ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2013, 73: 338–353

Cholinergic and nitrergic neuronal networks in the goldfish telencephalon

Rosa María Giraldez-Perez, Susana Pilar Gaytan, and Rosario Pasaro*

Department of Physiology, Faculty of Biology, University of Sevilla, Sevilla, Spain, *Email: mrpasaro@us.es

The general organization of cholinergic and nitrergic elements in the central nervous system seems to be highly conserved among vertebrates, with the involvement of these neurotransmitter systems now well established in sensory, motor and cognitive processing. The goldfish is a widely used animal model in neuroanatomical, neurophysiological, and behavioral research. The purpose of this study was to examine pallial and subpallial cholinoceptive, cholinergic and nitrergic populations in the goldfish telencephalon by means of histochemical and immunohistochemical techniques in order to identify neurons containing acetylcholinesterase (AChE), choline acetyltransferase (ChAT), NADPH-diaphorase (NADPHd), and neuronal nitric oxide synthase (nNOS), and to relate their distribution to their putative functional significance. Regions containing AChE-labeled neurons represented terminal fields of cholinergic inputs as well as a widespread distribution of AChE-related enzymes; these regions also usually contained NADPHd-labeled neurons and often contained small numbers of nNOS-positive cells. However, the ventral subdivisions of the medial and lateral parts of the dorsal telencephalic area, and the ventral and lateral parts of the ventral telencephalic area, were devoid of nNOS-labeled cells. ChAT-positive neurons were found only in the lateral part of the ventral telencephalic area. ChAT- and nNOS-positive fibers exhibited a radial orientation, and were seen as thin axons with *en-passant boutons*. The distribution of these elements could help to elucidate the role of cholinergic and nitrergic neuronal networks in the goldfish telencephalon.

Key words: acetylcholine, goldfish, histochemistry, immunohistochemistry, nitric oxide, telencephalon

ABBREVIATIONS

AChE – acetylcholinesterase
ChAT – choline acetyltransferase
CNS – central nervous system
Dc – dorsal telencephalic area, central part
Dd – dorsal telencephalic area, dorsal part
Dl – dorsal telencephalic area, lateral part
Dld – dorsal telencephalic area, dorsal subdivision of the lateral part
Dlv – dorsal telencephalic area, ventral subdivision of the lateral part
Dm – dorsal telencephalic area, medial part
Dmd – dorsal telencephalic area, dorsal subdivision of the medial part
Dmv – dorsal telencephalic area, ventral subdivision of the medial part
Dp – dorsal telencephalic area, posterior part
ENT – entopeduncular nucleus
Ep – ependymal cells
G – preglomerular complex
Gl – glomerular layer of the olfactory bulb
Gr – granular layer of the olfactory bulb
nAChR – nicotinic acetylcholine receptor
NADPHd – NADPH-diaphorase
NOS – nitric oxide synthase
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
Nt – nucleus taeniae
OB – olfactory bulb
OC – optic chiasm
Ppp – preoptic nucleus, parvocellular part
SC – suprachiasmatic nucleus
Tch – tela choroidea
Vd – ventral telencephalic area, dorsal part
Vl – ventral telencephalic area, lateral part
Vp – ventral telencephalic area, posterior part
Vs – ventral telencephalic area, supracommissural part
Vv – ventral telencephalic area, ventral part

Correspondence should be addressed to R. Pásaro, Email: mrpasaro@us.es

Received 05 February 2013, accepted 13 June 2013

INTRODUCTION

The distribution of cholinergic and nitrergic neural elements in the central nervous system (CNS) appears to be similar among vertebrates (Medina et al. 1993, Medina and Reiner 1994, Butcher 1995, Ichikawa et al. 1997, Marín et al. 1997, González et al. 2002, Clemente et al. 2004, Giraldez-Perez et al. 2008, 2009). However, controversy remains in relation to apparent intra-group and species-specific differences in the fish telencephalon (Brantley and Bass 1988, Arévalo et al. 1995, Adrio et al. 2000, Anadón et al. 2000, Rodríguez-Moldes et al. 2002, Norcutt 2006, 2008).

Cholinergic neurotransmission plays an important role in the activation and modulation of telencephalic circuitries, and is also involved in aspects of sensory, motor and cognitive processing (Butcher 1995, Havekes et al. 2011). However, in order to characterize the neuronal networks that mediate these functions, it is important to ensure that the most appropriate techniques are chosen. In this regard, immunohistochemistry against choline acetyltransferase (ChAT, the synthesizing enzyme of acetylcholine) antibodies is the most reliable marker for identifying cholinergic elements and has been used to determine cholinergic neuronal networks in the CNS of the fish (Brantley and Bass 1988, Anadón et al. 2000, Clemente et al. 2005, Giraldez-Perez et al. 2008, 2009). Although acetylcholinesterase (AChE, the carboxylesterase enzyme that hydrolyzes acetylcholine) histochemistry does not serve as a specific marker for cholinergic cells (Clemente et al. 2004), it is useful for revealing the presence of cholinoceptive neurons and terminal fields. On the other hand, neuronal nitric oxide synthase (nNOS) immunocytochemistry can be used to identify neuronal cells that contain the enzyme required for nitric oxide (NO) production, while the enzyme NADPH-diaphorase (NADPHd) serves as a reliable indicator of nitrergic cells (Dellacorte et al. 1995, Giraldez-Perez et al. 2008, 2009).

It has been postulated that cholinergic and nitrergic neuronal systems may modulate synaptic transmission, and several subtypes of the nicotinic acetylcholine receptor (nAChR) are known to be widely expressed in the central and peripheral nervous systems (Bond et al. 2009). Other studies have also indicated that nAChRs are activated by intracellular Ca^{2+} signals mediated by NOS-induced NO production, with the regulation of NOS activity being achieved through binding with calmodulin. The nAChR-calmodulin-NOS-NO pathway may therefore be involved in the regulation of both the nitrergic and cholinergic systems (Gilbert et al. 2009).

The aim of this study was to examine the distribution pattern of telencephalic cholinoceptive, cholinergic and nitrergic elements in the goldfish brain, to further elucidate the role of cholinergic and nitrergic neuronal networks in this species.

METHODS

Subjects

Experiments were performed on 20 adult goldfish (*Carassius auratus)* (8–15 cm body-length; both sexes) in accordance with the European Community Council directive of 22 September 2010 (2010/63/EU) for the Care and Use of Laboratory Animals, and following approval by the Animal Ethics Committee of the University of Seville. The animals were maintained in controlled-temperature (18–20°C) aquaria on a 14:10 h dark–light cycle (lights on at 07:00 AM) until the day of the experiment. Goldfish were placed under deep anesthesia with a 0.4% solution of 3-aminobenzoic acid ethyl ester methane sulfonate salt (MS-222, Sigma-Aldrich, St. Louis, MO) in freshwater. They were then perfused with heparinized saline followed by 4% cold paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 mL/50 g body weight). After perfusion, the brains were removed and postfixed in the same cold fixative solution for 4 h and then immersed in a cold 30% sucrose solution in PB for 24 h. The brains were sectioned in the transverse plane at a thickness of 30 μm using a cryostat (Leica CM1850, Nussloch, Germany), and mounted on double gelatinized slides.

AChE and NADPHd histochemistry

The Karnovsky and Roots (1964) method was used to determine AChE activity. For inhibition of nonspecific esterases, the slide-mounted sections were incubated in 0.2 mM etopropazine chloride (Sigma-Aldrich), followed by incubation in a solution of 1.7 mM acetylthiocholine iodide (Sigma-Aldrich), 5 mM potassium ferricyanide (Merck, Whitehouse Station, NJ), 30 mM copper sulfate

Table I

Average size, AChE-labeled, ChAT-positive, NADPHd-labeled and, nNOS-positive neurons in the goldfish telencephalon

Telencephalic regions	AChE-labeled	ChAT-positive	NADPHd-labeled	nNOS-positive
Olfactory bulb				
- Granular layer - Glomerular layer	6.9 ± 0.2 [a] (7.1–6.2)[b]	–	5.6 ± 0.5 (6.5–5.0)	–
- Magnocellular. cells	–	–	33.4 ± 0.2 (33.9–33.2)	–
- Large cells	19.9 ± 0.2 (20.1–19.2)	–	18.9 ± 0.2 (20.0–18.2)	–
- Small cells	6.0 ± 0.2 (6.2–5.3)	–	4.2 ± 0.2 (4.7–4.0)	–
Dorsal telencep. area				
- Dorsal part	10.2 ± 0.2 (10.4–9.5)	–	8.4 ± 0.1 (8.6–8.3)	8.3 ± 0.1 (8.6–8.1)
- Medial part (Dmd, Dmv)	9.8 ± 0.2 (10–9.1)	–	8.1 ± 0.2 (9.1–8.1)	8.2 ± 0.2 (9.1–8.1)
- Central part	17.9 ± 0.2 (18.1–17.2)	–	14.7 ± 0.2 (15.3–14.4)	14.8 ± 0.1 (15.1–14.6)
- Lateral part	7.1 ± 0.2 (7.3–6.4)	–	5.3 ± 0.1 (5.6–5.1)	5.4 ± 0.1 (5.6–5.2)
- Posterior part	6.8 ± 0.2 (7.0–6.1)	–	5.5 ± 0.1 (5.6–5.3)	5.5 ± 0.1 (5.6–5.4)
Ventral telencep. area				
- Dorsal part	9.3 ± 0.2 (9.5–8.6)	–	7.6 ± 0.1 (7.7–7.5)	7.5 ± 0.1 (7.7–7.3)
- Ventral part	7.1 ± 0.3 (7.2–6.3)	–	5.7 ± 0.2 (6.0–5.3)	–
- Lateral part	10.5 ± 0.3 (10.6–9.7)	8.3 ± 0.2 (8.5–7.6)	8.4 ± 0.1 (8.9–8.2)	–
- Posterior part	6.8 ± 0.8	–	6.8 ± 0.1 (7.1–6.3)	6.8 ± 0.2 (7.2–6.4)
- Supracomissural nucleus	12.4 ± 0.1 (12.6–12.4)	–	12.3 ± 0.1 (12.6–12.2)	12.4 ± 0.2 (12.7–12.3)

(a) mean of the major axis measures of the positive neurons in μm ± standard deviation; (b) interval of the largest and shortest measures

(Merck), and 0.1 M sodium citrate (Panreac, Barcelona, Spain) in 0.1 M PB (pH 7.4) for 10–30 min at 35°C. The reaction was stopped with 0.1 M PB (pH 7.4) when Hatchett's brown deposits had formed. To test the accuracy of the histochemical protocol, controls were carried out by omission of the acetylthiocholine iodide, whereupon no AChE-labeled cells were observed.

