, 1994a,b) and in laryngeal motoneurons of rat (Saji and Miura, 1991).

Although the cerebellar nucleus contains ChAT-ir cells in the dogfish, no ChAT-ir populations corresponding to the pedunculopontine nuclei of mammals and other tetrapods have been identified in this species. One possibility to explore is whether the cerebellar nucleus of the dogfish may correspond to the laterodorsal tegmental-pedunculopontine nuclei of other vertebrates. The projection of the cerebellar nucleus to the optic tectum (Smeets, 1982) suggests similarity to populations of the isthmus of teleosts (Grover and Sharma, 1981; Ekström, 1987; Molist et al., 1993a), amphibians (Desan et al., 1987; Marín and González, 1999), and reptiles (Kunzle and Snyder, 1984). However, although this nucleus in elasmobranchs receives fibers from the cerebellar cortex (Paul and Roberts, 1984; Alvarez-Otero et al., 1996) and also projects to the cerebellum (Fiebig, 1988), these features have not been reported for the isthmic nuclei in teleosts (Murakami and Morita, 1987) or for cholinergic pontomesencephalic cell populations in rat (Woolf and Butcher, 1989).

Motor nuclei. The distribution of ChAT-ir motoneurons in the dogfish brainstem corresponds with that described previously in elasmobranchs by using general methods (see Smeets et al., 1983) and tract-tracing experiments (Rosiles and Leonard, 1980; Montgomery and Housley, 1983; Barrett and Taylor, 1985; Withington-Wray et al., 1986). The motor nuclei also are AChE-positive in other sharks (Kusunoki et al., 1973). The presence of ChAT immunoreactivity in these nuclei (oculomotor, trochlear, trigeminal, facial, abducens, glossopharyngeal, and vagal nuclei) is in agreement with immunohistochemical observations in other vertebrates (teleosts: Ekström, 1987; Brantley and Bass, 1988; amphibians: Marín et al., 1997; reptiles: Medina et al., 1993; Powers and Reiner, 1993; birds: Medina and Reiner, 1994; mammals: Kimura et al., 1980, 1981; Armstrong et al., 1983; Hedreen et al., 1983; Houser et al., 1983; Satoh et al., 1983; Kimura et al., 1984; Jones and Beaudet, 1987; Maley et al., 1988; Mufson and Cunningham, 1988; Tago et al., 1989). The distribution of ChAT-ir in motor nuclei of the dogfish is coincident with the distribution of CGRP reported in motor nuclei of this species (Molist et al., 1995), in agreement with the codistribution of these two substances in motor nuclei of other vertebrates (Takami et al., 1985; Batten et al., 1989; Hietanen et al., 1990). Moreover, the efferent neurons of the octavolateral system of the dogfish (Meredith and Roberts, 1986) were both CGRP-ir (Molist et al., 1995) and ChAT-ir (current results), in agreement with observations in teleosts (Roberts et al., 1994) and in octaval efferents of mammals (Vetter et al., 1991).

Other medullary nuclei. The octavolateral area of the dogfish shows a characteristic pattern of ChAT-ir neurons. These cells were observed only in the vestibular nuclei (ventral octavolateral nuclei) and lateral-line electroreceptive area (dorsal octavolateral nucleus) but not in the mechanoreceptive lateral-line area (medial octavolateral area). Previous experimental studies have indicated that the mechanoreceptive and electroreceptive primary afferents of the lateral-line nerves are segregated in the octavolateral region of elasmobranchs (Paul and Roberts, 1977a,b; Bodznick and Northcutt, 1980; Koester, 1983; Boord and Montgomery, 1989). The distribution and abundance of catecholaminergic fibers also was found to be different in these two lateral-line centers (Roberts, 1992), and ultrastructural studies indicate that the synaptic organization of these two regions is quite different (Díaz-Regueira et al., 1990). The current results point in the same direction, suggesting that processing of sensory information is very different in the two regions of the lateral-line area of elasmobranchs. A comparison of cholinergic structures of these lateral-line centers with those of teleosts is very difficult, because the central electroreceptive nuclei (in the teleost species that possess them) are not related evolutionarily with the dorsal lateral-line area of elasmobranchs (McCormick, 1983). Although some adult urodeles (such as *Pleurodeles waltl*) possess both electroreceptive and mechanoreceptive lateral-line centers (Muñoz et al., 1992), no ChAT-ir cells have been observed in any of the two portions (Marín et al., 1997).

