Pattern of Nitrergic Neuronal System Organization in the Brain of Two Holostean Fishes (Actinopterygii: Ginglymodi)

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Abstract

The study of the nitrergic system, formed by the networks of neurons containing the enzyme nitric oxide synthase (NOS), has been extremely useful in unraveling neuroanatomical features of the organization of the central nervous system of vertebrates. Thus, data are available for representatives of most vertebrate classes and, in particular, several studies have detailed the organization of this system in teleosts. In contrast, no information is available regarding this neurotransmission system in the brains of holosteans, an early diverged and poorly understood group of actinopterygian fishes, currently considered a sister group of teleosts that contains only 8 species. The present study provides the first detailed information on the distribution of nitrergic cell bodies and fibers in 2 holostean species of the genus *Lepisosteus*, the spotted gar *L. oculatus* and the Florida gar *L. platyrhincus*. NOS immunohistochemistry and the NADPH diaphorase (NADPH-d) histochemical reaction were used, and both

techniques yielded identical results, with the exception of the primary olfactory and terminal nerve fibers, which only labeled for NADPH-d exclusively in *L. oculatus*. Double immunohistochemistry was conducted for the simultaneous detection of NOS with tyrosine hydroxylase, choline acetyltransferase, calbindin, calretinin, and serotonin to accurately establish the localization of the nitrergic neurons and fibers in the brain of holosteans, the neuroanatomy of which has been mostly neglected, and to assess possible interactions between these neuroactive substances. Distinct groups of nitrergic cells were located in subpallial areas, the basal hypothalamus, posterior tubercle, optic tectum and mesencephalic tegmentum, reticular formation, solitary tract nucleus, spinal cord, and amacrine cells in the retina. In addition, low numbers of nitrergic cells were observed in the pallium, suprachiasmatic nucleus, prethalamic and thalamic areas, torus lateralis and torus semicircularis, cerebellar and laterodorsal tegmental nuclei, and the ventral octavolateral area. Comparison of these results with those from other classes of vertebrates, and including a segmental analysis to correlate cell populations, reveals that the pattern of the nitrergic system in holosteans is very close to that in ancestral actinopterygian fishes and highlights conserved and derived traits.

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List of abbreviations

ac	anterior commissure	p1–p3	prosomerens 1–3
aCb	cerebellar auricle	POA	preoptic area
Cb	cerebellum	PT	pretectum
cc	central canal	PTh	prethalamus
cCb	crista cerebellaris	r	rostral
Cg	rhombencephalic central gray	r0–r8	rhombomeres 0–8
Cn	central nucleus of the inferior hypothalamic lobes	Rai	inferior raphe nucleus
d	dorsal	Ram	median raphe nucleus
Dd	dorsal zone of the dorsal telencephalic area	Ras	superior raphe nucleus
DF	dorsal funiculus	rHb	right habenula
dh	dorsal horn of the spinal cord	Rhom	rhombencephalon
DL	lateral zone of the dorsal telencephalic area	Ri	inferior reticular nucleus
Dld	dorsolateral zone of the dorsal telencephalic area	RM	retromammillary hypothalamic area
Dlv	ventrolateral zone of the dorsal telencephalic area	Rm	middle reticular nucleus
Dm	medial zone of the dorsal telencephalic area	Rs	superior reticular nucleus
Dn	diffuse nucleus of the inferior hypothalamic lobes	sac	stratum album centrale of the mesencephalic tectum
Dp	posterior zone of the dorsal telencephalic area	SC	suprachiasmatic nucleus
fb	forebrain bundle	sco	subcommissural organ
flm	fasciculus longitudinalis medialis	sfgs	stratum fibrosum et griseum superficiale of the mesencephalic tectum
fr	fasciculus retroflexus	sg	granular layer of the cerebellum
GCL	ganglion cell layer of the retina	sm	molecular layer of the cerebellum
gl	glomerular layer of the olfactory bulb	smn	somatomotor neurons of the spinal cord
Hb	habenula	so	stratum opticum of the mesencephalic tectum
Hyp	hypophysis	sol	solitary tract
ICL	internal cellular layer of the olfactory bulb	son	spino-occipital motor nucleus
INL	inner nuclear layer of the retina	son-smn	spino-occipital motor nucleus-somatomotor neurons of the spinal cord
Ip	interpeduncular nucleus	spc	spinal cord
IPL	inner plexiform layer of the retina	spv	periventricular layer of the mesencephalic tectum
Ipn	interpeduncular neuropil	Tegm	tegmentum
Is	nucleus isthmi	Tel	telencephalon
Lc	locus coeruleus	Th	thalamus
LDT	laterodorsal tegmental nucleus	Tl	torus longitudinalis
LF	lateral funiculus	Tor	torus semicircularis
lHb	left habenula	Torl	torus lateralis
lih	inferior hypothalamic lobe	TP	nucleus of the posterior tubercle
Ln	nucleus of the lateral hypothalamic recess	Tu	tuberal hypothalamus
<i>L.o</i>	<i>Lepisosteus oculatus</i>	v	ventricle
<i>L.p</i>	<i>Lepisosteus platyrhincus</i>	Vd	dorsal part of the ventral telencephalic area
lr	lateral hypothalamic recess	Vdc	caudodorsal part of the ventral telencephalic area
MC	Mauthner cell	VF	ventral funiculus
MCa	Mauthner cell axon	vh	ventral horn of the spinal cord
MTP	nucleus medianus of the posterior tubercle	VL	lateral part of the ventral telencephalic area
nCb	cerebellar nucleus	Vm	trigeminal motor nucleus
nso	spino-occipital nerve	Vn	'nother nucleus of the ventral telencephalic area
Nsol	nucleus of the solitary tract	Vp	posterior nucleus of the ventral telencephalic area
nt	terminal nerve	Vs	supracommissural part of the ventral telencephalic area
nI	olfactory nerve	VTA	ventral tegmental area
nII	optic nerve	Vv	ventral part of the ventral telencephalic area
nIII	oculomotor nerve	III	oculomotor nucleus
nV	trigeminal nerve	IV	trochlear nucleus
nVIII	octaval nerve	VI	abducens nucleus
nX	vagal nerve	VIIIm	facial motor nucleus
ob	olfactory bulb	VIII-Ila	anterior octavolateral area
oc	optic chiasm	VIII-Ili	intermediate octavolateral area
ONL	outer nuclear layer of the retina	VIIIv	ventral octavolateral area
OPL	outer plexiform layer of the retina	IXm	glossopharyngeal motor nucleus
OT	optic tectum	Xm	vagal motor nucleus
ot	optic tract		
Pa	paraventricular hypothalamic area		
pc	posterior commissure		

Introduction

Nitric oxide (NO) is a gaseous neurotransmitter and neuromodulator produced in the central nervous system (CNS) by the neuronal NO synthase (nNOS) after the oxidation of arginine into citrulline. Its main target is the soluble guanylate cyclase, which increases the intracellular levels of cyclic guanosine monophosphate. A second action of NO is the production of a posttranslational modification of proteins by S-nitrosylation of cysteine residues [Bredt and Snyder, 1992; Dawson et al., 1998; Jaffrey et al., 2001; Bartus et al., 2013; Dzoljic et al., 2015; Raju et al., 2015; Bradley and Steinert, 2016]. In addition to nNOS, 2 other isoforms of this enzyme have been identified (endothelial and inducible) [Alderton et al., 2001], and the molecular structure of these isoforms is highly conserved throughout evolution [González-Domenech and Muñoz-Chapuli, 2010; Andreakis et al., 2011]. The importance of the nitrergic system, formed by the networks of neurons containing nNOS, in the control of other “classical” neurotransmitter systems has been established. Thus, the NO-mediated signal can modulate glutamatergic [Garthwaite, 1991; Lawrence and Jarrott, 1993; Rudkouskaya et al., 2010; Neitz et al., 2011; Raju et al., 2015] and dopaminergic [Zhu and Luo, 1992; Bugnon et al., 1994; Chaparro-Huerta et al., 1997; Kiss et al., 2004] neurotransmission, via presynaptic regulation of neurotransmitter release and/or postsynaptic regulation of signal action. In addition, NO also acts on GABAergic synaptic transmission [Yang et al., 2007; Maggesissi et al., 2009; Tarasenko et al., 2014; Gasulla and Calvo, 2015; Yamamoto et al., 2015] by reducing the strength of inhibition, which allows the fine tuning of information processing [Yassin et al., 2014]. Therefore, NO plays a key role in synaptic plasticity, being involved in long-term potentiation and long-term depression, and in the regulation of the sleep-wake cycle. It is also related to processes of neuroprotection and neurodegeneration [Shibuki and Okada, 1991; Haley et al., 1992; Mizutani et al., 1993; McCann, 1997; Calabresi et al., 1999; Hars, 1999; Bon and Garthwaite, 2003; Stanton et al., 2003; Wang et al., 2005; Sultana et al., 2006; Mancuso et al., 2007; Sergeeva et al., 2007; Rudkouskaya et al., 2010; Hardingham et al., 2013; Rafalovich et al. 2015], as an excessive release of NO produces nitrosative stress and has a neurotoxic effect [Brown, 2010; Steinert et al., 2010; Nakamura et al., 2013; Bradley and Steinert, 2016].

The neuroanatomical organization of the nitrergic systems was first investigated by the NADPH diaphorase (NADPH-d) histochemical reaction, a quick and easy

technique that generally labels the nitrergic structures [Bredt et al., 1991; Hope et al., 1991], although a more consistent labeling of this system is achieved by the immunodetection of nNOS with specific antibodies. Thus, the anatomy of the nitrergic system has been extensively studied by histochemical and immunohistochemical methods in the CNS of most groups of vertebrates [for references see Alonso et al., 2000; López et al., 2016]. In particular, several studies have described the organization of the nitrergic system in teleosts [Holmqvist et al., 1994, 2000; Arévalo et al., 1995; Brüning et al., 1995; Villani and Guarnieri, 1995; Anken and Rahmann, 1996; Bell et al., 1997; Virgili et al., 2001; Singru et al., 2003; Giráldez-Pérez et al., 2008, 2013] but little information is available for other actinopterygian groups [López et al., 2016].