A variation of the histochemical methods of Sims and coauthors (1974) was used to determine NADPHd activity (Giraldez-Perez et al. 2008). Sections were first incubated in a solution of 0.1 M Tris buffer (pH 8.0), 0.05% Triton X-100 (Sigma-Aldrich), 1 mM β-NADPH (Sigma-Aldrich) and 0.8 mM nitroblue tetrazolium (Sigma-Aldrich), at 37°C for 1–2 h, under observation. Following incubation, the sections were rinsed three times in PB saline (PBS) for 5 min. To test the accuracy of the histochemical protocol, control sections were prepared by omitting the β-NADPH or nitroblue tetrazolium from the incubation step. In both cases, no NADPHd-labeled cells were observed.

Finally, all sections were mounted on gelatin-coated glass slides. Following each of the histochemical protocols, half of the sections were counterstained with Neutral Red (1% in acetate buffer) to permit identification of the different telencephalic areas. After drying overnight, the sections were dehydrated, cleared in xylene and then coverslipped with DPX mounting medium (Panreac).

ChAT and nNOS immunohistochemistry

To detect the expression of ChAT and nNOS, slide-mounted sections were first rinsed in PBS and then pretreated with 2% H_2O_2 and 5% methanol in Tris-buffered saline (TBS, 0.05M, pH 7.4) for 10 min to inactivate endogenous peroxidases. After three rinses in TBS the sections were treated with a solution of 5% donkey serum (DS) in PBS containing 0.3% Triton X-100 (Sigma-Aldrich, PBS+) for 1 h, followed by a further three rinses for 10 min each in PBS.

To reveal ChAT-positive elements, the sections were incubated with a cold solution of goat anti-ChAT serum (1:50, AB144P, Chemicon International, Billerica, MA) in PBS+ for 72 h at 4°C, rinsed three times in PBS (10 min each), and incubated at room temperature (RT) with mouse anti-goat secondary antibody (1:10 00 biotin-SP-conjugated (H+L) 705-065-003, Jackson ImmunoResearch Europe Ltd., Newmarket, UK) in PBS+ and 5% DS for 2 h. They were then rinsed three times in TBS (10 min each), incubated with the ABC kit (Vector Labs., Burlingame, CA) using 35 µL of solution A and 35 µL of solution B in 10 mL TBS plus 0.3% Triton X-100 (TBS+, pH 8.8) for 1.5 h at RT, and rinsed sequentially in TBS (10 min) and Tris-HCl buffer 0.05 M (pH 7.6) for 10 min, before being stained with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl with 0.033% H_2O_2 for 30 min, and rinsed a final three times in Tris-HCl (10 min each).

To reveal nNOS-positive elements, the sections were incubated with a cold solution of rabbit anti-nNOS (1:3 000 AB5380, polyclonal antibody, Chemicon International) in PBS+ for 72 h at 4°C, rinsed three times in PBS (10 min each), and incubated at RT with mouse anti-rabbit secondary antibody (1:2 000 biotin-SP-conjugated (H+L) 211-065-109, Jackson ImmunoResearch Europe Ltd) in PBS+ and 5% DS for 2 h. They were then rinsed three times in TBS (10 min each), incubated with the ABC kit (Vector Labs) using 35 µL of solution A and 35 µL of solution B in 5 mL TBS+ for 1.5 h at RT, rinsed sequentially in TBS (10 min) and Tris-HCl buffer 0.05 M (pH 7.6) for 10 min, stained with 0.05% DAB in Tris-HCl with 0.033% H_2O_2 for 30 min, and rinsed three times in Tris-HCl (10 min each).

Following the immunohistochemical protocols, half of the sections were counterstained with Neutral Red (1% in acetate buffer) to permit identification of the different telencephalic areas. Finally, the sections were

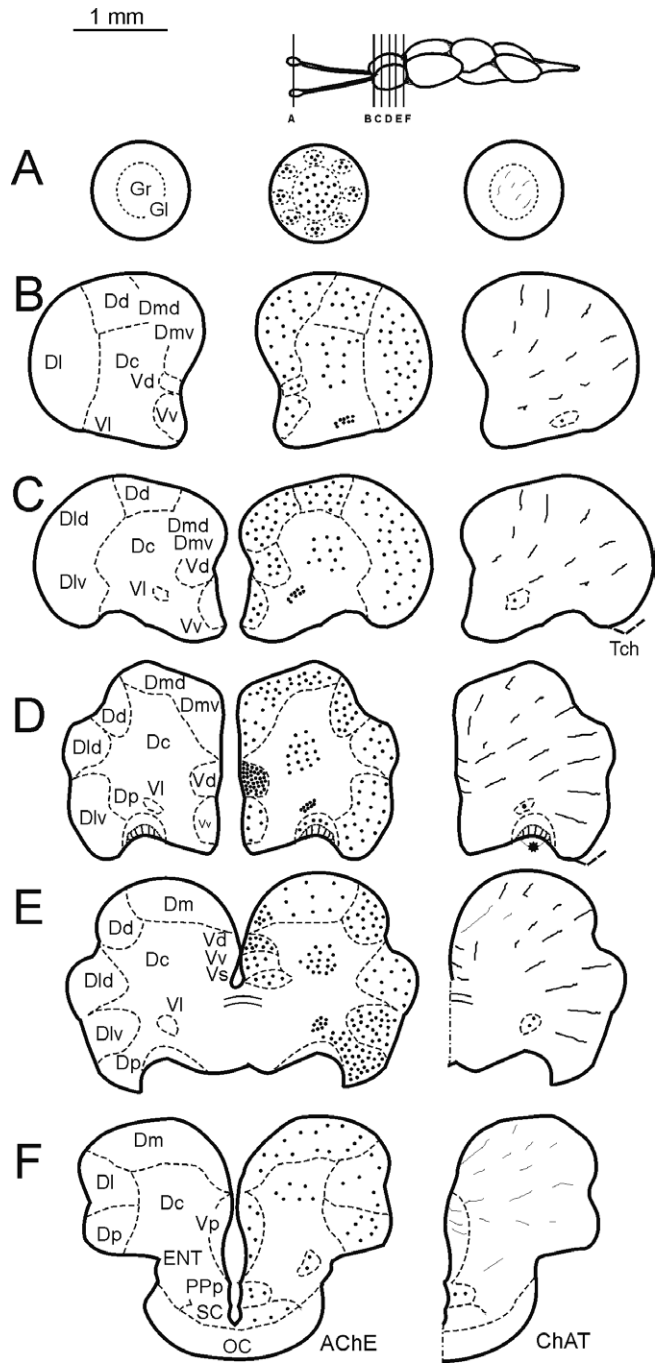

Fig. 1. Schematic drawings of transverse sections through the goldfish telencephalon. These schemas (A–F) illustrate the localization of AChE-labeled and ChAT-positive cells and fibers (thin black lines). The level of each drawing of the brain representation is shown at the top of the panel. Each dot represents 10 stained cells. The star symbol represents the sulcus externus. The tela choroidea is indicated by a discontinuous line.

