Organization of the Orexin/Hypocretin System in the Brain of Holostean Fishes: Assessment of Possible Relationships with Monoamines and Neuropeptide Y

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Abstract

Holosteans form a small group of actinopterygian fishes considered the sister group of teleosts. Despite this proximity to the biggest group of vertebrates, relatively few studies have been conducted to investigate the organization of the central nervous system of this group of fishes. In this study, the neuroanatomical distribution of orexin/hypocretin-like immunoreactive (OX-ir) cell bodies and fibers was analyzed in the brain of 3 representative species of the 2 orders of extant holosteans, the spotted gar *Lepisosteus oculatus*, the Florida gar *Lepisosteus platyrhincus*, and the bowfin *Amia calva*. Antibodies against orexin-A (OXA) and orexin-B (OXB) were used, which labeled the same cells and fibers throughout the brain. In addition, double immunohistofluorescence was performed for the simultaneous detection of OXA and OXB with tyrosine hydroxylase, serotonin, and neuropeptide Y (NPY), in an attempt to localize the orexinergic structures precisely and study the possible interactions between these

neuroactive substances. The pattern of distribution of OX-ir cells in the 3 species was largely similar, showing labeled cells in the preoptic area (POA), and the tuberal and retrotuberal hypothalamic regions, with only subtle differences between species in the density of labeled cells. OX-ir fibers were found in all main brain subdivisions of the 3 species, mostly in the ventral subpallial areas, POA, hypothalamus, posterior tubercle, thalamus, and mesencephalic tectum. Different densities of orexinergic fibers were observed in relation to catecholaminergic and serotonergic cell groups, as well as an absence of colocalization between orexins and NPY in the same hypothalamic neurons. The comparison of these results with those obtained in other vertebrates highlights a constant pattern of distribution of this system of neurotransmission among different groups of actinopterygian fishes, especially in teleosts. Conserved features shared by all vertebrates studied were also observed, such as the presence of OX-ir cells in the basal hypothalamus, reflecting the preserved functions of these neuropeptides over the course of evolution.

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Abbreviations used in this paper

A.c.	<i>Amia calva</i>	PB	phosphate buffer
ac	anterior commissure	pc	posterior commissure
aCb	cerebellar auricle	POA	preoptic area
cCb	crista cerebellaris	PT	pretectum
Cb	cerebellum	PTh	prethalamus
cc	central canal	PVO	paraventricular organ
Cg	central gray	Rai	inferior raphe nucleus
Dc	central zone of the dorsal telencephalic area	Ram	median raphe nucleus
DF	dorsal funiculus	Ras	superior raphe nucleus
dh	dorsal horn of spinal cord	Ri	inferior reticular nucleus
DI	lateral zone of the dorsal telencephalic area	RM	retromamillary region
Dld	dorsolateral zone of the dorsal telencephalic area	Rm	median reticular nucleus
Dlv	ventrolateral zone of the dorsal telencephalic area	Rs	superior reticular nucleus
Dm	medial zone of the dorsal telencephalic area	RTu	retrotuberal hypothalamus
Dp	posterior zone of the dorsal telencephalic area	sac	stratum album centrale of mesencephalic tectum
flm	fasciculus longitudinalis medialis	SC	suprachiasmatic nucleus
fr	fasciculus retroflexus	SG	nucleus subglomerulosus
gl	glomerular layer of the olfactory bulb	sg	granular layer of the cerebellum
Hb	habenula	smn	somatomotor neurons of spinal cord
5-HT	serotonin	sm	molecular layer of the cerebellum
Hyp	hypophysis	so	spino-occipital motor nucleus
igl	internal granular layer	sol	solitary tract
inf	infundibulum	spv	periventricular layer of the mesencephalic tectum
Ip	interpeduncular nucleus	Tegm	tegmentum
Ipn	interpeduncular neuropile	TH	tyrosine hydroxylase
Is	isthmus nucleus	Th	thalamus
LC	locus coeruleus	TI	torus longitudinalis
LDT	laterodorsal tegmental nucleus	to	optic tract
L.o.	<i>Lepisosteus oculatus</i>	Tor	torus semicircularis
L.p.	<i>Lepisosteus platyrhincus</i>	Torl	torus lateralis
LF	lateral funiculus	TP	nucleus of the posterior tuberculum
lih	inferior hypothalamic lobe	Tu	tuberal hypothalamus
lot	lateral optic tract	v	ventricle
lr	lateral hypothalamic recess	Vd	dorsal nucleus of the ventral telencephalic area
MCA	Mauthner cell axon	vh	ventral horn of spinal cord
me	median eminence	VL	lateral nucleus of the ventral telencephalic area
ml	mitral cell layer	Vp	posterior nucleus of ventral telencephalic area
NPY	neuropeptide Y	Vs	supracommissural nucleus of the ventral telencephalic area
Nsol	nucleus of the solitary tract	Vv	ventral nucleus of the ventral telencephalic area
nII	optic nerve	VF	ventral funiculus
nVIII	octaval nerve	III	oculomotor nucleus
ob	olfactory bulb	IV	trochlear nucleus
oc	optic chiasm	Vm	trigeminal motor nucleus
OT	optic tectum	VIIIa	anterior octaval zone
OX-ir	orexin-immunoreactive	VIIIi	intermediate octaval zone
OXA/OXB	orexin-A/orexin-B	IXm	glossopharyngeal motor nucleus
Pa	paraventricular region	Xm	vagal motor nucleus
PAP	peroxidase antiperoxidase		

Introduction

Within extant vertebrates, the group Osteichthyes (bony vertebrates) is formed by the ray-finned fishes (actinopterygians) and the lobe-finned fishes (sarcopterygians), 2 large groups that diverged in the mid-Silurian, about 425 million years ago. The lobe-finned fishes separated in the early Devonian (approx. 400 million years ago) into coelacanth, lungfishes, and tetrapods. At about the same time, actinopterygians gave rise to the basal group of polypteriforms (Cladistia), and another basal group that diverged only later into chondrosteans and 2 groups of neopterygian fishes, the teleosts and the holosteans [Broughton et al., 2013]. With >30,000 species, teleosts are by far the most successful group of aquatic vertebrates whereas holosteans are a small group constituted by 2 orders: Lepisosteiformes, commonly known as gars, which includes 2 genera, *Atractosteus* (3 species) and *Lepisosteus* (4 species), and Amiiformes, known as bowfins, with a single living species, *Amia calva* [Nelson, 2006]. Based on the analysis of multiple nuclear gene sequences [Near et al., 2012], whole-genome duplication events [Pasquier et al., 2016], and phylotranscriptomics [Irisarri et al., 2017], holosteans are now regarded as a monophyletic sister group of teleosts. These data are consistent with traditional morphologically based inferences [Stiasny et al., 2004] and previous neuroanatomical studies [Nieuwenhuys, 1967, 2011; Nieuwenhuys and Meek, 1990]. Given the large diversification of teleosts, variation in brain region proportions and adaptations is not surprising [Meek and Nieuwenhuys, 1998]. Therefore, because teleosts exhibit many evolutionary novelties, understanding the ancestral condition of neopterygians appears easier by analyzing and resolving particular traits in the sister group of holosteans.

Despite the evolutionary importance of holosteans, there is little information about their neuronatomy, in contrast to the extensive experimental data about the organization of the nervous system of teleosts [Meek and Nieuwenhuys, 1998]. In particular, modern approaches such as tract-tracing and immunohistochemical techniques were used in several studies related to the visual and lateral-line systems [Northcutt and Butler, 1976, 1980; McCormick, 1981; Song and Northcutt, 1991a, 1991b; Butler and Northcutt, 1992; Collin and Northcutt, 1995] and some neurotransmitter systems [Parent and Northcutt, 1982; Chiba and Oka, 1999; Malz et al., 1999; Baker and Bird, 2002; Chiba, 2005].

