Distribution of Choline Acetyltransferase (ChAT) Immunoreactivity in the Brain of the Adult Trout and Tract-Tracing Observations on the Connections of the Nuclei of the Isthmus

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ABSTRACT

The distribution of cholinergic neurons and fibers was studied in the brain and rostral spinal cord of the brown trout and rainbow trout by using an antiserum against the enzyme choline acetyltransferase (ChAT). Cholinergic neurons were observed in the ventral telencephalon, preoptic region, habenula, thalamus, hypothalamus, magnocellular superficial pretectal nucleus, optic tectum, isthmus, cranial nerve motor nuclei, and spinal cord. In addition, new cholinergic groups were detected in the vascular organ of the lamina terminalis, the parvocellular and magnocellular parts of the preoptic nucleus, the anterior tuberal nucleus, and a mesencephalic tegmental nucleus. The presence of ChAT in the magnocellular neurosecretory system of trout suggests that acetylcholine is involved in control of hormone release by neurosecretory terminals. In order to characterize the several cholinergic nuclei observed in the isthmus of trout, their projections were studied by application of 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (DiI) to selected structures of the brain. The secondary gustatory nucleus projected mainly to the lateral hypothalamic lobes, whereas the nucleus isthmi projected to the optic tectum and parvocellular superficial pretectal nucleus, as previously described in other teleost groups. In addition, other isthmic cholinergic nuclei of trout may be homologs of the mesopontine system of mammals. We conclude that the cholinergic systems of teleosts show many primitive features that have been preserved during evolution, together with characteristics exclusive to the group. J. Comp. Neurol. 428:450–474, 2000. © 2000 Wiley-Liss, Inc.

Indexing terms: cholinergic system; retrograde labeling; immunohistochemistry; preoptic nucleus; isthmic complex; teleost

Acetylcholine (ACh) is a neurotransmitter that is widely used in efferent systems and also in some central circuits (Woolf, 1991). It is one-step-synthesized in the cytoplasm of cholinergic neurons by the enzyme choline acetyltransferase (ChAT) and is degraded at the synapse by the enzyme acetylcholinesterase (AChE). The cholinergic systems of the central nervous system (CNS) have attracted increasing interest, due to the involvement of acetylcholine in processes such as learning, memory (Deutsch, 1971; Hagan and Morris, 1988; Levin and Simon, 1998; van der Zee and Luiten, 1999), and sleep (Sitaram et al., 1978; Jones, 1993; Perry et al., 1999). Until the 1980s, biochemical assays for ACh and ChAT and histochemical techniques for AChE were the methods used to locate putative cholinergic neurons. Histochemical methods for

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Grant sponsor: Xunta de Galicia; Grant number: PGIDT99BIO20002; Grant sponsor: Spanish Education Ministry; Grant number: PB96-0945-C02-01.

Received 22 February 2000; Revised 7 August 2000; Accepted 22 August 2000

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ChAT IMMUNOREACTIVITY IN TROUT BRAIN

AChE (Koelle and Friedenwald, 1949; Karnovsky and Roots, 1964) revealed a number of putative cholinergic perikarya and fibers in the CNS of vertebrates (Jacobowitz and Palkovits, 1974; Silver, 1974). However, the studies of Levey et al. (1983a) demonstrated that AChE is not a good marker for cholinergic neurons, because it also occurs in noncholinergic cells. In contrast, ChAT appears to be exclusive to acetylcholine-synthesizing neurons.

The development of antisera to ChAT has enabled immunocytochemical studies of the distribution of cholinergic cells in the brains of several vertebrates (Eckenstein et al., 1981; Levey et al., 1981, 1983b). Specific antibodies have also been developed against ACh (Geffard et al., 1985a, b), cholinergic receptors (Andre et al., 1984; Wenthold et al., 1990), AChE (Rakonczay and Brimijoin, 1985a, b), cholinergic receptors (Andre et al., 1984; Wenthold et al., 1990), AChE (Rakonczay and Brimijoin, 1986; Liao et al., 1991), and vesicular acetylcholine transporter (VACHT: Arvidsson et al., 1997; Ichikawa et al., 1997), contributing significantly to our knowledge of cholinergic systems. The distribution of cholinergic systems in mammals has been characterized in detail by ChAT.

Abbreviations

| AC       | anterior commissure               | OEN   | octalateral effenter nucleus |
| ans      | commissura ansulata              | OT    | optic tract                 |
| AP       | area postrema                     | OVT   | vascular organ of the lamina terminalis |
| ATh      | anterior thalamic nucleus of Holmgren (= nucleus glo- | P     | pineal organ                |
| cc       | cerebellar cortex                 | PC    | posterior commissure        |
| cc       | cerebellar cortex                 | PG    | preglomerular complex       |
| CcI      | central gray                      | PHt   | preoptic-hypophyseal tract  |
| cVln     | caudal abducens subnucleus        | PON   | magnocellular preoptic nucleus |
| DC       | central nucleus of the dorsal telencephalic area | PONm  | magnocellular preoptic nucleus, magnocellular part |
| Dd       | dorsal zone of the dorsal telencephalic area | Ppr   | preoptic prepositi on nucleus, anterior part |
| Dl-d     | dorsolateral zone of the dorsal telencephalic area | Ppp   | paraventricular preoptic nucleus, posterior part |
| Dl-p     | posterolateral zone of the dorsal telencephalic area | pr    | posterior recess            |
| Dl-v     | ventrolateral zone of the dorsal telencephalic area | FSi   | superficial preoptic nucleus, intermediate part |
| DDL      | diffuse nucleus of the lateral hypothalamic lobes   | PSm   | superficial preoptic nucleus, magnocellular part |
| Dm       | medial zone of the dorsal telencephalic area         | PSp   | superficial preoptic nucleus, parvocellular part |
| DMTm     | dorsomedial thalamic nucleus       | PVoAs | paraventricular organ, anterior part |
| Dp       | posterior zone of the dorsal telencephalic area       | PVoP  | paraventricular organ, posterior part |
| dIV      | trigeminal descending tract        | PVoP  | paraventricular organ, posterior part |
| ENT      | entopeduncular nucleus            | Res   | reticulospinal neurons      |
| FR       | fascicular retroflexus            | Ri    | inferior division of the reticular formation |
| GE       | granular eminence                 | Rm    | medial division of the reticular formation |
| Gl       | granular layer of the cerebellum  | Rs    | superior division of the reticular formation |
| h        | habenula                          | RSI   | superior reticular nucleus  |
| H        | hypothysis                        | rVln  | rostral abducens subnucleus |
| HL       | lateral hypothalamic lobe         | SAC   | stratum album centrale      |
| hsoC     | horizontal commissure            | SFGS  | stratum fibrosum et griseum superficiale |
| Hy       | hypothalamus                      | SG    | nucleus subglomerulosus    |
| i        | infundibulum                      | SGC   | stratum griseum centrale   |
| III      | third ventricle                   | SGN   | secondary gustatory nucleus |
| IIIIN    | oculomotor nerve                  | SGP   | stratum griseum periventriculare |
| IIIr     | oculomotor nucleus                | SGt   | secondary gustatory tract   |
| IIN      | optic nerve                       | SM    | stratum marginale           |
| Ip       | interpeduncular nucleus           | sN    | spinal nerve                |
| IV       | fourth ventricle                  | SO    | stratum opticum            |
| iVln     | intermediate abducens subnucleus  | spmc  | spinal motor column         |
| IVN      | trochlear nerve                   | st    | solitary tract              |
| IVr      | trochlear nerve root              | SV    | saccus vasculosus           |
| IXN      | glossopharyngeal motor nucleus    | T     | telencephalon               |
| IX-Xn    | glossopharyngeal and vagal motor nuclei |
| LN       | nucleus dorsomedialis tegmenti    | TAN   | tangential nucleus of the octavolateral area |
| LNN      | laminar nucleus                   | tcm   | cerebellomotor tract        |
| LOt      | lateral optic tract               | Th    | thalamus                    |
| lr       | lateral hypothalamic recess       | tl    | torus longitudinalis        |
| LV       | lateral nucleus of the valvula cerebelli | ts    | torus semicirculars         |
| Lj       | lemniscus lateralis               | ts    | torus semicirculars         |
| MI       | molecular layer of the cerebellum | tv    | telencephalic ventricle     |
| mlf      | medial longitudinal fascicle      | VC    | valvula cerebelli           |
| MLP      | nucleus of the medial longitudinal fascicle | Vd    | dorsal nucleus of the ventral telencephalic area |
| MO       | medulla oblongata                 | VEM   | magnocellular vestibular nucleus |
| mv       | mesencephalic ventricle           | VIII  | octval nerve                |
| NAT      | anterior tuberal nucleus          | VIII  | facial motor nucleus        |
| nI       | nucleus isthmi                    | VIf   | facial nerve root           |
| NIL      | neurointermediate lobe of hypophysis | VIn   | abducens nucleus            |
| NPT      | posterior tuberal nucleus         | VIO    | facial nerve root           |
| NSV      | nucleus of the saccus vasculosus | VIN    | abducens nucleus            |
| O       | olivary nucleus                   | VI     | lateral part of the ventral telencephalic area |
| OB       | olfactory bulb                    | VMTh  | ventromedial thalamic nucleus |
| OC       | optic chiasma                     | Vm    | trigeminal motor nucleus    |
|          |                                   | Vp    | postcomissural nucleus of the ventral telencephalic area |
|          |                                   | Vr    | trigeminal motor root       |
|          |                                   | Vsm   | supracomissural nucleus of the ventral telencephalic area |
|          |                                   | Vv    | ventral nucleus of the ventral telencephalic area |
|          |                                   | Xn    | vagal motor nucleus         |
immunohistochemistry (Kimura et al., 1981; Armstrong et al., 1983; Houser et al., 1983; Meininguer et al., 1983; Mesulam et al., 1983a, b, 1984; German et al., 1985; Satoh and Fibiger, 1985; Vincent and Reiner, 1987; Maley et al., 1988; Tago et al., 1988; St-Jacques et al., 1996). Studies with ChAT immunohistochemistry have also been carried out in other tetrapods (birds: Sorenson et al., 1988; Medina and Reiner, 1994; reptiles: Mufson et al., 1984; Brauth et al., 1985; Hoogland and Vermeulen-Van der Zee, 1990; Powers and Reiner, 1993; amphibians: Desan et al., 1987; Marin et al., 1997). In the majority of the vertebrates studied, ChAT-immunoreactive (ChAT-ir) cells have been detected in the basal telencephalon, habenula, isthmic tegmentum, and cranial nerve motor nuclei.