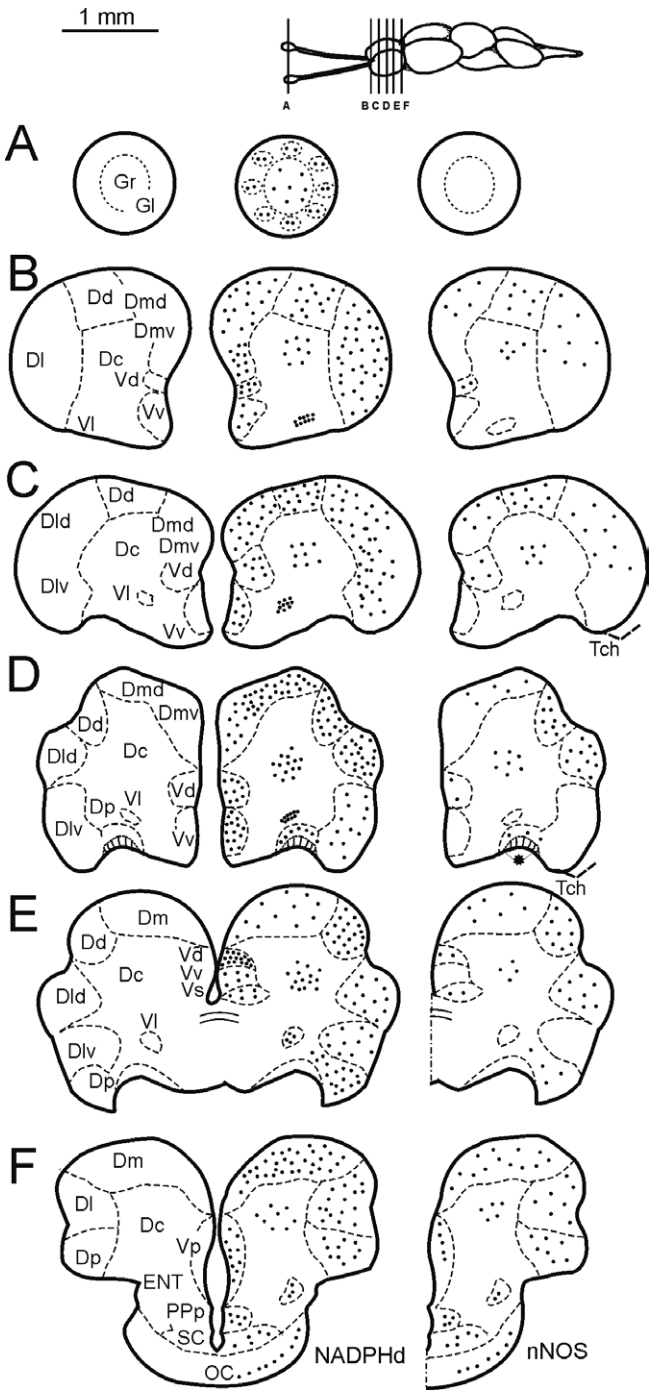

Fig. 2. Schematic drawings of transverse sections through the goldfish telencephalon. These schemas (A–F) illustrate the localization of NADPHd-labeled and nNOS-positive cells. The level of each drawing of the brain is shown in the top panel. Each dot represents 10 stained cells. The star symbol represents the sulcus externus. The tela choroidea is indicated by a discontinuous line.

dried overnight, dehydrated, and coverslipped with DPX mounting medium (Panreac). We verified the specificity of the antibodies used in the present work by two routine control procedures: replacement of the primary or secondary antibodies with PBS. In both cases, no ChAT- or nNOS-positive neurons were observed. The antibodies against ChAT (Medina and Reiner 1994, Adrio et al. 2000, Anadón et al. 2000, Giraldez-Perez et al. 2009) and nNOS (Giraldez-Perez et al. 2008, 2009) have also been tested and used successfully in our previous studies.

Morphometric and statistical analysis

All sections were analyzed and photographed using an Olympus BX61 microscope (Olympus, Tokyo, Japan). Bright-field photomicrographs were captured with a digital camera (DP70, Olympus). Sections were used to count AChE-, ChAT-, NADPHd-, and nNOS-labeled cells (those with a clearly identified nucleus) at 60 μm intervals (every two sections) along the rostro-caudal extent of the telencephalon, and charted onto outline diagrams of sections (Figs 1 and 2), taking the same reference points along the rostro-caudal axis for all animals. The drawings were made using a microscope (Nikon Alphaphot-2 YSZ, Tokyo, Japan) fitted with a camera lucida (Nikon drawing tube) over Nissl-stained brain sections. To measure the major axis (either AChE-labeled, ChAT-positive, NADPHd-labeled, or nNOS-positive soma), five sections were systematically chosen, at regular intervals, in the rostro-caudal axis of each observed nucleus. In each of the chosen sections, a random sample of 40 labeled cells, each with a clearly distinguished nucleus, was measured (Table I). Measurement of the cell profiles of positively stained neurons was performed directly on the screen of a monitor coupled to the microscope, using the Scion Image Beta 4.02 software program (Scion Corp. MD). All data were expressed as mean ± SD. The nomenclature and arrangement of brain structures were taken from Meek and Nieuwenhuys (1998), Mueller and coauthors (2011), and Northcutt (2011), unless otherwise stated. For statistical purposes, five fish from each experimental group were used, and all sections containing nuclei were counted. To generate panels of images, the brightness and contrast of photomicrographs were adjusted with CorelDRAW software (v.3X Corel Corporation, Ottawa, Canada).

RESULTS

The distributions of AChE-, ChAT-, NADPHd- and nNOS-labeled cells per structure (each dot represents 10 cells) in the telencephalon are illustrated in Figures 1 and 2. A direct comparison of the number of labeled cells based on the four different labeling techniques can be scored from the dots represented in the drawings.

Localization of AChE activity and ChAT immunoreactivity

In the olfactory bulb (OB), only AChE-labeled cells and fibers and ChAT-positive fibers were found (Figs 1A and 3A–B). The cells within the granular layer were rounded and sparse, whereas in the glomerular layer they were packed, showing two different populations of neurons with respect to their size (Table I). AChE-positive fibers entered the OB (Fig. 3A), forming compact bundles that terminated in distinct glomeruli of the glomerular layer (Fig. 3B).

The nomenclature for the parcellation of the telencephalic lobes is derived from the topological analysis of Nieuwenhuys (2009, 2012); however we have further subdivided several of the described regions. AChE-labeled cells were found in the pallium (Fig. 1B–F), forming strings of neurons which presented different sizes in the different parts of the dorsal pallium (Table I).

AChE-labeled neurons were found within the medial part (Dm; Fig. 4B), which contained small neurons rostrally, with a diffuse organization in layers, and which could be subdivided into dorsal and ventral subregions (Table I, Fig. 1B–D); however, no subdivisions were observed caudally (Fig. 1E–F). The dorsal part (Dd) contained AChE-labeled neurons, densely packed (Table I, Fig. 5A) between the ipsiliform sulcus and Dm. The lateral part (Dl) also contained a high density of AChE-labeled neurons, and could be subdivided into dorsal (Dld; Fig. 5A) and ventral (Dlv; Fig. 5 E) sectors. A large number of AChE-labeled neurons, with a round soma, were also found within the more posterior part of the dorsal telencephalic area (Dp; Fig. 6F), close to the nucleus taeniae (Nt). The central part (Dc) extended along the dorsal telencephalic area, and contained a central mass of cells, which were larger in size (Table I) and had a pyriform body with several dendrites (Fig. 6B). In the subpallium, AChE positive neurons were compactly arranged within the dorsal part of the ventral telencephalic area (Vd), where small rounded cells (Table I) were found (Fig. 4E), and in the ventral part of the ventral telencephalic area (Vv), where the cells had the same morphology and a similar size, although they were more densely packed than in Vd (Table I, Fig. 1B–E); those near the surface of the ventricle were larger (15.6 ± 0.9 µm) and had distinct apical dendrites. AChE-labeled neurons were also observed in the lat-

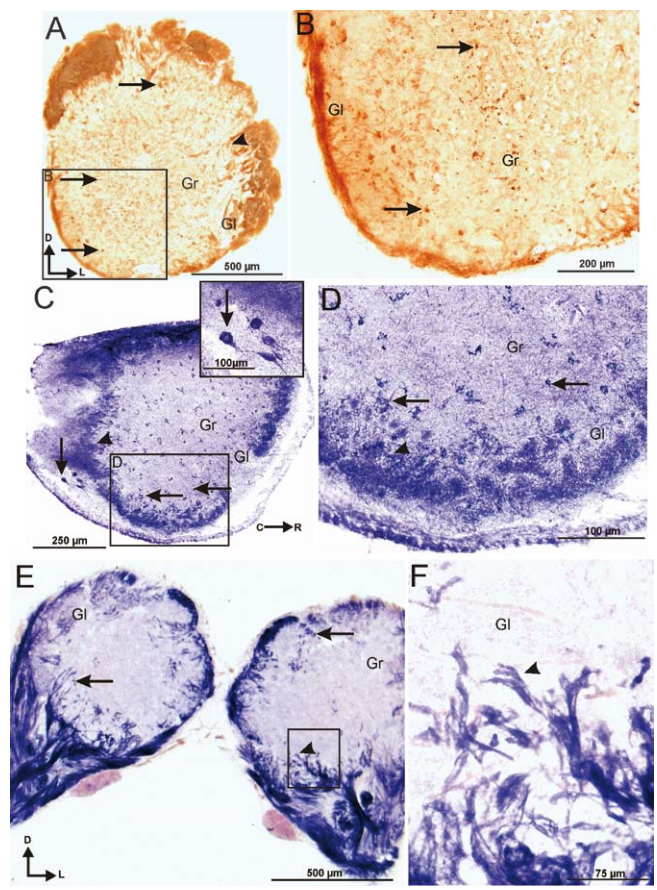

Fig. 3. Photomicrographs of transverse sections of the goldfish olfactory bulb (OB). (A–B) AChE-labeled neurons (black arrows) and fibers (black arrowheads) within the granular (Gr) and glomerular (Gl) layers of the OB. (B) Higher magnification of the AChE-labeled neurons shown in the box in A. (C–F) NADPHd-labeled neurons and fibers within the Gr and Gl layers of the OB. The labeled ganglionic cells of the terminal nerve are shown at higher magnification in the insert. (E) Higher magnification of the NADPHd-labeled neurons shown in the box in C. (E–F) NADPHd-labeled neurons and thick bundles of labeled fibers entering the OB and terminating in the Gl layers. (F) Higher magnification of the NADPHd-labeled fibers shown in the box in E. Anatomical directions indicate the orientation of the photographed section.

