Distribution of choline acetyltransferase (ChAT) immunoreactivity in the brain of the teleost *Cyprinus carpio*

Arianna Casini,[1] Rosa Vaccaro,[1] Mattia Toni,[2] Carla Cioni[2]

[1]Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, Sapienza University of Rome
[2]Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Italy

Abstract

Cholinergic systems play a role in basic cerebral functions and its dysfunction is associated with deficit in neurodegenerative disease. Mechanisms involved in human brain diseases are often approached by using fish models, especially cyprinids, given basic similarities of the fish brain to that of mammals. In the present paper, the organization of central cholinergic systems have been described in the cyprinid *Cyprinus carpio*, the common carp, by using specific polyclonal antibodies against ChAT, the synthetic enzyme of acetylcholine, that is currently used as a specific marker for cholinergic neurons in all vertebrates. In this work, serial transverse sections of the brain and the spinal cord were immunostained for ChAT. Results showed that positive neurons are present in several nuclei of the forebrain, the midbrain, the hindbrain and the spinal cord. Moreover, ChAT-positive neurons were detected in the synencephalon and in the cerebellum. In addition to neuronal bodies, afferent varicose fibers were stained for ChAT in the ventral telencephalon, the preoptic area, the hypothalamus and the posterior tuberculum. No neuronal cell bodies were present in the telencephalon. The comparison of cholinergic distribution pattern in the *Cyprinus carpio* central nervous system has revealed similarities but also some interesting differences with other cyprinids. Our results provide additional information on the cholinergic system from a phylogenetic point of view and may add new perspectives to physiological roles of cholinergic system during evolution and the neuroanatomical basis of neurological diseases.

Introduction

Cholinergic cell groups are widely distributed in the nervous system of all vertebrates in which are involved in the control of motor functions. Several studies have demonstrated that cholinergic systems are also implicated in complex cognitive functions, such as learning and memory, in both vertebrates[1-8] and invertebrates[9-11] and may be involved in human neurodegenerative disorders, including Alzheimer's and Parkinson's diseases.[12-14] The association of cholinergic systems to neurodegenerative diseases was first postulated by Frederic Lewy, who found that neuronal loss is accompanied with the accumulation of amyloid inclusion bodies (Lewy bodies, Lb) in cholinergic neurons of the vagal nucleus and nucleus basalis of Meynert of patients with Parkinson's disease.[15] The main component of the Lb are aggregates of α-synuclein (α-syn)[16] that are recognized as the key feature of neurodegenerative diseases known as synucleinopathies. In recent years, detailed mapping of cholinergic nuclei and *in vivo* magnetic resonance imaging morphometry have provided strong evidence for the implication of cholinergic dysfunctions in the pathogenesis of the cognitive decline occurring in Alzheimer's[17] and Parkinson's[18] diseases.

Several studies have proposed teleost fishes as valuable models for investigating brain functions and human neurological disorders.[19-21] The research from our group is aimed at this area. We have recently reported that α-syn-like proteins are expressed in the carp CNS and are quite selective for cholinergic neurons.[22,23] This evidence encouraged the possible use of this fish as vertebrate model alternative to mammals for investigating synucleinopathies on cholinergic system.

The organization of cholinergic systems was described in mammals (cat,[24] guinea pig,[25,26] hyrax,[27] macaque,[28] monotremes,[29] rabbit,[30] rat[31-36]) including humans[37-41] and non-mammalian vertebrates[42-53] by means of choline acetyltransferase (ChAT) immunohistochemical assay (IHC).[54] The comparative analysis demonstrated that ChAT immunoreactive (ChATir) cell groups are conserved in the brainstem and the spinal cord of all vertebrates whereas the distribution of putative cholinergic neurons is much less conserved in other brain regions (*i.e.*, forebrain, optic tectum and cerebellum).[55] In particular, the organization of cholinergic system shows differences in fish compared to tetrapods and between different fish groups. Literature data indicated that a certain degree of variability is also present in close-related species, as demonstrated in Ciprinidae.[19,56-64] Indeed, ChAT expression differs in the diencephalon and the cerebellum among European minnows,[56,57] goldfish,[58-61] tench[62] and zebrafish[19,62-64]. Other differences emerged in the other species studied so far, *i.e.* eel[65] (Anguillidae), trout[66] (Salmonidae), midshipmen[67] (Batrachoididae) and some cichlids[68] (Cichlidae) (Table 1). Based on these studies, the diversification of cholinergic systems in the extant teleosts is not negligible.

Given the species variability, we have described the cholinergic system in the brain and the spinal cord of the carp *Cyprinus carpio* by ChAT IHC, with the aim of providing the background for future studies on synucleinopathies affecting cholinergic neurons in this fish model. This study also contributes to the evolutionary perspective on the organization of cholinergic systems in teleosts.

Correspondence: Arianna Casini, Sapienza University of Rome, Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, Via Alfonso Borelli 50, 00161 Rome, Italy.
E-mail: arianna.casini@uniroma1.it

Keywords: ChAT; immunohistochemistry; cyprinid; central nervous system.

Contributions: AC, CC, RV, study concept and design; AC, RV, data acquisition; AC, CC, analysis and interpretation of data; AC, RV, MT, CC, drafting of the article; MT, obtained funding; CC, study supervision. All authors had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

Conflict of interest: The authors declare no conflict of interest.

Funding: This study was supported by the Sapienza University of Rome (Progetti di ricerca 2016 to MT).

Received for publication: 27 March 2018.
Accepted for publication: 6 July 2018.

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

©Copyright A. Casini et al., 2018
Licensee PAGEPress, Italy
European Journal of Histochemistry 2018; 62:2932
doi:10.4081/ejh.2018.2932

Materials and Methods

Tissue preparation

Four adult individuals of *Cyprinus carpio* (Taxon 7962) (s.l. 9 cm), obtained by local authorized providers, were anesthetized by adding 2-phenoxyethanol to the fish tank (final concentration of 1.5 mL/L)

and successively transcardially perfused by PFA fixative (4% para-formaldehyde in 0.1M phosphate buffer), pH 7 at 4°C. The brains were quickly dissected out and post-fixed in the same fixative for 24 h, then stored at 4°C in 0.01 M phosphate buffer (PB) containing 15% of sucrose, embedded in PB containing 10% gelatin and frozen. Samples were cut on a cryostat (HM 505 E, Microm, Walldorf, Germany) into 30 µm-thick coronal serial sections that were stored until use in 24-well plates containing cold 15% sucrose PB, each well containing a single section to allow the sections to thaw and float in the buffer; sections were enumerated to avoid misplacement, maintaining the seriality. Before immunohistochemical staining, the free-floating sections were

Table 1. Summary of ChATir structures in the CNS of *Cyprinus carpio* and literature data from other teleosts investigated so far.

	C. carpio (this paper)		*A. anguilla*[65*]		*C. auratus*[58-61***]		*D. rerio*[62,63**]		*D. rerio*[19**]		*D. rerio*[64**]		*H. lifalili, H. guttatus*[68****]	*O.mykiss, S. trutta*[66**]		*P. phoxinus*[56,57*****]		*P. Notatus*[67***]		*T. tinca*[62**]	
ChATir	CB	NF	CB	NF	CB	NF	CB	NF	CB	NF	CB	NF	CB	CB	NF	CB	NF	CB	NF	CB	NF
Olfactory bulb (OB)	-	+			-	+	-	-	-	-	-	+		-		-	-			-	-
Pallium	-	+			-	+	-	+	-	+				+	+	-	+	-	+	-	+
Subpallium	+	+			+	+	-	+	+	+				+	+	+	+	+	+	+	+
Preoptic nuclei (PM,PPa,PPp)	+	+	+		+	+	+	+	+	+				+	+	-	+			+	+
Suprachiasmatic nucleus (SCN)	+	+			+	+	-	-	-	-				+	+	+	+				
Periventricular hypothalamus (Hd, Hv)	-	+			-	-	-	+	+	-				+	+	+	+			-	+
Tuberal hypothalamic region (NAT, NLT, PTN)	-	+			-	-	-	+	-	-				+	+	-	-			-	+
Inferior hypothalamic lobe (IL)	-	+		+	-	+	-	+	-	+				-	+	-	+			-	+
Posterior tuberculum	-	+	+		-	+	-	+	+	-						+	+			-	+
Prethalamus	-	+	+		-	+	-	-	-	-						-	+			-	+
Dorsal thalamus	-	-			+	+	-	-	+					+	+	+	+			+	
Pineal organ	+	+			+	-	-	-	-	-				-	-	+	-				
Habenula	+	+			+	-	-	-	+					+	+	-	+			-	+
Habenulo-interpeduncolar tract (fr)		+				+		-		-					+		-				
Nucleus of the medial longitudinal fascicle (Nmlf)	+				+	-	-	+						-		-	-				
Medial longitudinal fascicle (mlf)		+				+		-							+		-				
Pretectum	-	+		+	-	+	-	+	-	+			+	+	+	+	+				
Optic tectum (OT)	+	+	+	+	+	+	+	+	+	-			+	+	+	+	-	+	+	+	+
Mesencephalic rostral tegmentum (RTN)	+	+			-	-	+	+	+	+										+	
NIII	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+		+	+
Nucleus lateralis valvulae (NLV)	+	+	+	+	+				+					-	+	+					
NIV	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+		+	+
Nucleus isthmi (NI)	+	+	+	+	+		+	+	+	+			+	+	+	+	+	+	+	+	+
Secondary gustatory nucleus (SGN)	+	+	+		+	+	+	+	+	+				+	+					+	+
Superior reticular formation (SRN)	+	+	+	+	-	-	+	+	+	+				+		+		+		+	+
Cerebellum	+	+			+	+	-	-	-	-				-	+	-	+	+			
Motor nuclei V-VI-VII-IX-X	+	+			+	+	+	+	+	+			+	+	+	+	+	+		+	+
Octaval column	+	+			+	-	+	+	-	+										+	
Octavolateralis efferent system (OEN)	+						+		+				+	+				+		+	
Rombencephalic reticular formation	+	+			+	-	-	-	+					+	+	+	+	+		+	
Spinal cord	+	+			+	+	+		+	+				+						+	+

CB, cell bodies; NF, nerve fibers; -, absence; +, presence; empty squares, data not available; light gray squares highlight the similarity of our data with other teleosts (superscript numbers for references). Antibodies used: *Rat monoclonal antibody anti-ChAT (Incstar); **AB144p (Chemicon); *** monoclonal antibody AB8 (provided by Dr. A.I. Levey, University of Chicago, USA); ****polyclonal antibody anti-chicken ChAT (provided by Dr. M.L. Epstein, University of Wisconsin, USA); *****polyclonal antibody anti-chicken ChAT (provided by Dr. F. Eckenstein, Harvard University, USA).

treated with 0.1 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST) at 4°C for 3 days, to improve tissue permeability. All experiments were performed in accordance with the Directive 2010/63/EU (EU 2010) and were approved by the Italian Decree DM 70/96 of the Ministry of Health.