Ray-finned fishes constitute the largest and most versatile group of vertebrates (Lauder and Liem, 1983). There have been a few general studies on cholinergic systems in the brain of teleosts (Phoxinus phoxinus: Ekström, 1987; Porichthys notatus: Brantley and Bass, 1988; Anguilla anguilla: Molist et al., 1993). In addition, there have been specific studies of ChAT in some neural centers of teleosts (pineal organ and retina: Ekström and Korff, 1986; Mauthner neuron system: Rhodes et al., 1986; optic tectum: Zottoli et al., 1987, 1988; posterior lateral line efferent system: Danielson et al., 1988; cortical nucleus: Wullman and Roth, 1992). Only recently, the cholinergic systems of some non-teleost fishes have been investigated with immunocytochemistry (lampreys: Pombal et al., 1997; elasmobranchs, Anadón et al., 2000).

The teleost species belong to a group of Euteleosts with a generalized structural pattern considered to be close to that of the ancestral lineage of teleosts. Although salmonid species have been widely used to investigate the chemical neuroanatomy of the fish brain, as far as we are aware there have been no studies of their cholinergic systems. The aim of the present study was to investigate the distribution of these systems in the brain of adults of two species of salmonids, the rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri) and the brown trout (Salmo trutta fario). To characterize further the cholinergic populations observed in the isthmus, we also performed tract-tracing experiments with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) in fixed tissue. The results obtained in this study will contribute to knowledge of phylogeny of cholinergic systems of vertebrates.

MATERIALS AND METHODS

Four adult rainbow trout (18–30 cm long) and seven brown trout (20–30 cm long) were used in this immunohistochemical study. Animals were obtained from two hatcheries (Centro Ictioxeno de Soboardo dos Monxes, A Coruña, Spain; Piscifactoría Farítia, Coristanco, A Coruña, Spain). All the animals were deeply anesthetized with a 0.05% solution of tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in fresh water and perfused through the conus arteriosus with a Ringer's solution containing 0.1% procaine, followed by a 4% solution of paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The animals and solutions were cooled on ice during the perfusion. All experiments were conducted in accordance with European Community guidelines on animal care and experimentation.

Immunohistochemistry

After perfusion, the brains were removed and kept in the same fixative for 2–3 hours. Nine brains were immersed at 4°C in 30% sucrose in PB solution until they sank, then embedded in OCT Compound (Tissue Tek, Torrance, CA), frozen with liquid-nitrogen-cooled isopentane, and serially sectioned in transverse and sagittal planes on a cryostat. The sections (12–16 μm thick) were mounted on chrome-alum-gelatin-coated slides. Two additional brains were processed as free-floating sections. They were embedded in a 30% sucrose solution in PB containing 15% gelatin and then stored overnight in 4% paraformaldehyde at 4°C. These brains were cut on a freezing microtome (40 μm thick) in transverse and sagittal planes and collected in PB.

Both free-floating and slide-mounted sections were processed for ChAT immunohistochemistry following the peroxidase-antiperoxidase (PAP) method. After pretreatment with 1% H2O2 in PB saline (PBS; pH 7.4) for 15–30 minutes to eliminate endogenous peroxidase activity, sections were incubated in a purified goat anti-ChAT serum (1:100; Chemicon, Temecula, CA) for 40 hours at 4°C (free-floating sections were in continuous agitation). The sections were rinsed in PBS (three rinses of 10 minutes each), incubated in rabbit anti-goat serum (1:50, Chemicon) for 2 hours, and then rinsed in PBS and incubated in goat PAP complex (1:600, Chemicon) for 90 minutes. The antibodies and the PAP complex were diluted in PBS containing 0.2–0.5% Triton X-100 (PBS-T), 15% normal rabbit serum (NRS), and 2% bovine serum albumin (BSA).

After the sections were rinsed again, the PAP complex was developed with 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% H2O2 in 0.05 M Tris-HCl buffer (Tris, pH 7.6) for 5–15 minutes. In some series of free-floating sections, visualization of the immunostaining was improved by adding nickel to the developing solution (0.05% DAB, 0.01% H2O2, 0.04% nickel ammonium sulfate in PB). The slide-mounted sections were dehydrated and coverslipped and the free-floating sections were mounted on slides, dried overnight, and coverslipped.

The specificity of this antiserum for ChAT has been tested previously (Shiromani et al., 1987; Medina and Reiner, 1994; Grosman et al., 1995). This antibody has also been checked by Western blot analysis of brain extracts of trout (Anadón et al., 2000): it recognizes similar bands in rat, dogfish, sturgeon, and rainbow trout. Moreover, some sections were processed without primary antiserum, which resulted in no specific labeling of perikarya or fibers.

Tract-tracing experiments

Tract-tracing experiments were done in paraformaldehyde-fixed brains of twenty juvenile specimens of rainbow trout (28–80 mm long). Small crystals of DiI (Molecular Probes, Eugene, OR) on the tip of an electro-lytically sharpened 000 insect pin were applied to the investigated regions (cerebellum, optic tectum, parvocellular pretectal superficial nucleus, lateral hypothalamic lobes, secondary gustatory nucleus, and ventromedial tegmental region of the isthmus) either through the brain surface (for those structures directly accessible from the brain surface), or after sectioning the brain at the required level. The brain was then embedded with melted agarose
and left in fixative for between 20 and 35 days, which was sufficient to allow the diffusion of the dye along the different tracts. The fixative was changed every 2 days. After the diffusion period, the brain was sectioned transversely (50–100 μm thick) on a vibration microtome (Campden Instruments, Cambridge, UK). The sections were photographed with an epifluorescence photomicroscope equipped with a rhodamine filter set. Images were scanned from photographic films and digitalized. Contrast and brightness were adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA).

For our description of the different brain nuclei of the trout we have mainly followed the nomenclatures of Northcutt and Davis (1983), Bradford and Northcutt (1983), and Nieuwenhuys and Pouwels (1983). For lateral nuclei of the thalamo-pretectal region we followed the nomenclature of Corujo and Anadón (1990) and Butler et al. (1991).

RESULTS

The distribution of ChAT-ir structures was very similar in the two species of trout. In what follows we therefore refer collectively to the two species as “trout.” ChAT-ir perikarya were identified in all divisions of the CNS, with the exception of the cerebellum (Fig. 1).