Actinopterygians (ray-finned fishes) are among the most successful radiations in the long evolutionary history of vertebrates that contains more than 30,000 species, of which only 50 are not teleosts [Nelson et al., 2016]. The group commonly known as “gars” is formed by 7 extant species of nonteleost actinopterygian fishes belonging to the superorder Ginglymodi, which includes a single family (Lepisosteidae) and 2 genera: *Atractosteus* (with 3 living species) and *Lepisosteus* (with 4 living species). Gars and bowfins (superorder Halecomorphi with the single species *Amia calva*) are included in the “Holostei” (holosteans), which are restricted to the fresh waters of eastern North America [Nelson, 2006]. Although the phylogenetic relationships among actinopterygian fishes have been controversial, recent studies, in which multiple nuclear DNA-encoded genes and whole genome duplication events have been analyzed, showed that holosteans form a monophyletic group (Fig. 1a). This lineage diverged from the lineage leading to teleosts before the teleost-specific round of whole genome duplication occurred more than 320–350 million years ago [Hoegg et al., 2004; Kikugawa et al., 2004; Amores et al., 2011; Near et al., 2012; Pasquier et al., 2016]. Thus, holosteans and teleosts are currently grouped in the “Neopterygii” (neopterygians). These data are consistent with traditional morphologically based inferences [Stiassny et al., 2004] and previous neuroanatomical studies that observed peculiar features present only in the telencephalon and cerebellum of holosteans [Nieuwenhuys, 1967; Nieuwenhuys and Meek, 1990], and absent in other actinopterygian fishes [Nieuwenhuys, 2011]. In contrast to the extensive experimental data about the organization of the CNS of teleosts, there is little information concerning the brain of holosteans. In particular, there are limited hodological data related to the visual and the lateral line

Fig. 1. a Cladogram illustrating Holostei as the sister group of Teleostei and the relationship to other actinopterygian fishes; approximate dates are from Near et al. [2012]. **b** Lateral view of the brains of the Florida gar (*L. platyrhincus*) and the spotted gar (*L. oculatus*) dissected after fixation so that the major divisions of its CNS can be observed. See list of abbreviations.

systems [Northcutt and Butler, 1976, 1980; Song and Northcutt, 1991; Collin and Northcutt, 1995], and several mapping studies of the distribution of monoamines [Parent and Northcutt, 1982; Chiba and Oka, 1999] and diverse neuropeptides [Malz et al., 1999; Baker and Bird, 2002; Chiba, 2005]. In addition, in a previous study we described in detail the organization of the brain cholinergic system [Morona et al., 2013].

Given the phylogenetic importance of holosteans as the sister group of teleosts, the aim of the present study was to provide a comprehensive description of the organization of the nitrergic system in 2 holostean species of the genus *Lepisosteus*, the spotted gar *L. oculatus* and the Florida gar *L. platyrhincus*. In a previous review article, some information based on the NADPH-d method was provided for the telencephalon [Braford, 2009]. We used nNOS immunolocalization and NADPH-d histochemistry to confirm the consistency of both approaches in the detection of this gaseous neurotransmission system, as in previous studies conducted by our group in different vertebrates [González et al., 1996, 2002b; López and González, 2002; López et al., 2016]. This neuroanatomical information is a useful tool for distinguishing and defining classes of neurons and cell groups within the poorly investigated brain of gars. In addition, double-labeling experiments for NOS or NADPH-d and choline acetyltransferase (ChAT; the synthesis enzyme of acetylcholine), tyrosine hydroxylase (TH; the first and rate-limiting enzyme for catecholamine synthesis), serotonin (5-HT), and the calcium binding proteins calbindin (CB) and calretinin (CR), were conducted to locate more precisely the nitrergic cell populations and to evaluate some neurochemical features of the NOS immunoreactive (NOS-ir) neurons, given the previous descriptions of the localization of these neuroactive molecules in the brain of gars [Chiba and Oka, 1999; Morona et al., 2013; González et al., 2014]. The results are compared with those reported in other groups of fishes and tetrapods to establish conserved and specific features along their evolution, and therefore to increase the current knowledge of this neurotransmitter system. To facilitate these comparisons, the study was framed within the neuromeric model of the vertebrate brain, which serves to compare structures that are topologically homologous although their topography in the adult vertebrate brain may appear different [Puelles, 1995; Puelles and Rubenstein, 2003, 2015].

Materials and Methods

For the present study, a total of 9 spotted gars, *L. oculatus*, and 5 Florida gars, *L. platyrhincus*, were used. The animals, young adults 10–16 cm in length, were obtained from licensed suppliers (PezyCia, Madrid, Spain) and were maintained in aquaria with a controlled temperature (24–28°C) and natural light conditions. The original research reported herein 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 Complutense University to conduct the experiments described.

The animals were deeply anesthetized by immersion in 0.1% tricaine methanesulfonate solution (MS222, Sandoz, Basel, Switzerland; pH 7.3) and perfused transcardially with physiological saline followed by 100–150 mL of cold 4% paraformaldehyde in a 0.1 M phosphate buffer (PB; pH 7.4). The brain, olfactory sacs, eyes, and 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. They 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 and the rest of the biological material were cut on a freezing microtome at a thickness of 30–40 µm in the transverse or sagittal plane, and sections were collected and rinsed in cold PB.

NADPH-d Histochemistry

Free-floating sections were rinsed in fresh PB and incubated in a medium made up of 1 mM β-NADPH (Sigma), 0.8 mM nitro blue tetrazolium (Sigma), and 0.06% Triton X-100 in PB, at 37°C in darkness for 1–2 h. The reaction was stopped by successive rinses in cold PB. Some sections were incubated in a medium without β-NADPH. A second group of control sections was heated in PB to 70°C for 10 min. In both cases, no reaction was observed. 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.

NOS Immunohistochemistry

In another series of experiments, the free-floating sections were rinsed twice in PB, treated with 1% H₂O₂ in PB for 15 min to eliminate endogenous peroxidase activity, and rinsed again 3 times in PB. The sections were then incubated in a sheep antiserum against neuronal NOS (K205 antibody, kindly donated by Dr. Piers Emsen), diluted 1:15,000 in PB containing 0.5% Triton X-100 (PB-T), for 48–60 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-sheep biotinylated IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:100. After rinsing a further 3 times for 10 min, the sections were incubated for 90 min in Vectastain ABC-peroxidase system (Vector). Secondary antiserum and ABC complex were diluted in PB containing 0.5% Triton X-100 and 2% BSA. Finally, the sections were rinsed 3 times for 10 min in PB and subsequently stained in 0.5 mg/mL of 3,3'-diaminobenzidine (DAB; Vector; catalog No. SK4100) intensified with nickel [Adams, 1981], with 0.01% H₂O₂ in PB for 5–10 min.

In addition, after incubation in the sheep antiserum against neuronal NOS (in the same conditions mentioned above), another set of free-floating sections were processed for immunohistochemistry using Alexa 594-conjugated donkey anti-sheep (Molecular Probes, Eugene, OR, USA; catalog No. A11058) diluted 1:300 in PB-T or FITC-conjugated donkey anti-sheep (Vector Laboratories, Burlingame, CA, USA; catalog No. FI-6000) diluted 1:100 in PB-T, applied for 2 h at room temperature. These sections were mounted on glass slides with 0.25% gelatin in 0.1 M Tris-HCl buffer and coverslipped with Vectashield (Vector).

Double Labeling for NOS or ChAT and NADPH-d

NOS immunofluorescence was conducted in sections as described above. Similarly, ChAT-immunofluorescence detection

was accomplished with a first incubation of the sections in a primary goat anti-ChAT (diluted 1:100; Millipore; catalog No. AB144P) in PB-T for 48–60 h at 4°C, and revealed by using Alexa 594-conjugated donkey anti-goat (Molecular Probes) diluted 1:300 or FITC-conjugated donkey anti-goat (Vector) diluted 1:100, both in PB-T, applied for 2–4 h at room temperature. The sections were mounted on glass slides and studied, plotted, and photographed without drying. They were then recovered from the slides and rinsed thoroughly in PB for 1 h. The histochemical technique for NADPH-d was subsequently performed. The results of both labels were compared throughout the brain after plotting and photographing the NADPH-d reactive elements.

Double NOS and TH, 5-HT, CB or CR Immunofluorescence

To analyze the possible codistribution/colocalization of NOS and TH, 5-HT, CB or CR, combinations of antibodies were used in a procedure based on immunohistochemistry as follows: (1) first incubation for 72 h at 4°C in a mixture of sheep anti-NOS (diluted 1:15,000) and mouse anti-TH (diluted 1:1,000; ImmunoStar; catalog No. 22941), rabbit anti-5-HT (diluted 1:1,000; ImmunoStar; catalog No. 20080), mouse anti-CB (diluted 1:1,000; Swant; catalog No. 300) or rabbit anti-CR (diluted 1:1,000; Swant; catalog No. 7699/3H); (2) second incubation for 90 min at room temperature in a mixture of secondary antisera: donkey anti-sheep Alexa 594 (red fluorescence; diluted 1:300; Molecular Probes) and Alexa 488-conjugated chicken anti-mouse (green fluorescence; diluted 1:300; Molecular Probes; catalog No. A21200) or Alexa 488-conjugated chicken anti-rabbit (green fluorescence; diluted 1:300; Molecular Probes; catalog No. A21441). After rinsing 3 times in PB, the sections were mounted on glass slides and coverslipped with UltraCruz™ mounting medium containing DAPI for fluorescent labeling of DNA (sc-24941; Santa Cruz Biotechnology).

Double Brightfield Labeling for NADPH-d and ChAT or TH

These series of experiments were conducted to correlate in brightfield microscopy the nitroergic structures with the cholinergic and catecholaminergic cell groups. Following the histochemical reaction for NADPH-d which produced a blue reaction product, the free-floating sections were rinsed twice in PB, treated with 1% H₂O₂ in PB for 15 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-ChAT (diluted 1:100; Millipore) or mouse anti-TH (diluted 1:1,000; ImmunoStar) antisera, for 48–60 h at 4°C. The sections were then processed immunohistochemically according to the PAP technique using rabbit anti-goat (Chemicon; diluted 1:50) or goat anti-mouse (Dakopatts, diluted 1:50) in PB for 1 h at room temperature. After further rinsing 3 times in PB for 10 min, the sections were incubated for 90 min in goat PAP complex (Chemicon) or mouse PAP complex (Chemicon) diluted 1:500 for 90 min. Secondary antiserum and PAP complex were diluted in PB containing 0.5% Triton X-100 and 2% BSA. Finally, the sections were rinsed 3 times for 10 min in PB and subsequently stained in 0.5 mg/mL of DAB (SK4100; Vector), with 0.01% H₂O₂ in PB for 3–5 min, giving rise to a brown reaction product. The sections were then mounted, dehydrated, and coverslipped as for NADPH-d histochemistry.

Controls and Specificity of the Antibodies

There were 2 general controls for the immunohistochemical reaction: (1) staining some selected sections with preimmune normal serum, and (2) controls in which either the primary and/or the secondary antibody or the ABC/PAP complex was omitted. The immunostaining was eliminated in all these negative controls. The antibodies used have been tested under identical conditions in tissues devoid of antigen, as a negative control, and in tissues positive for the antibodies. The controls were shown to be satisfactory in all cases.

The polyclonal NOS antiserum was raised in sheep against recombinant rat NOS, and its specificity has been previously described [Herbison et al., 1996; Smeets et al., 1997]. Antibody specificity against neuronal NOS was assessed by liquid-phase adsorption experiments [Simonian and Herbison, 1996]. In addition, the specificity of the antiserum for the detection of NOS was warranted by the observation of a staining pattern that was identical to that in previous reports in which it matched the NADPH-d histochemical reaction [González et al., 1996; Smeets et al., 1997; Muñoz et al., 2000; Brüning and Mayer, 2001; López and González, 2002; Moreno et al., 2002; Moreno and González, 2004, 2005a, 2005b; López et al., 2016]. The immunoreactivity is abolished by absorption of the K205 antiserum with recombinant neuronal NOS protein (1 mM overnight at 4°C).

The specificity of the ChAT, TH, CB, and CR antisera has been previously described in *L. platyrhincus* [Morona et al., 2013], and a similar pattern of labeling for these markers has been detected in *L. oculatus* (present results).