Immunohistochemistry

To inactivate the endogenous peroxidase activity, the sections were pre-treated for 30 min at room temperature with PBS containing 0.3% Triton X-100, 0.1% sodium azide and 0.5% H_2O_2 and, to avoid non-specific binding of serum proteins, incubated for 30 min at room temperature with normal donkey serum 1:50 in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA). Serial sections were then incubated for 4 days at 4°C in the primary polyclonal antibody solution rabbit anti ChAT (EMD Millipore, Burlington, MA, USA, Cat. no. AB143, RRID: AB 2079760 diluted 1:5,000). The sections were then incubated with a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA; diluted 1:1,000) for 2 h at room temperature and then for 1h at room temperature with avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, CA, USA; diluted 1:2,000). PBS containing 0.3% Triton X-100 was used for diluting all the reagents and washing sections after each step. The localization of peroxidase activity was visualized by reacting the sections for 3 min at room temperature with a solution containing 0.04% 3-3' diaminobenzidine tetrahydrochloride (DAB; Fluka, Buchs, Switzerland), 0.4% nickel ammonium sulfate, and 0.003% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6 giving a dark blue granular precipitate. The stained sections were mounted on glass slides and air-dried. After staining, the sections were then dehydrated, cleared and coverslipped with Permount (Fisher Scientific, Pittsburg, PA, USA). Some sections were counterstained with the Nuclear Fast Red (Kernechtrot) solution (Sigma-Aldrich, Cat# 368458-500G) after immunohistochemistry procedure. For control experiments, the primary antiserum was substituted with buffer or normal rabbit serum or primary antiserum was pre-absorbed overnight at 4°C with the antigen used for its production (2 µg/mL antiserum at working dilution). None of the control sections showed positive immuno-staining.

Image acquisition and processing

Photomicrographs were captured using an AX70 Provis microscope (Olympus Optical, Tokyo, Japan). Images were captured using a cooled CCD digital camera (Spot, Diagnostic Instruments, Sterling Heights, MI, USA) and the IAS 2000 software (Delta Sistemi, Rome, Italy) and saved as tiff files. Images were digitally processed in Adobe Photoshop CS5 (San Jose, CA, USA). Only general contrast adaptations were made, and figures were not otherwise manipulated. The final figure composition was done using Microsoft Office Powerpoint 2008 software (Redmond, WA, USA).

Results

In this paper, the forebrain regions of the carp are described in the rostrocaudal sequence proposed by the neuromeric model[69] recently updated by Puelles and Rubenstein.[70] According to this model, the telencephalon, the preoptic region and the hypothalamus (the secondary prosencephalon) originate from the cranial prosomeres P6-P4 (updated to hp2 and hp1 by Puelles and Rubenstein[70]), the diencephalic structures (posterior tuberculum, prethalamus, dorsal thalamus and epithalamus) derive from prosomeres P3-P1 (that also give rise to the pretectum) and the midbrain structures, the isthmus and the medulla oblongata derive from mesomeres m 1-2 and rhombomeres r 0-11, respectively.

ChAT immunoreactive elements in the brain of the carp are represented in the schematic drawings of Figure 1 A-Q and in Table 1.

Olfactory bulb

Neuronal cell bodies were not labeled for ChAT in the stratified olfactory bulb (OB). However, varicose axons were stained (Figures 1A, 2A) in both the OB and the olfactory tract (Figure 2A insert).

Dorsal and ventral telencephalon

Scarce ChATir neuronal bodies of bipolar appearance were only observed in the lateral nucleus of the ventral telencephalic area (Vl) (Figure 1B). The carp telencephalon also showed a moderate cholinergic innervation. ChATir varicose fibers were scarce in the dorsal regions and more abundant in the medial region of the ventral telencephalon. Scarce ChATir axons were also present in the commissural region. In the ventral nucleus of the ventral telencephalic area (Vv), ChATir varicose axons were interspersed between unlabeled neurons. Bouton-like contacts were seen bordering the immunonegative perikarya (not shown). We could not follow the labeled fibers to their cellular origin but some of them, at least, seemed to be continuous with ChATir neurons labeled in the Vl (Figure 1B).

Preoptic region

Abundant varicose axons were immunolabeled for ChAT in the entire preoptic region, from the anterior to the posterior region (PPa, PPp, PM) and in the suprachiasmatic nucleus (SCN) (Figures 1 C-E and 2 C,D). A moderate number of neuronal bodies were also labeled for ChAT in the periventricular PPa, PPp (Figure 1 C-E), in the SCN and magnocellular preoptic nucleus (PM) (Figure 1D).

Hypothalamus

Thin ChATir varicose fibers were observed in both the dorsal (Hd) and the ventral (Hv) periventricular hypothalamus, around the laterocaudal ventricular recess (LR) of the inferior lobe (IL) and from the anterior and lateral tuberal region (NAT, NLT) to the caudal hypothalamus (Figures 1 E,F,H and 2 E-G). No neuronal perikarya were labeled for ChAT in the hypothalamus.

Posterior tuberculum

Varicose axons were labeled for ChAT in the posterior tuberculum. In particular abundant labeled axons have been observed in the periventricular nucleus (TPp) and the posterior tuberal nucleus (PTN) (Figures 1F and 2G). In addition, ChATir varicose fibers appeared to outline the medial preglomeral nucleus (PGm) (Figures 1G and 2H).

Prethalamus

ChAT immunoreactive material was distributed in the prethalamus (VL, VM): in nerve fibers and terminal varicosities surrounding unlabeled perikarya (Figures 1E and 3A arrows).

Dorsal thalamus, epithalamus and epiphysis

No ChAT immunoreactivity was observed in the dorsal thalamus whereas ChATir cells and fibers were abundant in the habenular nuclei (dorsal, Had, and ventral, Hav), especially in the Hav (Figures 1E and 3 A,B,E). The superficial layer of the pineal organ was also labeled for ChAT. Moreover, immunoreactive axons were seen in the fiber tract connecting the pineal organ with the Hav and in the commissura habenularis (Chab) (Figure 3B). The fasciculus retroflexus (habenulo-interpeduncolar tract, fr) was also intensely labeled for ChAT (Figure 1G and 3 C,D).

Synencephalon and pretectum

In the carp synencephalon, several large multipolar neurons were labeled for ChAT in the nucleus of the medial longitudinal fascicle (Nmlf) (Figures 1G and 3 C,D). No ChATir neuronal bodies were found in the superficial, central and periventricular pretectum. However, thick ChATir fibers of the