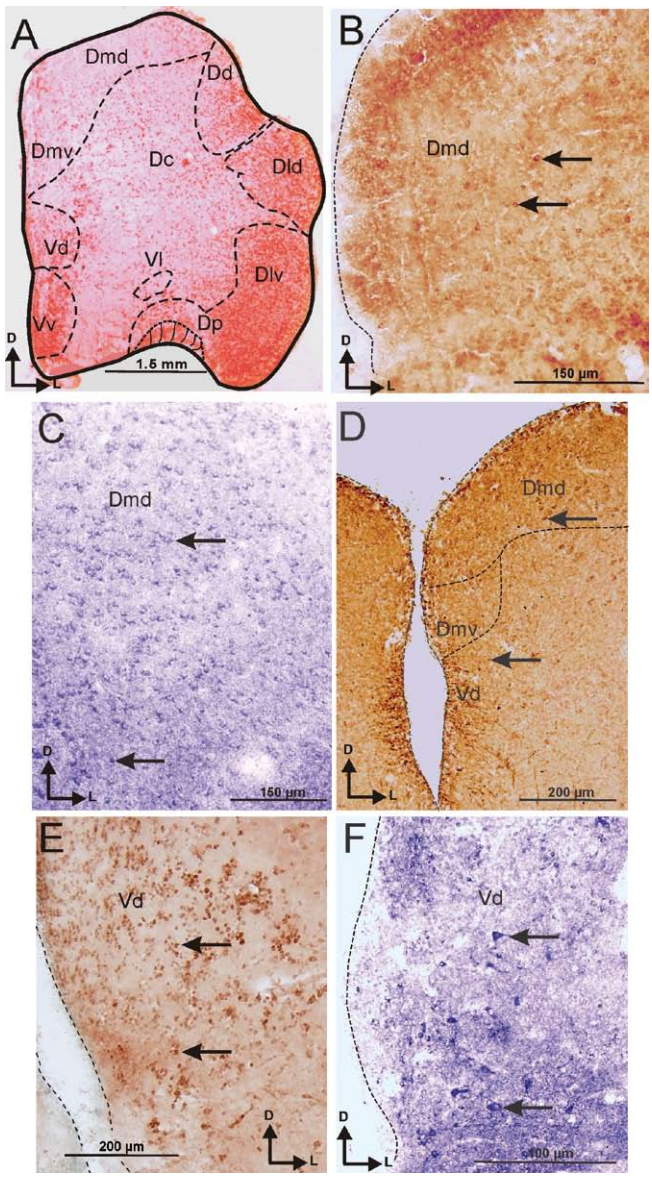

Fig. 4. (A) Transverse Nissl-stained section through the telencephalic hemisphere of the goldfish (at the level of Figs 1D and 2D) showing the limits of the different regions outlined by dashed lines; the coordinates were the same as those for the bright field photomicrographs shown in B–F and in Fig. 5. (B) AChE-labeled cells (black arrows) within the dorsal subdivision of the medial part of the dorsal telencephalic area (Dmd). (C) NADPHd-labeled neurons within Dmd. (D) nNOS-positive neurons within Dmd and the dorsal part of the ventral telencephalic area (Vd). Note that the ventral subdivision of the medial part of the dorsal telencephalic area (Dmv) is devoid of labeled cells. (E) AChE-labeled cells within Vd. (F) NADPHd-labeled neurons within Vd. Anatomical directions indicate the orientation of the photographed section.

eral part of the ventral telencephalic area (Vl; Fig. 1B–E), as well as in the posterior part (Dp), with their dendrites oriented dorsolaterally (Figs 1F and 6F), in the supracommissural part (Vs), where the cells had rounded somata (Fig. 1E), and in Nt (Table I, Figs 1D–E and 6F). In contrast, ChAT-positive neurons, which had several dendrites, were only found in Vl (Table I, Figs. 1B–E and 6E), and not in the dorsal telencephalic area. However a high density of ChAT-positive fibers and *en-passant boutons* was found distributed throughout the above described areas of the pallium and subpallium (Figs 1A–F, 5B, 6C and G). Statistically significant differences were found between the size of the AChE-labeled and ChAT-positive neurons ($P<0.005$) (Table I) in all of the studied nuclei.

Localization of NADPHd activity and nNOS immunoreactivity

In the OB, the NADPHd histochemical technique labeled not only fibers but also some neuronal bodies with a dark reaction product (Figs 2A and 3C–F). The cells within the granular layer presented similar sizes and shapes to those described above for the AChE-positive cell bodies (Table I). In addition, a few NADPHd-positive pyriform neurons with one or two dendrites and a larger soma, the so-called ganglionar cells of the terminal nerve, were observed close to the fibers that entered the OB (Table I, Fig. 3C insert). These fibers formed dense compact bundles, terminating in distinct glomeruli of the glomerular layer (Fig. 3E–F). No immune-positive labeling for nNOS was found in this area. NADPHd- and nNOS-labeled cells with similar morphologies to those previously described were found in the pallium (Fig. 2B–F).

In general, the number of labeled cells was higher for NADPHd than for nNOS. Regions containing NADPHd- and nNOS-positive neurons included Dd (Figs 2B–E and 5D), where strings of labeled cells were observed, and Dc (Figs 2B–F, 5C and 6D), where the distribution was more dispersed (Table I). NADPHd- and nNOS-labeled neurons (Table I) were also found in Dm, where two subdivisions could be differentiated, one more dorsally (Dmd; Figs 2B–D and 4C–D) and the other more ventrally (Dmv), the latter being devoid of nNOS-positive neurons (Figs 2B–d and 4D). In Dmd, the NADPHd- and nNOS-labeled neurons were arranged in strings of cells; however in Dmv, the NADPHd-labeled neurons were more densely packed.

NADPHd- and nNOS-labeled neurons were also found within Dl (Fig. 2B–F), which could be also subdivided into a dorsal subdivision (Dld; Fig. 5C–D) and another more ventral subdivision (Dlv; Fig. 5F) that was devoid of nNOS-positive cells (Table I, Fig. 2C–E). Dp, located above Nt, contained both NADPHd- and nNOS-labeled neurons (Table I, Figs. 2D–F and 6H).

In the subpallium, NADPHd- and nNOS-positive neurons (Table I) were found highly compacted in Vd (Figs 2D–E, 4D and F), and were also identified in Vp and Vs, as well as in Nt (Table I, Fig. 2B–E). NADPHd-labeled neurons were also found within Vv and Vl (Table I, Figs 2B–E and 6H); however, these areas contained no nNOS-positive cells (Fig. 2B–E). The labeled neurons observed in Vl comprised groups of densely compacted cells. Statistically significant differences were found between the two population sizes of NADPHd-labeled and nNOS-positive neurons ($P<0.005$), although the sizes were very similar (Table I) in all of the studied nuclei. The distribution of NADPHd and AChE activity and nNOS and ChAT immunoreactivity overlapped in most of the regions (Figs 1 and 2). Regions with AChE-positive neurons also generally contained NADPHd-positive neurons. These regions included Dm, Dd, Dl, Dc, and Dp in the pallium, and Vd, Vv, Vl, Vp, Vs and Nt in the subpallium (Figs 1B–F and 2B–F). In contrast, Vl, the only region that contained ChAT-positive neurons, was devoid of nNOS-positive cells (Figs 1 and 2). ChAT- and nNOS-positive fibers exhibited a radial orientation towards the dorsal areas (Figs 1B–F and 5B–D) where thin axons with *en-passant boutons* (Figs 5B, 6C, E and G) were observed, with a higher density evident for the ChAT-positive than the nNOS-positive ones.

DISCUSSION

Regions with AChE-labeled neurons represent terminal fields of cholinergic inputs as well as the widespread distribution of AChE-related enzymes. In this study, these areas also usually contained NADPHd-labeled neurons and often small numbers of nNOS-positive neurons. However, the ventral subdivisions of the medial and lateral parts of the dorsal telencephalic area, as well as the ventral and lateral parts of the ventral telencephalic area, were devoid of nNOS-labeled cells. ChAT-positive neurons were found only in Vl, but this region contained no nNOS-positive cells. Descriptions of the cholinergic and nitrergic systems of the telencephalon of several actinopterigian and nonactinopterigian fishes, as well as a number of tetrapods, have been published previously (Brantley and Bass 1988, Medina et al. 1993, Medina and Reiner, 1994, Arévalo et al. 1995, Butcher 1995, Muñoz et al.

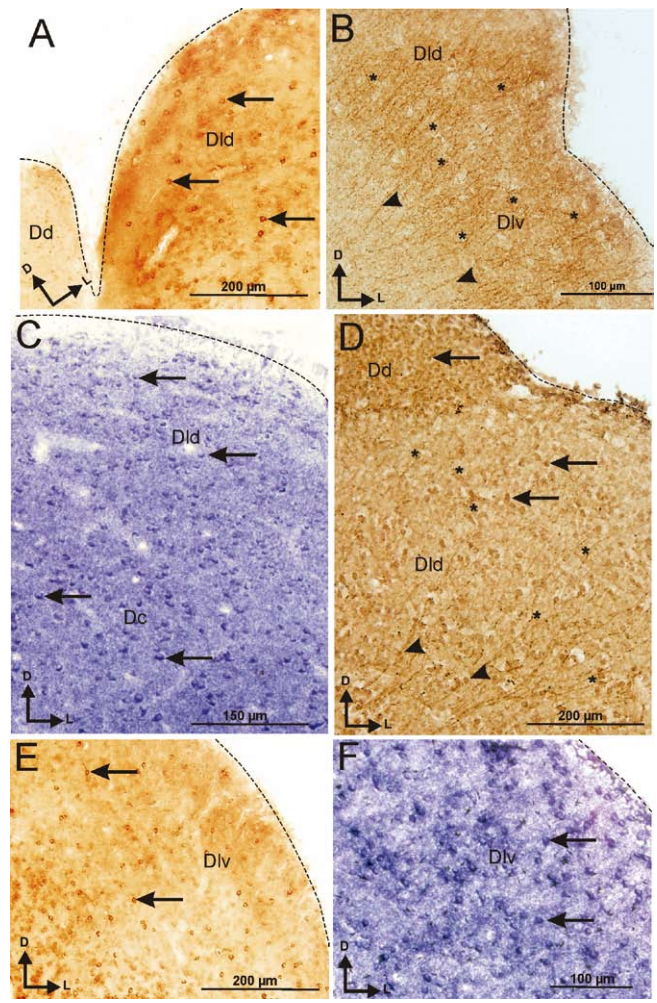

Fig. 5. (A–F) Bright field photomicrographs taken at the same coordinates as the Nissl-stained section shown in 5A. (A) AChE-labeled cells (black arrows) within the dorsal part of the dorsal telencephalic area (Dd) and the dorsal subdivision of the lateral part of the dorsal telencephalic area (Dld). (B) ChATpositive fibers (black arrowheads) and *en-passant boutons* (asterisks) within Dld and the ventral subdivision of the lateral part of the dorsal telencephalic area (Dlv). (C) NADPHd-labeled neurons within Dld and the central part of the dorsal telencephalic area (Dc). (D) nNOS-positive neurons, fibers and *en-passant boutons* within Dd and Dld. (E) AChE-labeled cells within Dlv. (F) NADPHd-labeled neurons within Dlv. Anatomical directions indicate the orientation of the photographed section.