Evaluation and Presentation of the Results

The distribution of nitrergic cell bodies and fibers in the brain was carefully analyzed in both the single- and double-labeled sections. The correspondence between the NADPH-d method and NOS immunohistochemistry was first assessed in the double-labeled sections (Fig. 2). The pattern of labeling was charted in representative transverse sections of *L. platyrhincus*, from rostral to caudal levels (Fig. 3). Drawings were made by means of a 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 with a digital camera (Olympus DP70). Contrast and brightness were adjusted in Adobe Photoshop CS4 (Adobe System, San Jose, CA, USA) and figures were mounted in Canvas 11 (ACS System International). Selected single-labeled sections were photographed and the pattern of nitrergic structures is presented from rostral to caudal levels (Fig. 4–6). Double-labeled sections for NADPH-d and TH or CHAT analyzed with brightfield microscopy are shown in Figure 7, whereas images of double immunofluorescence are illustrated in Figures 8 and 9. Finally, the main groups of nitrergic neurons detected in the present study are summarized for the case of *L. platyrhincus* in a sagittal schematic drawing, with special reference to the neuromeric regionalization of the brain (Fig. 10).

The nomenclature used is essentially the same as in previous studies of the brain in the genus *Lepisosteus* [Northcutt, 1982; Parent and Northcutt, 1982; Song and Northcutt, 1991; Northcutt and Butler, 1993; Collin and Northcutt, 1995; Meek and Nieuwenhuys, 1998; Chiba and Oka, 1999; Baker and Bird, 2002; Chiba, 2005; Morona et al., 2013], including the terminology of the prosomeric model for the forebrain [Puelles and Rubenstein, 2003, 2015].

Results

The antibody against neuronal NOS used in the present study revealed patterns of immunoreactivity that, for each of the 2 species examined, were constant from animal to animal. This pattern of labeling was widely coincident with the staining of the NADPH-d technique (Fig. 2), with the exceptions found in the olfactory system and terminal nerve of *L. oculatus* detailed below. In both transverse and sagittal sections, labeling of neuronal cell bodies and fibers was observed in distinct groups of the forebrain, brainstem, and spinal cord (charted for *L. platyrhincus* in Fig. 3). In addition, the regional localization of nitrergic cell groups was summarized in a schematic sagittal drawing in Figure 10, according to a segmental interpretation of the brain. In the following sections, we describe the rostrocaudal regional distribution of the nitrergic system (neuronal bodies and fibers) taking *L. platyrhincus* as the core species for which the complete pattern is detailed, but commenting on the small differences obtained in particular brain regions of *L. oculatus* (Table 1). The widespread nitrergic structures are presented in selected photomicrographs in Figures 4–6 and will be described in the following sections. The doubly labeled sections for NOS/NADPH-d and TH, ChAT, 5-HT, CB or CR were mainly used to corroborate the actual position of certain cell groups in the brain and to establish some neurochemical features of nitrergic populations, and the selected images are shown in Figures 7–9.

Forebrain

The brain of gars shows a discretely everted telencephalon, which differs from the greatly developed optic tectum, the inferior hypothalamic lobes, and the cerebellum (Fig. 1b). In the most rostral part of the telencephalon, the large olfactory bulbs of *L. platyrhincus* lacked NOS immunoreactivity and NADPH-d activity (Fig. 4g), whereas the olfactory receptor cells in the olfactory mucosa of *L. oculatus*, the axons of which form the olfactory nerve ending in the glomerular layer of the olfactory bulbs, showed intense NADPH-d reactivity, as well as the coarse fibers that

Fig. 2. Photomicrographs of transverse sections through the telencephalon (a), mesencephalon (c), and rhombencephalon (e, g) of *L. platyrhincus* (a, c) and *L. oculatus* (e, g) illustrating the perfect match of cell bodies and processes immunolabeled with NOS antiserum and NADPH-d histochemistry in the Vdc (b, b'), the mesencephalic tegmentum (Tegm; d, d'), the Rm (f, f'), and the Nsol (h, h'). See list of abbreviations. Scale bars, 50 μ m (b, b', d, d', f, f', h, h'), 200 μ m (a, c, e, g). See online version for colors.

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Fig. 3. a–t Diagrams of transverse sections through the brain of *L. platyrhincus* at the rostrocaudal levels indicated in the photograph of the lateral view of the brain. NOS-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. The thick line in each diagram demarcates the ventricular surface. See list of abbreviations. Scale bar, 1,000 μ m.

Table 1. Localization and relative abundance of NOS-immunoreactive cells and fibers in the CNS of the gars *L. platyrhincus* and *L. oculatus*

	<i>L. platyrhincus</i>		<i>L. oculatus</i>	
	C	F	C	F
<i>Forebrain</i>				
Olfactory bulb	–	–	–	–
Pallium (DI)	+	–	+	–
Subpallial area Vd	++++	+++	++++	+++
Subpallial area Vv	+++	++	++	++
Subpallial areas Vn and VL	–	–	–	–
Subpallial areas Vs	++	++	++	++
Subpallial area Vp	+++	++	+++	++
Preoptic area	+++	++	+++	++
Paraventricular area	+	+	+	+
Suprachiasmatic nucleus	+	+	++	+
Tuberal hypothalamus	+++	++	+++	++
Inferior hypothalamic lobe	+++	++	+++	++
Retromammillary hypothalamic region	++	++	++	++
Hypophysis	–	–	–	–
Prethalamus	++	+	++	+
Posterior tubercle	+++	++	+++	++
Habenula	++	+	++	+
Thalamus	++	+	++	+
Pretectum	+	++	+	+
<i>Midbrain</i>				
Optic tectum	++++	++	++++	++
Torus semicircularis	++	+	++	+
Torus lateralis	++	+++	+	++
Torus longitudinalis	–	–	–	–
Mesencephalic tegmentum	+++	++	+++	++
Oculomotor nucleus	–	–	–	–
<i>Hindbrain</i>				
Trochlear nucleus	–	–	–	–
Interpeduncular nucleus	–	+	–	+
Cerebellum	–	+	–	+
Cerebellar nucleus	++	+	+	+
Central gray	+++	++	+++	++
Laterodorsal tegmental nucleus	+	++	++	++
Superior reticular nucleus	++	++	+++	++
Trigeminal motor nucleus	–	–	–	–
Mauthner cells	–	–	–	–
Abducens nucleus	–	–	–	–
Median reticular nucleus	+++	+++	+++	+++
Ventral octavolateral area	++	+	+	+
VIIIm-IXm-Xm	–	–	–	–
Nucleus of the solitary tract	+++	+++	+++	+++
Raphe nuclei	–	+	–	+
Inferior reticular nucleus	++	+	+	+
Spino-occipital motor nucleus	–	–	–	–
<i>Spinal cord</i>	+++	++	++	++
<i>Retina</i>	++	++	++	+
<i>Sensory ganglia of cranial nerves</i>	++	+	++	+

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

might represent the terminal nerve (Fig. 4b, d). However, all these structures were NOS immunonegative and, therefore, were not considered nitrergic structures. The intense NADPH-d labeling throughout the glomerular layer served to delimitate the caudal extent of the olfactory bulb, and also to trace the projections of the putative terminal nerve, only in *L. oculatus*. Thus, these thick fibers were observed in ventromedial locations of the olfactory bulbs, coursing caudally through the ventral part of the ventral telencephalic area (Vv; Fig. 4b, d) and reaching the rostral preoptic area in the caudal telencephalon.

The pallial part of the slightly everted telencephalic hemispheres of *Lepisosteus* possessed a scarce and faintly NOS-ir population of small cells located in the dorsolateral and ventrolateral zones of the dorsal telencephalic area (Dld, Dlv; Fig. 3a–c, 4g). These cells were separated from the ventricle and were intermingled with CB-ir cells, with some being doubly labeled (Fig. 8a). In clear contrast with the scarce presence of NOS-ir cells in the pallium of gars, the subpallium was the telencephalic region with a more prominent presence of nitrergic elements (Fig. 3a–d). In particular, the most conspicuous nitrergic cell group was located in the dorsal part of the ventral telencephalic area (Vd; Fig. 3a, b, 4b–e). At rostral levels, this group was formed by round, intensely NOS-ir cells that were densely packed and showed long processes directed laterally and laterodorsally (Fig. 4b, c, 8b, d). The tight clustering of these cells makes it difficult to recognize individual neurons, but more caudally in the Vd (the caudodorsal part of ventral telencephalic area; Vdc) the cell density was lower and the nitrergic cells were more separated, making their observation easier (Fig. 2a, 4d–f). Some of these cells in the Vdc were doubly labeled with CB (Fig. 8e) but not with CR, the cells of which were intermingled with the nitrergic population. In contrast, dorsal to the Vd and just at the pallial-subpallial border, the ‘nother nucleus (Vn), and the lateral part of the ventral telencephalic area (VL) were devoid of nitrergic elements (Fig. 3a, b, 4b–g, 8b). In the double-labeling experiments, the NOS-ir part of the Vd showed a prominent plexus of 5-HT-ir varicose fibers (Fig. 8c), and remarkable TH-ir cells and fibers were detected in the lateral part of Vd (Fig. 8b). In addition, CB-ir cells, which did not colocalize with NOS, were found in the Vd, although they were especially notable in the Vn (Fig. 8d). The Vv also housed a population of nitrergic cells (Fig. 3a, b, 4c, g), some of which were near the ventricle and exhibited short processes contacting the cerebrospinal fluid (CSF). These NOS-ir cells, which were more numerous in *L. platyrhincus* than in *L. oculatus*, were strongly intermingled with

CB-ir cells, although no colocalization was detected (Fig. 8d).

In the caudal telencephalon, several populations of nitrergic neurons were observed in the supracommissural part and posterior nucleus of the ventral telencephalic area (Vs and Vp; Fig. 3c, d, 4g–i). The cells of the Vs were generally multipolar neurons of large size and intense immunoreactivity that formed a disperse group (Fig. 4i, 8f). At this level, many nitrergic fibers were seen in the anterior commissure (Fig. 4i). Despite their proximity, CB and CR were not found in the NOS-ir cells in the Vs. The nitrergic cells in the Vp were small, rounded, and tightly packed near the ventricle (Fig. 3d, 4h), forming a prominent group in the caudal telencephalon (Fig. 4g, 9a).

In the caudoventral telencephalon, a group of moderate to intense immunoreactive nitrergic cells was observed near the ventricle of the preoptic recess, showing long lateral and short CSF-contacting processes (Fig. 3c, d, 4g, i, j, 8n). These cells were near to or intermingled with other cells labeled for TH, CB or ChAT, but no double-labeled cells were observed (Fig. 7a, 8f).

According to the prosomeric model [Puelles and Rubinstein, 2003, 2015], a small group of faintly immunoreactive cells was observed in the paraventricular area that constitutes the dorsalmost part of the alar hypothalamus (Pa; Fig. 3d, 8n). These round and small nitrergic cells were periventricularly located and some of them showed short CSF-contacting processes. Lateral to the paraventricular hypothalamic area, thick and intensely immunoreactive fibers were seen in the forebrain bundle (fb; Fig. 3d, e, 4k). In the ventral part of the alar hypothalamus, a group of nitrergic cells was moderately labeled in the suprachiasmatic nucleus (SC; Fig. 3e, 8g). This cell population, which was more prominent in *L. oculatus* than in *L. platyrhincus*, showed double NOS/TH cells (Fig. 8g–i, n) and the cholinergic cell population in the suprachiasmatic region [Morona et al., 2013; present results] was located ventromedial to the NOS-ir neurons.