The vestibular region of the dogfish has many cholinergic neurons, including large, magnocellular, octaval neurons and populations of smaller cells in the descending and anterior octaval nuclei. In amphibians, ChAT immunoreactivity has been observed in large neurons of the ventral vestibular nucleus (homologous to Deiters's nucleus) and in a number of smaller cells of the caudal vestibular nucleus (Marín et al., 1997). Small, ChAT-ir cells also are present in the descending vestibular nucleus of turtle (Powers and Reiner, 1993) and in the medial vestibular nucleus of birds (Medina and Reiner, 1994). In some mammals, some vestibular and cochlear nuclei also have neurons that are immunoreactive to ChAT (Kimura et al., 1984; Carpenter et al., 1987, 1990; Jones and Beaudet, 1987; Tago et al., 1989; Barmack et al., 1992). The abundance of cholinergic vestibular cells in the dogfish is in contrast with the lack of cholinergic cells in the octaval region of teleosts (Ekström, 1987; Brantley and Bass, 1988). These results indicate that the presence of ChAT-ir cells in the octaval region may be a primitive feature of vertebrates. A tendency to a reduction of these populations is observed in amniotes, whereas teleosts may have lost these populations secondarily.

In anamniotes, the reticular formation is the main source of descending fibers from the brainstem to the spinal cord. Several groups of medium-sized to very large, ChAT-ir reticular cells were observed in the reticular region of the rhombencephalon that gives rise to spinal

projections in elasmobranchs (Smeets and Timerick, 1981; Cruce et al., 1999). These correspond in part to the gigantocellular reticular nuclei studied in other elasmobranch species by Cruce et al. (1999). Our results suggest that most large neurons of the reticulospinal system of the dogfish (the superior, intermediate, and inferior reticular rhombencephalic nuclei) use acetylcholine. In other sharks, these reticular cells are AChE-positive (Kusunoki et al., 1973). Other reticular populations of medium-sized cells, such as the superior and inferior raphe groups, were serotoninergic or enkephalinergic in some elasmobranchs (Stuesse et al., 1991a,b, 1995; Stuesse and Cruce, 1992). These populations, which were located in the raphe or its neighborhood, did not show ChAT immunoreactivity in the dogfish, with the exception of a few medium-sized, fusiform, ChAT-ir cells just lateral to the medial longitudinal fascicle at the level of the octaval nerve. The large, ChAT-ir reticular cells may be similar to the cholinergic reticular cells observed in the rhombencephalon of other vertebrates (teleosts: Ekström, 1987; Brantley and Bass, 1988; amphibians: Marín et al., 1997; birds: Medina and Reiner, 1994; mammals: Jones and Beaudet, 1987; Tago et al., 1989; Holmes et al., 1994). In cats, there are a number of ChAT-ir neurons in the gigantocellular and magnocellular portions of the reticular formation (Holmes et al., 1994), similar to what is observed in the dogfish. Moreover, ChAT-ir cells practically are absent from the raphe region of the cat.

Another cholinergic nucleus that is found in the dogfish rhombencephalon, the nucleus of the lateral funiculus, is known to project to the spinal cord and the optic tectum of the dogfish (Smeets and Timerick, 1981; Smeets, 1982). Studies in a skate also have demonstrated projections from this nucleus to the vestibulolateral cerebellum (Schmidt and Bodznick, 1987). Our immunohistochemical results also indicated that the nucleus of the lateral funiculus gives rise to both ascending and descending ChAT-ir projections that could be followed caudally to the spinal cord and rostrally to the isthmus, where a subset of their fibers ascend toward the cerebellum. In other elasmobranch species, this nucleus does not appear to project to the spinal cord (Cruce et al., 1999). A cholinergic lateral reticular nucleus has been reported in a similar position in reptiles (Brauth et al., 1985; Medina et al., 1993; Powers and Reiner, 1993) and birds (Medina and Reiner, 1994). Thus, the dogfish nucleus of the lateral funiculus may be similar to some cholinergic nuclei of the caudal medulla of other vertebrates, although more information on its connections is clearly needed.