1996, Ichikawa et al. 1997, Marín et al. 1997, Adrio et al. 2000, Rodríguez-Moldes et al. 2002, González et al. 2002, Clemente et al. 2004). The distributions described here for the cholinergic and nitrergic elements found in the goldfish telencephalon to some extent match those reported for other teleosts; however, our observations also provide new insights concerning species differences.

The existence of a relationship between nitrergic and cholinergic neurons could be postulated on the basis of the present results, with NO possibly regulating the level of acetylcholine released by the terminal endings of cholinergic neurons. The nAChR, acting *via* the nAChR-calmodulin-eNOS-NO pathway, may be involved in this interaction (Gilbert et al. 2009). In this way, the mapping of cholinergic and nitrergic neuronal networks could be a valuable tool for understanding function in the goldfish telencephalon.

Classically, AChE has been used as a valuable tool to delineate many nuclei and fiber tracts the boundaries of which are difficult to distinguish in Nissl-stained sections. In addition, AChE is transported by many neurons, including monoaminergic neurons, and its accumulation or depletion can be used for the anatomical tracing of these systems (Paxinos et al. 1980). Due to its high sensitivity, AChE histochemistry is a good marker to identify cholinergic as well as cholinoceptive neurons in the CNS (Butcher 1995). In the present results, areas lacking ChAT-positive neurons but containing AChE-labeled somata were identified, revealing dense ChAT-positive innervation.

NOS, which generates the physiological second messenger molecule NO, and its associated NADPHd activity, are distributed throughout selective neuronal populations of the goldfish telencephalon (Giraldez-Perez et al. 2008). Considerable evidence has accumulated to indicate that NADPHd labeling might be detected in cells lacking nNOS, i.e., the specificity of the reaction has to be considered for the reliable detection of the enzyme in both neuronal and non-neuronal tissue. The high correspondence between NADPHd and nNOS has also been assessed using classical models of transgenic knockout mice for NOS which do not exhibit NADPHd labeling (Huang et al. 1993). Furthermore, a cell line transfected with nNOS cDNA has been shown to exhibit de novo expression of both the enzyme and NADPHd activity (Dawson et al. 1991). However, studies of the co-localization and regional distribution of NADPHd- and nNOS-positive cells have reported some discrepancies in nonmammalian vertebrates, including fish (Arévalo et al. 1995, Virgili et al. 2001, Giraldez-Perez et al. 2008, 2009). Elsewhere, the various degrees of dissociation of NADPHd activity and nNOS immunoreactivity detected in fishes have been related to the expression of different subtypes of nNOS in their forebrain (Bordieri et al. 2003), with the nNOS-positive cells representing only a fraction of the total population of NADPHd-labeled cells. Furthermore, nNOS is activated by calmodulin and Ca^{2+} (Nathan and Xie 1994), the levels of which can vary depending on the physiological status of the animal (Jadhao et al. 1999). On this basis, nNOS immunohistochemistry is a more reliable technique for the identification of nitrergic neurons in the fish brain (Ando et al. 2004, Giraldez-Perez et al. 2008).

The present results indicate that the OB of the goldfish is devoid of intrinsic cholinergic and nitrergic neurons, although AChE- and NADPHd-reactive neurons as well as ChAT-positive fibers were observed. The OB is the first central relay station in the vertebrate olfactory system (MacLeod and Lowe 1976, Yoshihara et al. 2001). The sensory information received there is processed by a diverse array of local interneurons that have different spatial distributions, neurochemical expression profiles and synaptic connections of GABAergic, dopaminergic and glutamatergic origin.

Cholinergic activity affects OB information processing and associated learning and memory. However, the presence of intrinsic cholinergic interneurons in the OB remains controversial. Although a small population of intrinsic AChE-positive cells has been observed in the OB of elasmobranches (Anadón et al. 2000) and teleosts (Ekström 1987, Brantley and Bass 1988, Clemente et al. 2004, Giraldez-Perez et al. 2008, 2009, López et al. 2012), no ChAT-immunoreactive somas or fibers were identified. The presence of ChAT-labeled cells has, however, been reported in elasmobranches (Anadón et al. 2000). The presence of cholinergic signaling in the OB of zebrafish has also been established by the identification of a varicose network of cholinergic fibers originating from the terminal nerve ganglion and the demonstration that nAChR agonists stimulate the activity-dependent labeling of bulbar neurons (Edwards et al. 2007), which is in agreement with the presence of ChAT-positive fibers in the OB.

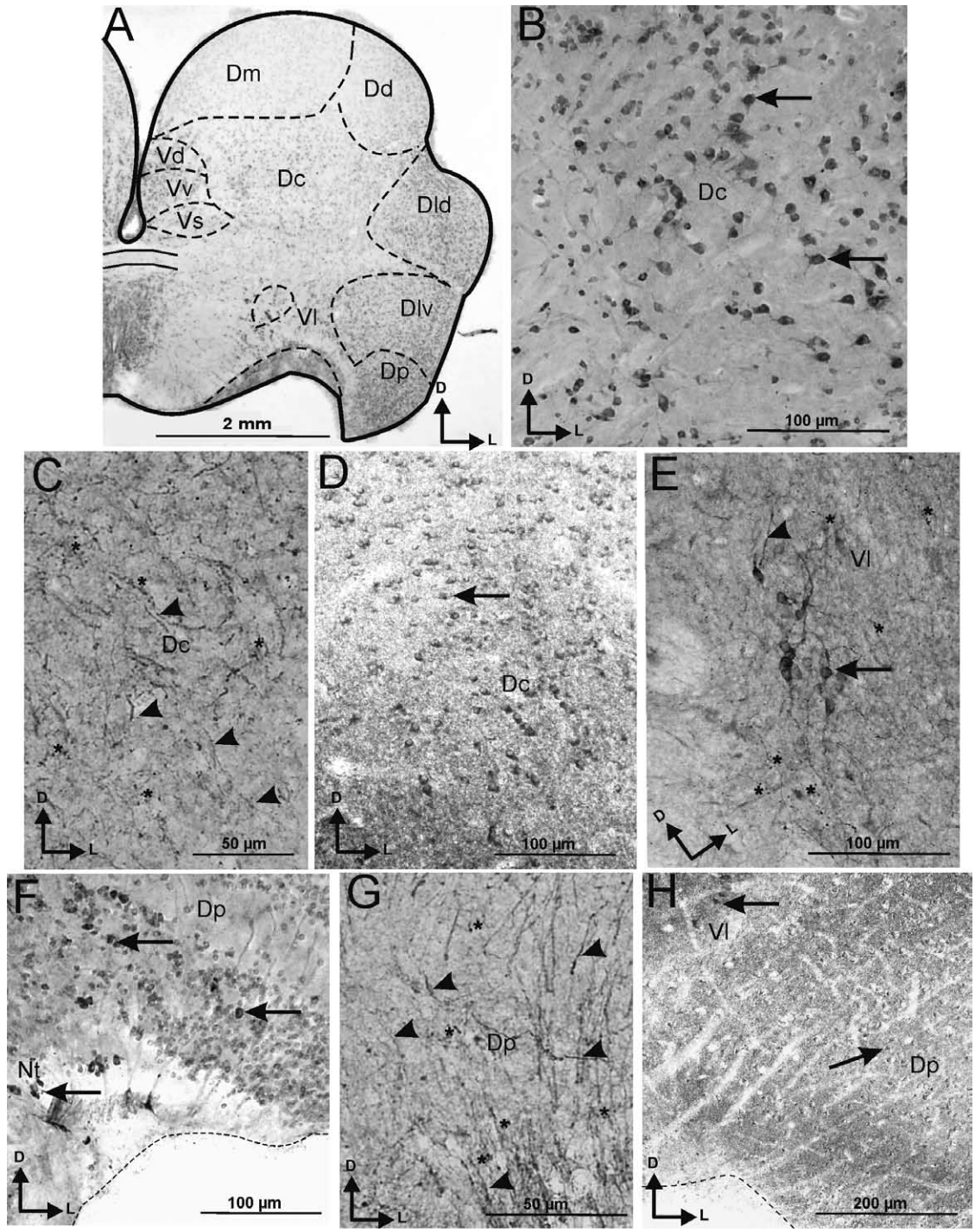

Fig. 6. (A) Transverse Nissl-stained section through the telencephalic hemisphere of the goldfish (at the level of Figs 1E and 2E) showing the limits of the different regions outlined by dashed lines; the coordinates are the same as those in the bright field photomicrographs shown in B–H. (B) AChE-labeled cells (black arrows) within the central part of the dorsal telencephalic area (Dc). (C) ChAT-positive fibers (black arrowheads) and *en-passant boutons* (asterisks) within Dc. (D) NADPHd-labeled neurons within Dc. (E) ChAT-positive neurons, fibers and *en-passant boutons* within the lateral part of the ventral telencephalic area (Vl). (F) AChE-labeled cells within the posterior part of the dorsal telencephalic area (Dp), and the nucleus taeniae (Nt). (G) ChAT-positive fibers and *en-passant boutons* within Dp. (H) NADPHd-labeled neurons within Dp and Vl. Anatomical directions indicate the orientation of the photographed section.