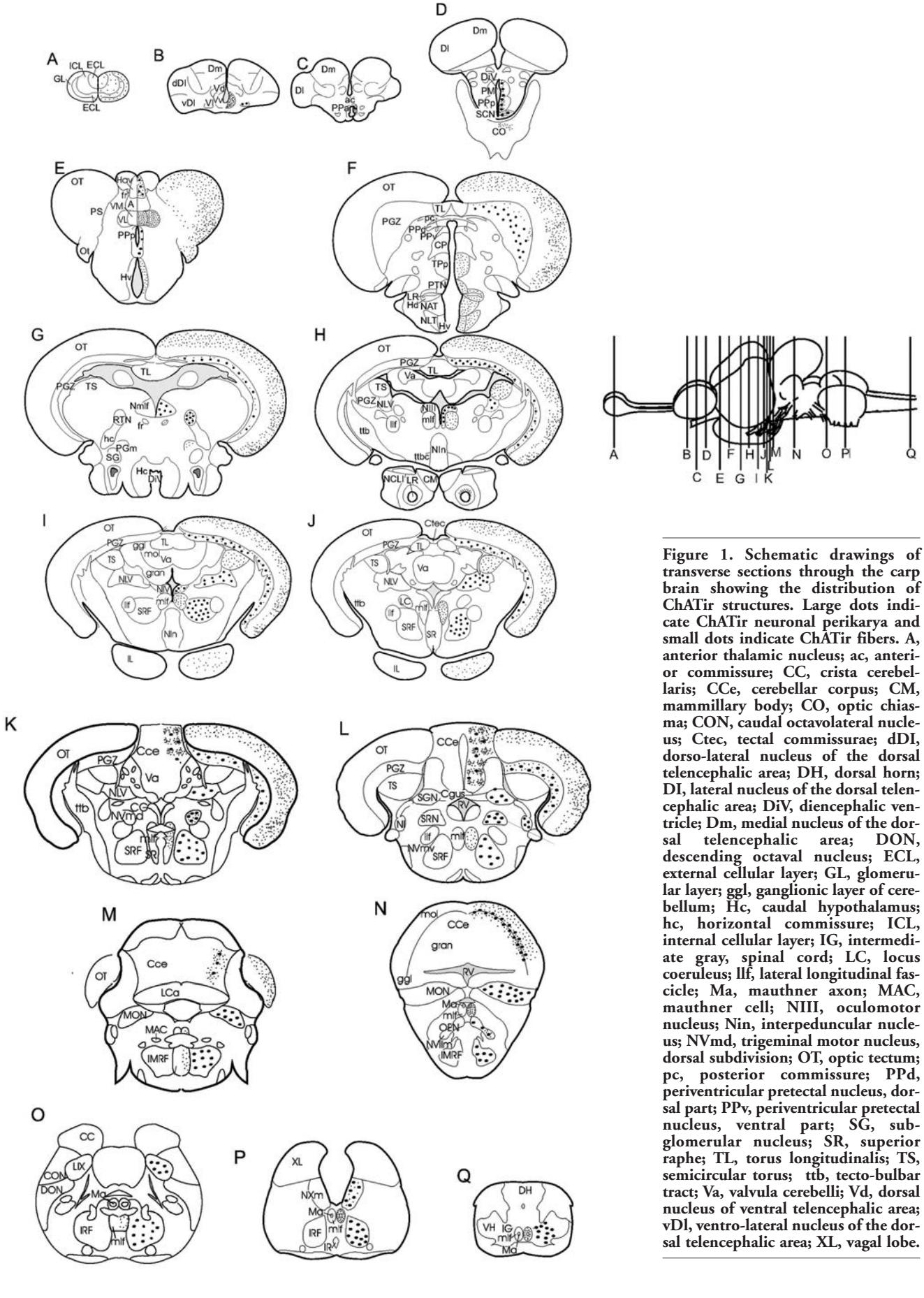

Figure 1. Schematic drawings of transverse sections through the carp brain showing the distribution of ChATir structures. Large dots indicate ChATir neuronal perikarya and small dots indicate ChATir fibers. A, anterior thalamic nucleus; ac, anterior commissure; CC, crista cerebellaris; CCe, cerebellar corpus; CM, mammillary body; CO, optic chiasma; CON, caudal octavolateral nucleus; Ctec, tectal commissurae; dDl, dorso-lateral nucleus of the dorsal telencephalic area; DH, dorsal horn; Dl, lateral nucleus of the dorsal telencephalic area; DiV, diencephalic ventricle; Dm, medial nucleus of the dorsal telencephalic area; DON, descending octaval nucleus; ECL, external cellular layer; GL, glomerular layer; ggl, ganglionic layer of cerebellum; Hc, caudal hypothalamus; hc, horizontal commissure; ICL, internal cellular layer; IG, intermediate gray, spinal cord; LC, locus coeruleus; llf, lateral longitudinal fascicle; Ma, mauthner axon; MAC, mauthner cell; NIII, oculomotor nucleus; Nin, interpeduncular nucleus; NVmd, trigeminal motor nucleus, dorsal subdivision; OT, optic tectum; pc, posterior commissure; PPd, periventricular pretectal nucleus, dorsal part; PPv, periventricular pretectal nucleus, ventral part; SG, subglomerular nucleus; SR, superior raphe; TL, torus longitudinalis; TS, semicircular torus; ttb, tecto-bulbar tract; Va, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; vDl, ventro-lateral nucleus of the dorsal telencephalic area; XL, vagal lobe.

optic tract appeared to profusely innervate the superficial pretectal region (PS) (Figure 1E and 3E).

Mesencephalon

ChATir neuronal bodies were observed in both the mesencephalic tectum and the ventral tegmentum. In the optic tectum, a large number of neurons were immunoreactive for ChAT. These neurons have their perikarya in the periventricular grey zone (PGZ) and give rise to a single apical process extending in the superficial layers of the optic tectum (Figures 1 F-L and 3F). Varicose axons were also ChAT-labeled in the superficial layers of the optic tectum being more abundant in the stratum opticum (SO) than in the superficial marginal layer (SM) (Figure 3F). In addition, a profuse ChATir innervation was seen in the torus semicircularis (Figure 1H). In the midbrain tegmentum, immunopositive ChAT neurons were found in the rostral tegmental nucleus (RTN) (Figures 1G and 3C), in both the dorsal and the ventral subdivision of the oculomotor nucleus (Nlllsd and Nlllsv) (Figures 1H and 3G) and in the nucleus lateralis valvulae (NLV) (Figures 1 H-K and 3H).

Isthmus

Neuronal bodies and processes were labeled for ChAT in the trochlear nucleus (NIV) (Figure 1I and 4A). Moreover, a large number of monopolar neurons strongly immunoreacted for ChAT in the nucleus isthmi (NI) (Figures 1L and 4B) and the secondary gustatory nucleus (SGN) (Figures 1L and 4 B,C). Other neuronal bodies positive for ChAT could be localized in the superior reticular formation (SRF) (Figure 1I-L), extending from the midbrain to the isthmic tegmentum (Figure 4B).

Cerebellum

The molecular layer of the carp cerebellum was diffusely labeled for ChAT whereas the immunolabeling was scarce in the granule cells layer (Figures 1N and 4D). The immunoreactive material in the molecular layer had a granular appearance that might be referred to a large accumulation of terminal varicosities. ChAT labeling was more well-defined in other cerebellar regions, as at the boundary between the molecular and granular layer and in the proximal region of the molecular layer where numerous ChATir cell bodies were seen (Figure 4 D,E). Labeled neurons showed a single dendrite-like process extending towards the granular layer (Figure 4D insert, 4E) and ChAT immunoreactivity was observed in terminal

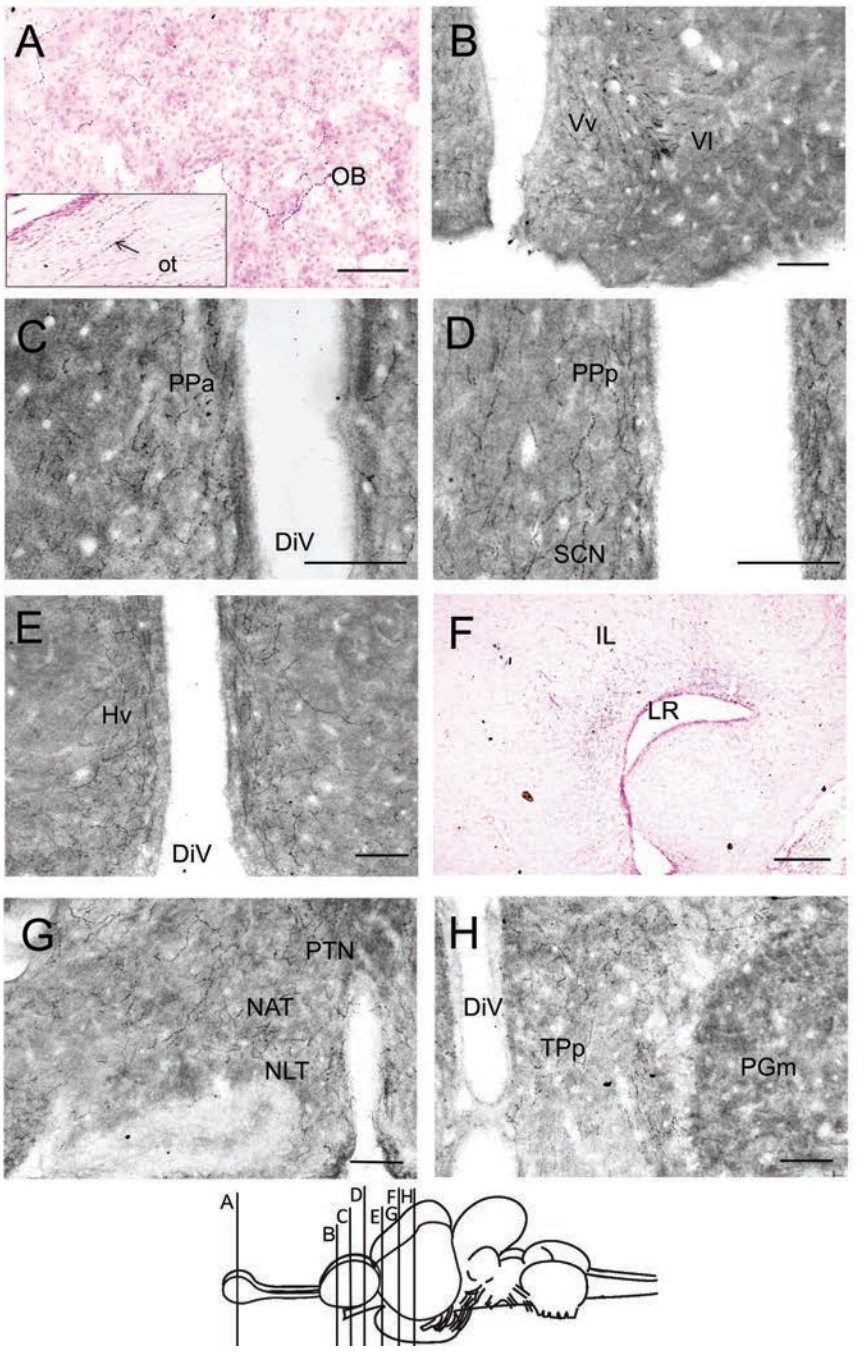

Figure 2. Distribution of ChAT immunoreactivity in transverse sections through the carp brain. The level of the sections is indicated in the small diagram of the lateral view of the brain at the bottom of the page. Varicose ChATir axons in the olfactory bulb (OB) (A) and in the olfactory tract (ot) (A insert, arrow). ChATir cells and fibers in the ventral telencephalic area, lateral (Vl) and ventral (Vv) nucleus (B). ChATir varicose fibers in the parvocellular preoptic nucleus anterior part (PPa) (C), posterior part (PPp) and in the suprachiasmatic nucleus (SCN) (D). Thin ChATir varicose fibers in the ventral zone of periventricular hypothalamus (Hv) (E) and around the laterocaudal ventricular recess (LR) of the inferior lobe (IL) (F). Abundant labeled axons in the anterior and lateral tuberal region (NAT, NLT) and in the posterior tuberal nucleus (PTN) (G). Several positive axons in the periventricular nucleus (TPp) and in the medial preglomeral nucleus (PGm) (H). DiV, diencephalic ventricle. Panels A and F were counterstained with Nuclear Fast Red Solution. Scale bars: 100 μm.