Telencephalon

In the telencephalon we observed scarce cholinergic neurons, most of them located in the lateral part of the ventral telencephalic area (subpallium) (VI) (Figs. 2A,B, 3A). Most of these cells were small to medium-sized ChAT-ir neurons of bipolar appearance. The VI also contained larger ChAT-ir neurons among the medium-sized ones, at a level rostral to the anterior commissure (Figs. 2B, 3A). In the dorsal part of the dorsolateral and dorsal zones of the dorsal telencephalic area (pallium), scarce medium-sized ChAT-ir neurons were seen contiguous with those of VI (Figs. 2A, 3A).

The telencephalon showed moderate cholinergic innervation, ChAT-ir fibers being very scarce in the dorsal telencephalic area and more abundant medially and laterally in the ventral telencephalic area (Fig. 2A, B). Caudally, some ChAT-ir fibers were also observed surrounding the entopeduncular nucleus (Fig. 2C).

Preoptic region

Five ChAT-ir neuronal groups were observed in the preoptic region (Fig. 2C–F). The most rostral group contained small ChAT-ir neurons that were located near the preoptic recess in its rostrolateral walls. These cells belong to the anterior parvocellular preoptic nucleus (Ppa) (Figs. 2C, 3B). Processes of these neurons were directed dorsally and ventrally, coursing parallel to the ventricle. Ventral to the Ppa, some ChAT-ir neurons were located in the vascular organ of the lamina terminalis (OVLT) (Figs. 2C, 3B). This organ occupied the thin ventral wall of the preoptic recess. The ChAT-ir cells were observed within a dense plexus of ChAT-ir fibers associated with the abundant capillaries that characterize this organ (Fig. 3B).

Numerous ChAT-ir neurons were observed in both the parvocellular and magnocellular parts of the magnocellular preoptic nucleus (PON) (Figs. 2D,E, 3C–F). These ChAT-ir cells were seen extending from a level caudal to the Ppa to the suprachiasmatic level (Figs. 2D,E, 3C–F). The size of these ChAT-ir cells increased in the caudal
Fig. 2. A–R: Schematic drawings of transverse sections through the brain of an adult trout showing the distribution of ChAT-ir perikarya (solid circles) and fibers (fine lines and dashes). For abbreviations, see list.
Figure 2 (Continued)
direction, and the nucleus shifted dorsally (Fig. 3C). In supra-chiasmatic levels, the ChAT-ir magnocellular cells were very large and scarce (Fig. 3E). The majority of the ChAT-ir preoptic neurons showed labeled axonal processes directed laterally (Fig. 3D), which could be followed caudally in the preoptic-hypophyseal tract that courses through the ventromedial region of the hypothalamus (Fig. 2F,G). Some of the ChAT-ir neurons of this nucleus showed several dendritic processes, and one of them often contacted the preoptic recess (Fig. 3F). A few small ChAT-ir neurons were observed lateral to the PON (Fig. 2D,E). These could be either displaced PON ChAT-ir neurons or part of an independent lateral preoptic nucleus. At supra-chiasmatic levels, another group of small ChAT-ir neurons were seen ventral to the PON. These neurons were identified as belonging to the posterior parvocellular preoptic nucleus (Figs. 2E, 3F). Processes of these ChAT-ir neurons originated dorsolaterally from the perikaryon. Most ChAT-ir fibers coursed in the preoptic region, where they occupied a central or ventral position (Fig. 2C–E).
Fig. 4. Photomicrographs of transverse (A, B, E, G) and sagittal (C, D, F) sections through the diencephalon. A: ChAT-ir neurons in the ventromedial (vh) and dorsolateral (dh) regions of habenula. Note the light staining of the neuropil and several ChAT-ir tracts (arrowheads) in the ventromedial zone. B: Detail of the inset of A showing small ChAT-ir neurons in the dorsomedial region. C: ChAT-ir neurons of the dorsal thalamus. Rostral is to the left. D: Section through the anterior tuberal nucleus showing small ChAT-ir neurons. Note fibers coursing through the hypothalamic floor (arrows). E: Section showing a dense ChAT-ir plexus of the posterior part of the paraventricular organ as well as ChAT-ir fibers in the tract associated with paraventricular organ (arrowhead). F: Section through the hypophysis showing numerous ChAT-ir fibers in the neurointermediate lobe. Rostral is to the left. G: Detail of a ChAT-ir neuron near the magno cellular superficial pretectal nucleus. Note thick axonal processes. Scale bars = 250 μm in A; 100 μm in B, D, E; 300 μm in C; 400 μm in F; 50 μm in G.
Some of these fibers showed swellings along their trajectory.

**Diencephalon**

The diencephalon contained a few ChAT-ir elements. ChAT-ir neuronal groups were observed in the habenula, thalamus, pretectal region, and to a lesser extent, the hypothalamus (Fig. 2E–H).

**Epithalamus.** Abundant ChAT-ir neurons were observed in two different regions of the habenula, one dorsolateral and the other ventromedial, on both sides of the brain (Figs. 2E, 4A,B). The ChAT-ir neurons of the dorsolateral region were larger than those of the ventromedial region. The right habenula contained a further group of ChAT-ir neurons located close to its dorsal surface. The distribution of ChAT-ir in the ventromedial region was not homogeneous, some areas showing more densely grouped ChAT-ir neurons. Although the pineal organ of *Phoxinus* contained ChAT-ir cells (Ekström and Korf, 1986), the pineal organ of trout exhibited neither ChAT-ir neurons nor ChAT-ir fibers (Fig. 2D, E).

ChAT-ir fibers were especially abundant in the neuropil of the ventromedial region of the habenula (Fig. 4A). These fibers were organized in thin fascicles that coursed through the habenula to the fasciculus retroflexus (Figs. 2F–I, 4A). The ChAT-ir fibers of this fascicle could be followed to the neuropil of the intermediodorsal nucleus, in the tegmentum of the isthmus (Figs. 2F–K, 5C).

**Thalamus.** Small ChAT-ir neurons were seen in a band extending in a thalamic region medial to the anterior thalamic nucleus of Holmgren (1920) (= nucleus glomerulosus; = posterior pretectal nucleus of Butler et al., 1991) (Figs. 2G,H, 4C). These ChAT-ir cells and their processes were oriented in rostrocaudal direction (Fig. 4C). The position of this nucleus corresponds with that of the accessory optic nucleus of salmonids (Shiga et al., 1989). This cell group, as the anterior thalamic nucleus of Holmgren (1920), can be considered a migrated part of the dorsomedial thalamus (Bergquist, 1932; Corujo and Anadón, 1990).

**Hypothalamus.** Two groups of ChAT-ir neurons were observed in the hypothalamus. At the level of the anterior tuberal nucleus, some faintly ChAT-ir neurons occupied the ventrolateral walls of the infundibular recess (Figs. 2F,G, 4D). The second ChAT-ir group, containing a few small perikarya, was observed lateral to the anterior portion of the paraventricular organ (Fig. 2G). At a rostral hypothalamic level, scattered ChAT-ir fibers were seen in the periventricular walls of the paraventricular organ (Fig. 2G). Ependymofugal ChAT-ir processes originating from these paraventricular organ-associated ChAT-ir neurons formed a conspicuous tract that connected the anterior and posterior parts of the paraventricular organ (Figs. 2G,H, 4E). More caudally, a dense plexus of ChAT-ir fibers was observed close to the ventricular recess in the dorsal wall of the posterior part of the paraventricular organ (Figs. 2H, 4E). Other hypothalamic areas, such as the lateral hypothalamic lobes, showed moderate innervation by ChAT-ir fibers (Fig. 2H, I).

Numerous ChAT-ir fibers were seen coursing along the hypothalamus-hypophyseal tract in the floor of the infundibulum (Figs. 2G, 4D). Study of sagittal sections indicated that most of these ChAT-ir fibers came from the magnocellular preoptic nucleus. These ChAT-ir fibers could be followed to the neurointermediate lobe of the hypophysis, where they formed a dense plexus of terminals in the cords associated with its glandular portion (Figs. 2H, 4F).

The saecus vasculosus, a characteristic neuroependymal organ that forms a caudal diverticle of the infundibular recess, did not contain ChAT-ir neurons (Figs. 2L,J, 4F). Moreover, the tract of the saecus vasculosus, which is formed by the axons of cerebrospinal fluid-contacting neurons (see Yáñez et al., 1997), was not ChAT-ir.