A few studies have shown a small number of bulbar ChAT-expressing cells in rats (Phelps et al. 1992), but most studies failed to label cholinergic cell bodies in the OB using either immunolabeling or *in situ* hybridization techniques in other vertebrate (anurans: Marín et al. 1997, reptiles: Medina et al. 1993, birds: Medina and Reiner 1994, and mammals: Le Jeune and Jourdan 1994, Butcher et al. 1995, Ichikawa et al. 1997). In addition, many studies have reported various types of bulbar neurons, including output and local AChE-labeled interneurons in the OB, with the overall results being inconclusive for the presence of cholinergic interneurons given that AChE is present in both cholinergic and cholinoceptive neurons (Nickell and Shipley 1988, Le Jeune and Jourdan 1994). Without consistent results showing the presence of ChAT-positive neurons in the OB, the conclusion is that AChEpositive neurons are cholinoceptive. It has been proposed that NO plays a role in olfactory signal transduction (Breer and Shepherd 1993). The presence of NADPHd-positive neurons in the OB of vertebrates (cyclostomes: Schober et al. 1994, elasmobranches: Dellacorte et al. 1995, teleosts: Jadhao et al. 1999, Lema and Nevitt 2001, Singru et al. 2003, and mammals: Alonso et al. 1998, Schoenfeld and Knott 2002) has been previously demonstrated; however, in contrast to the present results, only a few authors have demonstrated, using different labeling techniques, the presence of a small number of nNOS-positive neurons in the teleostean OB (Virgili et al. 2001, Ando et al. 2004).

The boundary between the pallium and the subpallium of the telencephalon in teleost fish has been defined by precise neurochemical and connectional data (Ito and Yamamoto 2009, Nieuwenhuys 2011, Ganz et al. 2012). The dorsal region (D) represents the pallium and the ventral region (V) represents the subpallium (Nieuwenhuys 2009).

The dorsal telencephalon of teleosts can be divided into seven or more divisions on the basis of developmental, genetic, hodological, neurochemical and functional criteria. These divisions comprise three longitudinal columns: Dm, Dd and Dc, considered together, and Dl, which converge into a uniform posterior part Dp (Northcutt 2008). Soma sizes were similar in Dl and Dp, and in Dm and Dd. The columns can also be divided into several subregions: Dmd and Dmv, as well as Dld and Dlv, precommissurally (Northcutt 2006, 2008). These divisions of the dorsal telencephalic area of teleost fish have been compared with different tetrapod structures by different authors.

All the studied areas of the goldfish telencephalic pallium contained AChE-positive cells and ChAT-positive axons and terminal fields, which could indicate a cholinoceptive role for the AChE-positive pallial neurons in goldfish as well as other vertebrates (elasmobranches: Anadón et al. 2000, teleosts: Brantley and Bass 1988, Clemente et al. 2004, 2005, anurans: Marín et al. 1997, birds: Medina and Reiner 1994, and mammals: Butcher 1995, Ichikawa et al. 1997). The pallial areas studied in the present report received projections from cholinergic cells in the basal forebrain, although a cholinergic projection from the brainstem has also been described in teleosts (Ekström 1987, Brantley and Bass 1988). ChAT-positive cells are absent in the goldfish pallium. The absence of cholinergic cells in the pallium of the telencephalon is a characteristic shared by teleosts (Ekström 1987, Brantley and Bass 1988, Pérez et al. 2000, Clemente et al. 2004, Mueller et al. 2004, Wullimann and Mueller 2004, López et al. 2012) and most tetrapods (Butcher 1995), and could thus be considered as a primitive feature of vertebrates.

The Dl, Dd and Dm parts of the goldfish telencephalon (Giraldez-Perez et al. 2008, 2009), like the pallial areas of other vertebrates, are rich in neurons producing NADPHd and nNOS (lamprey: Schober et al. 2004, teleosts: Arévalo et al. 1995, Jadhao and Malz 2004, present results, amphibians: Muñoz et al. 1996, González et al. 2002, López and González 2002, birds: Suárez et al. 2006, and mammals: Guirado et al. 2008).

Dm appears to be heterogeneous along its rostrocaudal axis (Murakami et al. 1983, Briñón et al. 1994, Castro el al. 2003, Northcutt 2006, Yamamoto et al. 2007, Giassi et al. 2012); however, in the present results a more dorsally located area (Dmd) was identified, where NADPHd- and nNOS-labeled neurons were organized in strings of neurons. Another more ventrally located area (Dmv) was also characterized, where NADPHd-labeled neurons were more densely packed and no nNOS-positive neurons were seen. These two subdivisions have also been found in teleosts with the aid of different immunohistochemical techniques (Castro et al. 2003, Giassi et al. 2012). Other authors consider that the Dmv subdivision represents the dorsal part of Vd (Northcutt 2006) due to the lack of calretinin-positive immunoreactivity. In both cases, a differentiated region of Dm has been demonstrated. On the basis of its connectivity and the topological relationships of its subdivisions, several

authors have also associated Dm with the ventral pallium (Wulliman and Mueller 2004, Northcutt 2006, 2008, Braford 2009, Mueller et al. 2011). It has been proposed that Dm is involved in emotional learning, because lesions within Dm induce selective deficits in emotional (but not spatial) learning in goldfish, indicating that this area could be the ventral pallium (pallial amygdala) (Salas et al. 2006, Portavella and Vargas 2005, Portavella et al. 2004a,b). In the present study, Dmd contained, but Dmv was devoid of nNOS-positive cells. However, considerable controversy remains, with Nieuwenhuys (2009) proposing, based mainly on topological grounds, that Dm is homologous to the lateral pallium, whereas other authors have suggested, on the basis of developmental and topological data, that it is homologous to the dorsal pallium (Yamamoto et al. 2007) or that it corresponds to the medial pallium (Harvey-Girard et al. 2012).

Dl has been reported to be homologous to the medial pallium of tetrapods (Northcutt 2008, Braford 2009, Nieuwenhuys 2009, Mueller et al. 2011, Harvey-Girard et al. 2012). The ventral part of Dl in goldfish (Dlv, Northcutt 2006) is considered to be involved in the processing of olfactory information, as it receives a minor OB input, as well as inputs from the preglomerular complex, projects to Dc, and has reciprocal connections to Dd (Northcutt 2006). NADPHd- and nNOS-positive neurons, of similar soma size were found within Dl, which could also be subdivided into a more dorsal subdivision (Dld) and another more ventral region (Dlv) that was devoid of nNOS-positive cells. These subdivisions have also been identified using several other immunohistochemical techniques in teleosts (Castro et al. 2003, Yamamoto et al. 2007, Giassi et al. 2012). However, Harvey-Girard and colleagues (2012) reported strong differences in the expression of transcription factors in Dld and Dlv, which is consistent with our finding of two different nNOS-positive inmunohistochemical distributions for Dld and Dlv.

Three other pallial divisions have been considered for teleosts: one is more centrally located (Dc), a second is the narrower Dd area located between Dm and Dl, and the third subdivision is Dp, the pallial target of massive projections from the OB (Northcutt 2008). AChE-, NADPHd- and nNOS-positive neurons were identified in all of these regions. Dc is the most ventral of the different parts of the dorsal telencephalic area, where NADPHd- and nNOS-positive neurons were found to have larger soma sizes than those seen in other subdivisions. These neurons are considered to send abundant efferent connections to several extratel-encephalic centers, including the paracomissural nucleus, optic tectum, torus semicircularis, thalamus, preglomerular complex, posterior tubercle nuclei, and inferior hypothalamic lobes, indicating that Dc is involved in multisensorial processing and modulation of brain activity (Folgueira et al. 2004); some authors have even considered Dc to be the deep, efferent layers (V, VI) of the mammalian isocortex (Harvey-Girad et al. 2012). Dd has been described as a potential homolog of the dorsal pallium of tetrapods (Nieuwenhuys 2009). However, other authors support the theory that this region could be the dorsal pallium or a uniquely evolved pallial region in teleosts (Northcutt 2008, Braford 2009). Dp has been characterized as homologous to the lateral pallium of amphibians by some authors, or even the piriform cortex based on its molecular anatomy and expression pattern of genes involved in the regulation of neurotransmitters (Mueller and Wullimann 2009); however, other authors suggest that this region is homologous with the medial pallium of inverted forebrains (Nieuwenhuys 2009).

The subpallium exhibits several subdivisions, whose homologies to nuclei in other vertebrates have been the subject of debate (Nieuwenhuys 2009). ChAT-immunoreactive neuronal cell bodies were found only in the subpallial Vl area of the goldfish telencephalon, which represents the most rostral population of cholinergic neurons in the brain of this species, similar to findings described in other reports (stickleback fish: Ekström 1987, trout: Pérez et al. 2000, Clemente et al. 2005, goldfish: Giraldez-Perez et al. 2009, zebrafish: Mueller et al. 2004). However, this situation is different from that found in the midshipman teleost fish (Brantley and Bass 1988), which has cholinergic neurons within Vv. The Vl nucleus sends extensive ascending projections to the dorsal, medial and lateral divisions of the pallium (Murakami et al. 1983, Pérez et al. 2000, Northcutt 2006). These cholinergic cells appear to represent septal populations comparable to cholinergic neurons in the amniote basal forebrain (Wullimann and Mueller 2004), and might have a functional role in the activation and modulation of the teleost pallium, similar to the basal forebrain cholinergic populations in tetrapods (Butcher 1995, Pérez et al. 2000). However, comparable cholinergic cells seem to be absent in the telencephalon of elasmobranches

(Anadón et al. 2000). Thus, the cholinergic basal (septal) forebrain population in teleost fish could have evolved homoplastically to those found in tetrapods (Wullimann and Mueller 2004).