varicosities of thin labeled axons contacting their perikarya and dendritic processes (Figure 4D insert, arrows). Thin axons apparently coursed in parallel from the granular layer. Among them, few varicose axons were also labeled for ChAT (Figure 4D insert). A substantially similar type of labeling was observed in the three main subdivisions of the cerebellum, the corpus (CCe) and the lobus caudalis (LCa) (Figures 1 K-N and 4 D,E).

Medulla oblongata

In the medulla oblongata of the carp, ChATir neuronal perikarya and fibers were observed in the dorsal and ventral motor nuclei of the trigeminal and facial nerves (Figure 1 K,N, 4F and 5 A,B), in the abducens nucleus (Figure 4G), in the motor zone of the vagal lobe and in the glossopharyngeal/vagal motor nuclei (Figures 1P and 5 C,D). The medial longitudinal fascicle (mlf) was also immunoreactive for ChAT (Figures 1 H-Q and 4A). Mauthner cell bodies were not stained for ChAT, but Mauthner axons (Ma) often appeared slightly labeled (Figure 5 A-F arrows), with a decreasing or lacking immunoreactivity in the caudal oblongata and in the spinal cord (Figure 5E). ChAT positive neurons were present in the sensory medial octavolateralis nucleus (MON) (Figures 1 M,N and 4H) and in the octavolateralis efferent neurons (OEN), that is localized at the midline close to the facial motor nucleus (Figures 1N and 5B). Positive neurons were also observed in the intermediate and inferior reticular formation (IMRF, IRF) (Figures 1 M-P and 5 B-D) and in the supracommissural Cajal nucleus (*not shown*).

Rostral spinal cord

Large primary and smaller secondary motoneurons were stained by ChAT in the ventral horn (VH) of the carp spinal cord, in a dorsomedial and ventrolateral position respectively (Figure 1Q and 5E).

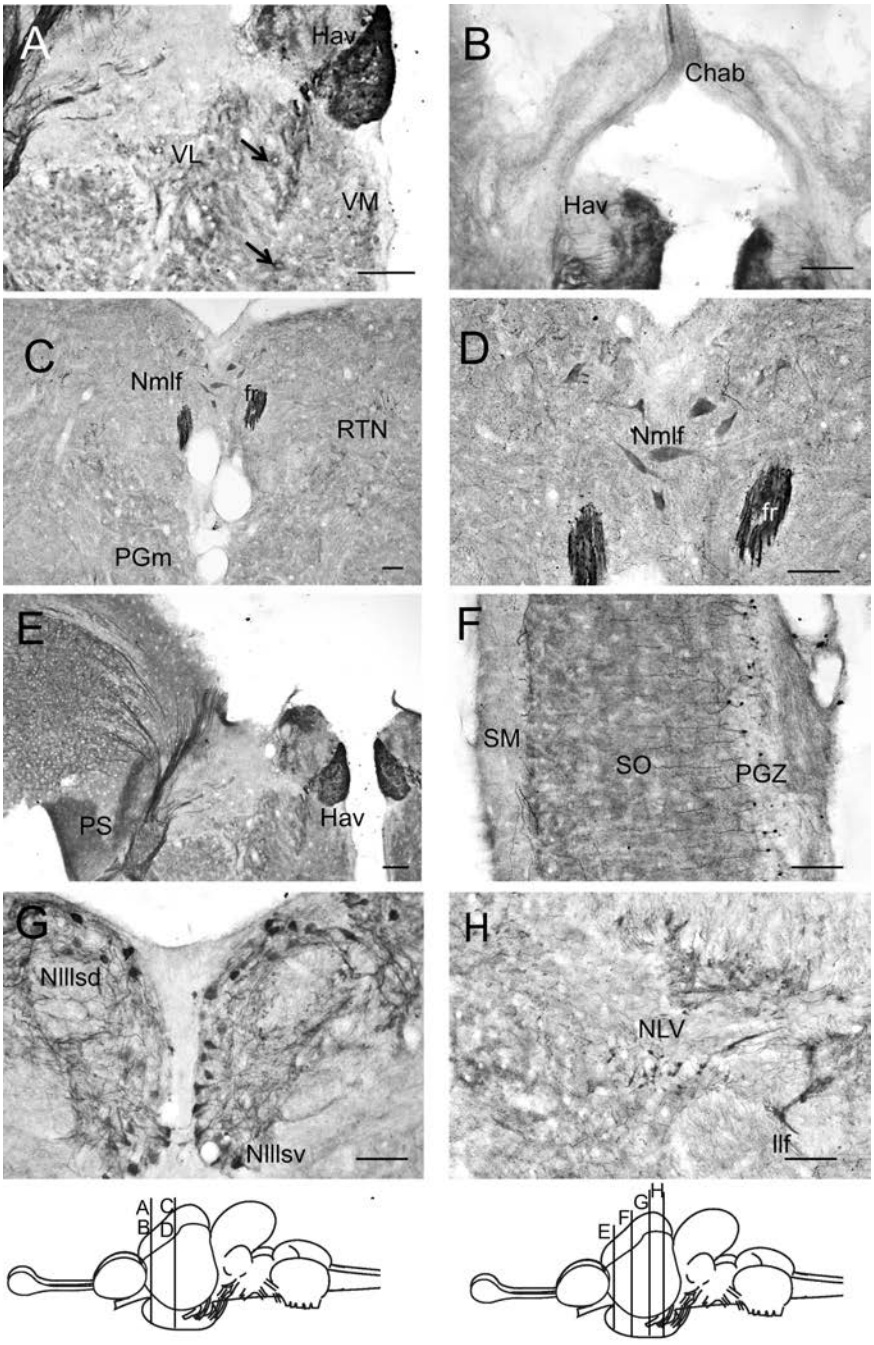

Figure 3. ChAT immunoreactive elements in transverse sections through the carp brain. The level of the sections is indicated in the small diagrams of the lateral view of the brain at the bottom of the page. ChATir fibers in the ventrolateral and ventromedial thalamic nucleus (VL, VM) and terminal varicosities surrounding unlabeled perikarya (arrows) (A). Strong ChATir cells and fibers in the ventral habenular nucleus (Hav) (A, B, E) and thin positive fibers in the commissura habenularis (Chab) (B). Strong ChAT immunoreactivity in the fasciculus retroflexus (habenulo-interpeduncolar tract, fr) and several large ChATir multipolar neurons in the nucleus of the medial longitudinal fascicle (Nmlf) (C and detail D). Thin ChATir axons of the optic tract innervating the superficial pretectal region (PS) (E). ChATir neuronal perikarya in the periventricular grey zone (PGZ) with the apical dendrite extending in the superficial layers of the optic tectum. Abundant ChATir varicose axons in the stratum opticum (SO), but scarce in the superficial marginal layer (SM) (F). ChATir cells in both the dorsal and the ventral subdivision of the oculomotor nucleus (NIIIsd and NIIIsv) (G). ChATir elements in the nucleus lateralis valvulae (NLV) (H). Scale bars: 100 μm.

Discussion

The organization of cholinergic system has been studied in representatives of all vertebrate taxa by means of ChAT IHC that is recognized as the most reliable assay to reveal cholinergic neurons and fibers. In this study, ChAT IHC is used to describe the distribution of putative cholinergic cell groups and axons in the brain and the rostral spinal cord of *Cyprinus carpio* and this distribution is compared to that studied in other teleosts (*Anguilla anguilla,*[65]

Phoxinus phoxinus,[56] *Porichthys notatus,*[67] *Oncorynchus mykiss* and *Salmo trutta,*[66] *Carassius auratus,*[58,59,61] *Hemicromis lifalili* and *H. guttatus,*[68] *Danio rerio,*[19,63,64] *Tinca tinca*[62]) and in mammals.[24-36] ChAT distribution in the teleost species is summarized in Table 1.

On describing the location of the major ChATir structures in the carp, this paper provides new data for understanding the evolutionary diversification of cholinergic systems in teleosts. Moreover, the comparison of cholinergic system in the carp with that of mammals sustains the possible use of this fish as vertebrate model for studying neurological disorders to cholinergic system known as synucleinopathies.

ChAT distribution in the carp and comparison to other teleosts

Olfactory bulb and telencephalon

The carp OB is devoid of ChATir neuronal cell bodies but it receives cholinergic innervation by varicose axons coursing in the olfactory tract. ChATir structures were not described in all the species studied so far (Table 1). Such discrepancy may be due to the scarce cholinergic innervation of the teleost OB, which makes it difficult to identify ChATir structures. Indeed, different studies in zebrafish disagreed in revealing ChAT immunoreactivity in the OB (positive results[64] *versus* negative results[19,63]).