**Pretectal region**

In the pretectal region, the only ChAT-ir neurons were those surrounding the magnocellular superficial pretectal nucleus (PSm; see Butler et al., 1991; Figs. 2F, 4G). Thick axonal processes of these ChAT-ir spindle-shaped cells coursed either laterally to the ventrolateral wall of the optic tectum, or ventrally through the thalamus and hypothalamus to cross in the postoptic commissure.

The pretectal region showed rich innervation by ChAT-ir fibers mainly originating from the cell groups of the isthmus. These fibers were seen innervating the V-shaped neuropil of the parvocellular superficial pretectal nucleus (PSp; Fig. 2E), whose immunonegative neurons were covered by small ChAT-ir boutons. The cholinergic fibers that innervated the PSp appeared to originate from the isthmus region, coursed below the torus semicircularis, and branched in the region of the PSp (Fig. 2E–J). The major portion of the fascicle coursed through the pretectal region to the lateral optic tract, reaching the optic tectum rostrally and then extending caudally in the stratum opticum (Fig. 2E–I).

**Mesencephalon**

Abundant ChAT-ir neurons and fibers were seen in the optic tectum of trout. The optic tectum contained numerous pear-shaped ChAT-ir cells with perikarya located in the stratum griseum periventriculare (Figs. 2F–K, 5A). Each tectal ChAT-ir neuron bore a thick radial dendrite that was directed toward the tectal surface and that branched in the stratum fibrosum et griseum superficiale and stratum opticum (Fig. 5A). The thick ChAT-ir axons originating from cholinergic isth-
Fig. 6. Photomicrographs of sagittal (A, B) and transverse (C–F) sections through the rhombencephalon. A: Section showing ChAT-ir motoneurons and a root of the oculomotor nerve. More caudally, a few ChAT-ir neurons of the trigeminal motor nucleus (arrows), abducens motor nucleus, as well as the facial and glossopharyngeal-vagal motor nuclei can be observed. Note the different columnar organization of ChAT-ir neurons of the VIIth, IXth, and Xth motor nuclei and the three abducens subnuclei. B: Detail of a sagittal section through the trigeminal motor nucleus showing ChAT-ir neurons in its rostral (r) and caudal (c) parts. Small ChAT-ir neurons are also seen in the post-trigeminal nucleus (arrows). C: Section at an intermediate level of the medulla oblongata showing ChAT-ir neurons of the caudal abducens subnucleus, the facial motor nucleus, and the octavolateral efferent nucleus (OEN). Note the spindle-shaped cells of the OEN, and the presence of large ChAT-ir reticulo-spinal neurons (arrow). D: Section through the medulla oblongata at the level of the area postrema showing ChAT-ir cells in the vagal motor nucleus and the spinal motor column. E: Detail of the central gray at the level of Figure 2L showing a few small ChAT-ir neurons in lateral positions (arrows) and their ChAT-ir fibers, some of them decussating (arrowheads). F: Detail of a caudal section through the medulla oblongata showing a small ChAT-ir neuron in the trigeminal descending nucleus (arrow), as well as ChAT-ir cells of the vagal motor nucleus, which is crossed by a tract of non-ChAT-ir internal arcuate fibers. Note the presence of several ChAT-ir axons coursing in the medial longitudinal fascicle (arrowheads). The asterisk indicates the fourth ventricle. Scale bars = 600 μm in A; 200 μm in B–D; 100 μm in E,F.
mus neurons could be observed coursing in the lateral parts of the stratum marginale and stratum opticum. A moderate number of thin ChAT-ir fibers was seen in the different strata of the optic tectum and also in the torus longitudinalis (Figs. 2F–K, 5A).

In the mesencephalic tegmentum we identified two ChAT-ir neuronal groups (Fig. 2H, I). The most conspicuous consisted of large ChAT-ir neurons of the oculomotor nucleus that appeared caudal to the immunonegative cells of the nucleus of the medial longitudinal fascicle (MLF; Figs. 2I, 6A). The rostral portion of the oculomotor nucleus appeared as a paired nucleus located dorsal to the medial longitudinal fascicle (mlf), whereas caudally and ventrally it approached the midline medial and ventral to the mlf. In this location, the oculomotor nuclei of both sides were separated only by a thin midline glial region. The oculomotor axons were prominently labeled and coursed ventrally in the oculomotor nerve (Figs. 2I, 6A).

At levels of the mesencephalic tegmentum rostral to the torus semicircularis and lateral to the MLF, we observed a few ChAT-ir neurons with ventrally directed processes. We identified this ChAT-ir nucleus as the laminar nucleus (Figs. 2H, 5B).

**Isthmus**

The isthmus contained several ChAT-ir cell groups. The isthmic tegmentum, dorsal to the mlf, exhibited the intensely ChAT-ir motoneurons of the trochlear nucleus (Figs. 2J, 5C). Bundles of ChAT-ir trochlear axons could be seen ascending around the ventricle to decussate in the ventral portion of the valvula cerebelli, emerging from the brain contralaterally between the tegmentum of the isthmus and the optic tectum (Figs. 2J,K, 5D,F).

The most conspicuous group of ChAT-ir cells was the nucleus isthmi, which was located ventrolateral to the lateral nucleus of the valvula cerebelli at the level of the trochlear nucleus (Figs. 2J, 5C). This nucleus consisted of ChAT-ir cells and neuropil, and showed a tear-shaped appearance in cross sections (Fig. 5D). The cells occupied the medial portion of the nucleus, at some places appearing as two parallel bands, sending processes to an extensive region of ChAT-ir neuropil located more superficially (Fig. 5D). ChAT-ir fibers of the nucleus isthmi could be followed to the pretectum, although other fibers coursed to the anterior hypothalamus.

Dorsal and lateral to the nucleus isthmi and ventral to the torus semicircularis, there was an elongated group of medium-sized ChAT-ir neurons that extended rostrally to levels of the oculomotor nucleus (Figs. 2I,J, 5D,E). These neurons had processes directed dorsolaterally or ventrally (Fig. 5E). These ChAT-ir neurons were interspersed among ChAT-ir isthmo-tegmental bundles (Fig. 5E). Here, this group is referred to as the nucleus dorsolateralis tegmenti.

Medial and caudal to the nucleus isthmi and ventral to the lateral nucleus of the valvula, close to the ventricle, there was a rounded group of ChAT-ir neurons that we have identified as the secondary gustatory nucleus, and (e) the corpus cerebelli. The approximate location of the photomicrographs of Figure 8 (squared areas A-H) is indicated at the right. For anatomical details, see the corresponding levels of Figure 2.
Figure 8
two portions, medial and principal, could be distinguished. In sagittal sections, a ChAT-ir tract formed of SGn axons could be followed to the lateral hypothalamic lobes. The SGn of the two sides were related by ChAT-ir fibers that crossed the midline in the subcerebellar commissure (Fig. 2K).

In the tegmentum ventral and caudal to the nucleus isthmi, numerous large ChAT-ir neurons formed a cholinergic nucleus here referred to as the superior reticular nucleus (Figs. 2J,K, 5D). This nucleus extended to rostral levels of the trigeminal motor nucleus, where its neurons were smaller. The thick ChAT-ir processes of these cells were preferentially oriented ventrally and ascended in the ventrolateral tegmentum toward the pretectum and postoptic commissure.

The isthmus region showed a large number of ChAT-ir processes, most of them projecting dorsolaterally to the pretectum and tectum (Fig. 2E–J). In the lateral nucleus of the valvula, some tiny ChAT-ir fibers and small boutons appeared bordering the immunonegative cells. Characteristically, the ventral neuropil of the interpeduncular nucleus contained a dense field of ChAT-ir fibers and terminals (Figs. 2J,K, 5C).

Cerebellum

We have not observed any ChAT-ir neurons in the cerebellum, although scarce CHAT-ir fibers could be seen coursing in the granular layer of the valvula cerebelli.

Medulla oblongata

In the medulla oblongata, ChAT-ir neurons were observed in all efferent nuclei and in several nonmotor nuclei. In general, the ChAT-ir afferent innervation was scarce in the medulla.

The motor nuclei of the trigeminal, facial, glossopharyngeal, and vagal (Vth, VIIth, IXth, and Xth) nerves exhibited moderate to strong ChAT immunoreactivity (Figs. 2L–P, 6A). In the motor nucleus of the Vth nerve it was possible to differentiate two portions, one rostral and dorsal consisting of large ChAT-ir cells, and the other caudal, consisting of smaller and more intensely stained cells (Fig. 6B). The trigeminal motoneurons extended long ChAT-ir dendrites laterally and ChAT-ir axons ventrolaterally following the motor root of this nerve. The facial, glossopharyngeal, and vagal motoneurons formed a long ChAT-ir column of medium-sized cells near the ventricle (Figs. 2N–P, 6A). The three abducens motor subnuclei (rostral, intermediate, and caudal) exhibited ChAT-ir cells near the ventral meninges (Figs. 2M,N, 6A,C). In addition, the octavolateral efferent nucleus, which is located ventromedial to the VIIth motor nucleus and lateral to the mlf at the level of the IXth and Xth motor nuclei, consisted of spindle-shaped ChAT-ir neurons (Figs. 2N, 6C).