Segmental organization of cholinergic nuclei of the brain

The organization of the embryonic brain segments (neuromeres) in vertebrates is highly conservative, so that the territories of origin of given nuclei may be useful for establishing homologies (Vaage, 1969; Lumsden and Keynes, 1989; Puelles, 1995). Studies of cholinergic nuclei in several vertebrates have found a clear segmental pattern of distribution, and this has been considered useful for comparative approaches to the organization of the brain (Medina et al., 1993; Medina and Reiner, 1994; Marín et al., 1997).

Several nonmotor cholinergic nuclei exhibit a clear segmental distribution in the dogfish. Segmental analysis of the cholinergic neurons of the Nflm reveals that they occupy the basal plate of prosomere 1 (P1; synencephalon) and P2 (posterior parencephalon). These two segments also bear cholinergic cells in the alar plate derivatives, namely, habenular and pineal cells in P2 and the superficial pretectal nucleus in P1 (see Fig. 2). Of these, only the habenular cholinergic population appears to be present widely in vertebrates (Houser et al., 1983; Mesulam et al., 1984; Medina et al., 1993; Medina and Reiner, 1994; Villani et al., 1994; Marín et al., 1997). The ventral tegmental area and substantia nigra of the dogfish extend in the basal plate of P1 and the mesencephalic neuromere, a distribution similar to that occupied by cholinergic populations in amphibians, birds, and mammals (Medina and Reiner, 1994; Marín et al., 1997). Whereas the EW nucleus of Smeets et al. (1983) extends only in the mesencephalic neuromere, the strongly cholinergic pre-EW nucleus extends in caudal P1 and the rostral mesencephalon. Thus, the segmental distribution of the latter nucleus appears to differ somewhat from that reported for the cholinergic EW nucleus of amphibians, reptiles, birds, and mammals (Medina et al., 1993; Medina and Reiner, 1994; Marín et al., 1997). The mesencephalic neuromere also contains the cholinergic trigeminal mesencephalic nucleus. The nonmotor cholinergic populations of the isthmus (rhombomere 1; r1) consist of nucleus G, the cerebellar nucleus, and the putative locus coeruleus. The nonmotor cholinergic populations of the rhombencephalon, like those of the reticular formation and the nucleus of the lateral funiculus, do not show a clear segmental distribution, which is in agreement with observations in other vertebrates (Medina and Reiner, 1994; Marín et al., 1997). Although most cholinergic neurons observed in the octavolateral region of the adult dogfish extend between r3 and r5, they do not exhibit any clear segmental distribution.

The motor nuclei exhibit a conspicuous segmental distribution in vertebrate embryos (see Gilland and Baker, 1993). In the adult dogfish, the ChAT-ir oculomotor and trochlear nuclei are located in the basal mesencephalic and isthmus segments, respectively, which is in agreement with observations in other vertebrates (Medina et al., 1993; Medina and Reiner, 1994; Marín et al., 1997). A small discontinuity observed between these two nuclei represents the boundary between the mesencephalon and isthmus.