A possible source for the cholinergic input to the fish OB is the terminal nerve ganglion, as suggested in zebrafish.[64] However, since the OB is reciprocally connected with the ventral telencephalon in teleosts,[67] ChATir fibers in the carp OB may also represent afferent projections from cholinergic neurons located in the ventral telencephalon (see below). In the carp telencephalon, ChATir neurons are scarce and limited to the ventrolateral area (Vl), whereas the dorsal pallium is devoid of cholinergic cells. Cholinergic cell bodies are restricted to the ventral telencephalon in almost all the species studied so far (Table 1). Data in the carp thus confirms that the ventral telencephalon contains basal cholinergic systems in ray-finned fishes.[71,72]

The ventral telencephalon of the carp also receives a moderate cholinergic innervation that might originate from cholinergic neurons of posterior brain regions (see below) whereas the cholinergic innervation to the dorsal telencephalon is very sparse as reported in other teleosts.

Preoptic region and diencephalon

The preoptic region of the carp contains ChATir in the periventricular parvocellular and magnocellular nuclei. Moreover, the entire preoptic region and the periventricular hypothalamus are innervated by ChATir

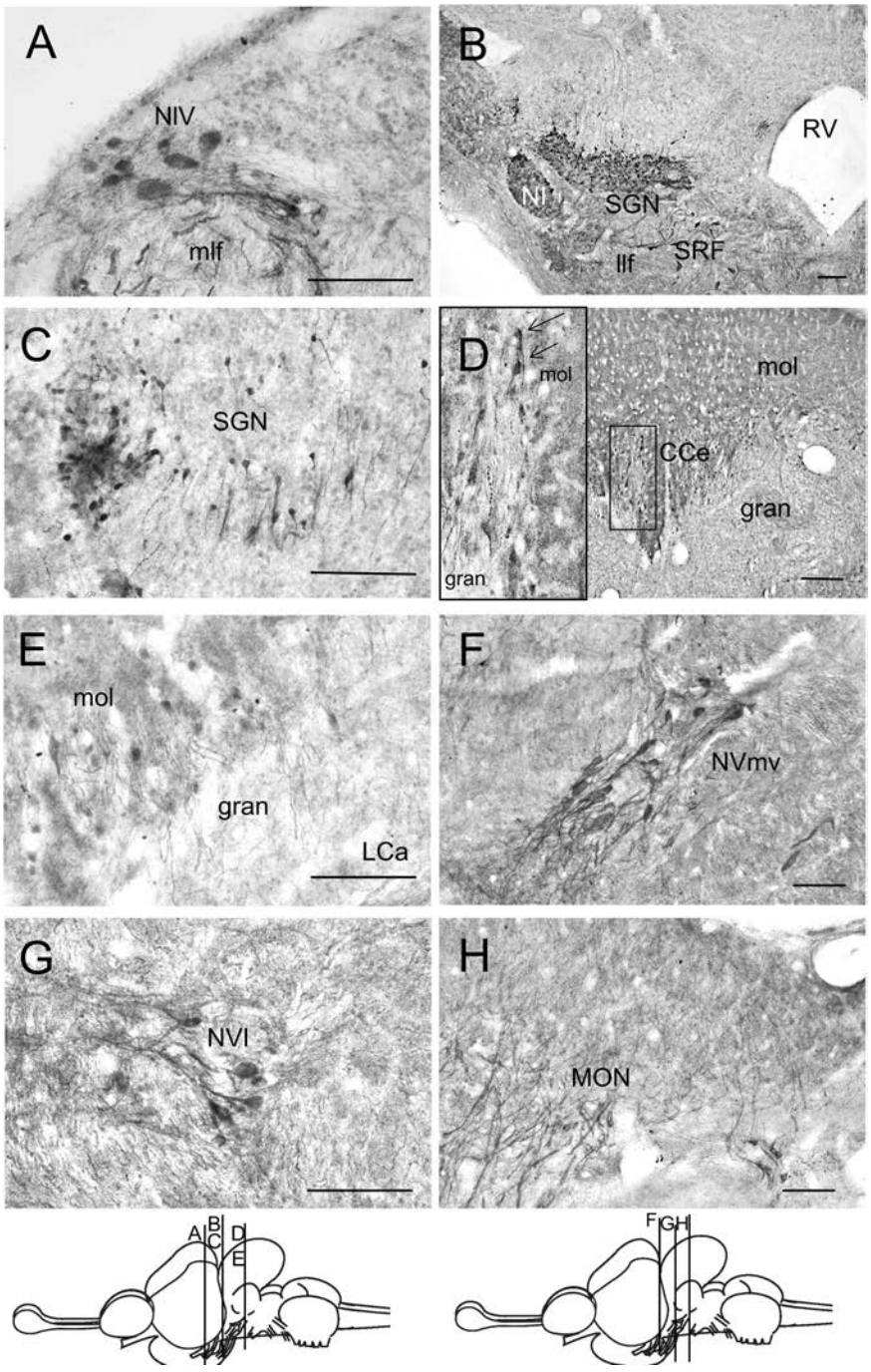

Figure 4. Immunoreactivity for ChAT in transverse sections of the carp brain. The level of the sections is indicated in the small diagram of the lateral view of the brain at the bottom of the page. ChATir in neuronal bodies and fibers in the trochlear nucleus (NIV) and ChATir fibers in the medial longitudinal fascicle (mlf) (A). Some ChATir neuronal bodies in the superior reticular formation (SRF) (B). Numerous and intensely ChATir neurons in the nucleus isthmi (NI) (B) and in the secondary gustatory nucleus (SGN) (B, C). Intense ChAT positivity in the molecular layer of the cerebellum (mol), but scarce in the granule cells layer (gran) (D). ChATir perikarya showed a single dendrite-like process extending towards the granular layer (arrows) (D insert, arrows). Several ChATir neurons between the molecular and granular layer (E). ChATir neuronal perikarya and fibers in the ventral subdivision of trigeminal motor nucleus (NVmv) (F). ChATir neurons in the abducens nucleus (NVI) (G) and in the medial octavolateralis nucleus (MON) (H). llf, lateral longitudinal fascicle; RV, rombencephalic ventricle. Scale bars: 100 μm.

varicose axons. The preoptic region and the hypothalamus receive a dense cholinergic innervation in all teleosts and contain ChATir neuronal cell bodies in most ciprinids[19,58,63] and in trout.[66] However, few positive neurons were described in the European minnows *Phoxinus phoxinus*[56] and no immunoreactive cells in the midshipmen *Porichthys notatus*.[67] In most teleosts, magnocellular preoptic neurons projecting to the neurohypophysis are cholinergic and the same was reported in lampreys,[73] dogfish,[49] sturgeon,[50] dipnoans[53] and polipterids.[51] These data suggest that the cholinergic nature of the preoptic-hypothalamic neurosecretory system is conserved in all major fish radiations and only secondarily lost in some species.

Cholinergic cells are not present in the ventral (prethalamus) and dorsal thalamus or in the posterior tuberculum of the carp, but these regions receive abundant ChATir varicose fibers. Differently, habenular nuclei, the fasciculus retroflexus and the pineal organ are strongly immunoreactive for ChAT. The same was not found in all teleosts (Table 1).

The diencephalon is the brain region of teleosts where the presence of cholinergic neurons is more variable among species and this variability seems to be largely independent from their systematic position. Given that cholinergic habenular neurons were reported in lampreys,[74] elasmobranchs,[49] primitive actinopterygians (condrosteans,[50] polypterids,[5] holosteans,[52] dipnoans[53]) we agree with Clemente *et al.*[63] who suggested that cholinergic cells in the habenular complex are a plesiomorphic feature of vertebrates that has been secondarily modified within the radiation of teleosts.

Pretectum

The presence of cholinergic cell bodies also varies in the pretectum of teleosts (Table 1). In the carp, cholinergic neuronal somata are exclusively observed within the synencephalon, in the nucleus of the medial longitudinal fascicle (Nmlf). However, the superficial pretectal region is richly innervated by thick ChATir fibers from the optic tract. Cholinergic cells have been also reported in the Nmlf in goldfish,[58] but not in zebrafish[63] and trout.[62] However, the zebrafish Nmlf is contacted by ChATir terminals and all the pretectal regions are innervated by ChATir fibers as observed in the carp. The pretectum contains few ChATir neurons but it is richly innervated by ChATir fibers also in non-cyprinids.[66] In the cichlid *Hemichromis,* both the pretectal magnocellular and the corticalis nuclei contain ChATir neurons and it has been shown that secondary visual projections from the pretectum to the hypothalamus are generally cholinergic in fish displaying complex visually guided behaviors.[68]

In spite of species differences, cholinergic pathways are probably involved in the functional modulation of the superficial pretectum in all fish. Indeed, ChATir neurons have been reported in the dogfish pretectum[49] (including in the Nmlf) and cholinergic fibers are abundant in the pretectal region of condrosteans,[50] polipterids,[51] holosteans[52] and dipnoans.[53]

Midbrain optic tectum

Within the carp optic tectum, a large number of monopolar neurons were strongly immunoreative for ChAT in the periventricular grey zone. These were the single neuronal types labeled for ChAT in the stratified tectum. Cholinergic periventricular neurons have been reported in the optic tectum of all teleosts and in a primitive actinopterygian (*Polypterus*[51]). However, ChATir cells are not present in the optic tectum of lampreys,[74] elasmobranchs,[49] condrosteans[50] and dipnoans.[53] These variations indicate that the presence of cholinergic cells in the optic tectum is a derived feature of the vertebrate brain that appeared independently in the actinopterygians and possibly in other vertebrate lines.