The nitrergic cells in the basal hypothalamus were significantly more numerous than in the alar region. Thus, the most prominent NOS-ir cell population in the hypothalamus was found in the tuberal region and within the conspicuous large inferior lobes characteristic of this group of fishes (Fig. 3f–j, 4l–n, 8k, l, n, 10). Most cells in these regions were periventricular NOS-ir neurons and many of them were bipolar with short CSF-contacting processes and long lateroventrally oriented processes (Fig. 4p, 8r). The tuberal nitrergic cells were intermingled with CB-ir cells without colocalization of both markers in the same neurons (Fig. 8j). In the ventral part of the tu-

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beral hypothalamus (caudal in classical transverse sections), many of the NOS-ir cells were doubly labeled with CR (Fig. 8s). In addition, nitrergic fibers were found in the outer fiber zone of the tuberal region and many of them coursed ventrocaudally toward the median eminence (Fig. 3g–j). The large inferior hypothalamic lobes of *Lepisosteus* represent a lateral expansion of the tuberal region and housed a remarkable nitrergic cell population periventricularly in the nucleus of the lateral recess (lih; Fig. 3h–j, 4l, n, 8k, l, 9a), whereas the central and diffuse nuclei were devoid of labeling. Of note, these intense NOS-ir cells lacked CSF-contacting processes (Fig. 4o) and were intermingled with CB-ir and CR-ir cells constituting separate subpopulations (Fig. 8j). The last nitrergic cell group detected in the hypothalamus of gars was observed in the caudoventral part of the basal hypothalamus (dorsocaudal in classical transverse sections) within the retromammillary area (RM; Fig. 3j, 4n, 8s, 10). These cells were moderately NOS-ir and showed laterally oriented processes (Fig. 4q), distinct from the TH-ir cells located in the RM, which showed CSF-contacting processes (Fig. 8q). It is noteworthy that the hypophysis of gars was devoid of NOS-ir cells.

In the caudal portion of the forebrain, the diencephalon is divided into 3 caudorostral segments or prosomeres (p1–p3), the alar plates of which give rise to the pretectal area, thalamus (former dorsal thalamus), and prethalamus (former ventral thalamus), respectively, and the basal plates form different tegmental regions that are generally smaller than the alar regions (Fig. 10). Following the description from rostral to caudal regions, in the ventral part (caudal in classical transverse sections) of alar plates of p3, a group of small and intensely NOS-ir cells was detected near the ventricle in the prethalamus (Fig. 3g, 8l, m, 10). These cells lacked CSF-contacting processes and were located medial to the prominent serotonergic group of the paraventricular organ (Fig. 8r). A distinct cell population of large TH-ir cells was observed lateral to the prethalamus

nitrergic group (Fig. 7b, 8l). In addition, CB-ir cells were largely intermingled with the NOS-ir cells, although colocalization of both markers was not detected in double-labeled sections (Fig. 8m). The most conspicuous nitrergic cell group of the diencephalon was detected in the posterior tubercle (TP), within the basal plate of p3 (Fig. 3h, i, 4l, m, 10). These intensely NOS-ir cells exhibited thick and long laterodorsal processes and formed a separate group from the TH-ir cells located more dorsally in the TP (Fig. 7c, 8k, o, p). In addition, small and rounded CB-ir cells were observed in the TP intermingled with the NOS-ir cells, but no double-labeled cells were detected (Fig. 8j).

In the dorsal part of the alar p2 (epithalamus), the asymmetric habenula showed a nitrergic group of rounded and moderately immunoreactive cells in the ventrolateral part of both the large right habenula and the small left habenula (Fig. 3e, 5a, b, 10). Of note, the cholinergic (ChAT-ir) cell group that characterizes the habenula of gars was located dorsal to the NOS-ir cells [Morona et al., 2013; present results] forming distinct subpopulations, whereas some NOS-ir cells were doubly labeled with CB (Fig. 8t). Ventral to the habenula, a group of slight to moderate NOS-ir cells was seen in the thalamus (Fig. 3e–g, 10). This group constituted a column of small cells located close to the ventricle, with long lateral processes but without CSF-contacting processes (Fig. 5c). In the dorsal part (rostral in conventional transverse sections) of this thalamic group, many NOS-ir cells coexpressed CB, but no colocalization was observed for NOS and CR (Fig. 8u).

The most caudal diencephalic NOS-ir cells were observed at the level of the posterior commissure in the alar plate of p1 (Fig. 3f, 10). They formed a small group of faintly immunoreactive cells with lateral oriented processes located immediately dorsal to the fasciculus retroflexus (Fig. 3f, see arrowheads in Fig. 5c). At this level a plexus of nitrergic fibers was observed in the dorsolateral zone of the pretectal area (Fig. 5c), which was more prominent in *L. platyrhincus* than in *L. oculatus*.

Midbrain

A remarkably numerous nitrergic cell population was localized throughout the optic tectum within the alar mesencephalon (Fig. 3e–k, 5g, 9a, 10). These moderate immunoreactive cells showed pear-shaped somata and were mainly located in the inner zone of the periventricular tectal layer [Meek and Nieuwenhuys, 1998]. They possessed long apical processes directed toward the pial surface (spv; Fig. 5d–g) and were identified as neurons of type XIV [according to Meek and Nieuwenhuys, 1998; Kinoshita et al., 2006]. The apical processes ramified es-

Fig. 4. Photomicrographs of transverse (a–c, f, h–k, n–q) and sagittal (d, e, g, i, m) sections through the forebrain of *L. oculatus* (*L.o.*) and *L. platyrhincus* (*L.p.*; indicated in the upper right corner of each photomicrograph), illustrating NADPH-d-positive cell bodies and fibers in the olfactory bulb (a), rostral and caudal parts of Vd (b–f), terminal nerve (arrowheads in b, d), Vp (g–i), Vs (i), preoptic area (i–k), paraventricular area (k), tuberal hypothalamus and TP (l, m, p), retromammillary hypothalamic area (m, n, q), and inferior hypothalamic lobes (n, o). Asterisks in c, j, p indicate CSF-contacting cell processes. See list of abbreviations. Scale bars, 100 μ m (b, c, e, f, j, k, o–q), 200 μ m (a, d, h, i, l, m), 500 μ m (g, n).

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pecially in the stratum album centrale (sac; Fig. 5e) and showed varicose swellings. In double-immunolabeling experiments, ChAT-ir, CB-ir, and CR-ir cell populations have also been detected in the optic tectum of gars [Morona et al., 2013; present results], which were generally located in the outer zones of the periventricular layer of the mesencephalic tectum and constituted separate populations from the nitrergic group because no colocalization with these neuromarkers was observed (Fig. 7d; 9b, c).

In contrast to the optic tectum, the torus semicircularis housed a small group of slight to moderate immunoreactive nitrergic cells in its caudal part (Fig. 3i, j, 5f). In the large torus lateralis, a group of scarce and faintly NOS-ir cells was observed (Fig. 3i, 4n), and these cells were more numerous in *L. platyrhincus* than in *L. oculatus*, whereas the torus longitudinalis of both species lacked NOS-ir elements (Fig. 2c, 3g–j).

Within the basal part of the midbrain, a numerous population of strongly immunoreactive cells was observed in the tegmentum at rostral mesencephalic levels (Fig. 2c, d, 3h, 5g, 9a). These tegmental cells were located near the ventricle and showed very long lateroventral processes (Fig. 2c, 5h). They were profusely innervated by TH-ir fibers and were clearly separated from the tegmental cholinergic cells that were farther from the ventricle and more caudally in the mesencephalon [Morona et al., 2013; present results]. CB-ir and CR-ir cell populations were also observed in the tegmentum, but only some nitrergic cells were doubly labeled with CR (Fig. 9a).

Hindbrain

Several groups of NOS immunopositive and NADPH-d reactive cells were identified in the hindbrain of the gar species studied and will be described from rostral to caudal in relation to the segmental organization of the rhombencephalon into rhombomeres 0–8 (r0–r8; Fig. 10). Interestingly, the cholinergic cells in the rhombencephalic motor nuclei and the serotonergic cells in the

raphe column described in this group of fishes [Parent and Northcutt, 1982; Chiba and Oka, 1999; Morona et al., 2013] were devoid of nitrergic labeling (Fig. 5l, 6a, e, j, 7h, i, l–n). In the basal part of the rostral rhombencephalon, the interpeduncular nucleus also lacked nitrergic cells (Fig. 3k, l, 5l). In the alar plate of r1 a group of small nitrergic cells with long lateral and laterodorsal processes was labeled in the putative cerebellar nucleus, considering their position and nitrergic nature (Fig. 3k; 5k, l; 7f), whereas the corpus and the auricles of the cerebellum were devoid of NOS-ir cells. This nitrergic cerebellar group was more prominent in *L. platyrhincus* than in *L. oculatus*. Also in the alar plate of r1, a population of NOS-ir cells was located in the region close to the locus coeruleus (TH-ir) and the prominent cholinergic laterodorsal tegmental nucleus (LDT) [Morona et al., 2013] (Fig. 3l, 5i, 7f), but the double-labeling technique revealed that the nitrergic cells were located more dorsolaterally without colocalization (Fig. 7e–g). In contrast, CB-ir and CR-ir cells were observed highly intermingled in the LDT, and some nitrergic cells were doubly labeled with CB and CR (Fig. 9d). Of note, the NOS-ir cell group located close to the cholinergic LDT was more numerous in *L. oculatus* than in *L. platyrhincus* (Fig. 5j, k).

In the basal part of r1–r2 in *L. oculatus* and slightly more caudally in *L. platyrhincus*, a remarkable group of nitrergic cells was observed in the rhombencephalic central gray (Cg; Fig. 3m; 5k; 6a). These intensely NOS-ir cells with long ventral processes (Fig. 6b) formed a distinct subpopulation more laterally located to the CB-ir, CR-ir, and ChAT-ir cell groups located in this area [Morona et al., 2013; present results] (Fig. 9e). In the reticular formation, the superior, middle, and inferior nuclei possessed nitrergic cells (Fig. 10). Thus, large cells with long ventral and ventrolateral processes were observed in the superior reticular nucleus (Rs) within the basal part of caudal r1 and r2, just lateral and extending caudal to the group of the central gray (Fig. 3l; 5j; 6a). This population was more conspicuous in *L. oculatus* than in *L. platyrhincus* and was coincident with the nitrergic cells of the rhombencephalic central gray (Fig. 6b). Several populations of NOS-ir, ChAT-ir, and CR-ir neurons were seen in the Rs, although no double-labeled cells were detected (Fig. 9e).

At mid rhombencephalic levels, the most prominent reticular NOS-ir group was found in the middle reticular nucleus (Rm; Fig. 2e, 3n, o, 6d), constituting of large and intensely immunoreactive multipolar cells (Fig. 2e, f). This group formed a long and continuous column from r3, coinciding with the caudal pole of the trigeminal mo-

Fig. 5. Photomicrographs of transverse (**a–f, h, i, l**) and sagittal (**g, j, k**) sections through the diencephalon, midbrain, and rostral rhombencephalon of *L. oculatus* (*L.o.*) and *L. platyrhincus* (*L.p.*; indicated in the upper right corner of each photomicrograph), illustrating NADPH-d-positive cell bodies and fibers in the asymmetric habenula (**a**), left habenula (**b**), thalamus and pretectum (**c**; arrowheads point to faintly reactive pretectal cells), optic tectum (**d–g**), torus semicircularis (**f**), tegmental mesencephalic nucleus (**g, h**), LDT (**i–k**), Rs (**j**), central rhombencephalic gray (**k**), and cerebellar nucleus (**l**). See list of abbreviations. Scale bars, 50 μ m (**e**), 100 μ m (**b–d, f, h, i**), 200 μ m (**a, g, j–l**).