Among the numerous neurotransmitters that characterize specific brain systems, orexins (hypocretins) are

well conserved across vertebrates, and so studies of orexinergic cells and fibers in the brain have been carried out in representatives of most vertebrate groups (see below). Orexin-A (OXA) and OXB (hypocretins 1 and 2, respectively) are 2 neuropeptides that belong to the incretin peptide superfamily and are derived from the common precursor prepro-orexin [de Lecea et al., 1998; Sakurai et al., 1998; Alvarez and Sutcliffe, 2002]. In mammals, only a few hypothalamic cell populations contain orexin-producing neurons, which show extensive projections to most brain regions [de Lecea et al., 1998; Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; McGranaghan and Piggins, 2001; Mintz et al., 2001; Zhang et al., 2001, 2004; Ferguson and Samson, 2003; Kirouac et al., 2005; Nixon and Smale, 2007; Kruger et al., 2010; Bhagwandin et al., 2011a, 2011b; Gravett et al., 2011; Dell et al., 2012, 2013]. This may explain the diversity of the brain functions of these peptides, such as regulation of food intake, reproduction, the sleep-wake cycle, cardiovascular control, and thermoregulation [Hoskins et al., 2008; Teske et al., 2010; Sakurai and Mieda, 2011; Volkoff, 2012].

Due to the conserved molecular structure of orexins [Ohkubo et al., 2002; Shibata et al., 2008; Wong et al., 2011], it is possible to localize orexin-like immunoreactive (OX-ir) cells and fibers in the brains of nonmammalian vertebrates by using antibodies against mammalian orexins. The distribution of orexins in the brain has been reported in birds [Ohkubo et al., 2002; Singletary et al., 2006; Miranda et al., 2013], reptiles [Domínguez et al., 2010], amphibians [Shibahara et al., 1999; Galas et al., 2001; López et al., 2009b, 2016], and lungfishes [López et al., 2009a]. In the case of teleosts, there are many studies regarding the orexinergic system [Kaslin et al., 2004; Huesa et al., 2005; Amiya et al., 2007; Panula, 2010; Matsuda et al., 2012; Pérez Sirkin et al., 2013] and its functions [Volkoff et al., 2005; Matsuda et al., 2012; Volkoff, 2012; Chiu and Prober, 2013; Nissembaum et al., 2014; Shahjahan et al., 2014], which were demonstrated to be basically comparable to those in mammals. In a previous study, we reported the distribution of the OX-ir cells and fibers in the brain of cladistians, in an attempt to highlight shared primitive features of this system in ray-finned fishes [López et al., 2014]. Surprisingly, the orexinergic system has not been previously addressed in holosteans.

Given the phylogenetic importance of holosteans as the sister group of teleosts, in our laboratory we have a line of research into holosteans that aims to clarify the neuroanatomical features that aid in the understanding of the basic condition of neopterygians and enable comparisons with more primitive actinopterygians, such as

cladistians. We have described the cholinergic [Morona et al., 2013] and nitrergic systems [López et al., 2017] and some transcription factors in the subpallium of holosteans [González et al., 2014]. Following this research, we have conducted this study to provide detailed information about the location of OX-ir cells and fibers in the brain of 3 representative species of holosteans: the spotted gar (*Lepisosteus oculatus*), the Florida gar (*L. platyrhincus*), and the bowfin (*A. calva*). We carried out immunohistochemical studies with the same methodology previously used in the brain of several tetrapods [López et al., 2009a, 2016; Domínguez et al., 2010], lungfishes [López et al., 2009b], and cladistians [López et al., 2014]; this reinforces the reliability of results and facilitates their comparison across vertebrates.

In addition, we investigated, by means of double-labeling experiments, the possible colocalization/codistribution of orexins with tyrosine hydroxylase (TH; the first and rate-limiting enzyme in the synthesis of catecholamines), serotonin (5-HT), and neuropeptide Y (NPY). These experiments were prompted by previous studies on tetrapods and fishes, which reported interactions with some catecholaminergic groups in the brainstem, serotonergic cells in the raphe nuclei [Peyron et al., 1998; Baldo et al., 2003; Wang et al., 2003; Kaslin et al., 2004; Zhang et al., 2004; Balcita-Pedicino and Sesack, 2007; López et al., 2009a, 2009b, 2014, 2016; Domínguez et al., 2010; Panula, 2010], and NPY elements in the hypothalamus [Volkoff, 2006; López et al., 2014].

Finally, as in our previous studies of holosteans, our comparative analysis was framed within the current neuromeric model of the brain regionalization that has now been validated for most vertebrates (forebrain: Puelles and Rubenstein [2003, 2015]; brainstem: Gilland and Baker [1993]; Aroca and Puelles [2005]; Straka et al. [2006]).

Materials and Methods

Eighteen young adult gars, 9–16 cm in length (11 spotted gars, *L. oculatus*, and 7 Florida gars, *L. platyrhincus*), and 3 adult bowfins, *A. calva*, 28–35 cm in length, were used. The animals were obtained from licensed suppliers (PezyCia, Madrid, Spain) and maintained in aquaria with a controlled temperature (24–28°C) and natural light conditions. Although the sex of each animal was not determined, it was established that both males and females were studied. The original research reported here was performed according to the regulations and laws established by the European Union (2010/63/EU) and Spain (Royal Decree 53/2013) for the care and handling of animals in research, and after approval from the Complutense University to conduct the experiments described.

The animals were deeply anesthetized by immersion in 0.1–0.2% tricaine methanesulfonate solution (MS222, pH 7.3; Sandoz, Basel, Switzerland), and perfused transcardially with physiological saline, followed by 100–150 mL of cold 4% paraformaldehyde in 0.1-M phosphate buffer (PB; pH 7.4). The brain and upper spinal cord were removed and kept in the same fixative for 2–3 h. Subsequently, they were immersed in a solution of 30% sucrose in PB for 4–6 h at 4°C until they sank, and were then embedded in a solution of 20% gelatin with 30% sucrose in PB, and stored for 6 h in a 10% formaldehyde solution at 4°C. The brains were cut on a freezing microtome (30–40 µm) on the transverse or sagittal plane, and sections were collected and rinsed in cold PB.

Orexin Immunohistochemistry

The free-floating sections were rinsed twice in PB, treated with 1% H₂O₂ in PB for 20 min to reduce endogenous peroxidase activity, rinsed again 3 times in PB, and processed by the peroxidase antiperoxidase (PAP) method [Sternberger, 1979]. This included a first incubation of the sections in a primary goat anti-OXA serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA; code sc-8070) or goat anti-OXB serum (Santa Cruz Biotechnology; code sc-8071), diluted 1:500 in PB containing 0.5% Triton X-100, 15% normal rabbit serum, and 2% bovine serum albumin, for 48 h at 4°C. Subsequently, the sections were rinsed 3 times in PB for 10 min and incubated for 60 min at room temperature in rabbit anti-goat serum (Merck-Millipore, Darmstadt, Germany) diluted 1:50. After rinsing again 3 times in PB for 10 min, the sections were incubated for 90 min in goat PAP complex (diluted 1:500; Merck-Millipore). Secondary antiserum and PAP complex were diluted in PB containing 0.5% Triton X-100, 15% normal rabbit serum and 2% bovine serum albumin. Finally, the sections were rinsed three times for 10 min in PB, and subsequently developed for 3–6 min in 0.5 mg/mL DAB (Vector SK4100) intensified with nickel [Adams, 1981], with 0.01% H₂O₂ in PB. Transverse and sagittal series were mounted on glass slides (mounting medium: 0.25% gelatin in 0.1 M Tris-HCl buffer, pH 7.6) and, after dehydration, coverslipped with Entellan (Merck, Darmstadt, Germany). Some sections were counterstained with cresyl violet to facilitate the analysis of the results.