The embryonic elasmobranch rhombencephalon exhibits eight well-delimited rhombomeres. The segmental origin of motor nuclei in shark embryos has been studied recently with tract-tracing methods (Gilland and Baker, 1992, 1993). The localization of the abducens nucleus caudal to the octaval region is also in agreement with its position in r6 in a shark embryo (Gilland and Baker, 1992, 1993). This nucleus is located in r5 and r6 in amphibians, reptiles, and birds (Medina et al., 1993; Medina and Reiner, 1994; Marín et al., 1997), but it is located in r5 in mammals (see Gilland and Baker, 1993; Medina and Reiner, 1994). The rostral position of the trigeminal motor nucleus in the adult dogfish is in agreement with the postulated origin of this nucleus from segments r2–r3 in all vertebrates (Lumsden and Keynes, 1989; Gilland and Baker, 1992, 1993; Medina et al., 1993; Medina and Reiner, 1994; Marín et al., 1997). Similar to other vertebrates, no separation between the cells originated from each of these segments was observed in the adult dogfish. It is noteworthy that the position of the facial motor nu-

cleus observed in adult elasmobranchs (Kappers et al., 1936; Anadón, 1978; current results) is far more caudal than in embryonic shark (Gilland and Baker, 1992, 1993). According to the latter authors, the facial motor nucleus originates from segments r4–r5, which is in agreement with the bimeric pattern reported in chick (Lumsden and Keynes, 1989), whereas its position observed in adult dogfish (current results) is clearly r6–r7, forming a continuous column with the glossopharyngeal and vagal motor nuclei. Thus, either the putative rule of Lumsden and Keynes (1989; motor axons do not cross the boundaries r3–r4, r5–r6, and so on) is violated during dogfish development, or these boundaries are crossed by migrating neuroblasts. On the basis of our observations in adults, the possibility of a caudal migration of facial motoneurons from r4–r5 primordia to r6–r7 segments, as suggested for elasmobranchs by Gilland and Baker (1992), appears rather improbable. The exit through r2 of axons of the abducens motoneurons of lamprey that originate in r5–r6 (Pombal et al., 1994) also clearly violates Lumsden and Keynes' rule. In one elasmobranch (the electric ray), the facial motor root has been reported even to exit alongside the trigeminal nerve (Anadón, 1978). These observations suggest that boundaries between segments r3 and r4 and segments r5 and r6 can be crossed by some types of growing motor axons in fishes. Thus, the cells of the dogfish facial motor nucleus probably originate from the same segments that they occupy in the adult. Such a caudal origin of facial motoneurons in the dogfish would be similar to the origin of this nucleus from r6 in mammals and urodeles but two segments caudal to the position occupied in anurans (Marín et al., 1997).

In some teleosts, the reticulospinal neurons exhibit a clear segmentation: They are distributed in 14 clusters that are postulated to lie in 8 metameric segments (Kanwal and Finger, 1997). However, a similar metameric pattern was not reported in the reticulospinal system of the dogfish (Smeets and Timerick, 1981; Timerick et al., 1992), and we have not found a distribution of ChAT-ir reticular cells in groups different from the cell groups reported by those authors.

Rostral spinal cord

Like the spinal motoneurons in other vertebrates (Barber et al., 1984; Phelps et al., 1984; Borges and Iversen, 1986; Rhodes et al., 1986; Hietanen et al., 1990; Ichikawa and Hirata, 1990; Thiriet et al., 1992), the motoneurons of the dogfish spinal cord were ChAT-ir. These large cells were located in the ventral horn, as reported previously for elasmobranchs on the basis of general methods (Lenhossék, 1894; Smeets et al., 1983). In addition to the cholinergic motoneurons, other ChAT-ir cells were observed in the rostral spinal cord of the dogfish, namely, in the dorsal horn, the marginal nucleus, and the ventral funiculus. Cholinergic cells are common in laminae III–V of the spinal dorsal horn of mammals, and the dorsal horn contains a number of cholinergic boutons (Barber et al., 1984; Phelps et al., 1984; Borges and Iversen, 1986; Kosaka et al., 1988; Ribeiro da Silva and Cuello, 1990; Wetts and Vaughn, 1994). Likewise, small, ChAT-ir cells, together with ChAT-ir fibers and boutons, are fairly abundant in the dorsal horn of the dogfish and mammals. This suggests that acetylcholine is related to sensory processing in the spinal cord of elasmobranchs. Although ChAT-ir cells also have been reported in the superficial dorsal horn of the spinal cord of birds and reptiles (Thiriet et al., 1992; Medina et al., 1993; Medina and Reiner, 1994), they were not found in amphibians or teleosts. Thus, whether there is any evolutionary correlation between the cholinergic cells of the dorsal horn of elasmobranchs and those of reptiles, birds, and mammals or whether they have arisen separately could not be determined.