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tor nucleus (Fig. 7h), to the caudal part of the abducens nucleus in r6 (Fig. 7i). In r4, this group was observed lateral to the giant Mauthner cells, which were devoid of nitrergic labeling (Fig. 6c). The reticular NOS-ir cells were intermingled with the CB-ir and CR-ir cell populations also found in the Rm, but no double-labeled cells were observed (Fig. 9f, g). In the alar plate of the r3, a scarce group of moderate to intense NOS-ir cells was observed in the ventral octavolateral area, dorsal to the conspicuous Rm (Fig. 3n, o, 6d, 7h). The cells in this group extended caudally to the rostral pole of the abducens nucleus in r5, were more numerous in *L. platyrrhincus* than in *L. oculatus*, and were intermingled with CB-ir and CR-ir cells without colocalization (Fig. 9f, g).

At caudal rhombencephalic levels, within the alar plate of r6–r8, a long column of nitrergic cells was observed medial and ventromedial to the solitary tract, in the nucleus of the same name (Nsol; Fig. 3p–s, 6e, h, i, 7l–n). This group extended caudal to the obex and was formed by small and strongly immunoreactive cells with long ventrolateral processes, some of which crossed in the midline (Fig. 2g, 6e–h, j, 7n). Some of these nitrergic cells were situated near the ventricle, whereas other slightly larger cells were observed in inner locations (Fig. 2g, h, 6f). In addition, a few cells were intermingled with the fibers coursing in the solitary tract (Fig. 6g). At the level of the glossopharyngeal motor nucleus, the nitrergic cells of Nsol were located medially to the cholinergic cells of the nucleus intermedius of the octavolateral zone [Morona et al., 2013; present results]. TH-ir and CB-ir cell populations were also present in the Nsol, and only some double-labeled cells were detected in the caudal part of the nitrergic column, near the obex, that also received a strong serotonergic innervation (Fig. 9m). The TH-ir and CR-ir cells of Nsol were located ventrally to the nitrergic cell population (Fig. 9h, i, l), whereas the CB-ir cells were highly intermingled with the NOS-ir cells (Fig. 9j, k).

Finally, a scarce cell group was NOS-ir in the caudal reticular formation (r8) in the inferior reticular nucleus (Ri; Fig. 3r, s). This group of small and moderately reac-

tive cells (Fig. 6e) was more numerous in *L. platyrrhincus* than in *L. oculatus*, and extended caudally to the obex (Fig. 6j). These cells were intermingled with the cholinergic population of the Ri [Morona et al., 2013; present results], but no double-labeled cells were found (Fig. 7j).

Spinal Cord

The spinal cord of both species was studied along its rostrocaudal extent revealing a nitrergic cell population situated in the dorsal horn of the spinal gray (Fig. 3t). These small cells with moderate to intense immunoreactivity showed long lateral and laterodorsal processes (Fig. 6k), and formed a long column in the dorsal horn, more numerous in *L. platyrrhincus* than in *L. oculatus* (Fig. 6i, l), from rostral to caudal spinal levels, although the number of nitrergic cells clearly decreased caudally (Fig. 7p). Some of these cells were doubly labeled with CR. In addition, nitrergic cells with long lateral and lateroventral processes were detected in the ventral horn and intermediate spinal gray (Fig. 6k, l), but the large cholinergic neurons of the ventral somatomotor column lacked nitrergic labeling (Fig. 7p, q). Scarce NOS-ir cells were observed in the midline or around the fasciculus longitudinalis medialis in the ventral aspect of spinal cord (Fig. 7o). In double immunohistochemistry experiments, these cells were doubly labeled with CB (Fig. 9n). The spinal catecholaminergic and serotonergic cells, located ventral to the central canal and with liquor contacting processes [Chiba and Oka, 1999; present results], were clearly separated from the NOS-ir cells (Fig. 7o), confirming separated populations of spinal nitrergic and monoaminergic cells.

The nitrergic innervation was more prominent in rostral than caudal spinal levels, and was abundant in the dorsal and lateral funiculi (Fig. 3t, 6k) and reduced in the ventral funiculus. Most of this innervation probably represented the caudal continuation of the nitrergic fiber tracts observed in the ventrolateral aspect of the rhombencephalon (Fig. 3m–s), mainly arising in the NOS-ir reticular nuclei.

Retina

The presence of nitrergic cells in the retina of gars was demonstrated using the immunodetection of NOS and the NADPH-d histochemical techniques. Thus, a population of nitrergic amacrine cells was detected in the inner nuclear layer (INL; Fig. 7r, s, 9o). These cells of round aspect showed short processes that entered the inner plexiform layer. No nitrergic ganglion cells, bipolar cells or displaced amacrine cells within the ganglion cell layer were detected in the retina of the 2 species studied. Dou-

Fig. 6. Photomicrographs of transverse (**a–c, e–h, j, k**) and sagittal (**d, i, l**) sections through the rhombencephalon and spinal cord of *L. oculatus* (*L.o.*) and *L. platyrrhincus* (*L.p.*; indicated in the upper right corner of each photomicrograph), illustrating NADPH-d-positive cell bodies and fibers in the Rs and central gray (**a, b**), Rm (**c, d**), Nsol (**e–j**), and spinal cord (**i, k, l**). See list of abbreviations. Scale bars, 100 μ m (**b–d, f–i, k, l**), 200 μ m (**a, e, j**).

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ble-labeling experiments revealed other subpopulations of TH-ir, ChAT-ir, 5-HT-ir, CB-ir, and CR-ir amacrine cells in the inner nuclear layer. The catecholaminergic and serotonergic amacrine cells were larger in size and less numerous than the nitrergic cells (Fig. 7r, 9o), whereas the CB-ir, CR-ir, and cholinergic amacrine subpopulations were more abundant and highly intermingled with the NOS-ir cell subpopulation (Fig. 7s). All these retinal cells generally formed separated amacrine subpopulations with the exception of some nitrergic cells doubly labeled with CR (Fig. 9p).

Discussion

The present study on the brain of the small fish group of holosteans expands the knowledge of the organization of the nitrergic system in vertebrates from the point of view of comparative neuroanatomy. The phylogenetic position of this group of fishes is important in the analysis of primitive brain features likely present in the earliest neopterygian fishes. Although the actual NOS protein has not yet been isolated and characterized in this group, the control series of experiments and the similar and conserved structure of this enzyme through vertebrates [Andreakis et al., 2011] support the specificity of NOS immunodetection. In addition, the pattern of labeling obtained with the NADPH-d histochemical technique matches that of NOS immunohistochemistry, with mismatches only observed in the olfactory system and terminal nerve of *L. oculatus*, strongly reactive for NADPH-d histochemistry but NOS immunonegative. The general colocalization of NOS immunolabeling and NADPH-d activity in the same neuronal elements supports that NOS catalytic activity is revealed by the NADPH-d staining [Darius et al., 1995]. This is reinforced by the lack of NADPH-d staining observed in transgenic knockout

mice for NOS [Huang et al., 1993]. The lack of correlation in the olfactory system, noted in several species, including cladistians, teleosts, amphibians, and mammals [González et al., 1996; Porteros et al., 1996; Virgili et al., 2001; López and González, 2002; Moreno et al., 2002; Giráldez-Pérez et al., 2008, 2013; López et al., 2016], was explained by the presence of cytochrome P-450 reductase, which has close homology with NOS [Bredt et al., 1991] and presents NADPH-d activity. It should be mentioned that although we have referred here to “terminal nerve” fibers, it might be the case that these fibers represent extrabulbar olfactory projections, demonstrated for other actinopterygians and amphibians [Pinelli et al., 2004; Gayoso et al., 2011]. Of note, in some cases mismatches between both techniques were noted in the cerebral cortex [Kharazia et al., 1994; Sobreviela and Mufson, 1995] and spinal cord [Vizzard et al., 1994] of mammals. However, these mismatches are not observed in the pallium or spinal cord of gars. Furthermore, in the rainbow trout, NADPH-d staining was reported in oligodendrocytes [Pérez et al., 1996] but they were not labeled in our study. For reasons of comparability, the methods used in this study were the same as in our former studies. The detailed pattern of the nitrergic system obtained in the 2 species of the genus *Lepisosteus* enables direct comparison with the profuse data in other groups of vertebrates (Table 2), thus highlighting shared and derived traits of its organization. According to the organization of the results, the pattern of distribution of nitrergic cell bodies and fibers is discussed below following a regional rostrocaudal order.

Forebrain

The olfactory bulbs in holosteans, like in cladistians, chondrosteans, and some teleosts, arise as rostral evaginations of the telencephalic hemispheres and enclose central ventricles. NOS-ir cells or fibers were not observed in the olfactory bulbs of gars, in line with results obtained in most actinopterygians [López et al., 2016]. However, nitrergic cells in the olfactory bulbs have been reported in a teleost fish [Singru et al., 2003], a cartilaginous fish [Ferrando et al., 2012], a lungfish [Northcutt, 2009a], and urodele and gymnophionan amphibians [González et al., 1996, 2002b]. In addition, they are present in all species of mammals studied [Kishimoto et al., 1993; Hopkins et al., 1996; Kendrick et al., 1997; Alonso et al., 1998]. Consequently, this is a variable feature that appears several times in evolution, and it is generally absent in actinopterygian fishes.

Holosteans have hemispheric hypertrophy and in gars the dorsal (pallial) part shows slight eversion [Northcutt

Fig. 7. Photomicrographs of transverse (a–d, f–l, n, p, r, s) and sagittal (e, m, o, q) double-labeled sections through the brain, spinal cord, and retina of *L. oculatus* (*L.o.*) and *L. platyrhincus* (*L.p.*; see the upper right corner of each photomicrograph), showing staining for NADPH-d (blue) and TH or ChAT (brown), indicated in the upper left corner of each photomicrograph. The relationship between the distinct reactive elements is illustrated for the preoptic area (a), prethalamus (b), TP (c), optic tectum (d), LDT (e–g), Rm (h, i), Ri (j), Nsol (k–n), spinal cord (o–q), and retina (r, s). The asterisks in o indicate CSF-contacting cell processes to the central canal. See list of abbreviations. Scale bars, 50 μ m (a, c, d, g, j, o–s), 100 μ m (f, h, i, k–m), 200 μ m (b, e, n). See online version for colors.