The specificity of the immunohistochemical reaction was corroborated with controls that included: (1) staining some selected sections with preimmune goat serum; (2) controls in which either the primary antibody, secondary antibody or the PAP complex was omitted; (3) homologous and heterologous preabsorptions of the primary antibody with synthetic blocking peptides for OXA or OXB (both from Santa Cruz Biotechnology; codes sc-8070P and sc-8071P respectively; 0.1, 1.0, or 10 µM). In all these negative controls, the immunostaining was eliminated, even when the goat anti-OXA or goat anti-OXB was preabsorbed with the blocking peptides at a low concentration (0.1 µM).

Double Orexin and NPY, TH, or 5-HT Immunolabeling

A procedure based on immunohistochemistry was used as follows: (1) a first incubation for 72 h at 4°C in a mixture of goat anti-OXA or goat anti-OXB (diluted 1:500) and rabbit anti-NPY (diluted 1:1,000; gift from Dr. J.D. Mikkelsen, University of Copenhagen, Denmark), mouse anti-TH (diluted 1:1,000; Immunostar, USA; code P22941), or rabbit anti-5-HT (diluted 1:1,000; Immunostar, USA; code 20080), and (2) a second incubation for 90 min at room temperature in a mixture of secondary antisera: don-

Fig. 1. a–u Diagrams of transverse sections through the brain of *L. platyrhincus* at the levels indicated in the lateral view of the brain. OX-ir cell bodies (large dots) and fibers (small dots, wavy

lines) are represented in the left half of each section. Faintly labeled cells are drawn empty. For abbreviations, see list.

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key anti-goat Alexa 594 (red fluorescence; diluted 1:300; Molecular Probes, Denmark) and FITC-conjugated chicken anti-rabbit (green fluorescence; diluted 1:100; Merck-Millipore) or chicken anti-mouse Alexa 488 (green fluorescence; diluted 1:300; Molecular Probes). After rinsing 3 times in PB, the sections were mounted on glass slides and coverslipped with UltraCruz™ mounting medium containing DAPI for fluorescence labeling of DNA (Santa Cruz Biotechnology, sc-24941). The specificity of the TH and 5-HT antisera has been previously described [Morona et al., 2013; González et al., 2014; López et al., 2017], and the pattern of staining observed for NPY was consistent with that reported for *L. oculatus* [Chiba, 2005].

Evaluation and Presentation of the Results

The distribution and localization of OX-ir cell bodies and fibers in the brains was carefully analyzed, and the pattern of labeling was charted in representative transverse sections at different brain levels for *L. platyrhincus* and *A. calva* (Fig. 1, 2). Drawings were made by means of camera lucida in which the sections counterstained with cresyl violet facilitated the interpretation of the localization of the labeled structures. The sections were analyzed with an Olympus BX51 microscope equipped for fluorescence with appropriate filter combinations for the identification of orexins and NPY/TH/5-HT-ir cells and fibers in the double-labeled sections. Photomicrographs were adjusted for contrast and brightness with Adobe Photoshop CS4 (Adobe Systems, San José, CA, USA) and were mounted on plates using Canvas 11 (ACS Systems International, Santa Clara, CA, USA). Selected photomicrographs of single-stained sections are presented in Figures 3, 4, and double-stained images are arranged in Figure 5.

The nomenclature is the same used in our previous studies on holosteans [Morona et al., 2013; López et al., 2017], and essentially follows previously used terms in these species [Northcutt, 1982; Parent and Northcutt, 1982; Song and Northcutt, 1991a, 1991b; Butler and Northcutt, 1992; Northcutt and Butler, 1993; Heijdra and Nieuwenhuys, 1994; Collin and Northcutt, 1995; Meek and Nieuwenhuys, 1998; Chiba and Oka, 1999; Baker and Bird, 2002; Chiba, 2005], but incorporates the terminology of the prosomeric model for the forebrain [Puelles and Rubenstein, 2003, 2015].

Results

Similar patterns of immunoreactivity were revealed with the antibodies against OXA and OXB used in this study and, for each of the 3 species examined, these were constant from animal to animal. Therefore, throughout the text, we will refer to OX-ir structures independently of the antibody used. The labeling observed was restricted to neuronal cell bodies located in specific forebrain groups, whereas fibers were widely distributed in the central nervous system, albeit more densely in the forebrain. The brain distribution of OX-ir cells and fibers in the 3 holostean species is described below, and the main data are summarized in Table 1 (where species variations can be observed). The schematic chartings in Figures 1 and 2

depict the distribution of the labeled cells and fibers for the cases of *L. platyrhincus* and *A. calva*, and are meant to help the description, which follows a rostrocaudal organization.

OX-ir Cell Bodies

The 3 species showed OX-ir neurons at comparable locations, although some differences in cell density were noted (Table 1). Cells in the preoptic area (POA) were the most rostrally located, and were observed close to the ventricular layer (Fig. 1d, 2d, 3c). They were small and rounded with cerebrospinal fluid-contacting (CSF-c) processes that reached the preoptic recess of the third ventricle (Fig. 3c, asterisks). *A. calva* showed more cells in this area than both *Lepisosteus* spp. (Table 1). The double-labeling techniques revealed that OX-ir and 5-HT-ir cells do not coexist in the same rostrocaudal levels of the POA, and that OX-ir cells occupy a dorsolateral position in relation to the TH-ir cells, with no coexpression of orexins and TH in the same cells (fig. 5a, a').

Another group of OX-ir cells was located in the retrotuberal area within the basal hypothalamus (RTu), and it extended from the most dorsal to the most ventral levels of this area (rostral to caudal hypothalamic levels in classical transverse sections). This population was the most numerous of the brain, and was formed by conspicuously intense OX-ir cells (Fig. 1g, h, 2g, h, 3f, g, j, 4a, b, 5d–i). Mainly bipolar cells with long CSF-c processes that reach the ventricle formed this OX-ir population (Fig. 3g, j, 4b, 5e). There were no significant differences in the labeling intensity and the number of OX-ir cells of this group across the 3 species studied, although the number of orexinergic cells was higher at the rostral level (Fig. 3f, g) than at the caudal hypothalamic level where the number of cells was clearly reduced (Fig. 3h, j). The double-labeling experiments showed that this group was located lateroventral to the paraventricular organ identified by its 5-HT-ir cells close to the ventricle (Fig. 5f, g), and ventromedial to the prethalamic TH-ir cell group from which it was distinctly separated (Fig. 5e, h, i). The double-staining procedure also showed that a small population of NPY-ir cells in the RTu intermingled with the OX-ir cells only at the rostral hypothalamic level, but actual colocalization of both neuropeptides in the same cells was never observed (Fig. 5d). However, a noticeable NPY-ir innervation of the OX-ir cell groups in the preoptic, retrotuberal, and tuberal hypothalamic regions was observed, and also a moderate OX-ir innervation of the NPY-ir hypothalamic cell population (Fig. 5d, d').

The last population of orexinergic neurons was located along the tuberal hypothalamus (Tu) of *A. calva* (Fig. 2h, i), and was restricted to the caudal tuberal hypothalamic levels in the case of *Lepisosteus* (Fig. 1i–k). These faintly OX-ir cells were small and showed bipolar morphology with a short CSF-c process (Fig. 3i) observed close to the ventricle of the infundibulum (Fig. 5k). Comparing the 3 species, *L. oculatus* had fewer OX-ir cells in the Tu (Table 1).