We have also observed three groups of nonmotor ChAT-ir neurons in the medulla oblongata, two rostral groups located in the region of the periventricular gray (Figs. 2L,M), and one situated at more caudal levels (Fig. 2O). In the medial region of the central gray, just caudal to the interpeduncular nucleus, there was a small group of ChAT-ir neurons that surrounded the mlf dorsally and extended to a level rostral to the trigeminal motor nucleus (Figs. 2L, 6E). These cells gave rise to thin smooth fibers that decussated in the central gray close to the fourth ventricle (Fig. 6E). Some ChAT-ir processes could be seen coursing to the interpeduncular nucleus. More caudally, there was a posttrigeminal group formed by small ChAT-ir neurons occupying a lateral region of the periventricular gray (Figs. 2M, 6B). ChAT-ir processes of these cells coursed to the isthmus region. In addition, between the levels of the IXth and Xth motor nuclei, we identified the third ChAT-ir group associated with the trigeminal descending tract (Figs. 2O, 6F). This sparse group contained a few small spindle-shaped cells and their processes (Fig. 6F).

In addition to well-defined cholinergic nuclei between the levels of the VIIth and Xth motor nuclei and dorsolateral to the mlf, we also observed a few large ChAT-ir neurons, some of them even located among motoneurons, that were identified as reticulospinal cholinergic neurons (Figs. 2N, 6C). Up to eight thick ChAT-ir axons, probably originating from these neurons, could be followed in the mlf to the spinal cord (Figs. 2K–R, 6F).

Rostral spinal cord

The only ChAT-ir neurons of the rostral spinal cord were observed in the ventral horn. Most ChAT-ir cells were large to medium-sized motoneurons located in dorsal areas of the ventral horn (Figs. 2P–R, 6D). In addition, small intensely ChAT-ir neurons, often with a bipolar appearance, were observed among large motoneurons.

Dil labeling

The connections of the isthmus of the trout have not been studied with tract-tracing methods, and thus the equivalence of the nuclei described in this region of trout and those found in other teleosts has not been demonstrated. In order to elucidate the significance of the cholinergic nuclei of the isthmus region, we analyzed their connections on the basis of application of Dil to fixed...
brains, following a published procedure (Yañez and Anadón, 1996). The applications were made to several structures: secondary gustatory nucleus, corpus cerebelli, optic tectum, lateral hypothalamic lobes, parvocellular pretectal superficial nucleus, and ventromedial temporal region of the isthmus (Fig. 7).

Application of DiI to the secondary gustatory nucleus led to intense labeling of fibers in a triangular region medial to the diffuse nucleus of the lateral hypothalamic lobes (Fig. 8A,B). This labeled region of the preglomerular complex, which was ventral to a conspicuous negative preglomerular region, corresponds to a tertiary gustatory nucleus. The labeling was intense on the ipsilateral side, but there were contralateral projections to this region. In addition, fields with some labeled fibers were observed in the dorsomedial nucleus of the thalamus and in the torus lateralis mesencephali. Some retrogradely labeled neurons were also observed in the tertiary gustatory nucleus (Fig. 8B) and also in the nucleus of the lateral hypothalamic recess.

Application of DiI to the secondary gustatory nucleus sometimes led to some labeling of the cerebellar projections, as ascertained by comparison of results of DiI application to the secondary gustatory nucleus with those of application of DiI to the corpus cerebelli. According to this second type of DiI application (results not shown), rich cerebellar projections were observed to the nucleus lateralis valvulae, oculomotor nucleus, laminar nucleus, nucleus of the medial longitudinal fascicle and thalamic regions adjacent to it, and posterior tubercle. No projections to the tertiary gustatory nucleus, to the torus lateralis, or to the diffuse nucleus were observed after DiI application to the cerebellum. Among the nuclei labeled after DiI application to the cerebellum, the most prominent were some nuclei of the pretectum, the lateral mesencephalic reticular nuclei, the nucleus lateralis valvulae, and the inferior olivary nucleus. A conspicuous pretectocerebellar tract passed through the torus semicircularis, but the torus itself and the tectum were not labeled. The nucleus isthmi and the secondary gustatory nucleus contained no labeled neurons. In general, these results were similar to those obtained in the goldfish by Wullimann and Norden (1987), with notable exceptions such as the absence of any labeling (afferent and efferent) in the nucleus isthmi and torus semicircularis.

We also made control applications of DiI to the lateral hypothalamic lobes, to distinguish further the origin of the tertiary gustatory projections. This type of application led to retrograde labeling of neurons in the main and medial portions of the secondary gustatory nucleus (Fig. 8C,D), largely ipsilateral to the application side but also on the contralateral side. These cells were pear-shaped and had branched processes directed to the inner neuropil. Some fibers of the commissure between the secondary gustatory nuclei of both sides (crossing in the ventral region of the cerebellum) were also labeled. In addition, some cells in the superior reticular nucleus were labeled. No labeled structures were observed in the nucleus isthmi after this type of application, and only occasional labeled neurons were observed in the cerebellum. In the thalamus, this type of application massively labeled cells of the anterior thalamic nucleus of Holmgren (1920) (= nucleus glomerulosus, = posterior pretectal nucleus; Butler et al., 1991), and also labeled a pretectal nucleus below the rostral lateral end of the mesencephalic ventricle.

Application of DiI to the optic tectum strongly labeled cells and neuropil in the nucleus isthmi, indicating that these two structures are interconnected (Fig. 8E). In addition, this type of DiI application labeled cells in the superior reticular nucleus (Fig. 8E), in the region of the lateral mesencephalic tegmental nucleus, and in the laminar nucleus of the mesencephalon, i.e., nuclei containing ChAT-ir cells (see above). The torus semicircularis was also rather intensely labeled. The tectomesencephalic and tectobulbar tracts were strongly labeled. No labeling of the secondary gustatory nucleus was observed with this type of application.

Application of DiI to the ventromedial region of the isthmus labeled cells in the optic tectum with a morphology similar to that of ChAT-ir cells (Fig. 8G), in addition to other types of tectal neurons. The nucleus isthmi or the secondary gustatory nucleus were not labeled with this type of DiI application.

Application of DiI to the parvocellular superficial pretectal nucleus strongly labeled numerous cells in the nucleus isthmi and cells in the superior reticular nucleus (Fig. 8F). A few tectal cells were also labeled (Fig. 8H). No labeling was observed in the secondary gustatory nucleus.

DISCUSSION

Telencephalon

The telencephalon of the trout contains scarce cholinergic elements, as observed in other teleosts (Ekström, 1987; Brantley and Bass, 1988). As reported in Phoxinus (Ekström, 1987) and Porichthys (Brantley and Bass, 1988), no ChAT-ir perikarya was observed in the olfactory bulb of trout. In contrast, the olfactory bulb contains some ChAT-ir cells in elasmobranchs (Anadón et al., 2000), amphibians (Marín et al., 1997), reptiles (Medina et al., 1993), birds (Medina and Reiner, 1994), and mammals (Ichikawa et al., 1997). The main telencephalic ChAT-ir population of trout was located in the lateral part of the ventral telencephalic area (subpallium, VI). This population is similar to that described by Ekström (1987) in the cyprinid Phoxinus. However, no telencephalic cholinergic neurons were detected in Porichthys notatus (Brantley and Bass, 1988), indicating the existence of considerable between-species differences in teleosts. The VI of teleost fishes has been suggested to be homologous to the olfactory tubercle of vertebrates with evaginated telencephalic hemispheres (Northcutt and Davis, 1983). ChAT-ir cells have also been described in the olfactory tubercle of several mammals (Kimura et al., 1984; Mesulam et al., 1984; Tago et al., 1989; Ichikawa et al., 1997) and some reptiles (Mufson et al., 1984; Powers and Reiner, 1993), but not in amphibians (Marín et al., 1997). The absence of similar telencephalic cholinergic groups in lampreys (Pombal et al., 1997), elasmobranchs (Anadón et al., 2000), and sturgeons (Adrio et al., in press) suggests that the presence of cholinergic cells in the olfactory tubercle is not a primitive feature, but that these populations have evolved independently in different lines of vertebrates.

