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

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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, affarent 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 vertebrates1-8 and invertebrates9-11 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 morphology have provided strong evidence for the implication of cholinergic dysfunctions in the pathogenesis of the cognitive decline occurring in Alzheimer’s17 and Parkinson’s18,19 diseases.

Several studies have proposed teleost fishes as valuable models for investigating brain functions and human neurological disorders.20-21 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 rat31-36) including humans37-41 and non-mammalian vertebrates42-53 by means of histochemical 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).54 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.19,30,46 Indeed, ChAT expression differs in the diencephalon and the cerebellum among European minnows,56,57 goldfish,58-61 tench62 and zebrafish19,62,63. Other differences emerged in the other species studied so far, i.e., eel64 (Anguillidae), trout66 (Salmonidae), midshipmen67 (Batrachoideidae) and some cichlids68 (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.

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. |
|---------------------------------------------------------------|
| **ChATir**          | **C. carpio (this paper)** | **A. anguilla** | **C. auratus** | **D. rerio** | **D. rerio** | **D. rerio** | **H. hippopotami** | **L. rostratus** | **S. trutta** | **P. phoxinus** | **P. notatus** | **T. triton** |
|---------------------|----------------------------|----------------|----------------|-------------|-------------|-------------|---------------------|----------------|-------------|----------------|-------------|-------------|
| Offactory bulb      | -                          | +             | -              | -           | -           | -           | +                   | -              | +           | +              | +           | -           |
| Parvocellular      | -                          | -             | +              | +           | +           | +           | +                   | -              | +           | +              | +           | -           |
| Suprachiasmatic     | +                          | +             | -              | -           | -           | -           | -                   | -              | -           | -              | -           | -           |
| Hypothalamic region | -                          | -             | +              | +           | +           | +           | +                   | +              | +           | +              | +           | +           |
| Lateral thalamus    | -                          | -             | -              | -           | -           | -           | -                   | -              | -           | -              | -           | -           |
| Pons                | -                          | -             | -              | -           | -           | -           | -                   | -              | -           | -              | -           | -           |
| Medulla             | +                          | +             | -              | -           | -           | -           | -                   | +              | +           | +              | +           | +           |
| Reticular formation | +                          | +             | -              | -           | -           | -           | -                   | +              | +           | +              | +           | +           |
| Cerebellum          | +                          | +             | -              | -           | -           | -           | -                   | +              | +           | +              | +           | +           |
| Motor nuclei        | +                          | +             | +              | +           | +           | +           | +                   | -              | +           | +              | +           | +           |
| Octavolateralis     | +                          | +             | -              | -           | -           | -           | -                   | +              | +           | -              | -           | -           |
| Rombencephalic      | +                          | +             | +              | +           | +           | +           | +                   | +              | +           | +              | +           | +           |
| 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 at A.I. Levey, University of Chicago, USA); ****polyclonal antibody anti-chicken ChAT (provided by at M.L. Epstein, University of Wisconsin, USA); *****polyclonal antibody anti-chicken ChAT (provided by at 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% H2O2 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 1 h 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% H2O2 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, Pittsburgh, PA, USA).

Results
In this paper, the forebrain regions of the carp were described in the rostrocaudal sequence proposed by the neuromeric model recently updated by Rubenstein and Puelles. 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), 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 thalamus and the medulla oblongata derive from mesomeres m1-m2 and rhombomeres r0-11, 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 (VI) (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 system. 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 (PA, PP, 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 latero caudal 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 preglomerular 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 3A,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-interpeduncular 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; Cte, tectal commissures; dDI, dorso-lateral nucleus of the dorsal telencephalic area; DH, dorsal horn; DI, 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; NH, 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, sub-gemlular nucleus; SR, superior raphé; TL, torus longitudinalis; TS, semicircular torus; ttb, tecto-bulbar tract; Va, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; vDi, 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 (NIIlsd and NIIlsv) (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 1H-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

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**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 (VI) 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 lateroventral 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 preglomerular 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).

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

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 (Nllsd and Nllsv) (G). ChATir elements in the nucleus lateralis valvulae (NLV) (H). Scale bars: 100 μm.
Phoxinus phoxinus,56 Porichthys notatus,67 Oncorhynchus mykiss and Salmo trutta,66 Carassius auratus,56,59,61 Hemichromis lifalili and H. guttatus,68 Danio rerio,19,63,64 Tinca tinca62) 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 results64 versus negative results19,63).

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 (VI), 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 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 (NVI) (F). ChATir neurons in the abducens nucleus (NVI) (G) and in the medial octavolateralis nucleus (MON) (H). III, lateral longitudinal fascicle; RV, rombencephalic ventricle. Scale bars: 100 μm.

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 (NVI) (F). ChATir neurons in the abducens nucleus (NVI) (G) and in the medial octavolateralis nucleus (MON) (H). III, 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,68 in zebrafish,69 and in trout.64 However, few positive neurons were described in the European minnows Phoxinus phoxinus56 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 dipnoans53 and polypterids.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,51 holosteans,52 dipnoans53) we agree with Clemente et al.53 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 neurons have been also reported in the Nmlf in goldfish,58 but not in zebrafish53 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 general-
Cholinergic projections to the optic tectum have been traced in goldfish from the nucleus isthmi and the nucleus reticularis mesencephalicus 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 and in the granule layer in Porichthys. By contrast, ChATir cells were not detected in the cerebellum of Phoxinus, zebrafish, tench and trout. Cerbellar ChATir cells have not been detected in other fish, as primitive actinopterygians and dipnoans (data summarized by Lopez), with the exception of dogfish. 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 teleost species. A cholinergic RTN nucleus was also identified in zebrafish 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 to the optic tectum. Therefore, the NRMT/RTN may be considered relay centers for gustatory inputs from the vagal lobe to higher brain centers in cyprinids and other teleost species (Table 1). They were detected in many cyprinids, oldfish, zebrafish, tench, trout. However, the cholinergic nature of the cranial nerve motor nuclei has appeared several times during the evolution but it is not shared by all mammals or amniotes.

Comparison with mammals

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. 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. In particular, tract tracing studies in zebrafish suggested that Vv and Vl belong to the septum and VI is the homologous of sepal nuclei sending cholinergic projections to the dorsal telencephalon in amniotes. 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 and mouse, but they are lacking in guinea pig and cat and dog.

Furthermore, ChATir neurons have been identified in the fetal monkey cerebral cortex but not in the cortex of adult primates, including humans. 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.

ChATir cell populations were described in the preoptic region of monotremes and rat but are not conserved in all amniotes. Cholinergic habenular neurons were reported in mammals, including humans and most tetrapods. Cholinergic cells are not present in the prefrontal cortex containing basal cholinergic systems in all the vertebrate taxa. However, ChATir cells have been detected in the cat cerebellum, 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. Thus, cholinergic neurons projecting to the midbrain tectum are a brain feature that has been maintained from fish to mammals. The RNMT is homologous to the subpallium and is considered homologous to the pedunculopontine nucleus of the mesencephalic locomotor region of mammals projecting to the caudate-putamen. Motor neurons of the cranial nerve nuclei represent one of the most conservative cholinergic cell groups in all vertebrates, 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...
tuberculosis, 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, 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 a new model for basic biomedical research on α-synuclein pathologies of the cholinergic system.

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