OX-ir Fibers

In the rostral telencephalon of the 3 species studied, sparse OX-ir varicose fibers and terminal-like structures were observed in the olfactory bulbs, located mainly in the internal granular layer (Fig. 1a, 2a, 3a). The pallial areas were poorly innervated by OX-ir fibers (Fig. 1b–e, 2b–e), being the ventrolateral (Dlv), central (Dc), and medial (Dm) zones of the dorsal telencephalic area, the regions more densely innervated (Fig. 3b, 5b). In contrast, the subpallium of holostean fishes showed a rich orexin innervation (Fig. 1b–e, 2b–d) that was especially relevant on the ventral part of the ventral telencephalic area (Vv) and, to a lesser extent, on the lateral part of the ventral telencephalic area (VL) of fishes of the genus *Lepisosteus* (Fig. 1b, c, 3b, 5b). Comparatively, the presence of OX-ir fibers and terminal-like structures in the dorsal (Vd), supracommissural (Vs) and posterior (Vp) parts of the ventral telencephalic area of the 3 species was markedly more restricted (Fig. 1b–e, 2b–d, 3b, 5b). The POA, together with the Vv, was the telencephalic zone with more OX-ir fibers (Fig. 1d, 2d), surrounding the preoptic orexinergic cells (Fig. 3c, 5a, a') and laterally separated from the ventricle. Comparatively, *L. platyrhincus* was the species with the largest number of immunoreactive fibers in this area (Table 1).

In the alar hypothalamus, remarkable OX-ir innervation was observed in the paraventricular and subparaventricular areas (Fig. 1e, f, 2e, f, 3d, e). The latter region, which includes the suprachiasmatic nucleus (SC), was the alar hypothalamic area with the highest density of OX-ir varicose fibers and terminal-like structures, especially in fishes of the genus *Lepisosteus* (Fig. 3e), some of which crossed the midline through the optic chiasm. In the double-labeling experiments, this prominent orexinergic innervation was observed close to the TH-ir cells of the SC (Fig. 5c). The basal hypothalamus was also widely innervated by OX-ir varicose fibers and terminal-like structures, with the Tu, RTu, and retromamillary region (RM) being the areas with more innervation in *Lepisosteus* (Fig. 1g–k, 2g–I, 4a–c, 5h, k) and less conspicuous in *A.*

calva (Fig. 3f, h). In contrast, the large inferior hypothalamic lobes were almost devoid of orexinergic innervation (Fig. 1i–k, 2i, j). By means of the double-labeling technique, the OX-ir fibers in the RM were observed intermingled with the prominent TH-ir cell group present in this area (Fig. 5k). Although the median eminence showed orexin innervation, no OX-ir fibers were observed entering the hypophysis (Fig. 1i–k, 2g, i, 5k).

According to the prosomeric model [Puelles and Rubenstein, 2003, 2015], in the caudal part of the forebrain, the diencephalon is divided into 3 prosomeres (p1–p3, from caudal to rostral) containing the pretectum (p1), the thalamus (p2; formerly dorsal thalamus), and the prethalamus (p3; formerly ventral thalamus) in their alar plates, and smaller tegmental regions in their basal plates. In the diencephalic alar plate, moderate OX-ir innervation of fine varicose fibers and terminal-like structures was observed mainly in the periventricular region of the 3 prosomeres (Fig. 1f–h, 2f–I, 3e, f, h, j, 4a, 5c', d', e, g', h, i). The asymmetric habenula situated dorsal to the thalamus was reached by a few OX-ir fibers only in the ventral part of both habenulae (Fig. 1f, 2g, 3e). In the basal plate of prosomere 3, a remarkable innervation of the TH-ir cell group located in the posterior tubercle (Fig. 1i, j, 2i, 4c, 5j) was observed, especially in the 2 *Lepisosteus* spp. (Table 1).

In the dorsal mesencephalon, moderate OX-ir innervation was found in deep and intermediate layers of the optic tectum along its rostrocaudal extent (Fig. 1g–m, 2h–m), whereas the superficial layers were almost devoid of immunoreactive fibers. These varicose fibers and terminal-like structures were located in the outer zone of the stratum griseum periventriculare and in the stratum album centrale (after Meek and Nieuwenhuys, 1998; Fig. 4a, d, 5d', l'). In all 3 species, moderate distribution of OX-ir fibers was also observed primarily in the periventricular region of the torus semicircularis and the mesencephalic tegmentum (Fig. 1h–k, 2j–l, 4d, 5l'). The torus lateralis of holosteans showed a few OX-ir varicose fibers (Fig. 1i, j, 2j–l) whereas the torus longitudinalis was devoid of orexinergic fibers (Fig. 1h–k, 2i–l 4a).

In general, the pattern of orexinergic innervation observed in the hindbrain was reduced compared to other

Fig. 2. a–u Diagrams of transverse sections through the brain of *A. calva* at the levels indicated in the lateral view of the brain. OX-ir cell bodies (large dots) and fibers (small dots, wavy lines) are represented in the left half of each section. Faintly labeled cells are drawn empty. For abbreviations, see list.

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Fig. 3. Photomicrographs of transverse sections through the forebrain of *L. platyrhincus* (*L.p.*), *L. oculatus* (*L.o.*), and *A. calva* (*A.c.*; indicated in the upper left corner of each photomicrograph) illustrating OX-ir cell bodies and fibers in the olfactory bulb (**a**), pallial and subpallial areas (**b**), preoptic area (**c**, asterisks indicate CSF-contacting cell processes), paraventricular hypothalamic area (**d** is a higher magnification of **d'**), suprachiasmatic nucleus and thalamus (**e**), and tuberal and retrotubal hypothalamic areas

from rostral (**f, g** [asterisks in **g** indicate the CSF-contacting cell processes]) to caudal levels (**h, j**). The higher magnification in **i** shows a detail of faintly OX-ir cells of tuberal hypothalamus. Photographs **a, b**, and **e** correspond to levels **a, b**, and **f** of Figure 1, whereas photographs **c, d**, and **f–j** correspond to levels **d, e**, and **h** of Figure 2. For abbreviations, see list. Scale bar, 500 μ m (**d', f, h**); 200 μ m (**a, b, d, e**); 100 μ m (**c, g, j**); 50 μ m (**i**).

Fig. 4. Photomicrographs of transverse sections through the brain of *L. platyrhincus* (*L.p.*), *L. oculatus* (*L.o.*), and *A. calva* (*A.c.*; indicated in the upper left corner of each photomicrograph) illustrating OX-ir cells and fibers in hypothalamic, diencephalic and mesencephalic levels (**a**), retrotuberal hypothalamic area (**b**, asterisks indicate CSF-contacting cell processes), retromamillary hypothalamic area and posterior tubercle (**c**), optic tectum, torus semicircularis, and mesencephalic tegmentum (**d**), central rhombencephalic gray and superior raphe nucleus (**e**, **f**), inferior raphe, inferior reticular and solitary tract nuclei (**g**, **h**), and spinal cord (**i**). Photographs **a–e**, **g–i** correspond to levels **h**, **j**, **n**, **r**, **s**, **u**, **f** of Figure 1, whereas photograph **f** corresponds to level **n** of Figure 2. For abbreviations, see list. Scale bar, 500 μ m (**a**, **f**); 200 μ m (**d–i**); 100 μ m (**b**, **c**).