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and Braford, 1980]. In the pallium, gars possess scattered and faintly NOS-ir cells separated from the ventricle in the lateral zone of the dorsal telencephalic area (Dl), largely comparable to the nitrergic population observed in the pallial field P3 of cladistians [López et al., 2016]. In teleosts, a common pattern is seen in the pallium with nitrergic cells in the Dl and in dorsal and medial zones of the dorsal telencephalic area, although in some species the total lack of nitrergic elements in the pallium has been reported [Arévalo et al., 1995; Gaikwad et al., 2009]. Because of the eversion process, the pallial Dl of gars, as the pallial P3 of cladistians, has been interpreted as the homolog of the medial pallium of the noneverted telencephalon of tetrapods [Bruce and Braford, 2008; Braford, 2009; Nieuwenhuys, 2011], and the presence of similar scattered nitrergic cells described in the medial pallium of lungfishes [González and Northcutt, 2009] and amphibians [González et al., 1996; Muñoz et al., 1996; López et al., 2002] supports this homology. Comparatively, in vertebrates (Table 2) the presence of pallial/cortical nitrergic cells seems to be a general pattern. However, using only the NADPH-d method, putative pallial nitrergic cells were not detected in larval lampreys [Schober et al., 1994a], 2 species of teleosts, and 2 reptiles [Bennis et al., 1996; Jiang and Terashima, 1996]. As demonstrated in mammals, the pallial/cortical nitrergic cells constitute an important population of interneurons that regulate the neuronal activity by lateral inhibition and are involved in synaptic plasticity [Shlosberg et al., 2012].

Almost all subpallial regions in the telencephalon of gars possess remarkable populations of nitrergic cells, with the exceptions of the Vn and VL nuclei of the ventral telencephalic area, which form a c-shaped configuration exclusive of this group of fishes [Braford, 2009]. The dorsorostral Vd showed similar staining patterns for TH, 5-HT, and calcium binding proteins as in *Polyp-*

terus [Chiba and Oka, 1999; González et al., 2014; López et al., 2016; present results], and the distribution of several transcription factors strengthened its homology with the striatum [González et al., 2014]. The conspicuous nitrergic staining observed in our study and in a previous analysis of this region [Braford, 2009] strongly supports this homology. However, the Vdc, which contains fewer nitrergic cells, is strongly NADPH-d reactive, and often coexpresses CB, seems to represent a distinct region. Comparison with similarly stained regions in topologically homologous subpallial areas leads to the proposition that the Vdc represents the central amygdala (striatal part of the amygdaloid complex) of other vertebrates [Moreno and González, 2005a; González and Northcutt, 2009].

In a previous study, staining for the transcription factor Nkx2.1 showed an intense labeling of the ventricular zone of all ventral parts of the ventral telencephalic area at mid and caudal levels, and the dorsal limit of expression marked the boundary with the region identified as the striatum of gars [González et al., 2014]. By comparison with tetrapods, this Nkx2.1-labeled region is topologically the counterpart of the septopallidal region of tetrapods [Moreno et al., 2009; Medina et al., 2014]. Therefore, the nitrergic cell populations observed in the Vv in our study would correspond to the septopallidal populations observed in tetrapods (Table 2). The nitrergic cells located in the Vs might correspond to similar cells in the medial amygdala of lungfishes and tetrapods because of its position and expression of specific transcription factors, such as Otp (orthopedia) [Moreno and González, 2003; Bardet et al., 2008; González et al., 2014]. In turn, the conspicuous nitrergic cells in the Vp might represent the lateral amygdala (pallial component of the amygdaloid complex), which in lungfishes and amphibians is characterized by a conspicuous population of nitrergic cells [Moreno and González, 2004, 2006, 2007a, b; González and Northcutt, 2009]. According to this view, the Vn-Vp complex would constitute the ventral pallial region in gars, and not the medial zone of the dorsal telencephalic area, as was previously proposed [Braford, 2009]. However, previous hodological studies challenge this proposal since torus lateralis projections to the medial zone of the dorsal telencephalic area (and not to Vn-Vp) have been described in gars [Northcutt, 2009b]. It should be noted that the homologies proposed are based on the scarce data available in holosteans and it is clear that more neurochemical, hodological, and developmental data are needed to confirm these ideas. Comparatively, with the special exception of the larval lamprey that lacks

Fig. 8. Photomicrographs of transverse (**b–m, p–u**) and sagittal (**a, n, o**) double-labeled sections through the brain of *L. oculatus* (*L.o.*) and *L. platyrhincus* (*L.p.*; see the upper right corner of each photomicrograph), showing staining for NOS (red fluorescence) and TH, 5-HT, CB or CR (green fluorescence), indicated in the upper left corner of each photomicrograph. The relationship between the immunoreactive cell populations is illustrated for the Dl (**a**), the rostral and caudal parts of Vd (**b–e**), the preoptic area (**f**), the suprachiasmatic nucleus (**g–i, n**), the tuberal hypothalamus (**j, s**), TP (**j, k, o, p**), the prethalamus (**l, m, r**), retromammillary hypothalamic area (**q, s**), the habenula (**t**), and the thalamus (**u**). Double-labeled cells in **a, e, i, n, s**, and **t** are indicated with arrowheads. See list of abbreviations. Scale bars, 100 μ m (**a, c, g–i, m, r, s**), 200 μ m (**b, d–f, j–l, n–q, t, u**). See online version for colors.

Fig. 9. Photomicrographs of transverse (**b–h, j–p**) and sagittal (**a, i**) double-labeled sections through the brain and retina of *L. oculatus* (*L.o.*) and *L. platyrhincus* (*L.p.*); see the upper right corner of each photomicrograph, showing staining for NOS (red fluorescence) and TH, 5-HT, CB or CR (green fluorescence), indicated in the upper left corner of each photomicrograph. The relationship between the immunoreactive cell populations is illustrated for the

inferior hypothalamic lobe (**a**), the optic tectum (**b, c**), the LDT (**d**), the Rs and central gray (**e**), the Rm (**f, g**), the Nsol (**h–m**), the spinal cord (**n**), and the amacrine cells in the retina (**o, p**). Arrowheads in **d, n**, and **p** indicate double-labeled cells. See list of abbreviations. Scale bars, 50 μ m (**o, p**), 100 μ m (**d, e, k–n**), 200 μ m (**b, c, f–j**), 500 μ m (**a**). See online version for colors.

Fig. 10. Schematic reconstruction of the sagittal series of sections of the brain showing the locations of the nitroergic cell groups in *L. platyrhincus* according to a segmental interpretation of the brain. See list of abbreviations.

nitroergic subpallial cells [Schober et al., 1994a], the existence of nitroergic cells in the subpallium is a commonly observed feature in the brain of vertebrates (Table 2), in line with the known relevant function of NO in motor control [West and Grace, 2000; Lorenc-Koci and Czarnecka, 2013] and in emotion/behavior control [Gulati and Ray, 2014].

The preoptic area of gars houses a remarkable population of nitroergic cells distinct from the TH-ir, ChAT-ir, and CB-ir populations also observed in this region. The presence of nitroergic cells in the preoptic area is a highly conserved feature observed in all anamniotes studied (Table 2), whereas it is a more variable feature in amniotes, being present in birds [Brüning, 1993; Cozzi et al., 1997; Atoji et al., 2001] and some species of reptiles [Smeets et al., 1997] and mammals [Hadeishi and Wood, 1996; Domínguez et al., 2006; Nutsch et al., 2014], where the role of NO in sexual behavior has been demonstrated [Nutsch et al., 2014]. It should be noted that gene expression data have highlighted that the preoptic area does not belong to the hypothalamus but it is part of the subpallial telencephalic territory [see Moreno and González, 2011; Domínguez et al., 2015] that is topologically adjacent to the dorsal hypothalamic territory, and similar observations have been obtained for gars [González et al., 2014].

In our description of the results, we have considered the main hypothalamic alar and basal regions currently interpreted by the prosomeric model [Puelles and Rubenstein, 2015]. From dorsal to caudal, the paraventricular and subparaventricular subdivisions constitute the alar

hypothalamus, and tuberal and mammillary regions form the basal portion. Within the paraventricular area of gars only a small group of nitroergic cells is slightly immunoreactive and projections to the hypophysis could not be confirmed. Comparatively, intensely NOS-ir cells are present in the paraventricular area of cladistians [López et al., 2016] and their projections could be followed towards the neurohypophysis. Similar nitroergic groups with hypothalamo-hypophysial projections have been reported in the electric ray [Pérez et al., 1995], in the teleost *Clarias batrachus* [Gaikwad et al., 2009], and in anuran amphibians [Allaerts et al., 1997; Prasada Rao et al., 1997], although some amphibian species may lack nitroergic cells in the paraventricular region [Pinelli et al., 2014]. In mammals, the NADPH-d-reactive cells of the neurosecretory hypothalamic nuclei coexpress vasopressin/oxytocin [Arévalo et al., 1992; Miyagawa et al., 1994; Sánchez et al., 1994], where the NO probably regulates the release of these neurohormones [Vanhatalo and Soinila, 1995], whereas this colocalization is absent in the hypothalamus of birds [Sánchez et al., 1996].

Nitroergic cells in the subparaventricular area of gars are found in the suprachiasmatic nucleus, the cells of which predominantly coexpress TH. Compared to other vertebrates, the presence of nitroergic cells in the suprachiasmatic nucleus is a generally conserved feature (Table 2) that has been reported in most species of teleosts and reptiles, although with some exceptions [Jiang and Terashima, 1996; Gaikwad et al., 2009], in birds and some species of amphibians [Lázár and Losonczy, 1999; González et al.,

Table 2. Summary of the distribution of nitrergic cells in different areas of the CNS of the vertebrate groups studied

	Agnathans	Chondrichthyes	Cladistians	Chondrosteans	Gars	Teleosts	Lungfishes	Amphibians	Reptiles	Birds	Mammals
Olfactory bulb	–	+	–	?	–	+/-	+	+/-	–	–	+
Pallium/cortex	–	?	+	?	+	+/-	+	+	+/-	+	+
Striatum	–	?	+	?	+	+/-	+	+	+/-	+	+
Septum	–	?	+	?	+	+/-	+	+	+/-	+	+/-
Pallidum	–	?	+	?	+	+/-	+	+	+/-	+	+/-
Amygdaloid complex	–	?	+	?	+	+/-	+	+	+/-	+	+
Preoptic area	–	?	+	?	+	+	+	+	+/-	+	+/-
Paraventricular area	–	+	+	?	+	+	?	+/-	+	+	+
Suprachiasmatic nucleus	–	?	+	?	+	+/-	?	+/-	+/-	+	+/-
Basal hypothalamus	–	?	+	+	+	+	?	+	+/-	+	+
Hypophysis	–	?	–	?	–	+/-	?	–	?	?	+/-
Prethalamus	–	?	+	–	+	+/-	?	+/-	–	–	+/-
Habenula	+	?	+	?	+	+/-	?	+/-	–	+/-	–
Thalamus	–	?	+	+	+	+/-	?	+/-	+/-	+/-	+/-
Pretegmentum	–	?	–	?	+	+/-	?	+/-	+/-	+	+/-
Posterior tubercle VTA/substantia nigra	?	?	+	?	+	+/-	?	+/-	+/-	+	+/-
Optic tectum/superior colliculus	?	?	+	+	+	+	?	+	+	+	+
Torus semicircularis/inferior colliculus	?	?	+	?	+	+	?	+	+/-	+/-	+/-
Mesencephalic tegmentum	?	?	+	?	+	+	?	+	+	+	+
Interpeduncular nucleus	?	?	+	?	–	+/-	?	+/-	+/-	+	+/-
Cerebellum	?	?	–	+	–	+	?	+/-	+/-	+/-	+
Cerebellar nucleus	?	+	+	?	+	?	?	+/-	+/-	+/-	+/-
Nonmotor nuclei in the upper rhombencephalon: LDT/PPT/Rs	?	?	–	?	–	+/-	?	+	+	+	+
Locus coeruleus	?	?	–	?	–	+/-	?	–	–	+/-	–
Reticular formation	+	?	+	+	+	+	?	+	+	+	+
Octavolateral area	?	?	+	?	+	+	?	+/-	+/-	+	+
Raphe nuclei	?	?	–	?	–	+/-	?	+/-	+/-	+/-	+/-
Motor nuclei of the cranial nerves	?	?	–	?	–	+/-	?	+/-	+/-	+/-	+/-
Solitary tract nucleus	?	?	+	?	+	+/-	?	+/-	+/-	+	+/-
Spinal cord	+	?	+	?	+	+	?	+	+	+	+
Retina	?	+	+	?	+	+	?	+	+	+	+

+, presence of NOS-ir cells; –, absence of NOS-ir cells; +/-, presence of NOS-ir cells in some species of the group; ?, no data available; PPT, pedunculo-pontine tegmentum.