In addition to cholinergic motoneurons and dorsal horn cells, the dogfish spinal cord contained two characteristic ChAT-ir populations located in peripheral regions of the cord. In a previous study, we reported the existence of a marginal nucleus in the lateral white matter of the spinal cord of the dogfish and other elasmobranchs, probably with a stretch-receptor function (Anadón et al., 1995a). The current results indicate that the cells of this nucleus are cholinergic in the dogfish. This also suggests that similar marginal neurons in the spinal cord of other vertebrates (edge cells of lampreys: Grillner et al., 1984; marginal nuclei of birds: Necker, 1997) may be cholinergic, a possibility that should be investigated.

The presence of ChAT-ir interstitial cells located among the coarse fibers of the ventral funiculus (at least at cervical levels) was rather unexpected, because neurons have not been described previously in this location (see Smeets et al., 1983). However, this simply may reflect the paucity of studies on the spinal cord of elasmobranchs. In the guitarfish, Cruce et al. (1999) recently described a ventral reticular nucleus pars alpha at the level of the obex near the ventrolateral pial surface. These neurons, which project to the spinal cord, may be similar to the ChAT-ir interstitial cells of the dogfish. A possible explanation for the finding of neurons scattered in the ventral funiculus is that the limits between the white matter and the gray matter are diffuse in elasmobranchs, with neurons sending dendrites that enter within bundles of fibers and even reach subpial regions (Lenhossék, 1894). The presence of white matter cells also has been reported in the spinal cord of lampreys (Vinay et al., 1998).

Peripheral ganglia

We found that ganglion cells of several dogfish cranial ganglia (trigeminal, octaval, and anterior lateral-line ganglia) exhibited intense ChAT immunoreactivity. This result is in agreement with the finding of ChAT immunoreactivity in primary sensory cells of the trigeminal mesencephalic nucleus (see above). Some authors also have reported ChAT immunoreactivity in cells of the sensory ganglia of rat (Palouzier et al., 1987; Sann et al., 1995) and chick (Tata et al., 1994), in agreement with our results in the dogfish. These results suggest that acetylcholine may be used as a transmitter by primary sensory cells in some sensory nuclei.

In addition to primary sensory cells of the cranial ganglia, small groups of ChAT-ir ganglion cells have been observed in the intracranial portion of the oculomotor nerve. The presence of ganglion cells in the oculomotor nerve has been reported previously in elasmobranchs (Nicholls, 1915; Anadón et al., 1980) and in man (Nicholson, 1924). We tried to find ChAT-ir beaded fibers in the third nerve, like those originated by cells of the presumptive EW nucleus, or ChAT-ir boutons contacting these ganglion cells. The presence of such boutons would indicate that they are parasympathetic neurons. We did not find structures of either type. Nevertheless, because ChAT immunoreactivity has been observed in both sensory neu-

rons (see above) and parasympathetic ganglion cells (Landis et al., 1987; Epstein et al., 1988), this result by itself does not resolve the question of whether the third nerve ganglion cells are sensory or motor.

CONCLUSIONS

The current study has shown that putative cholinergic neurons are distributed widely in the brain of an elasmobranch. In addition to nuclei like the motor nuclei, the habenula, medullary reticular nuclei, and some vestibular nuclei that contain cholinergic neurons in most vertebrates, ChAT-like immunoreactivity was observed in neurons of the olfactory bulb, pallium, pineal organ, superficial pretectal nucleus, Nflm, basal tegmentum, trigeminal mesencephalic nucleus, cerebellum, and electroceptive lateral-line nucleus. Together with these positive findings, ChAT-ir neurons were absent or were very scarce in regions such as the optic tectum and the isthmus. These findings suggest that, together with many features that are common to most vertebrates, elasmobranchs have evolved several characteristics that are unique to this group in their cholinergic systems.

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