Fig. 5. Photomicrographs of transverse double-labeled sections through the forebrain and brainstem of *L. platyrhincus* (*L.p.*), *L. oculatus* (*L.o.*), and *A. calva* (*A.c.*; indicated in the upper right corner of each photomicrograph) showing, in the same sections, staining for orexin (OX; red fluorescence) and TH (**a**, **a'**, **c**, **c'**, **e**, **h–k**, **n**, **o**), 5-HT (**b**, **f**, **g**, **g'**, **i**, **i'**, **m**), or NPY (**d**, **d'**) (green fluorescence), as indicated in the left corner of each photomicrograph. The relationship between the distinct immunoreactive elements in the brain is illustrated for the preoptic area (**a**), subpallial areas (**b**), suprachiasmatic nucleus (**c**, **c'**), retrotuberal hypothalamic area (**d**, arrowheads point to orexinergic cells separated from NPY-ir cells,

d'), prethalamic TH-ir cells in relation to the retrotuberal orexinergic cells (**e**, asterisks in **e** indicate CSF-contacting cell processes, **h**, **i**), serotonergic nucleus of the paraventricular organ in relation to the retrotuberal orexinergic cells (**f**, **g**, **g'**), and OX-ir fibers in the region of the posterior tubercle (**j**), retromamillary hypothalamic area (**k**), superior raphe nucleus (**l**, **l'**), inferior raphe nucleus (**m**), locus coeruleus (**n**), and the solitary tract nucleus (**o**). Photographs **a–d**, **g–j**, **o** correspond to levels **c**, **d**, **f**, **h–j**, **n**, **r**, **s** of Figure 1, whereas photographs **e**, **f** correspond to levels **h**, **n** of Figure 2. For abbreviations, see list. Scale bar, 500 μ m (**a'**, **d'**, **l'**), 200 μ m (**b**, **c'**, **e**, **g'**, **h**, **j**, **k**, **m**), 100 μ m (**a**, **c**, **d**, **f**, **g**, **i**, **n**, **o**).

(For figure see next page.)

Table 1. Comparative localization and relative abundance of OX-immunoreactive cells and fibers in CNS of the holostean fishes studied

	<i>L. platyrhincus</i>		<i>L. oculatus</i>		<i>Amia calva</i>	
	C	F	C	F	C	F
Forebrain						
<i>Telencephalon</i>						
Olfactory bulb	–	+	–	+	–	+
Pallial areas	–	+	–	+	–	+
Subpallial area Vd	–	+	–	+	–	+
Subpallial area Vv	–	+++	–	+++	–	++
Subpallial area VL	–	++	–	++	–	+
Subpallial areas Vs, Vp	–	+	–	+	–	+
Preoptic area	++	+++	+	++	+++	++
<i>Hypothalamus</i>						
Paraventricular region	–	++	–	++	–	++
Subparaventricular region	–	+++	–	+++	–	++
Tuberal region	++	+++	+	+++	++	++
Retrotuberal region	+++	+++	+++	+++	+++	++
Mamillary and retromamillary regions	–	+++	–	+++	–	++
Hypophysis	–	–	–	–	–	–
<i>Diencephalon</i>						
Prethalamus	–	++	–	++	–	++
Posterior tubercle	–	+++	–	+++	–	++
Habenula	–	+	–	+	–	+
Thalamus	–	++	–	++	–	++
Pretectum	–	++	–	++	–	++
Midbrain						
Optic tectum	–	++	–	++	–	++
Torus semicircularis	–	++	–	++	–	++
Torus longitudinalis	–	–	–	–	–	–
Torus lateralis	–	+	–	+	–	+
Mesencephalic tegmentum	–	++	–	++	–	++
Hindbrain						
Central gray	–	++	–	++	–	+++
Interpeduncular nucleus	–	++	–	++	–	++
Cerebellum	–	–	–	–	–	–
Laterodorsal tegmental nucleus	–	+	–	+	–	+
Locus coeruleus	–	–	–	–	–	+
Octavolateral area	–	+	–	+	–	+
Reticular formation	–	++	–	++	–	++
Superior raphe nucleus	–	++	–	++	–	+++
Median raphe nucleus	–	+	–	+	–	+
Inferior raphe nucleus	–	++	–	++	–	+
Nucleus of the solitary tract	–	++	–	++	–	+
Spinal cord						
	–	++	–	++	–	++

C, immunoreactive cell bodies; F, immunoreactive fibers; +, low density; ++, moderate density; +++, high density; –, no immunoreactive cell bodies or fibers.

Table 2. Summary of the distribution of OX-ir cells and fibers in different brain areas of the studied groups of vertebrates

	Cladistians		Holostei		Teleosts		Lungfishes		Amphibians		Reptiles		Birds		Mammals	
	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F
Forebrain																
Olfactory bulb	-	+	-	+	-	+/-	-	-	+	+	-	+	-	-	-	+/-
Pallium/cortex	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Striatum	-	+	-	+	-	+	+/-	+	+	+	-	+	-	+	-	+
Septum	-	+	-	+	-	+	+/-	+	+	+	-	+	-	+	-	+
Pallidum	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Amygdaloid complex	-	+	-	+	-	+	-	+	+	+	-	+	-	+	+/-	+
Preoptic area	+/-	+	+	+	+/-	+	+	+	+	+	+	+	-	+	-	+
Paraventricular region	-	+	-	+	+/-	+	-	+	+	+	-	+	+/-	+	-	+
Suprachiasmatic nucleus	+	+	-	+	+/-	+	+	+	+	+	-	+	+	+	+	+
Basal hypothalamus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Median eminence	-	+	-	+	-	+	-	+	+	+	-	+	-	?	-	+
Hypophysis	-	-	-	-	+/-	+/-	-	-	-	-	-	-	-	-	+/-	+/-
Prethalamus	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Habenula	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Thalamus	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Pretectum	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Brainstem																
Optic tectum/superior colliculus	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Torus semicircularis/inferior colliculus	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Mesencephalic tegmentum	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Nonmotor nuclei in the upper hindbrain: PPT/LDT/Is	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Locus coeruleus	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Reticular formation	-	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+
Raphe nuclei	-	+	-	+	-	+	-	+	+	+	-	+	-	?	-	+
Nucleus of the solitary tract	-	+	-	+	-	+	-	+	+	+	-	+	-	?	-	+
Spinal cord																
	-	+	-	+	-	+	-	-	+	+/-	-	+	-	?	-	+

Cladistians: López et al. [2014], *Teleosts*: Kaslin et al. [2004]; Huesa et al. [2005]; Faraco et al. [2006]; Amiya et al. [2007]; Pérez Sirkín et al. [2013]; Suzuki and Yamamoto [2013], *Lungfishes*: López et al. [2009b], *Amphibians*: Shibahara et al. [1999]; Galas et al. [2001]; Singletary et al. [2005]; Suzuki et al. [2008]; López et al. [2009a], *Reptiles*: Farrell et al. [2003]; Dominguez et al. [2010]. *Birds*: Ohkubo et al. [2002, 2003]; Phillips-Singh et al. [2003]; Singletary et al. [2006]; Miranda et al. [2013], *Mammals*: Sakurai et al. [1998]; Cutler et al. [1999]; Date et al. [1999]; Nambu et al. [1999]; Zhang et al. [2001]; McGranaghan and Piggins [2001]; Mintz et al. [2001]; Novak and Albers [2002]; Ciriello et al. [2003b]; Nixon and Smale [2007]; Kruger et al. [2010]; Bhagwandin et al. [2011a, 2011b]; Gravett et al. [2011]; Dell et al. [2012, 2013]; Gascuel et al. [2012]; Kruger et al. [2012]; Calvey et al. [2013].