As in other fishes (Ekström 1987, Brantley and Bass 1988, Anadón et al. 2000, Pérez et al. 2000), the Vd nucleus of the goldfish is devoid of ChAT-immunoreactive neurons, but it does show a particularly high density of AChE staining, as well as ChAT-positive axons and terminal arborizations. The Vd neurons are richly innervated by cholinergic boutons, indicating that this area is largely cholinoceptive, and it has been identified as homologous to the striatum in tetrapods (Wullimann and Mueller 2004), which is characterized by the presence of the enzymes associated with acetylcholine metabolism. This comparison is also supported by the presence of substance P-immunoreactive neurons, and high densities of GABA, and glutamate decarboxylase mRNA, as well as the dopamine receptor D2 mRNA, in teleost fish (Wullimann and Mueller 2004). The present results have also shown that neurons in the Vd nucleus of goldfish, like the striatum of tetrapods (González et al. 2002), are nitrergic. These results could help explain the different hypotheses concerning subpallium homologies with other vertebrates.

In the present results, NADPHd- and nNOS-labeled neurons were located in the Vd, Vp, and Vs subdivisions and in Nt. While only NADPHd-labeled neurons were found in the Vv and Vl parts, they presented slightly different soma sizes. There is some disagreement about which parts of the subpallial regions are striatal and which are striatal-septal domains (Wulliman and Mueller 2004, Ganz et al. 2012). It has been suggested that the Vd and Vv nuclei represent the striatum and septum, respectively, because dopaminergic innervation originating in the diencephalic posterior tubercle reaches Vd, whereas dense Vv efferents project to the midline hypothalamus in the adult zebrafish subpallium (Wullimann and Rink 2002). In the present study, Vv was found to be devoid of nitrergic cells, but these cells were present in Vd. Other reports have also described the presence of calretinin-immunoreactive cells in Vd and Vv, but only the presence of calretinin-rich processes in the Vd of zebrafish (Castro et al. 2006). These data are in contrast to those obtained for the trout (Castro et al. 2003), where Vd is devoid of calretinin-immunoreactive cells.

CONCLUSIONS

The cholinergic and nitrergic systems in the teleost brain could be involved in the activation and modulation of the pallial circuitries and play an important role in sensory processing, motor control, cognition, learning and memory. The distributions described here for the cholinergic and nitrergic elements in the goldfish telencephalon to some extent match those reported for other teleosts. However, our observations provide new insights concerning species differences, in particular the cholinoceptive nature of the telencephalic lobes. A putative relationship between nitrergic and cholinergic neurons could be inferred from the overlapping of terminal cholinergic fields and nitrergic neuronal populations, with NO possibly regulating the level of acetylcholine released by the terminal endings of cholinergic neurons.

The pallium and subpallium exhibit several subdivisions, whose homologies to nuclei in other vertebrates have been controversial. The present report has revealed some particular features of the goldfish pallium. Dm appears to be heterogeneous along its dorso-ventral axis, with NADPHd- and nNOS-positive neurons being found more dorsally in Dmd, and only NADPHd-labeled neurons being observed more ventrally in Dmv. Dl could be also subdivided into a more dorsal subdivision (Dld), which contained NADPHd- and nNOS-positive neurons, and another more ventral (Dlv) that was devoid of nNOS-positive cells. These results could help to elucidate the likelihood of the different hypotheses concerning pallial homologies with other vertebrates.

ACKNOWLEDGEMENTS

This research has been possible due to funding from the Junta de Andalucía (grant P09-CVI-4617).

The authors are grateful to the technical assistance provided by Ms. Angeles Bueso, as well as to the Servicio de Microscopía del Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS).

REFERENCES

Adrio F, Anadón R, Rodríguez-Moldes I (2000) Distribution of choline acetyltransferase (ChAT) immunoreactivity in the central nervous system of a chondrostean, the siberian sturgeon (*Acipenser baeri*). J Comp Neurol 426: 602–621.

Alonso JR, Porteros A, Crespo C, Arévalo R, Briñón JG, Weruaga E, Aijón J (1998) Chemical anatomy of the

macaque monkey olfactory bulb: NADPH-diaphorase/nitric oxide synthase activity. J Comp Neurol 402: 419–434.

Anadón R, Molist P, Rodríguez-Moldes I, López JM, Quintela I, Cervino MC, Barja P, González A (2000) Distribution of choline acetyltransferase immunoreactivity in the brain of an elasmobranch, the lesser spotted dogfish (*Scyliorhinus canicula*). J Comp Neurol 420: 139–170.

Ando H, Shi Q, Kusakabe T, Ohya T, Suzuki N, Urano A (2004) Localization of mRNAs encoding alpha and beta subunits of soluble guanylyl cyclase in the brain of rainbow trout: comparison with the distribution of neuronal nitric oxide synthase. Brain Res 1013: 13–29.

Arévalo R, Alonso JR, García-Ojeda E, Briñón JG, Crespo C, Aijón J (1995) NADPH-diaphorase in the central nervous system of the tench (*Tinca tinca* L, 1758). J Comp Neurol 352: 398–420.

Bond CE, Zimmermann M, Greenfield SA (2009) Upregulation of alpha7 nicotinic receptors by acetylcholinesterase C-terminal peptides. PLoS One 4: e4846.

Bordieri L, Persichini T, Venturini G, Cioni C (2003) Expression of nitric oxide synthase in the preoptic-hypothalamo-hypophyseal system of the teleost Oreochromis niloticus. Brain Behav Evol 62: 43–55.

Braford MR Jr (2009) Stalking the everted telencephalon: comparisons of forebrain organization in basal ray-finned fishes and teleosts. Brain Behav Evol 74: 56–76.

Brantley RK, Bass AH (1988) Cholinergic neurons in the brain of a teleost fish (*Porichthys notatus*) located with a monoclonal antibody to choline acetyltransferase. J Comp Neurol 275: 87–105.

Breer H, Shepherd GM (1993) Implications of the NO/cGMP system for olfaction. Trends Neurosci 16: 5–9.

Briñón JG, Alonso JR, García-Ojeda E, Arévalo R, Porteros A, Velasco A, Aijón J (1994) Parvalbumin immunoreactivity in the telencephalic hemispheres of the tench, *Tinca tinca*. Arch Ital Biol 132: 1–12.

Butcher LL (1995) Cholinergic neurons and networks. In: The Rat Nervous System, 2nd ed. (Paxinos G, Ed.). Academic Press, San Diego, CA, p. 1003–1015.

Castro A, Becerra M, Manso MJ, Anadón R (2003) Distribution and development of calretinin-like immunoreactivity in the telencephalon of the brown trout, *Salmo trutta fario*. J Comp Neurol 467: 254–269.

Castro A, Becerra M, Manso MJ, Anadón R (2006) Calretinin immunoreactivity in the brain of the zebrafish, *Danio rerio*: distribution and comparison with some neuropeptides and neurotransmitter-synthesizing enzymes. I. Olfactory organ and forebrain. J Comp Neurol 494: 435–459.

Clemente D, Porteros A, Weruaga E, Alonso JR, Arenzana FJ, Aijon J, Arévalo R (2004) Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis. J Comp Neurol 4742: 75–107.

Clemente D, Arenzana FJ, Sánchez-González R, Porteros A, Aijón J, Arévalo R (2005) Comparative analysis of the distribution of choline acetyltransferase in the central nervous system of cyprinids. Brain Res Bull 66: 546–549.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide syntase and neuronal NADPH diaphorase are identical in brain and peripheral tissue. Proc Natl Acad Sci U S A 88: 7797–7801.

Dellacorte C, Kalinoski DL, Huque T, Wysocki L, Restrepo D (1995) NADPH diaphorase staining suggests localization of nitric oxide synthase within mature vertebrate olfactory neurons. Neuroscience 66: 215–225.

Edwards JG, Greig A, Sakata Y, Elkin D, Michel WC (2007) Cholinergic innervation of the zebrafish olfactory bulb. J Comp Neurol 504: 631–645.

Ekström P (1987) Distribution of choline acetyltransferase-immunoreactive neurons in the brain of a cyprinid teleost (*Phoxinus phoxinus* L.). J Comp Neurol 256: 494–515.

Folgueira M, Anadón R, Yáñez J (2004) Experimental study of the connections of the telencephalon in the rainbow trout (*Oncorhynchus mykiss*). II: Dorsal area and preoptic region. J Comp Neurol 480: 204–233.

Ganz J, Kaslin J, Freudenreich D, Machate A, Geffarth M, Brand M (2012) Subdivisions of the adult zebrafish subpallium by molecular marker analysis. J Comp Neurol 520: 633–655.

Giassi AC, Harvey-Girard E, Valsamis B, Maler L (2012) Organization of the gymnotiform fish pallium in relation to learning and memory: I. Cytoarchitectonics and cellular morphology. J Comp Neurol 520: 3314–3337.

Gilbert D, Lecchi M, Arnaudeau S, Bertrand D, Demaurex N (2009) Local and global calcium signals associated with the opening of neuronal alpha7 nicotinic acetylcholine receptors. Cell Calcium 45: 198–207.