References: *agnathans*: Schober et al., 1994a; Kyriakatos et al., 2009; *chondrichthyes*: Shiells and Falk, 1992; Pérez et al., 1995; Álvarez-Otero et al., 1996; Funakoshi et al., 1997; Ferrando et al., 2012; Pose-Mendez et al., 2014; *cladistians*: López et al., 2016; *chondrosteans*: Pushchina and Obukhov, 2012; *teleosts*: Holmqvist et al., 1994, 2000; Osthalm et al., 1994; Arévalo et al., 1995; Brüning et al., 1995; Anken and Rahmann, 1996; Cioni et al., 1997; Pottek et al., 1997; Funakoshi et al., 1999; Baby et al., 2000; Oyan et al., 2000; Haamedi and Djamgoz, 2002; Bordieri et al., 2003, 2005; Singru et al., 2003; Ando et al., 2004; Bordieri and Cioni, 2004; Pushchina and Diuizen, 2004; Pushchina, 2007; Pushchina et al., 2007; Giráldez-Pérez et al., 2008, 2013; Gaikwad et al., 2009; Pushchina and Obukhov, 2010; Ganz et al., 2012; *lungfishes*: Schober et al., 1994b; Northcutt, 2009; *amphibians*: Nöll et al., 1994; Crowe et al., 1995; Kurenni et al., 1995; Brüning and Mayer, 1996; González et al., 1996, 2002b; Muñoz et al., 1996, 2000; Lázár and Losonczy, 1999; López and González, 2002; Pisu et al., 2002; López et al., 2005; Huynh and Boyd, 2007; Blom et al., 2009; Pinelli et al., 2014; *reptiles*: Luebke et al., 1992; Brüning et al., 1994b; Bennis et al., 1996; Jiang and Terashima, 1996; Blute et al., 1997; Radmilovich et al., 1997; Smeets et al., 1997; Haverkamp et al., 1998, 2000; Cuenca et al., 2000; Cao and Eldred, 2001; Morona et al., 2006, 2007; *birds*: Brüning, 1993; Brüning et al., 1994a; Panzica et al., 1994; Cozzi et al., 1997; Yamakawa et al., 1997; Fischer and Stell, 1999; Atoji et al., 2001; Nie and Wang, 2002; Necker, 2004, 2005; Scicolone et al., 2006; Wilson et al., 2011; Tekmen-Clark and Gleason, 2013; *mammals*: Mizukawa et al., 1989; Skinner et al., 1989; Dun et al., 1992, 1993, 1994; Vincent and Kimura, 1992; Wolff and Datto, 1992; Terenghi et al., 1993; Egberongbe et al., 1994; Harper et al., 1994; Rodrigo et al., 1994; Vizzard et al., 1994, 1997; Amir et al., 1995; Darius et al., 1995; Lloyd et al., 1995; Satoh et al., 1995; Vanhatalo and Soinila, 1995; Hadeishi and Wood, 1996; Wang and Morris, 1996; Bertini and Bentivoglio, 1997; Desvignes et al., 1997; Alonso et al., 1998; Iwase et al., 1998; Léger et al., 1998; Doone et al., 1999; Ng et al., 1999; Fabris et al., 2000; Saxon and Beitz, 2000; Takumida and Anniko, 2000a, b; Varathan et al., 2001; Kluchová et al., 2002; Lukácová et al., 2002; Wu et al., 2002; Golombek et al., 2004; Gotti et al., 2005; Papantchev et al., 2005; Domínguez et al., 2006; Abe et al., 2010; Blom et al., 2012; Vielma et al., 2012; Bombardi et al., 2013; Chertok and Kotsuba, 2013; Czarnecka et al., 2013; Nutsch et al., 2014; Santos-Lobato et al., 2016.

2002b; Huynh and Boyd, 2007] and mammals [Amir et al., 1995; Wang and Morris, 1996; Agostino et al., 2004], but not in other species of these groups [Mizukawa et al., 1989; Vincent and Kimura, 1992; Egberongbe et al., 1994;

Brüning and Mayer, 1996; González et al., 1996; Pinelli et al., 2014]. The colocalization of NOS and TH in the suprachiasmatic neurons of gars seems to be a specific feature of this group of fishes, since it has not been observed

by double-labeling techniques in cladistians, amphibians, and reptiles [Smeets et al., 1997; López et al., 2005, 2016].

Within the basal hypothalamus of gars, like in cladistians, nitrergic cell populations are located in the tuberal and mammillary areas. In particular, the brain part termed the retromammillary region houses an important nitrergic group that was demonstrated to be different from the catecholaminergic group (TH-ir) characteristically found in this region [López et al., 2016; present results]. The presence of nitrergic cells in specific locations of the basal hypothalamus is a shared and highly conserved feature reported in all vertebrate groups (Table 2), with the only exception reported in the snake *Trimeresurus flavoviridis*, which lacks NADPH-d-reactive cells in the whole basal hypothalamus [Jiang and Terashima, 1996]. In addition, the large inferior hypothalamic lobes of gars also show a prominent population of NOS-ir cells located in the nucleus of the lateral recess. This peculiar brain structure in the hypothalamus of teleosts shows similar nitrergic cells in the nucleus of the lateral recess, but also in the central and diffuse nuclei of the inferior hypothalamic lobes [Arévalo et al., 1995; Brüning et al., 1995; Bordieri et al., 2003; Ando et al., 2004; Giráldez-Pérez et al., 2008], with the exceptions of *C. batrachus* and *Haplochromis burtoni* [Jadhao and Malz, 2004; Gaikwad et al., 2009]. The inferior hypothalamic lobes of teleosts are related to feeding and aggressive biting [Demski and Knigge, 1971], receiving indirect visual information from the pretectum [Wullimann and Meyer, 1990; Wullimann et al., 1991], taste information from secondary gustatory centers [Finger and Kanwal, 1992; Lamb and Caprio, 1993; Folgueira et al., 2003] and general visceral centers [Yoshimoto and Yamamoto, 2010; Kato et al., 2011] to produce a brainstem motor response that implicates activation of the trigeminal and facial motor nuclei. In addition, the nucleus of the lateral recess gives rise to descending projections to the secondary gustatory and visceral nuclei in the isthmus region [Yáñez et al., 2017], and, thus, the NO could modulate these input and output connections.

In relation to the hypothalamus, the hypophysis of gars is devoid of NOS-ir cells, as is also the case for most vertebrates studied (Table 2), although exceptions have been reported for some teleosts [Anken and Rahmann, 1996; Ando et al., 2004; Gaikwad et al., 2009] and mammals [Wolff and Datto, 1992; Lloyd et al., 1995].

In the diencephalon, the prethalamus in the rostral prosomere p3 contains a discrete group of nitrergic cells in periventricular locations, separated from prethalamic TH-ir and CB-ir cell populations. Two groups of nitrergic

cells in the prethalamus, also separated from TH-ir and CB-ir cells, have been described in cladistians [López et al., 2016] and most teleosts studied also showed prethalamic nitrergic cells [Arévalo et al., 1995; Brüning et al., 1995; Ando et al., 2004; Giráldez-Pérez et al., 2008]. However, this feature appears only in some species of amphibians [Brüning and Mayer, 1996; Pinelli et al., 2014] and amniotes generally lack nitrergic prethalamic cells, with an exception found exclusively in the ventromedial thalamic nucleus of rats [Bertini and Bentivoglio, 1997]. Therefore, this trait seems to be a general feature present in actinopterygian fishes, whereas it is uncommon among tetrapods. The TP in the basal part of p3 houses the most prominent diencephalic NOS-ir cell group of gars, forming distinct and separated populations from the characteristic TH-ir and CB-ir cell populations observed in this brain area. Most of the species of cladistians, teleosts, and amphibians studied showed nitrergic cells in the TP, although a few exceptions have been noted [González et al., 1996; Gaikwad et al., 2009]. In the TP of teleosts, a dopaminergic cell group with ascending projections to the subpallium has been identified [Rink and Wulliman, 2001, 2002; Yamamoto et al., 2011], and it was considered equivalent to the ventral tegmental area/substantia nigra complex of amniotes. In amphibians, a comparable connectivity from TP/mesencephalic tegmentum to basal ganglia has been confirmed [Marín et al., 1998a, b]. Of note, some species of teleosts and anurans showed colocalization of NOS/TH in the TP [López et al., 2005; Pushchina and Obukhov, 2010]. Interestingly, NOS/TH colocalization was found in the ventral tegmental area/substantia nigra of reptiles and birds [Panzica et al., 1996; Smeets et al., 1997], whereas the presence of nitrergic cells in the substantia nigra of mammals remains controversial because lack of nitrergic reactivity was first described in rats, cats, and humans [Mizukawa et al., 1989; Vincent and Kimura, 1992; Egberongbe et al., 1994], but subsequent studies indicated the contrary, at least in mice and rats [Abe et al., 2010; Czarnecka et al., 2013].

In the dorsalmost part of alar p2, the asymmetric habenula of gars contains nitrergic cell populations in the ventrolateral part of the large right habenula and the smaller left habenula that are separated from the cholinergic population. In cladistians, nitrergic cells were only observed in the ventral part of left habenula also separated from the cholinergic population [López et al., 2016]. The presence of nitrergic cells in the habenula is variable in teleosts and amphibians. Thus, NOS-ir cells were observed dorsally located in the habenula of the goldfish and almost a third of them colocalized ChAT [Giráldez-Pérez

et al., 2008, 2009], whereas in amphibians nitrergic cells have been described only in the ventral habenula of *Rana* and *Dermophis* [Muñoz et al., 1996; González et al., 2002b], and nitrergic habenular asymmetry was found only in *R. esculenta* [Guglielmotti and Fiorino, 1999]. Habenular nitrergic cells in amniotes seem rare and have been described in just 2 avian species [Brüning, 1993; Atoji et al., 2001]. The NO in the habenula could be implicated in the regulation of fear and the reward system [Agetsuma et al., 2010; Jesuthasan, 2012]. Also in the alar plate of p2 and ventral to the habenula, the thalamus of gars houses a modest nitrergic cell population in rostral and median diencephalic levels. The existence of nitrergic cells in the thalamus is a shared feature with other anamniotes, like cladistians, chondrosteans, teleosts, and amphibians (especially remarkable in anurans; Table 2), with the exceptions of the teleost *C. batrachus* [Gaikwad et al., 2009] and the urodele amphibian *Pleurodeles waltl* [González et al., 1996]. In contrast, this feature is unusual in amniotes, and it has only been reported in the thalamus of the lizard *Gekko gecko* [Smeets et al., 1997], in the Japanese quail and budgerigar [Panzica et al., 1994; Cozzi et al., 1997], and in the rat [Vincent and Kimura, 1992]. Therefore, the importance of NO in the thalamic relay of sensory information to the telencephalon seems to be more relevant in anamniotes than amniotes.