C, immunoreactive cell bodies; F, immunoreactive fibers; Is, isthmus nucleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; +, presence of OX-ir cells or fibers; -, absence of OX-ir cells or fibers; +/-, presence of OX-ir cells and fibers only in some species of the group; ?, no data available.

areas of the forebrain and midbrain, with the exception of the rostral rhombencephalon of *A. calva* that showed a very intense innervation in the central gray, interpeduncular neuropil, and the superior raphe nucleus (Fig. 2m, n, 4f, f'). This innervation was low only in the 2 *Lepisosteus* spp. (Fig. 1m, n, 4e), although the double-labeling experiments also demonstrated OX-ir innervation of the 5-HT cells of the raphe, primarily at the rostral and caudal poles of the column (superior raphe nucleus: Fig. 1n, o, 5l, l'; inferior raphe nucleus: Fig. 1r, s, 2s, t, 5m). In contrast, no OX-ir fibers were observed in the cerebellum, and only scattered fibers were detected in the octavolateral area and the laterodorsal tegmental nucleus in all 3 species (Fig. 1m–q, 2n–q), and in the region of the locus coeruleus only in *A. calva* (Fig. 5n).

Along the rhombencephalon, the 3 species showed OX-ir varicose fibers in the reticular formation, and in descending tracts coursing at the ventral aspect of the rhombencephalon and reaching the spinal cord (Fig. 1m–t, 2n–t, 4g, h). In the dorsal alar plate of the caudal rhombencephalon, some scarce orexinergic fibers were seen in the region of the nucleus of the solitary tract (Fig. 1r, s, 2r–t, 4g, h), where TH-positive cells were also observed by double-immunolabeling techniques (Fig. 5o). Occasionally, some terminal-like structures were detected among the large cell bodies of the motor nuclei of the trigeminal, facial, glossopharyngeal, and vagal nerves, which innervate the jaw musculature and muscles of the branchial arches (Fig. 1o–t, 2o–t, 4g, h).

Finally, a moderate number of OX-ir fibers reached the spinal cord, and were primarily located in the laterodorsal and ventral funiculi with only a few in the dorsal funiculus (Fig. 1u, 2u, 4i). In addition, some varicose fibers and terminal-like structures were observed among the cell bodies of the dorsal horn and, more sparsely, in the intermediate and ventral spinal gray (Fig. 4i).

Discussion

The main aim of this study was to report for the first time the localization of orexin-like immunoreactivity in the brain of 3 representative species of the 2 orders of holostean fishes, *Lepisosteiformes* and *Amiiformes*. These results follow previous studies conducted by our group in cladistian fishes, lungfishes, amphibians, and reptiles [López et al., 2009a, 2009b, 2014, 2016; Domínguez et al., 2010]. The specificity of the antibodies was confirmed with control series, suggesting that the substances recognized are only orexins, although the presence of orexins

in holostean fishes remains unknown. The evolution of the structure of orexins through vertebrates shows some variations in the sequence of amino acids, particularly in teleosts [Kaslin et al., 2004; Tam et al., 2011; Wong et al., 2011], but the C-terminal sequence is highly conserved in all species of vertebrates studied [Sakurai et al., 1998; Shibahara et al., 1999; Kaslin et al., 2004; Panula, 2010; Wong et al., 2011]. In addition, it has been demonstrated that the synthetic human OXA has physiological activity in teleosts [Volkoff et al., 1999, 2003; Volkoff and Peter, 2001; Nakamachi et al., 2006; Yokogawa et al., 2007], which indicates that, despite the changes in the structure of orexins in vertebrates, there is still a high similarity between orexins in teleosts and humans. The anti-OXA and anti-OXB antisera used in this study were raised against a peptide mapping at the C-terminus of human OXA or OXA, whose invariability, together with the fact that orexins do not have structural homology with other biologically active peptides [Shibahara et al., 1999], accounts for the consistency in the immunolabeling pattern obtained with both antibodies in this and previous studies [Singletary et al., 2005, 2006; López et al., 2009a, 2009b, 2014, 2016; Domínguez et al., 2010; Miranda et al., 2013].

In the following section, we will discuss the organization of the orexinergic system in holosteans, and our results will be compared with those obtained for other groups of fishes and tetrapods (Table 2).

Localization of OX-ir Neurons in the Brain of Vertebrates

The pallial and subpallial areas of holosteans lack OX-ir cells, representing a general feature observed in all vertebrates, with the exception of the lungfish, *Neoceratodus forsteri*, that contains OX-ir cells in the caudal lateral septum and in the ventral striatum [López et al., 2009b], and the rat that shows OXB-ir cells in the lateral division of the central amygdala and the rostromedial part of the bed nucleus of stria terminalis [Ciriello et al., 2003b].

The only population of OX-ir neurons in the telenkephalon of holostean fishes is located in the POA. The presence of preoptic orexinergic cells seems to be a primitive and general feature shared by most anamniotes and reptiles, with exceptions in some species of cladistians [López et al., 2014], teleosts [Amiya et al., 2007] and amphibians [López et al., 2009a]. This cell population is probably related to neurosecretion and, particularly in reptiles, this system might act as a regulator of sexual behavior [Neary and Northcutt, 1983; Kaslin et al., 2004; Crews, 2005]. In contrast, no OX-ir cells have been observed in the POA of birds and mammals (Table 2).

The whole alar hypothalamus of holosteans studied is devoid of OX-ir cells. The absence of orexinergic cells in the paraventricular hypothalamic area is generally to be observed in all vertebrates with some specific exceptions reported in the teleost zebrafish [Kaslin et al., 2004], birds [Singletary et al., 2006; Miranda et al., 2013], and mammals [Nixon and Smale, 2007]. In contrast, the presence of orexinergic cells in the suprachiasmatic nucleus (within the subparaventricular hypothalamic area) is a primitive and generally conserved feature among anamniotes, with the exception of some teleosts [Huesa et al., 2005; Amiya et al., 2007] and holosteans (see Results). In contrast, no OX-ir cells have been reported in the suprachiasmatic nucleus of amniotes (Table 2).

The basal hypothalamus of holostean fishes houses 2 populations of orexinergic cells, situated in the Tu and RTu, with the latter being the most numerous and conspicuous OX-ir cell group in the brain. The presence of orexinergic cells in the basal hypothalamus is a primitive feature highly conserved in all vertebrates, although the number and precise localization of orexin cells vary specifically depending on the vertebrate group analyzed. Thus, the number of tuberal OX-ir cells is high and the cells are widely distributed in cladistian fishes [López et al., 2014]. In teleosts, however, OX-ir cells show restricted localizations of the basal hypothalamus, i.e., the anterior hypothalamus and lateral to the posterior tuberal nucleus of zebrafish [Kaslin et al., 2004; Faraco et al., 2006], the nucleus lateralis tuberis of the anterolateral hypothalamus and the nucleus posterioris periventricularis of the goldfish, and the cichlid *Cichlasoma dimerus* [Huesa et al., 2005; Pérez Sirkin et al., 2013], exclusively in the nucleus lateralis tuberis of *Pygocentrus nattereri* [Suzuki and Yamamoto, 2013], and in the nucleus posterioris periventricularis of medaka [Amiya et al., 2007]. Of note, some studies reveal the increase in levels of the orexin precursor mRNA in the posterior tuberal nucleus of zebrafish and winter flounder after fasting for 7–14 days [Novak et al., 2005; Buckley et al., 2010] as well as an increase in the number of orexinergic cells in the same nucleus [Yokobori et al., 2012], which allows us to relate the posterior tuberal nucleus to food intake behavior, a function that might also be shared by the basal hypothalamic OX-ir cells of holosteans.