The optic tectum of the carp also receives varicose ChATir fibers in the stratum opticum which is an afferent layer receiving minor projections from the

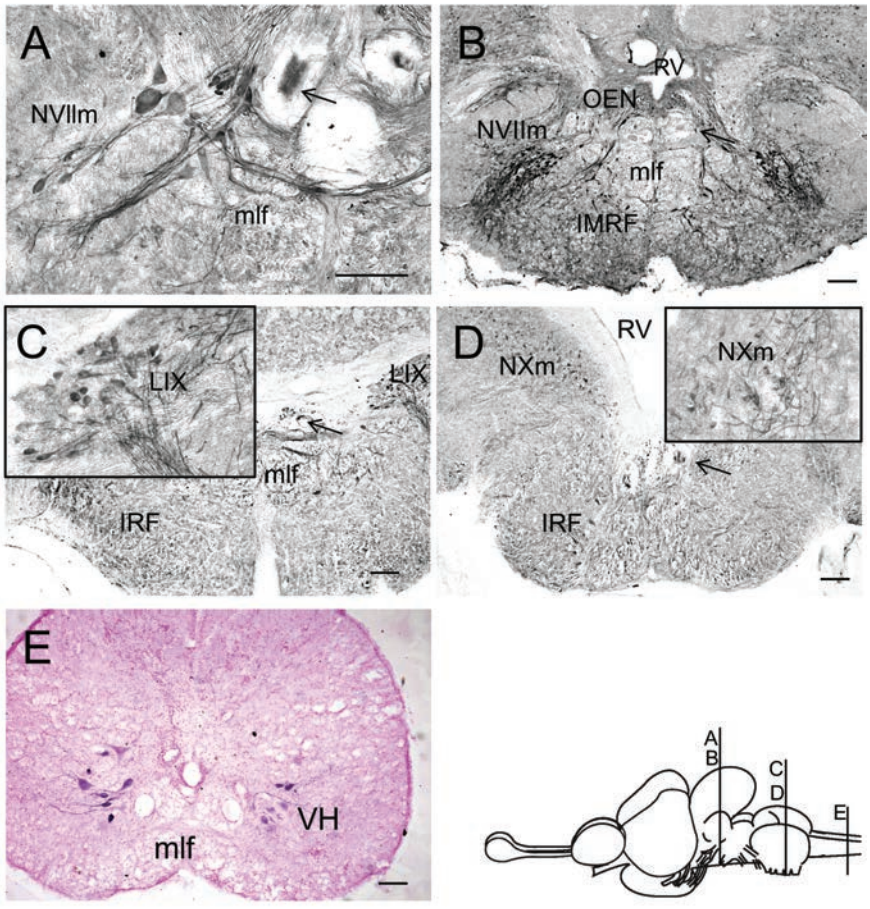

Figure 5. ChAT immunoreactivity in transverse sections of the carp brain. The level of the sections is indicated in the small diagram of the lateral view of the brain at the bottom of the page. The Mauthner axons (arrows) often appear slightly ChAT positive (A-E). ChATir perikarya and fibers in the facial motor nucleus (NVIIm) (A) and in the longitudinal fascicle (mlf) (A- C). ChATir elements in the octavolateralis efferent neurons (OEN) and in the intermediate reticular formation (IMRF) (B). ChATir neurons in the glossopharyngeal lobe (LIX) (C, insert) and in the vagal motor nucleus (NXm) (D insert). ChATir neurons in the inferior reticular formation (IRF) (D). Large ChATir primary and small secondary motoneurons in the ventral horn (VH) of the carp spinal cord (E). Panel E has been counterstained with Nuclear Fast Red Solution. Scale bars: 100 μm.

retina.[57,75] Cholinergic projections to the optic tectum have been traced in goldfish from the nucleus isthmi and the nucleus reticularis mesencephalic[61] and this cholinergic nucleus may be the source, in part at least, of the cholinergic innervation to the optic tectum of the carp.

Cerebellum

The carp cerebellum was reactive for ChAT. The immunoreactivity was observed in neuronal somata, axons and terminal varicosities in all the main subdivisions of the cerebellum. For their shape and position, the immunoreactive neurons of the carp cerebellum resembled Golgi-like cells. However, the cellular identity of ChATir cerebellar neurons remains to be determined.

The presence of ChATir neurons in the cerebellum is a feature observed in few teleost species (Table 1). They were detected in the ganglionic layer in the goldfish cerebellum[58] and in the granule layer in *Porichthys*.[67] By contrast, ChATir cells were not detected in the cerebellum of *Phoxinus,*[56] zebrafish,[63] tench[62] and trout.[66] Cerebellar ChATir cells have not been detected in other fish, as primitive actinopterygians and dipnoans (data summarized by Lopez[51]), with the exception of dogfish.[49] Available data thus suggest that the cholinergic circuitry in the cerebellum appeared several times during the evolution and is maintained in only few species.

Brainstem and spinal cord

Immunopositive ChAT neurons are present in the RTN and the NLV in the carp. A cholinergic RTN nucleus was also identified in zebrafish[19] and it probably corresponds to the nucleus of the rostral mesencephalic tectum (NRMT) described in goldfish. The NRMT was found to receive inputs from the vagal lobe and projecting them to the optic tectum.[76] Therefore, the NRMT/RTN may be considered relay centers for gustatory inputs from the vagal lobe to higher brain centers in cyprinids and perhaps in all teleosts.

ChATir neuronal cell bodies and fibers are abundant in the NI and in the SGN of the carp. The cholinergic nature of the NI is confirmed in all teleosts investigated so far and its projections to the optic tectum have been traced in goldfish, as already reported.[61] A ChATir homologue to the NI has been identified in polypterids,[51] chondrosteans[50] and dipnoans.[53] Thus, the presence of cholinergic isthmic neurons projecting to the midbrain tectum is a conserved feature of the brain in both actinopterygians and sarcopterygians bony fish.

A cholinergic SGN has been described in most cyprinids, oldfish,[58,61] zebrafish,[19,63] tench,[62] and in eel[65] and trout.[66] However, it was not identified in the minnow[56] and midshipman.[67] Main targets for the cholinergic SGN in teleosts are the preglomerular tertiary gustatory nucleus,[77] the torus lateralis[65] and the inferior lobe of the hypothalamus.[66].These findings suggested that acetylcholine is involved in the processing of taste and general visceral information in teleosts and this suggestion is confirmed in the carp.

ChATir neurons are also abundant in the superior, intermediate and inferior reticular formation in the carp. This evidence is confirmed in almost all teleosts investigated so far[56,58,62,67] with a difference in zebrafish,[63] which apparently lacks cholinergic neurons in the reticular formation of the oblongata. According to Clemente,[62] the cholinergic system in zebrafish has undergone a partly divergent evolution compared to other cyprinids. Present data agree with his view, showing several differences in cholinergic distribution between zebrafish and carp. Tracer studies have demonstrated that cholinergic neurons of the superior reticular nucleus (SRN), project to the basal forebrain in some teleosts.[66,78] Based on these data, ChATir reticular neurons may be one source of the cholinergic input to the ventral telencephalon in the carp. In the carp oblongata, Mauthner cell bodies were not labeled for ChAT. However, Mauthner axons showed a moderate staining that decreased or even disappeared caudally. Mauthner neurons are ChAT negative in other cyprinids[63,79] (zebrafish, goldfish) and in polypterids,[51] although indirect evidence suggest that they may use Ach as a neurotransmitter.[80,81] The extremely large size of the Mauthner cell soma might be responsible for the failure of ChAT staining to reveal immunoreactive products if they are not densely accumulated in the cell body. However, the cholinergic nature of Mauthner cells needs to be investigated in more species. In the carp brainstem, ChATir neurons are abundant in the motor nuclei of cranial nerves from III to X. The cholinergic nature of the cranial nerve motor nuclei has been confirmed in all actinopterygians studied so far[19,50,52,56,58,65,67] and it represents one of the most conservative cholinergic cell groups in all vertebrate brains. In the rostral spinal cord of the carp, large and small multipolar neurons immunoreacted for ChAT in the somatomotor columns of the VH. ChATir neurons have been described in the spinal cord motor columns in all fish.[55]

Comparison with mammals

As in the carp, the OB lacks cholinergic neurons in mammals. However, it receives cholinergic innervation from secondary olfactory areas as the olfactory tubercle which contain ChATir neurons.[35] The ventral telencephalon contains cholinergic neurons in the carp as in other teleosts. It is considered homologous to the subpallium of the evaginated brains containing basal cholinergic systems in all the vertebrate taxa.[71-75] In particular, tract tracing studies in zebrafish suggested that Vv and Vl belong to the septum and Vl is the homologous of septal nuclei sending cholinergic projections to the dorsal telencephalon in amniotes.[75] The telencephalic pallium does not contain cholinergic neurons in the carp as reported in most teleosts. Cholinergic cells are present in the cortex of rat[82-84] and mouse,[85] but they are lacking in guinea pig,[25] cat and dog.[24,86,87]

Furthermore, ChATir neurons have been identified in the fetal monkey cerebral cortex[88] but not in the cortex of adult primates,[28,89] including humans.[37,39] The presence of cholinergic neurons in the pallial regions may be a feature acquired by mammals during the evolution but it is not shared by all mammals or amniotes.[63]