Besides ChAT, other neuroactive substances have also been detected in cells of the VI of trout, such as somatostatin (Becerra et al., 1995) and neuropeptide Y (Castro et al., 1999). Although the possible colocalization of ChAT with these peptides has not been investigated in trout, the fact that most of the neurons of this area in teleosts contain
somatostatin and neuropeptide Y (Batten et al., 1990; Becerra et al., 1995; Castro et al., 1999) suggests that some of these cells are also cholinergic. In the present study, a few ChAT-ir cells were also observed in the dorsolateral and dorsal zones of the dorsal telencephalic area (Dl+d-Dd). Although application of carbocyanine dye to the hypophysis labels some cells in the V1 (Kah et al., 1993; Holmqvist and Ekström, 1995), most V1 cells appear to project to dorsal telencephalic areas (Murakami et al., 1983; Castro et al., 1999). In view of these projections to dorsal telencephalic areas, the cholinergic V1 population of trout might have a role similar to that of the basal forebrain cholinergic populations of mammals, which constitute an activating system in the pallium (Butcher, 1995).

Preoptic region

In the preoptic region of trout we observed numerous cholinergic neurons, which is in contrast with scarcity or absence in other teleosts (Phoxinus: Ekström 1987; Porichthys: Brantley and Bass, 1988). We distinguished five ChAT-ir preoptic groups: the vascular organ of the lamina terminalis (OVLT), the anterior parvocellular preoptic nucleus (Ppa), the magnocellular preoptic nucleus (PON), the ventral portion of posterior parvocellular preoptic nucleus (Ppp), and the neurons of the lateral preoptic area. ChAT immunoreactivity of the OVLT and PON has not been reported in other teleosts. The OVLT contained both ChAT-ir perikarya and a dense plexus of ChAT-ir processes among the blood vessels. This organ, which is present in all the classes of vertebrates (Wenger and Törf, 1968), is considered in fish to be a neurohemal organ similar to the median eminence (Weindl et al., 1968; Röhlich and Wenger, 1969; Gómez-Segade et al., 1991). In the trout, this organ also receives somatostatinergic fibers (Becerra et al., 1995) and primary olfactory projections (Becerra et al., 1994), and it has been suggested that it is involved in the control of reproduction. In tetrapods, the OVLT is highly developed and is associated with the control of water balance, blood pressure, and reproduction (Leonhardt, 1980).

The anterior and posterior parts of the parvocellular preoptic nucleus of trout contain ChAT-ir cells. In the preoptic region of Phoxinus, a cholinergic parvo cellular population comparable to the posterior parvo cellular preoptic nucleus of trout has been identified as the suprachiasmatic nucleus (Ekström, 1987), but in trout the ChAT-ir cells appear to be medial to the latter nucleus. In amphibians, ChAT-ir cells in a similar position have been described as part of the supra chiasmatic nucleus (Marín et al., 1997). In addition, ChAT-ir neurons were observed in the PON of trout. This has not been observed in other teleosts (Phoxinus: Ekström 1987; Porichthys: Brantley and Bass, 1988). We observed that ChAT-ir fibers from the PON course in the hypothalamic floor to the neurointermediate lobe of the neurohypophysis, where they form a conspicuous terminal region. Immunofluorescence study with double labeling for ChAT and neurophysin (Rodríguez-Moldes et al., 1999) indicated that these substances are co-localized, i.e., that these cholinergic cells are classical neurosecretory cells. The presence of arginine-vasotocin and isotocin in both parvocellular and magnocellular preoptic perikarya of Salmo gairdneri, as well as in the preoptic-hypophyseal tract, has been demonstrated previously (Van den Dungen et al., 1982; Holmqvist and Ekström, 1995). ChAT immunoreactivity in magnocellular preoptic neurosecretory cells and in their fibers coursing to the neurohypophysis is also observed in the lampreys (Pombal et al., 1999a) and sturgeon (Rodríguez-Moldes et al., 1999), suggesting that the classical preoptic-hypothalamic neurosecretory system of early vertebrates was cholinergic. In amphibians, however, ChAT immunoreactivity has been described in small cells located among the large neurosecretory cells of the preoptic nucleus (Marín et al., 1997). In reptiles (Medina et al., 1993; Powers and Reiner, 1993), birds (Sorenson et al., 1989; Medina and Reiner, 1994), and mammals (Mesulam et al., 1984; Tago et al., 1989; Ichikawa et al., 1997), ChAT immunoreactivity is not displayed by the magnocellular neurons of the supraoptic nucleus but is displayed by smaller cells located in adjacent areas (Theodosius and Mason, 1988).

Although the function of acetylcholine in the neurohypophysis is not clear, it has been suggested that it may play a role in the regulation of release of neurohypophyseal hormones by neurosecretory terminals (Bridges et al., 1976; Gregg, 1985; Marín et al., 1997). Ultrastructural studies of the neurointermediate lobe in teleosts and elasmobranchs have demonstrated the presence in these terminals of small clear round vesicles similar to those of typical synaptic terminals, in addition to the large dense-cored granules characteristic of the neurosecretory material of lampreys and belenky, 1965; Pollen, 1970; Van de Kam and Zandbergen, 1981). The presence of ChAT in magnocellular preoptic nucleus neurons raises the possibility that in fishes these small vesicles contain ACh, and thus that ChAT may act as an autocrine/paracrine signal in terminals of this neurosecretory system.

Diencephalon and pretectum

The diencephalon of teleosts, like that of other vertebrates, contains few cholinergic neurons. In trout, ChAT-ir cells are present in the habenula, where they form conspicuous groups in the dorsal and ventromedial regions. ChAT-ir fibers are also present in the fasciculus retroflexus and the neuropil of the interpeduncular nucleus, which is the main target of the trout habenula (Yánnez and Anadón, 1996). These results in trout are similar to those obtained in the goldfish (Villani et al., 1994) and in most other vertebrates (lampreys: Pombal et al., 1997; elasmobranchs: Anadón et al. 2000; chondrosteans: Adrio et al., in press; amphibians: Marín et al., 1997; reptiles: Medina et al., 1993; Powers and Reiner, 1993; birds: Sorenson et al., 1989; Medina and Reiner, 1994; mammals: Houser et al., 1983; Mesulam et al., 1984; Satoh and Fibiger, 1985; Kása, 1986; Vincent and Reiner, 1987; Maley et al., 1988; Tago et al., 1989; Woolf, 1991; Ichikawa et al., 1997).

We did not observe ChAT-ir cells in the pineal organ of trout. However, ChAT-ir nonganglionic cells have been reported in the pineal organ of Phoxinus (Ekström and Korf, 1986) and ChAT-ir photoreceptors in the pineal organ of lampreys and elasmobranchs (Pombal et al., 1997; 1999b; Anadón et al., 2000). Studies carried out in salmonids have revealed the presence of acetylcholine receptors in this organ (Samejima et al., 1994) and have demonstrated that ACh causes changes in the activity of gan glion cells (Brandstätter et al., 1995; Brandstätter and Hermann, 1996). If a cholinergic network is present in the pineal organ of trout, it was undetectable by present immunocytochemical techniques.
The ChAT-ir group of the thalamus of trout appears to correspond to a retinorecipient region of salmonoids, the accessory optic nucleus (Shiga et al., 1989). This nucleus is similar to that observed in other teleosts ("nucleus tractus rotundus" of Phoxinus: Ekström, 1987; eel: Molist et al., 1993). There have been reports in other teleosts of retinal projections to locations similar to that occupied by this population in the trout (Striedter, 1990a,b; Butler and Northcutt, 1992). In pigeon, but not in other vertebrates, ChAT-ir neurons have also been reported in three subdivisions of the nucleus dorsolateralis anterior thalami (Medina and Reiner, 1994), which receive projections from the retina and were collectively referred to as the principal optic nucleus of the thalamus (Karten et al., 1973). The absence of a similar nucleus in the thalamus of other nonmammalian vertebrates suggests that the thalamic ChAT-ir nuclei of trout and pigeon are not homologous.