Giraldez-Perez RM, Gaytan SP, Ruano D, Torres B, Pasaro R (2008) Distribution of NADPH-diaphorase and nitric oxide synthase reactivity in the central nervous system of the goldfish (*Carassius auratus*). J Chem Neuroanat 35: 12–32.

Giraldez-Perez RM, Gaytan SP, Torres B, Pasaro R (2009) Co-localization of nitric oxide synthase and choline acetyltransferase in the brain of the goldfish (*Carassius auratus*). J Chem Neuroanat 37: 1–17.

González A, Moreno N, López JM (2002) Distribution of NADPH-diaphorase/nitric oxide synthase in the brain of the caecilian *Dermophis mexicanus* (amphibia: gymnophiona): comparative aspects in amphibians, Brain Behav Evol 60: 80–100.

Guirado S, Real MA, Dávila JC (2008) Distinct immunohistochemically defined areas in the medial amygdala in the developing and adult mouse. Brain Res Bull 75: 214–217.

Harvey-Girard E, Giassi AC, Ellis W, Maler L (2012) Organization of the gymnotiform fish pallium in relation to learning and memory: IV. Expression of conserved transcription factors and implications for the evolution of dorsal telencephalon. J Comp Neurol. 520: 3395–3413.

Havekes R, Abel T, Van der Zee EA (2011) The cholinergic system and neostriatal memory functions. Behav Brain Res 221: 412–423.

Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC (1993) Targeted disruption of the neuronal nitric oxide synthase gene. Cell 75: 1273–1286.

Ichikawa T, Ajiki K, Matsuura J, Misawa H (1997) Localization 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 13: 23–39.

Ito H, Yamamoto N (2009) Non-laminar cerebral cortex in teleost fishes? Biol Lett 5: 117–121.

Jadhao AG, Malz CR (2004) Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity in the brain of a cichlid fish, with remarkable findings in the entopeduncular nucleus: a histochemical study. J Chem Neuroanat 27: 75–86.

Jadhao AG, Wilke I, Meyer DL (1999) NADPH-diaphorase expression in the hypothalamo-hypophysial system of different catfish. J Hirnforsch 39: 513–523.

Karnovsky MJ, Roots L (1964) A "direct-coloring" thiocholine method for cholinesterase. J Histochem Cytochem 12: 219–221.

Le Jeune H, Jourdan F (1994) Acetylcholinesterase-containing intrinsic neurons in the rat main olfactory bulb: cytological and neurochemical features. Eur J Neurosci 6: 1432–1444.

Lema SC, Nevitt GA (2001) Re-evaluating NADPH-diaphorase histochemistry as an indicator of nitric oxide synthase: an examination of the olfactory system of coho salmon (*Oncorhynchus kisutch*). Neurosci Lett 313: 1–4.

López JM, González A (2002) Ontogeny of NADPH diaphorase/nitric oxide synthase reactivity in the brain of *Xenopus laevis*. J Comp Neurol 445: 59–77.

López JM, Perlado J, Morona R, Northcutt RG, González A (2012) Neuroanatomical organization of the cholinergic system in the central nervous system of a basal actinopterygian fish, the Senegal bichir *Polypterus senegalus*. J Comp Neurol 521: 24–49.

MacLeod NK, Lowe GA (1976) Field potentials in the olfactory bulb of the rainbow trout (*Salmo gairdneri*): evidence for a dendrodendritic inhibitory pathway. Exp Brain Res 25: 255–266.

Marín O, Smeets WJ, González A (1997) Distribution of choline acetyltransferase immunoreactivity in the brain of anuran (*Rana perezi, Xenopus laevis*) and urodele (*Pleurodeles waltl*) amphibians. J Comp Neurol 382: 499–534.

Medina L, Reiner A (1994) Distribution of choline acetyltransferase immunoreactivity in the pigeon brain. J Comp Neurol 342: 497–537.

Medina L, Smeets WJ, Hoogland PV, Puelles L (1993) Distribution of choline acetyltransferase immunoreactivity in the brain of the lizard *Gallotia galloti*. J Comp Neurol 331: 261–285.

Meek J, Nieuwenhuys R (1998) Holosteans and teleosts. In: The Central Nervous System of Vertebrates. (Nieuwenhuys R, Ten Donkelaar HJ, Nicholson C, Eds) Springer-Verlag, Berlin, DE, p. 759–937.

Mueller T, Wullimann MF (2009) An evolutionary interpretation of teleostean forebrain anatomy. Brain Behav Evol 74: 30–42.

Mueller T, Vernier P, Wullimann MF (2004) The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish *Danio rerio*. Brain Res 1011: 156–169.

Mueller T, Dong Z, Berberoglu MA, Guo S (2011) The dorsal pallium in zebrafish, *Danio rerio* (Cyprinidae, Teleostei). Brain Res 1381: 95–105.

Muñoz M, Muñoz A, Marín O, Alonso JR, Arévalo R, Porteros A, González A (1996) Topographical distribution of NADPH-diaphorase activity in the central nervous system of the frog, *Rana perezi*. J Comp Neurol 367: 54–69.

Murakami T, Morita Y, Ito H (1983) Extrinsic and intrinsic fiber connections of the telencephalon in a teleost, *Sebastiscus marmoratus*. J Comp Neurol 216: 115–131.

Nathan C, Xie QW (1994) Nitric oxide synthases: roles, tolls, and controls. Cell 78: 915–918.

Nickell WT, Shipley MT (1988) Two anatomically specific classes of candidate cholinoceptive neurons in the rat olfactory bulb. J Neurosci 8: 4482–4491.

Nieuwenhuys R (2009) The forebrain of actinopterygians revisited. Brain Behav Evol 73: 229–252.

Nieuwenhuys R (2011) The development and general morphology of the telencephalon of actinopterygian fishes: synopsis, documentation and commentary. Brain Struct Funct 215: 141–157.

Northcutt RG (2006) Connections of the lateral and medial divisions of the goldfish telencephalic pallium. J Comp Neurol 494: 903–943.

Northcutt RG (2008) Forebrain evolution in bony fishes. Brain Res Bull 75: 191–205.

Northcutt RG (2011) Do teleost fishes possess a homolog of mammalian isocortex? Brain Behav Evol 78: 136–138.

Paxinos G, Watson CR, Emson PC (1980) AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. J Neurosci Methods 3: 129–149.

Pérez SE, Yánez J, Marín O, Anadón R, González A, Rodríguez-Moldes I (2000) 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 428: 450–474.

Phelps PE, Houser CR, Vaughn JE (1992) Small cholinergic neurons within fields of cholinergic axons characterize olfactory-related regions of rat telencephalon. Neuroscience 48: 121–136.

Portavella M, Vargas JP (2005) Emotional and spatial learning in goldfish is dependent on different telencephalic pallial systems. Eur J Neurosci 21: 2800–2806.

Portavella M, Torres B, Salas C (2004a) Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. J Neurosci 24: 2335–2342.

Portavella M, Torres B, Salas C, Papini MR (2004b) Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (*Carassius auratus*). Neurosci Lett 362: 75–78.

Rodríguez-Moldes I, Molist P, Adrio F, Pombal MA, Yañez SE, Mandado M, Marín O, López JM, González A, Anadón R (2002) Organization of cholinergic systems in the brain of different fish groups: a comparative analysis. Brain Res Bull 57: 331–334.

Salas C, Broglio C, Durán E, Gómez A, Ocaña FM, Jiménez-Moya F, Rodríguez F (2006) Neuropsychology of learning and memory in teleost fish. Zebrafish 3: 157–171.

Schober A, Malz CR, Schober W, Meyer DL (1994) NADPH-diaphorase in the central nervous system of the larval lamprey (*Lampetra planeri*). J Comp Neurol 345: 94–104.

Schoenfeld TA, Knott TK (2002) NADPH diaphorase activity in olfactory receptor neurons and their axons conforms to a rhinotopically-distinct dorsal zone of the hamster nasal cavity and main olfactory bulb. J Chem Neuroanat 24: 269-285.

Sims KL, Kauffman FC, Johnson EC, Pickel VM (1974) Cytochemical localization of brain nicotinamide adenine dinucleotide phosphate (oxidized)-dependent dehydrogenases. Qualitative and quantitative distributions. J Histochem Cytochem 22: 7–19.

Singru PS, Sakharkar AJ, Subhedar N (2003) Neuronal nitric oxide synthase in the olfactory system of an adult teleost fish *Oreochromis mossambicus*. Brain Res 977: 157–168.

Suárez J, Dávila JC, Real MA, Guirado S, Medina L (2006) Calcium-binding proteins, neuronal nitric oxide synthase, and GABA help to distinguish different pallial areas in the developing and adult chicken. I. Hippocampal formation and hyperpallium. J Comp Neurol 497: 751–771.

Virgili M, Poli A, Beraudi A, Giuliani A, Villani L (2001) Regional distribution of nitric oxide synthase and NADPH-diaphorase activities in the central nervous system of teleosts. Brain Res 901: 202–207.

Wullimann MF, Rink E (2002) The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. Brain Res Bull 57: 363–370.

Wullimann MF, Mueller T (2004) Teleostean and mammalian forebrains contrasted: evidence from genes to behaviour. J Comp Neurol 475: 143–162.

Yamamoto N, Ishikawa Y, Yoshimoto M, Xue HG, Bahaxar N, Sawai N, Yang CY, Ozawa H, Ito H (2007) A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. Brain Behav Evol 69: 96–104.

Yoshihara Y, Nagao H, Mori K (2001) Neurobiology. Sniffing out odors with multiple dendrites. Science 291: 835–837.