ChATir cell populations were described in the preoptic region of monotremes[29] mouse and rat[31] but are not conserved in all amniotes. Cholinergic habenular neurons were reported in mammals,[28,35,69] including humans[39] and most tetrapods.[42,45,48] Cholinergic cells are not present in the pretectum and mesencephalic tectum of mammals.[90] ChATir cells have been detected in the cat cerebellum,[76] in which they were identified as Golgi-like neurons. This might be a shared feature between carp and mammals. Ascending projections from NI to the midbrain have been found in all tetrapods, including mammals.[51] Thus, cholinergic isthmic neurons projecting to the midbrain tectum are a brain feature that has been maintained from fish to mammals.[51,55] The SRN is cholinergic in the carp, as in other species. The SRN in teleosts projects to the subpallium[78] and is considered homologous to the peduncolopontine nucleus of the mesencephalic locomotor region of mammals projecting to the caudate-putamen.[19] Motor neurons of the cranial nerve nuclei represent one of the most conservative cholinergic cell groups in all the vertebrate brains, as well as the rombencephalic reticular formation. The distribution of cholinergic structures, as summarized in Table 1, shows that major species differences are present in epithalamus, dorsal thalamus, posterior

tuberculum, pretectum and cerebellum of teleosts. Other regions, *i.e.* preoptic region, optic tectum, midbrain tegmentum, medulla oblongata and spinal cord, exhibit similar cholinergic cell populations in all species. This variability is not strictly related to the systematic position, given that different results were reported in closely related species, as among cyprinids. The organization of cholinergic system in the carp is quite similar to that described in *Carassius* and differs in some respects to that of zebrafish which is the model fish widely used for vertebrates

The cholinergic system of the carp shows several similarities to that of mammals that encourages the use of this fish as new model for basic biomedical research on α-synuclein pathologies of the cholinergic system.

References

1. Bellanger C, Dauphin F, Chichery MP, Chichery R. Changes in cholinergic enzyme activities in the cuttlefish brain during memory formation. Physiol Behav 2003;79:749-56.
2. Fibiger HC, Damsma G, Day JC. Behavioral pharmacology and biochemistry of central cholinergic neurotransmission. Adv Exp Med Biol 1991;295:399-414.
3. Rasmusson DD. The role of acetylcholine in cortical synaptic plasticity. Behav Brain Res 2000;115:205-18.
4. Semba K. Multiple output pathways of the basal forebrain: organization, chemical heterogeneity, and roles in vigilance. Behav Brain Res 2000;115:117–141.
5. Semba K. Phylogenetic and ontogenetic aspects of the basal forebrain cholinergic neurons and their innervation of the cerebral cortex. Prog Brain Res 2004; 145:3–43.
6. Everitt BJ, Robbins TW. Central cholinergic systems and cognition. Ann Rev Psychol 1997;48:649-84.
7. Sarter M, Bruno JP. Cognitive functions of cortical acetylcholine: Towards a unifying hypothesis. Brain Res Rev 1997;23:28-46.
8. Barraco DA, Eisenstein EM. Effects of pre- training administration of scopolamine on learning and retention in the cockroach, P. americana. Pharmacol Biochem Behav 1984;20:479-81.
9. Mpitsos GJ, Murray TF, Creech HC, Barker, DL. Muscarinic antagonist enhances one-trial food-aversion learning in the mollusc Pleurobranchea. Brain Res Bull 1988;21:169-179.
10. Gauthier M, Canolozano V, Zaoujal A, Richard D. Effects of intracranial injections of scopolamine on olfactory conditioning retrieval in the honeybee. Behav Brain Res 1994;63:145-9.
11. Pai TP, Chen CC, Lin HH, Chin AL, Lai JS, et al. Drosophila ORB protein in two mushroom body output neurons is necessary for long-term memory formation. Proc Natl Acad Sci USA 2013;110:7898-903.
12. Davies P, Maloney AJ. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 197;62: 1403.
13. Perry EK, Gibson PH, Blessed G, Perry RH, Tomlinson BE. Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. J Neurol Sci 1977;34:247-65.
14. Bartus RT, Dean RL, Beer B, Lippa AS. The cholinergic hypothesis of geriatric memory dysfunction. Science 1982;217: 408-414.
15. Lewy FH. Paralysis agitans. In: Pathologische Anatomie. Handbuch der Neurologie. M. Lewandowsky M, Editor. Springer-Verlag, Berlin; 1912; pp 920-33.
16. Spillantini MG, Schmidt ML, Lee Vm, Troianowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997;388:839-40.
17. Teipel SJ, Meindl T, Grinberg L, Grothe M, Cantero JL, Reiser MF, et al. The cholinergic system in mild cognitive impairment and Alzheimer's disease: an in vivo MRI and DTI study. Hum Brain Mapp 2011;32:1349-62.
18. Ray NJ, Bradburn S, Murgatroyd C, Toseeb U, Mir P, Kountouriotis JK, et al. In vivo cholinergic basal forebrain atrophy predicts cognitive decline in the novo Parkinson's disease. Brain 2018;141:165-76.
19. Mueller T, Vernier P, Wulliman MF. The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish Danio rerio. Brain Res 2004;1011:156-169.
20. Flinn L, Bretaud S, Lo C, Ingham PW, Bandmann O. Zebrafish as a new animal model for movement disorders. J Neurochem 2008;106:1991-7.
21. Kalueff AV, Echevarria DJ, Stewart AM. Gaining translational momentum: more zebrafish models for neuroscience research. Prog Neuropsychopharmacol Biol Psychiatry 2014;55:1-6.
22. Toni M, Cioni C. Fish synucleins: An update. Mar Drugs 2015;13:6665-86.
23. Vaccaro R, Toni M, Casini A, Vivacqua G, Yu S, D'este L, et al. Localization of α-synuclein in teleost central nervous system: immunohistochemical and Western blot evidence by 3D5 monoclonal antibody in the common carp, Cyprinus carpio. J Comp Neurol 2015; 523:1095-124.
24. Kimura H, McGeer PL, Peng JH, McGeer EG. The central cholinergic system studied by choline acetyltransferase immunohistochemistry in the cat. J Comp Neurol 1981;200:151-201.
25. Maley BE1, Frick ML, Levey AI, Wainer BH, Elde RP. Immunohistochemistry of choline acetyltransferase in the guinea pig brain. Neurosci Lett 1988;84:137-42.
26. Motts SD, Slusarczyk AS, Sowick CS, Schofield BR. Distribution of cholinergic cells in guinea pig brainstem. Neuroscience 2008;154:186-95.
27. Gravett N, Bhagwandin A, Fuxe K, Manger PR. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the rock hyrax, Procavia capensis. J Chem Neuroanat 2009;38:57-74.
28. Mesulam M-M, Mufson EJ, Levey AI, Wainer BH. Atlas of cholinergic neurons in the forebrain and upper brainstem of the macaque based on monoclonal choline acetyltransferase immunohistochemistry and acetylcholinesterase histochemistry. Neuroscience 1984;12:669-86.
29. Manger PR, Fahringer HM, Pettigrew JD, Siegel JM. The distribution and morphological characteristics of cholinergic cells in the brain of monotremes as revealed by ChAT immunohistochemistry. Brain Behav Evol 2002;60: 275-97.
30. Varga C, Hartig W, Grosche J, Keijser J, Luiten PG, Seeger J, et al. Rabbit forebrain cholinergic system: morphological characterization of nuclei and distribution of cholinergic terminals in the cerebral cortex and hippocampus. J Comp Neurol 2003;460:597-611.
31. Armstrong DM, Saper CB, Levey AI, Wainer BH, Terry RD. Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. J Comp Neurol 1983;216:53-68.
32. Kimura H, McGeer PL, Peng F, McGeer EG. Choline acetyltransferase-containing neurons in rodent brain demonstrated by immunohistochemistry. Science 1980;208:1057-9.
33. Ishida I, Ichikawa T, Deguchi T. Immunochemical and immunohistochemical studies on the specificity of a monoclonal antibody to choline acetyltransferase of rat brain. Neurosci Lett 1983;42:267-71.