The anterior tuberal nucleus of trout contains a ChAT-ir population that has not been described in other teleosts (Ekström, 1987; Brantley and Bass, 1988). However, ChAT-ir cells were recently observed in a similar region of an elasmobranch (Anadón et al., 2000). Cells of the anterior tuberal nucleus of teleosts project to the hypophysis (Kah et al., 1993; Holmqvist and Ekström, 1995) and are involved in the synthesis and liberation of hypothalamic factors. As suggested above for the preoptic nucleus, the cholinergic cells of the anterior tuberal nucleus might use ACh as an autocrine signal. In addition to the anterior tuberal nucleus, the hypothalamus of trout contains a cholinergic population lateral to the rostral part of the paraventricular organ, as well as ChAT-ir fibers coursing in a tract that seems to connect the anterior and posterior portions of the paraventricular organ and that is roughly similar to that previously described in Phoxinus (Ekström, 1987). A ChAT-ir tract occupies a similar location in the periventricular hypothalamic nucleus of a urodele amphibian (Marin et al., 1997).

The saccus vasculosus of teleosts contains a type of cerebrospinal fluid (CSF)-contacting neuron that projects to the posterior tubercle and medial thalamus through a conspicuous tract of the saccus vasculosus (Altner and Zimmermann, 1970; Yáñez et al., 1997). In several teleosts these cells and this tract have been considered to be cholinergic in view of their positivity to AChE (Zimmermann and Altner, 1970; Jansen and West, 1971; Vig et al., 1972). Recently, we have obtained evidence that this neuronal system uses γ-aminobutyric acid (GABA) and neuropeptide Y, raising the possibility that it is not cholinergic, despite the presence of AChE activity (Yáñez et al., 1997; Pérez, 1998). The present results suggest that this system is ChAT negative in trout, although in the eel the tractus sacci vasculosi is ChAT-ir (Molist et al., 1993), and in the sturgeons both ChAT-ir cells and fibers have been observed in this system (Adrio et al., in press).

The presence of cholinergic cells in the pretectum seems to be a feature shared by teleosts, although there are differences between species. In the trout pretectum, ChAT-ir neurons were observed surrounding the magnocellular superficial pretectal nucleus, which is in agreement with results in Phoxinus (Ekström, 1987), whereas in Hemichromis ChAT-ir neurons were observed within this nucleus (Wullimann and Roth, 1992). ChAT-ir cells were also described in the cortical nucleus of Hemichromis (Wullimann and Roth, 1992), whereas no ChAT-ir cortical nucleus has been observed in Phoxinus or trout, despite the possible existence in the central tectal zone of salmiformes of a cortical nucleus formed by scattered cells (Bazer and Ebbesson, 1987; Butler et al., 1991). Pretectal ChAT-ir neurons have also been observed in birds (Sorenson et al., 1989; Medina and Reiner, 1994) and an elasmobranch (Anadón et al., 2000), but not in amphibians (Marin et al., 1997), reptiles (Medina et al., 1993; Powers and Reiner, 1993), or mammals (Houser et al., 1983; Mesulam et al., 1984; Satoh and Fibiger, 1985; Kása, 1986; Vincent and Reiner, 1987; Maley et al., 1988; Woolf, 1991).

In view of these data, it seems probable that the cholinergic cells of the pretectum of fishes and birds have evolved independently, as suggested by Medina and Reiner (1994).

In both Phoxinus and trout (Ekström, 1987; present results), the parvocellular superficial pretectal nucleus (PSp) receives a rich cholinergic innervation whose origin has not been determined. Immunohistochemical studies carried out in Carassius (Zottoli et al., 1988) have suggested the existence of a cholinergic projection from the nucleus isthmi to the PSp. A nucleus isthmi projection on the PSp has been established in the teleost Navodon with tract-tracing methods (Murakami et al., 1986). The optic tectum also projects to the PSp in Navodon (Murakami et al., 1986), which might also originate the observed cholinergic projection.

Mesencephalon

The optic tectum of trout, like that of other teleost fishes (Tumosa et al., 1986; Ekström, 1987; Zottoli et al., 1987; Brantley and Bass, 1988; Molist et al., 1993), contains a large population of cholinergic neurons in the stratum griseum periventriculare. As in Carassius (Zottoli et al., 1987) and Phoxinus (Ekström, 1987), these cells have been identified as type XIV neurons in the classification of Meek and Schellart (1978). Other studies (Grover and Sharma, 1981; Ito et al., 1981, 1982) have described projections of some type XIV cells to the isthmus. However, Molist et al. (1993) were unable to label these cells from the isthmus. Although our results with DiI indicate that similar tectal cells project to the reticular region of the medulla oblongata, whether ChAT-ir tectal cells of trout are projection neurons or intrinsic neurons is unresolved. It has been demonstrated in teleosts that ACh is an important neurotransmitter in the optic tectum and that the retina is not the source of cholinergic afferents to the optic tectum (Migani et al., 1980; Tumosa et al., 1986; King and Schmidt, 1991; Schmidt, 1995). In agreement with these results, we did not detect any ChAT immunoreactivity in the optic tracts or in retinal ganglion cells. Our immunocytochemical and DiI results in trout indicate the presence of a rich cholinergic innervation of the optic tectum that probably originates from cholinergic neurons of the nucleus isthmi.

The optic tectum of lampreys (Pombal et al., 1997), elasmobranchs (Anadón et al., 2000), sturgeons (Adrio et al., in press), amphibians (Marin et al., 1997), and reptiles (Brauth et al., 1985; Medina et al., 1993; Powers and Reiner, 1993) does not contain cholinergic neurons, unlike that of teleosts (Ekström, 1987; Zottoli et al., 1987; Brantley and Bass, 1988; Molist et al., 1993; present results) and birds (Sorenson et al., 1989; Medina and Reiner, 1994). However, ChAT-ir fibers have been observed in the tectum of most of these vertebrates. In frogs, these fibers appear to innervate retino-receptive layers of the tectum.
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(Desan et al., 1987), as in trout. Only in some mammals have cholinergic cells been described in the superior colliculus (Vincent and Reiner, 1987; Tago et al., 1989). The presence of tectal cholinergic neurons in only teleosts, birds, and some mammals suggests that this is a secondary feature of these vertebrate lines.

The main cholinergic population of the mesencephalic tegmentum of trout, as in other teleosts (Ekström, 1987; Brantley and Bass, 1988; Molist et al., 1993), is the oculomotor nucleus. In addition, there is a small ChAT-ir group in the tegmentum rostral and dorsolateral to the oculomotor nucleus. This group probably corresponds to the laminar nucleus identified with immunohistochemistry in salmonids (Vecino and Ekström, 1992; Castro et al., 1999), which projects to the optic tectum (present results). A similar cholinergic nucleus has not been mentioned in other fishes.

Isthmus

The isthmus of the trout, like that of the other teleosts studied (Phoxinus: Ekström, 1987; Porichthys: Brantley and Bass, 1988; Carassius: Zottoli et al., 1988; eel: Molist et al., 1993), contains several populations of interneurons that show strong ChAT immunoreactivity, in addition to the motoneurons of the trochlear nucleus.

The most conspicuous group of cholinergic interneurons in this region is the nucleus isthmi, located in the homonymous region characterized by Nieuwenhuys and Pouwels (1983) in Salmo gairdneri. This nucleus, already described in teleosts by Ariëns Kappers et al. (1936), varies greatly in size in the different species. In the trout, as in eel (Molist et al., 1993), the nucleus isthmi is large and contains cells with strong ChAT immunoreactivity, whereas in other teleosts studied for ChAT it is smaller (Carassius: Zottoli et al., 1988; Porichthys: Brantley and Bass, 1988). Most studies carried out with neuronal tracers in teleosts (Grover and Sharma 1981; Ito et al., 1981, 1982; Luiten, 1981; Dunn-Meynell and Sharma, 1984; Zottoli et al., 1988; present results), amphibians (Gruberg and Uddin, 1978; Marín and González, 1999), reptiles (Künzle and Schneyder, 1984), and birds (Hunt et al., 1977) have shown isthmo-tectal projections, generally reciprocal. In these nonmammalian vertebrates, both the nucleus isthmi and its projection to the optic tectum are cholinergic (teleosts: Ekström, 1987; Brantley and Bass, 1988; Zottoli et al., 1988; Molist et al., 1993; amphibians: Desan et al., 1987; Marín et al., 1997; Marín and González, 1999; reptiles: Medina et al., 1993; Powers and Reiner, 1993; birds: Sorrenson et al., 1989; Bagnoli et al., 1992; Medina and Reiner, 1994).