As in actinopterygian fishes, sarcopterygian anamniotes (lungfishes and amphibians) also possess OX-ir cells in the Tu [López et al., 2009a, 2009b]. Reptiles show many orexinergic cells in this area, primarily in the periventricular hypothalamic nucleus, and, in the lizard *Gekko gecko* only, in the dorsolateral hypothalamic nucleus [Domín-

guez et al., 2010]. In the case of birds, several studies have described the presence of tuberal orexinergic cells within the periventricular hypothalamic nucleus and the lateral hypothalamic area [Ohkubo et al., 2002; Phillips-Singh et al., 2003; Singletary et al., 2006]. Likewise, populations of OX-ir cells are present in the basal hypothalamus of mammals. Thus, in the case of the rat, the orexinergic neurons have been observed in the perifornical nucleus, the dorsomedial hypothalamic nucleus, and the lateral and dorsal hypothalamic areas [Sakurai et al., 1998; Cutler et al., 1999; Date et al., 1999; Nixon and Smale, 2007; Bhagwandin et al., 2011a, 2011b; Kruger et al., 2012]. The perifornical nucleus and the lateral hypothalamus are the regions the most frequently identified with OX-ir cells in mammals, and they are involved in the control of feeding and energy balance [Winn et al., 1984; Stanley et al., 1996]. In addition, a population of orexinergic cells has been described in the ventrolateral hypothalamus, adjacent to the optic tract, in megachiropterans [Kruger et al., 2010; Dell et al., 2013], and exclusively in the medial hypothalamus of 2 Cetartiodactyla species, the giraffe and the harbor porpoise [Dell et al., 2012]. These hypothalamic populations might be involved in the roles given to orexins in the brain, i.e., food intake, reproduction, the sleep-wake cycle, and thermoregulation [Teske et al., 2010; Sakurai and Mieda, 2011].

Distribution of OX-ir Fibers in the Brain of Vertebrates

Despite the restricted number of orexinergic cells in the brains of holostean fishes and vertebrates in general, OX-ir fibers are widespread and reach all main regions of the brain, and this is shared by all the groups of vertebrates studied.

In the olfactory bulbs, holosteans show scattered OX-ir fibers and terminal-like structures, mainly located in the internal granular layer, similar to the situation observed in the olfactory bulbs of cladistians, teleosts, amphibians, and reptiles [Kaslin et al., 2004; Amiya et al., 2007; Domínguez et al., 2010; López et al., 2009a, 2014], although this innervation is indeed absent in some species of teleosts, lungfishes, and birds (Table 2). In the case of mammals, studies on rodents failed to detect OX-ir fibers in the olfactory bulbs [Nixon and Smale, 2007], but they were demonstrated in more recent studies on the same species [Shibata et al., 2008; Gascuel et al., 2012], suggesting that this innervation is the connection between olfactory and hypothalamic functions [Gascuel et al., 2012].

The pallial regions of holosteans that receive orexinergic fibers are the Dlv and Dm-Dc subdivisions. These ar-

eas were proposed to correspond to the medial and dorsal pallial regions, respectively, of the noneverted telencephalon of tetrapods [Yamamoto et al., 2007, 2017]. This limited distribution of OX-ir fibers in pallial/cortical regions is a general feature described in all groups of vertebrates studied (Table 2). On the contrary, the subpallium of holosteans possesses abundant innervation, especially in the Vv and VL, which would correspond to the septal-pallidal region of tetrapods, according to the pattern of labeling of some neuromarkers and transcription factors [Braford Jr, 2009; González et al., 2014].

Of note, in cladistians, not only the Vv but also the Vd shows abundant OX-ir fibers [López et al., 2014]. In the rest of the vertebrate groups, orexin innervation has been found in the striatum (equivalent to the Vd), septum, pallidum, and amygdaloid complex (equivalent to the Vs and Vp) [Kaslin et al., 2004; Singletary et al., 2005; Bisetti et al., 2006; Nixon and Smale, 2007; López et al., 2009a, 2009b; Domínguez et al., 2010; Schmitt et al., 2012]. These orexinergic fibers activate the basal forebrain cholinergic system in mammals in states of food deprivation [Wu et al., 2002, 2004; Fadel and Frederick-Duus, 2008]. This could also occur in reptiles, amphibians, and lungfishes, due to the codistribution of OX-ir fibers and cholinergic cells [López et al., 2009a, 2009b; Domínguez et al., 2010], as well as in holostean fishes because orexinergic fibers that reach the Vv and VL might innervate the cholinergic cells located in these areas [Morona et al., 2013].

In the alar hypothalamus of holosteans, the scarce orexinergic innervation observed in the paraventricular region and the dense innervation of the suprachiasmatic region concur with similar observations in all vertebrate groups studied [McGranaghan and Piggins, 2001; Mintz et al., 2001; Kaslin et al., 2004; Singletary et al., 2006; López et al., 2009a, 2009b, 2014; Domínguez et al., 2010]. In the case of the suprachiasmatic nucleus, this innervation can explain the tight relationship between the orexinergic system and the control of circadian rhythmicity [Klisch et al., 2009; Blasiak et al., 2017]. In the basal hypothalamus of holostean fishes, along with the OX-ir cells, abundant fibers course in the tuberal region and reach the median eminence, as has also been observed in cladistians, lungfishes, amphibians, reptiles, and mammals [Peyron et al., 1998; Date et al., 2000; López et al., 2009a, 2009b, 2014; Domínguez et al., 2010].

Previous studies have already dealt with the involvement of the orexinergic system in the control of the adeno-hypophysis [Kukkonen et al., 2002; Ferguson and Samson, 2003], regulating the liberation of ACTH, GH, gonadotropins, LH, and prolactin in rats [Pu et al., 1998;

Kuru et al., 2000; Blanco et al., 2001; Kohsaka et al., 2001; Porkka-Heiskanen et al., 2004; Seoane et al., 2004; Kiyokawa et al., 2011; Cataldi et al., 2014]. Of note, orexins inhibit the gonadotropin-releasing hormone system that produces reproductive behavior in goldfish [Hoskins et al., 2008]. These data suggest a role of orexins in the interaction between energy homeostasis and reproduction in teleosts and mammals, and this might extend to the rest of the vertebrates.

As in holostean fishes, the hypophysis of almost all vertebrates studied lacks orexinergic cells or fibers (Table 2), but there are some reports of the observation of hypophyseal OX-ir cells in some species of teleosts [Amiya et al., 2007; Suzuki et al., 2009; Pérez Sirkin et al., 2013; Suzuki and Yamamoto, 2013], in anurans [Yamamoto et al., 2004; Suzuki et al., 2007], and in humans [Blanco et al., 2003]. These differences most likely are related to technical variations in the use of different antibodies or the sensitivity of the immunostaining.

In the diencephalon of holosteans, the 3 prosomeres are moderately innervated with orexin varicose fibers, and this innervation is more conspicuous in the thalamic region. All vertebrates studied possess orexin innervation in the thalamus (Table 2), suggesting that this is a highly conserved feature. In mammals, thalamic innervation arises in the orexinergic cells located in the lateral hypothalamus, and is probably involved in feeding behavior [Lee and Lee, 2016] and in arousal states via projections to the limbic forebrain areas [Kirouac et al., 2005; Nixon and Smale, 2007; Pasumarthi and Fadel, 2008]. This may also occur in amphibians and reptiles, as the thalamic nuclei which are innervated by orexinergic fibers are connected to the nucleus accumbens, septum, and amygdala, all 3 basal limbic forebrain areas [Marín et al., 1997; Ten Donkelaar, 1998].

The mesencephalon of holosteans possesses a notable amount of OX-ir fibers and terminal-like structures in the optic tectum and torus semicircularis, observed in every group of vertebrates studied (Table 2), suggesting a role of orexins in visual and auditory processing and the integration of other sensory and motor information. The distribution of OX-ir fibers in the mesencephalic tegmentum also shows great similarities across all vertebrate groups.