34. Ichikawa T, Hirata Y. Organization of choline acetyltransferase-containing structures in the forebrain of the rat. J Neurosci 1986;6:281-92.
35. Ichikawa T, Ajiki K, Matsuura J, Misawa H. Location of two cholinergic markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: in situ hybridization histochemistry and immunohistochemistry. J Chem Neuroanat 1997;13:23-39.
36. Tago H, McGeer PL, Bruce G, Hersh LB. Distribution of choline acetyltransferase-containing neurons of the hypothalamus. Brain Res 1987;415:49-62.
37. Geula C, Schatz CR, Mesulam MM. Differential localization of NADPH-diaphorase and calbindin-D28k within the cholinergic neurons of the basal forebrain, striatum and brainstem in the rat, monkey, baboon and human. Neuroscience 1993;54:461-76.
38. Holt DJ, Hersh LB, Saper CB. Cholinergic innervation in the human striatum: a three-compartment model. Neuroscience 1996;74:67-87.
39. Oda Y, Nakanishi I. The distribution of cholinergic neurons in the human central nervous system. Histol Histopathol 2000;15:825-34.
40. Bernácer J, Prensa L, Giménez-Amaya JM. Cholinergic interneurons are differentially distributed in the human striatum. PLoS One 2007;2:e1174.
41. Mesulam MM. Cholinergic circuitry of the human nucleus basalis and its fate in Alzheimer's disease. J Comp Neurol 2013;521:4124-44.
42. Medina L, Reiner A. Distribution of choline acetyltransferase immunoreactivity in the pigeon brain. J Comp Neurol 1994;342:497-537.
43. Sorenson EM, Parkinson D, Dahl JL, Chiappinelli VA. Immunohistochemical localization of choline acetyltransferase in the chicken mesencephalon. J Comp Neurol 1989;281:641-57.
44. Hoogland PV, Vermeulen-VanderZee E. Distribution of choline acetyltransferase immunoreactivity in the telencephalon of the lizard Gekko gecko. Brain Behav Evol 1990;36:378-90.
45. Medina L, Smeets WJAJ, Hoogland PV, Puelles L. 1993. Distribution of choline acetyltransferase immunoreactivity in the brain of the lizard Gallotia galloti. J Comp Neurol 331:261-85.
46. Powers AS, Reiner A. The distribution of cholinergic neurons in the central nervous system of turtles. Brain Behav Evol 1993;41:326-45.
47. González A, López JM. A forerunner of septohippocampal cholinergic system is present in amphibians. Neurosci Lett 2002;327:111-4.
48. Marin O, Smeets WJ, Gonzalez A. Distribution of choline acetyltransferase immunoreactivity in the brain of anuran (Rana perezi, Xenopus laevis) and urodele (Pleurodeles waltl) amphibians. J Comp Neurol 1997;382:499-534.
49. Anadon R, Molist P, Rodriguez-Moldes I, Lopez JM, Quintela I, Cervino MC et al. Distribution of choline acetyltransferase (ChAT) immunoreactivity in the brain of an elasmobranch, the lesser spotted dogfish (Scyliorhinus canicula). J Comp Neurol 2000;420:139-70.
50. Adrio F, Anadon R, Rodrıguez-Moldes M. Distribution of choline acetyltransferase (ChAT) immunoreactivity in the central nervous system of a chondrostean, the siberian sturgeon (Acipenser baeri). J Comp Neurol 2000; 426:602-21.
51. López JM, Perlado J, Morona R, Northcutt RG, González A. Neuroanatomical organization of the cholinergic system in the central nervous system of a basal actinopterygian fish, the Senegal bichir Polypterus senegalus. J Comp Neurol 2013;521:24-49.
52. Morona R, López JM, Northcutt RG, González A. Comparative analysis of the organization of the cholinergic system in the brains of two holostean fishes, the Florida gar Lepisosteus platyrhincus and the bowfin Amia calva. Brain Behav Evol 2013;81:109-42.
53. López JM, Domínguez L, Morona R, Northcutt RG, González A. Organization of the cholinergic systems in the brain of two lungfishes, Protopterus dolloi and Neoceratodus forsteri. Brain Struct Funct 2012;217: 549-76.
54. Eckenstein F, Thoenen H. Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. EMBO J 1982;1:363-8.
55. Rodriguez-Moldes I, Molist P, Adrio F, Pombal MA, Yanez SE, Mandado M, et al. Organization of cholinergic systems in the brain of different fish groups: a comparative analysis. Brain Res Bull 2002;57:331-4.
56. Ekstrom P. Distribution of choline acetyltransferase-immunoreactive neurons in the brain of a cyprinid teleost (Phoxinus phoxinus L.). J Comp Neurol 1987;256:494-515.
57. Ekstrom P, Korf HW. Putative cholinergic elements in the photosensory pineal organ and retina of a teleost, Phoxinus phoxinus L. (Cyprinidae). Distribution of choline acetyltransferase immunoreactivity, acetylcholinesterase-positive elements, and pinealofugally projecting neurons. Cell Tissue Res 1986;246:321-9.
58. Giraldez-Perez RM, Gaytan SP, Torres B, Pasaro R. Colocalization of nitric oxide synthase and choline acetyltransferase in the brain of the goldfish (Carassius auratus). J Chem Neuroanat 2009;37:1-17.
59. Villani L, Battistini S, Bissoli R, Contestabile A. Cholinergic projections in the telencephalo-habenulo-interpeduncular system of the goldfish. Neurosci Lett 1987;76:263-8.
60. Zottoli SJ, Rhodes KJ, Mufson EJ. Comparison of acetylcholinesterase and choline acetyltransferase staining patterns in the optic tectum of the goldfish Carassius auratus. A histochemical and immunocytochemical analysis. Brain Behav Evol 1987;30:143-59.
61. Zottoli SJ, Rhodes KJ, Corrodi JG, Mufson EJ. Putative cholinergic projections from the nucleus isthmi and the nucleus reticularis mesencephali to the optic tectum in the goldfish (Carassius auratus). J Comp Neurol 1988;273:385-98.
62. Clemente D, Arenzana FJ, Sánchez-González R, Porteros A, Aijón Arévalo R. Comparative analysis of the distribution of choline acetyltransferase in the central nervous system of cyprinids. Brain Res Bull 2005;66:546-9.
63. Clemente D, Porteros A, Weruaga E, Alonso JR, Arenzana FJ, Aijon J, et al. Cholinergic elements in the zebrafish central nervous system: histochemical and immunohistochemical analysis, J Comp Neurol 2004;474:75-107.
64. Edwards JG, Greig A, Sakata Y, Elkin D, Michel WC. Cholinergic innervation of the zebrafish olfactory bulb. J Comp Neurol 2007;504:631-65.
65. Molist P, Maslam S, Velzing E, Roberts BL. The organization of cholinergic neurons in the mesencephalon of the eel, Anguilla anguilla, as determined by choline acetyltransferase immunohistochemistry, and acetylcholinesterase enzyme histochemistry. Cell Tissue Res 1993;271:555-66.
66. Perez SE, Yanez J, Marin O, Anadon R, Gonzalez A, Rodriguez-Moldes I. 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. J Comp Neurol 2000;428:450-74.
67. Brantley RK, Bass AH. Cholinergic neurons in the brain of a teleost fish (Porichthys notatus) located with a

monoclonal antibody to choline acetyltransferase. J Comp Neurol 1988;275: 87-105.
68. Wullimann MF, Roth G. Is the nucleus corticalis of teleosts a new cholinergic central nervous system for vertebrates? Neuroreport 1992;3:33-5.
69. Puelles L, Rubenstein JL. Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. Trends Neurosci 1993;16: 472-9.
70. Puelles L, Rubenstein JL. A new scenario of hypothalamic organization: rationale of new hypotheses introduced in the updated prosomeric model. Front Neuroanat 2015;9:27.
71. Northcutt RG, Davis RE. Telencephalic organization in ray-finned fishes. In: Davis RE, Northcutt RG, Editors. Fish neurobiology, vol. 2. Higher brain areas and functions. University of Michigan Press; Ann Arbor; 1983; pp. 203-36.
72. Wulliman MF. The central nervous system. In: Evans DH, Editor. The physiology of fishes. New York; CRC Press; 1997; pp. 245-82.
73. Pombal MA, Yanez J, Marin O, Gonzalez A, Anadon R. Cholinergic and GABAergic neuronal elements in the pineal organ of lampreys, and tract-tracing observations of differential connections of pinealofugal neurons. Cell Tissue Res 1999;295:215-23.
74. Pombal MA, Mar´ın O, Gonz alez A. Distribution of choline acetyltransferase-immunoreactive structures in the lamprey brain. J Comp Neurol 2001; 431: 105-26.
75. Meek J, Nieuwenhuys R. Holosteans and teleosts. In: The central nervous system of vertebrates. In: Nieuwenhuys R, Ten Donkelaar HJ, Nicholson C, Editors; Springer-Verlag, Berlin; 1998; pp. 759-937.
76. Grover BG, Sharma SC. Organization of extrinsic tectal connections in goldfish (Carassius auratus). J Comp Neurol 1981;196:471-88.
77. Wullimann MF. The tertiary gustatory center in sunfishes is not nucleus glomerulosus. Neurosci Lett 1988;86:6-10.
78. Wullimann MF, Rink E. The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. Brain Res Bull. 2002;57:363-70.
79. Rhodes KJ, Zottoli SJ, Mufson EJ. Choline acetyltransferase immunohistochemical staining in the goldfish (Carassius auratus) brain: evidence that the Mauthner cell does not contain choline acetyltransferase. Brain Res 1986;381:215-24.
80. Spira M, Model PG, Bennett MVL. Cholinergic transmission at a central synapse. J Cell Biol 1970;47:199-200.
81. Day JW, Hall DH, Hall LM, Bennett MV. alpha-Bungarotoxin labeling and acetylcholinesterase location at the Mauthner fiber giant synapse in the hatchetfish. J Neurosci 1983;3:272-9.
82. Eckenstein F, Thoenen H. Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. Neurosci Let. 1983;36:211-5.
83. Blaker SN, Armstrong DM, Gage FH. Cholinergic neurons within the rat hippocampus: response to fimbria-fornix transection. J Comp Neurol 1988;272: 127-38.
84. Reiner A. A comparison of neurotransmitter-specific and neuropeptide-specific neuronal cell types present in the dorsal cortex in turtles with those present in the isocortex in mammals: implications for the evolution of isocortex. Brain Behav Evol 1991;38:53-91.
85. Mufson EJ, Cunningham MG. Observations on choline acetyltransferase containing structures in the CD-1 mouse brain. Neurosci Lett 1988;84:7-12.
86. Vincent SR, Reiner PB. The immunohistochemical location of choline acetyltransferase in the cat brain. Brain Res Bull 1987;18:371-415.
87. St-Jacques R, Gorczyca W, Mohr G, Schipper HM. Mapping of the basal forebrain cholinergic system of the dog: a choline acetyltransferase immunohistochemical study. J Comp Neurol 1996; 366:717-25.
88. Hendry SH, Jones EG, Killackey HP, Chalupa LM. Choline acetyltransferase-immunoreactive neurons in fetal monkey cerebral cortex. Brain Res. 1987;465:313-7.
89. Alonso JR, Amaral DG. Cholinergic innervation of the primate hippocampal formation. I. Distribution of choline acetyltransferase immunoreactivity in the Macaca fascicularis and Macaca mulatta monkeys. J Comp Neurol 1995; 355:135-70.
90. Woolf NJ. Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 1991;37:475-524.