The nucleus isthmi has been considered the homolog of the parabigeminal nucleus of mammals (Sakamoto et al., 1981; Vanegas and Ito, 1983), which also contains cholinergic neurons (group Ch8; Mufson et al., 1986; Vincent and Reiner, 1987; Tago et al., 1989; Wolf, 1991; Ichikawa et al., 1997) that project to the superior colliculus (the optic tectum homolog; Beninato and Spencer, 1986; Mufson et al., 1986). The existence in the isthmus of a cholinergic nucleus that projects to the mesencephalic tectum therefore appears to be a feature that has been maintained throughout the evolution of vertebrates.

In the trout, we observed a ChAT-ir group lateral to the nucleus isthmi (nucleus dorsolateralis tegmenti) that continues to levels of the oculomotor nucleus. Based on its position, this nucleus could correspond to the nucleus dorsolateralis tegmenti described in Carassius (Grover and Sharma, 1981), and the “nucleus profundus mesencephali” of Cyprinus carpio (Luiten, 1981). The similar topology of the ChAT-ir pedunculopontine nucleus of the frog (Marín et al., 1997) and the nucleus dorsolateralis tegmenti of trout suggests the possibility that these nuclei are homologous. In other teleosts the presence of a similar ChAT-ir nucleus has not been reported.

We have also observed ChAT-ir cells in the superior reticular nucleus. This result is in agreement with those previously described in other teleosts (Ekström, 1987; Brantley and Bass, 1988; Zottoli et al., 1988; Molist et al., 1993). Injections of horseradish peroxidase (HRP) into the superior reticular nucleus of eel (Molist et al., 1993) revealed projections to the thalamus, pretectal region (parvocellular superficial pretectal nucleus), and telencephalon. Ekström (1987) in Phoxinus and Zottoli et al. (1988) in Carassius also described cholinergic fibers that project to the optic tectum, pretectal nucleus, and contralateral preoptic area; these latter probably crossed in the postoptic commissure. In the trout, cholinergic fibers of this nucleus can be easily followed to the postoptic commissure, and DiI applications to the optic tectum and the lateral hypothalamic lobe result in labeling of neurons of this nucleus. Although Molist et al. (1993) suggest that the superior reticular nucleus corresponds to the pedunculopontine nucleus of mammals, its position close to the locus coeruleus in trout (Manso et al., 1993) is in better agreement with that of the laterodorsal tegmental nucleus of amphibians (Marín et al., 1997; Marín and González, 1999). Our observations in trout, thus, suggest the presence of a mesopontine (pedunculopontine/laterodorsal tegmental) cholinergic complex similar to that observed in land vertebrates.

We found that the so-called secondary gustatory nucleus (SGn) of the trout isthmus was cholinergic, in agreement with results in Carassius (Zottoli et al., 1988) and eel (Molist et al., 1993). In Phoxinus (Ekström, 1987), however, a similar group of ChAT-ir cells has been described as the nucleus “a” of Nieuwenhuys and Pouwels (1983), which projects to the cerebellum. In trout, however, the SGNs does not project to the cerebellum (present results). In catfish and goldfish, Finger and Kanwal (1992) have distinguished between a SGn and a secondary visceral nucleus, because the latter receives projections from the general viscerosensory region of the medulla, not from gustatory centers. The topography of the ChAT-ir SGNs of goldfish (Zottoli et al., 1988) is similar to that of the secondary visceral nucleus of Finger and Kanwal (1992), suggesting that this is the general visceral region that contains ChAT-ir cells. The secondary visceral nucleus of trout, however, not the SGn proper, was also immunopositive to calcitonin gene-related peptide (CGRP; Finger and Kanwal, 1992).

We have found CGRP positivity in cells of all parts of the trout SGn (unpublished results; this peptide co-distributes with ChAT in many neuronal populations). Accordingly, it is probable that the ChAT-ir secondary gustatory nuclei described in eel (Molist et al., 1993) and trout (present results) only correspond to the secondary visceral nucleus of Finger and Kanwal (1992), although specific studies on their connections with the primary sensory areas appear necessary to resolve this question. Since the rostralmost portion of the SGn of trout was continuous with the caudal portion of the lateral nucleus.
of the valvula cerebelli, we cannot rule out the possibility that some cholinergic cells observed at this level belong to this latter nucleus and not to the SGn. Some cholinergic cells have been observed in the lateral nucleus of the valvula of *Phoxinus* (Ekström, 1987), eel (Molist et al., 1993), and sturgeon (Adrio et al., in press). In the trout, however, there is a conspicuous cholinergic projection from the isthmus to the lateral hypothalamic lobes, revealed by Dil tracing methods to originate from the SGn. In *Phoxinus* (Ekström, 1987) and eel (Molist et al., 1993), the lateral hypothalamic lobes are also innervated by cholinergic fibers. Tract-tracing studies in *Carassius* (Morita et al., 1980) and *Thamnacoconus* (Navodén; Shimizu et al., 1999) showed that the diffuse nucleus of the lateral hypothalamic lobes is a target of the neurons of the secondary gustatory nucleus. Moreover, studies in *Lepomis cyanellus* (Wullimann, 1988) showed that this nucleus also projects to the preganglionic tertiary gustatory nucleus, to the nucleus of the torus lateralis, to the central nucleus, and to the periventricular nucleus of the lateral hypothalamic lobes, and similar results were obtained in other teleosts (Lamb and Finger, 1996; Yoshimoto et al., 1998; Shimizu et al., 1999).

Our tract-tracing results in trout indicate that the main targets of the secondary gustatory nucleus are to the preganglionic tertiary gustatory nucleus and the torus lateralis. The presence in teleosts of a secondary gustatory (visceral) cholinergic projection to the hypothalamic region, together with the observation that the feeding behavior can be evoked by electrical stimulation of the lateral hypothalamic lobes (Demski, 1973), indicates that acetylcholine is involved in the processing of taste/general visceral information during feeding, as suggested by Molist et al. (1993).

In amphibians, the group referred to as the secondary visceral nucleus is noncholinergic. However, the secondary gustatory nucleus of teleosts may correspond to that reported in amniotes (Kimura et al., 1981; Hendry et al., 1987; Medina et al., 1993). The trigeminal descending nucleus of trout contains cholinergic cells, as observed in other teleosts (Ekström, 1987; Brantley and Bass, 1988). This nucleus also contains ChAT-ir cells in amniotes (Marín et al., 1997) and some reptiles (Medina et al., 1993).

In trout we also observed large ChAT-ir reticular cells. Similar cells were also present in other teleosts (Ekström, 1987; Brantley and Bass, 1988) and could correspond to some of the cholinergic reticulospinal cells described in lampreys (Pombal et al., 1997) and elasmobranchs (Anadón et al., 2000). These trout neurons also show ChAT activity (unpublished results). In amphibians, cholinergic neurons have been described in various reticular nuclei (Marín et al., 1997), and similar results have been obtained in amniotes (Kimura et al., 1981; Hendry et al., 1987; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994). The ventral horn of the rostral spinal cord of trout contains cholinergic motoneurons. Similarly, in *Porichthys notatus*, cholinergic cells were observed in the motor column and in a very specialized motor nucleus, the sonic motor nucleus (a spino-occipital nerve derivative; Brantley and Bass, 1988). In addition to motoneurons, small cholinergic cells were present in the ventral horn of trout. These cells may correspond to the interstitial cells observed in the ventral funiculus of elasmobranchs (Anadón et al., 2000).

**CONCLUSIONS**

The pattern of distribution of ChAT-ir neurons in the brain of trout is roughly similar to that observed in other teleost fishes, notably the euteleost *Phoxinus phoxinus*. However, we have detected ChAT-ir neuron groups in trout brain areas in which ChAT immunoreactivity has not been reported previously in other teleosts (namely, the preoptic region and the tuberal area). By comparison with the other major vertebrate groups, the organization of the

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**Medulla oblongata**

All the motor nuclei of the different cranial nerves (III, IV, V, VI, VII, IX, X, and XI) and the spinal motoneurons of trout, as in other teleosts (Ekström, 1987; Brantley and Bass, 1988), were ChAT immunoreactive. Similar results have been obtained in other vertebrate lines (elasmo-branchs: Anadón et al., 2000; amphibia: Marín et al., 1997; sauripsids: Sorenson et al., 1989; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994; mammals: Armstrong et al., 1983; Tago et al., 1989).
cholinergic systems of the trout brain showed great similarity with that in amphibians, but considerable differences with respect to the forebrain of amniotes. With these notable exceptions, most features of the distribution pattern of cholinergic systems appears to have developed in the fishes and to have been basically conserved through vertebrate evolution.

LITERATURE CITED

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ChAT IMMUNOREACTIVITY IN TROUT BRAIN