The rhombencephalon shows moderate orexinergic innervation in the rostral cholinergic groups, the reticular formation, and the nucleus of the solitary tract; not only in holostean fishes but also in most vertebrates (Table 2). Specifically, the OX-ir fibers in the cholinergic nucleus of alar rhombomere 1 of holosteans, identified as the equiv-

alment of the pedunculo-pontine and laterodorsal tegmental nuclei of amniotes [Morona et al., 2013], could innervate the cells of these nuclei, as has been described in other vertebrates [Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; McGranaghan and Piggins, 2001; Mintz et al., 2001; Kaslin et al., 2004; López et al., 2009a, 2009b; Domínguez et al., 2010; Kruger et al., 2010; Dell et al., 2013]. This innervation might account for the orexin regulation of the activity of these nuclei, thus controlling states of arousal and sleep-wakefulness [Kim et al., 2009; Hong et al., 2011; Kohlmeier et al., 2013]. Moreover, the OX-ir innervation observed in the nucleus of the solitary tract is very likely the basis of the orexinergic regulation of autonomic-cardiovascular responses, such as heart rate and arterial pressure [Smith et al., 2002; Ciriello et al., 2003a, 2013; de Oliveira et al., 2003; Shih and Chuang, 2007; Shahid et al., 2012].

In the rhombencephalon of holosteans, the motor nuclei of the trigeminal, facial, glossopharyngeal and vagal nerves showed scarce OX-ir innervation, which has been observed in the same nuclei of cladistians [López et al., 2014]. Of note, orexinergic innervation has been reported in trigeminal and hypoglossal motoneurons of mammals [Fung et al., 2001; McGregor et al., 2005]. In addition, the octavolateral area of holosteans shows a few orexinergic fibers, which is a feature that has been observed in some vertebrates, like cladistians [López et al., 2014] and lungfishes [López et al., 2009a], and in the medial vestibular nucleus of the hamster [Horowitz et al., 2005].

Finally, the spinal cord of holostean fishes is moderately innervated by orexin fibers located mainly in the laterodorsal and ventral funiculi, with terminal-like structures in the dorsal, intermediate, and ventral spinal gray, as has been described in cladistian fishes, teleosts, some amphibians, reptiles, and mammals (Table 2). It has been reported that in rodents, functionally, orexins modulate pain sensation and regulate the autonomous nervous system by the innervation in the marginal zone, lamina 10, and the intermediolateral column of the spinal cord [van den Pol, 1999; Date et al., 2000; Llewellyn-Smith et al., 2003], with antinociceptive actions demonstrated in the spinal and supraspinal levels [Mobarakeh et al., 2005; Chiou et al., 2010; Azhdari Zarmehri et al., 2011].

Possible Relationships between the Orexinergic, NPY, and Monoaminergic Systems

The simultaneous observation in the same sections of OX-ir elements and NPY-ir, TH-ir, or 5-HT-ir cells and fibers was useful to accurately characterize their localiza-

tion in the brain. In addition, the double-labeling experiments highlight possible interactions between these systems in the brains of holosteans. Although it was not the aim of our study to analyze and demonstrate connections between the labeled structures (no electron or confocal microscopy was used), we will comment on the possible places of interactions and compare them with previous data from other vertebrate groups.

NPY is a highly conserved neuropeptide with orexigenic properties, whose expression in the hypothalamus of teleosts is induced by orexins [Nisembaum et al., 2014]. One of the main functions of this neuropeptide in teleosts is the increase of food intake, in synergistic action with orexins [Volkoff, 2006, 2012; Pérez Sirkin et al., 2013]. Our results regarding the possible interaction of NPY and orexins in holostean fishes show the lack of colocalization of both peptides in the same cells, albeit that intermingled populations of NPY-ir and OX-ir cells are located in the RTu. However, the NPY-ir neurons are possibly innervated by OX-ir fibers because of the close relationship of these 2 elements observed in the double-labeled sections. Reciprocally, NPY-ir fibers may innervate OX-ir cells. These interactions are likely present in the POA, RTu, and Tu. A similar relationship between orexins and NPY has also been described in the Tu of cladistians [López et al., 2014] and in the nucleus posterioris periventricularis of the goldfish, with observations of possible mutual innervation [Kojima et al., 2009; Shahjahan et al., 2014]. Noteworthy experimental data indicate that the orexigenic actions of OXA and NPY are mediated by mutual signaling pathways in goldfish [Volkoff and Peter, 2001; Kojima et al., 2009; Matsuda, 2009]. In rodents, distinct and nonoverlapping groups of NPY-ir and OX-ir cells were observed in the lateral hypothalamus, and NPY-ir nerve terminals have been observed surrounding and in close relationship to perikarya and processes of OX-ir cells [Broberger et al., 1998; Marston et al., 2011]. Furthermore, the interactions of both systems were strengthened with the demonstration of the effective inhibition by NPY receptor antagonists of the feeding behavior induced by OXA [Dube et al., 2000; Yamanaka et al., 2000].

No actual colocalization in the same neurons of orexins with TH and 5-HT was observed at any location in the brain of holosteans, although several TH-ir and 5-HT-ir cell populations are innervated by orexinergic fibers. Conspicuous OX-ir fibers and terminal-like structures are distributed close to and around the TH-ir neurons of the suprachiasmatic nucleus and the posterior tubercle, the latter being the origin of the ascending dopaminergic system to the subpallium in teleosts, an equivalent struc-

ture to the dopaminergic ventral tegmental area/substantia nigra of mammals [Rink and Wullmann, 2001, 2002, 2004]. Several studies have shown that intraperitoneal injections of orexins increase the mRNA expression of TH in the brain of the cavefish [Penney and Volkoff, 2014], and intracerebroventricular administration of OXA in the goldfish increases locomotor activity [Nakamachi et al., 2006; Matsuda et al., 2011]. Furthermore, studies on rodents have shown that OXA stimulates hyperlocomotion, and that the effect is inhibited by dopamine antagonists [Nakamura et al., 2000]. This orexin-induced locomotor activity exerted by the mesencephalic dopaminergic cells of mammals has a probable counterpart in the TH-ir cells (dopaminergic; unpublished data) of the posterior tubercle of holosteans and other actinopterygian fishes.

Abundant orexinergic fibers and terminals could also be observed interspersed with the TH-ir cells of the POA, the RM and, to a lesser extent, the nucleus of the solitary tract, as well as with the serotonergic cells of the raphe column. These results are largely similar to those obtained in goldfish [Huesa et al., 2005] and cladistian fishes, with the exception of the actual colocalization of orexin and TH in cells of the POA of *Polypterus* [López et al., 2014]. Moreover, orexin interactions with the cholinergic and histaminergic systems have been reported in zebrafish [Kaslin et al., 2004; Chiu and Prober, 2013]. These interactions in actinopterygian fishes probably represent a primitive and incipient feature, which would have evolved more conspicuously in the brains of sarcopterygian vertebrates (lungfishes and tetrapods) [Peyron et al., 1998; Nambu et al., 1999; Fadel and Deutch, 2002; Baldo et al., 2003; Ferguson and Samson, 2003; Bubser et al.,

2005; Lee et al., 2005; Singletary et al., 2006; Yoshida et al., 2006; Balcita-Pedicino and Sesack, 2007; Nixon and Smale, 2007; Vittoz et al., 2008; López et al., 2009a, 2009b; Domínguez et al., 2010; Kruger et al., 2010; Gravett et al., 2011; Dell et al., 2013].

The neuroanatomical distribution of the orexinergic system found in the 3 species of holosteans showed a general pattern largely comparable to those of teleosts, their sister group. In some aspects, the highly conserved traits of this system are observed throughout vertebrates, such as the presence of OX-ir cells in the basal hypothalamus, suggesting a preservation of the physiological functions of orexins. From studies on teleosts, major functional roles of orexins would be related to food intake, regulation of the sleep/wakefulness cycle, reproduction, and locomotor activity, i.e., functions that most likely would be exerted in close relation with other neurotransmission systems, such as the catecholaminergic, the serotonergic, and the NPY systems, among others.

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