Finally, a small group of faintly NOS-ir cells is present in gars within the alar part of p1 in the pretectum, in the caudal diencephalon. In contrast, the pretectum of cladistians is devoid of nitrergic cells [López et al., 2016], whereas pretectal NOS-ir cells are common in teleosts and amphibians, although with some exceptions [Brüning et al., 1995; González et al., 1996; Pinelli et al., 2014]. This feature is also present in birds, and more rarely in reptiles and mammals, where pretectal cells have been described in a lizard [Smeets et al., 1997] and the rat [Vincent and Kimura, 1992].

Midbrain

Gars, like most teleosts, are highly visual predators with large, well-laminated optic tecta that mainly receive retinal projections [Northcutt and Butler, 1976, 1980, 1993]. An important nitrergic cell population is present in the inner zone of the periventricular layer of the optic tectum throughout its rostrocaudal and mediolateral extent. These cells are probably interneurons of type XIV morphology [Meek and Nieuwenhuys, 1998] and are intermingled with ChAT-ir, CB-ir, and CR-ir cells of similar morphology, but forming separated tectal subpopulations. Similar observations have been described in the op-

tic tectum of cladistians [López et al., 2016] and teleosts, although in the goldfish up to a 3% of NOS-ir cells in the optic tectum coexpress ChAT [Giráldez-Pérez et al., 2009]. The presence of nitrergic cells in periventricular tectal locations is a conserved feature observed in all groups of vertebrates (Table 2). However, in chondrosteans and birds there are also nitrergic cells in outer tectal layers [Brüning, 1993; Cozzi et al., 1997; Atoji et al., 2001; Scicolone et al., 2006; Pushchina and Obukhov, 2012], whereas in mammals these cells are present in inner, intermediate, and outer layers of the superior colliculus [Mizukawa et al., 1989; Vincent and Kimura, 1992; Egberongbe et al., 1994]. The tectal nitrergic cells are likely related to the processing of visual information, but may also be involved in multisensory integration, and in mammals these cells have been proposed to be interneurons that convey cortical influences onto superior colliculus output neurons [Fuentes-Santamaría et al., 2008].

Only modest numbers of nitrergic cells are detected in gars within the torus semicircularis and torus lateralis, as has also been observed in cladistians [López et al., 2016]. Tonal nitrergic cells, likely intervening in the processing of auditory information [Grassi et al., 1995; Wu et al., 2008], have been reported in most groups of vertebrates (Table 2), although it should be noted that NADPH-d reactive neurons could not be demonstrated in the torus of the snake *T. flavoviridis* [Jiang and Terashima, 1996], the budgerigar *Melopsittacus undulatus* [Cozzi et al., 1997], and the cat [Mizukawa et al., 1989].

A prominent group of intensely NOS-ir cells is located within the rostral mesencephalic tegmentum of gars, and some of these cells also contain CR. Conversely, the large cholinergic neurons of the oculomotor nucleus lack nitrergic labeling. A comparable nitrergic cell population has been observed in the mesencephalic tegmentum of cladistians, although it was described in the mid and caudal levels [López et al., 2016]. All groups of vertebrates studied show a characteristic nitrergic cell population in the mesencephalic tegmentum, representing another primitive and conserved feature (Table 2). A shared feature of amniotes is the presence of the dopaminergic ventral tegmental area/substantia nigra (VTA/SN) complex in the midbrain tegmentum [Smeets and González, 2000]. By means of double-labeling techniques, many of these dopaminergic cells have been demonstrated to coexpress NOS in some species of reptiles, birds, and mammals [Johnson and Ma, 1993; Panzica et al., 1996; Smeets et al., 1997]. However, as previously mentioned, the counterpart of the VTA/SN complex in gars most likely lies in the diencephalic TP, where abundant nitrergic cells are located.

Hindbrain

The segmental organization of the rhombencephalon can be inferred in gars by the localization of the cholinergic motor nuclei and other well-established landmarks [Morona et al., 2013]. Thus, we have referred the localization of the nitrergic structures to the rhombomeres 0–8 (r0–r8; Fig. 10). In the rostral rhombencephalon of gars, both the interpeduncular nucleus and the cerebellum (a dorsal derivative structure from r1) are devoid of nitrergic cells. The absence of this type of cell in the interpeduncular nucleus is a general feature observed in most anamniotes and reptiles, with the exceptions of cladistians [López et al., 2016], the teleost *Carassius auratus* [Giráldez-Pérez et al., 2008], the anuran amphibian *Hyla septentrionalis* [Pinelli et al., 2014], and the turtle *Pseudemys scripta elegans* [Brüning et al., 1994b]. In contrast, nitrergic cells were reported in all species of birds studied and in most mammals, with the remarkable exception of humans [Egberongbe et al., 1994]. The absence of cerebellar nitrergic cells is a variable trait reported in cladistians and gars [López et al., 2016; present results], in urodele and gymnophionan amphibians [González et al., 1996, 2002b], in a chameleon and a snake [Bennis et al., 1996; Jiang and Terashima, 1996], and in the pigeon [Atoji et al., 2001]. However, distinct nitrergic neurons were described in different cerebellar layers of a sturgeon [Pushchina and Obukhov, 2012], several teleosts [Arévalo et al., 1995; Brüning et al., 1995; Ando et al., 2004; Giráldez-Pérez et al., 2008], anurans [Lázár and Losonczy, 1999; Huynh and Boyd, 2007; Pinelli et al., 2014], reptiles [Brüning et al., 1994b; Smeets et al., 1997], birds [Brüning, 1993; Cozzi et al., 1997], and mammals [Vincent and Kimura, 1992; Egberongbe et al., 1994; Iwase et al., 1998].

Like in the early diverged cladistians, gars show a group of nitrergic cells in the alar part of r1, tentatively identified as a cerebellar nucleus according to its localization [López et al., 2016; present results], although hodological data are necessary to corroborate this designation. In elasmobranchs this nucleus is prominent and reactive for the NADPH-d histochemical technique [Álvarez-Otero et al., 1996; Pose-Méndez et al., 2014], whereas a similar nucleus has not been identified in teleosts. Nitrergic cells in cerebellar nuclei are more frequently described in tetrapods, including several species of amphibians [Lázár and Losonczy, 1999; Huynh and Boyd, 2007], reptiles [Brüning et al., 1994; Smeets et al., 1997], birds [Cozzi et al., 1997; Atoji et al., 2001], and mammals [Vincent and Kimura, 1992]. Therefore, the presence of nitrergic cerebellar nuclei in vertebrates supposes another variable feature.

In the dorsal part of r1 of gars, another group of NOS-ir cells is observed close to the prominent group of ChAT-ir cells identified as the LDT [Morona et al., 2013]. However, double-labeling experiments do not show nitrergic labeling in the cholinergic cells of this nucleus, in line with results obtained recently in cladistians [López et al., 2016]. In contrast, the LDT and its related pedunculopontine tegmental nucleus contain both ChAT and NOS in amphibians [González et al., 1996; Muñoz et al., 1996, 2000; Marín et al., 1997; González et al., 2002a, 2002b; López and González, 2002] and amniotes [Alonso et al., 2000; Veleanu et al., 2016]. This cholinergic and nitrergic complex is implicated in the control of the sleep-wake cycle, learning, motor control, and reward system [Wang and Morales, 2009], and given the absence of colocalization of NOS in the ChAT-ir cells of the LDT found in nontetrapod vertebrates, gars could represent a primitive neurochemical feature of this important cholinergic group.

The rhombencephalic reticular formation of the 2 species studied contains 3 subpopulations of nitrergic cells in the superior and middle nuclei (Rs and Rm), and to a lesser extent in the inferior nucleus. Nitrergic cells in the reticular formation constitute a highly shared feature among vertebrates (Table 2), and these cells are most probably reticulospinal neurons, as has been demonstrated in amphibians [McLean and Sillar, 2000; Sánchez-Camacho et al., 2001; González and ten Donkelaar, 2007].

At the mid-rostrocaudal level of the rhombencephalon, a group of nitrergic cells is found in the ventral part of the octavolateral area, which in gars is the medullary region containing the termination sites of vestibular as well as acoustic and lateral line fibers [McComick 1981; Song and Northcutt, 1991]. Among anamniotes the presence of nitrergic cells in the octavolateral area is a conserved feature reported in cladistians, teleosts, and most amphibians (Table 2), although with some exceptions [Brüning and Mayer, 1996; González et al., 1996; Pinelli et al., 2014]. In amniotes this feature is present in the vestibular nuclei of birds and mammals, whereas in reptiles similar cells have been observed in turtles and lizards [Brüning et al., 1994b; Smeets et al., 1997] but not in chameleons and snakes [Bennis et al., 1996; Jiang and Terashima, 1996].

The cholinergic neurons of the cranial motor nuclei and the serotonergic cells of the raphe column in the rhombencephalic midline of gars are devoid of nitrergic labeling [Parent and Northcutt, 1982; Chiba and Oka, 1999; Morona et al., 2013; present results], as is also observed in cladistians [López et al., 2016]. The presence of nitrergic staining (generally NADPH-d reactivity) in the

motor nuclei of the cranial nerves is a feature mentioned in some species of teleosts [Arévalo et al., 1995; Anken and Rahmann, 1996; Pushchina, 2007; Giráldez-Pérez et al., 2009], amphibians [Muñoz et al., 1996; Lázár and Losonczy, 1999; Huynh and Boyd, 2007; Pinelli et al., 2014], reptiles [Bennis et al., 1996; Jiang and Terashima, 1996], birds [Brüning, 1993; Atoji et al., 2001], and mammals [Vincent and Kimura, 1992; Varathan et al., 2001; Chertok and Kotsuba, 2013]. However, reliable evidence obtained with double-immunolabeling techniques for NOS and ChAT has only been performed in the case of goldfish [Giráldez-Pérez et al., 2009] and, therefore, the localization of NOS in rhombencephalic motoneurons is a feature that needs confirmation with the appropriate double-labeling techniques. In addition, the presence of nitrergic cells in the raphe nuclei has been repeatedly reported in representatives of most vertebrate groups, but using exclusively single-labeling techniques. These include teleosts [Arévalo et al., 1995; Pushchina and Diuizen, 2004; Giráldez-Pérez et al., 2008], amphibians [González et al., 1996; Muñoz et al., 1996; Lázár and Losonczy, 1999; Huynh and Boyd, 2007], reptiles [Smeets et al., 1997], birds [Atoji et al., 2001], and mammals [Vincent and Kimura, 1992; Egberongbe et al., 1994]. However, conclusive data obtained with double-labeling techniques revealed NOS in serotonergic neurons in the rostral raphe nuclei of the rat [Johnson and Ma, 1993; Dun et al., 1994; Wotherspoon et al., 1994; Wang et al., 1995; Simpson et al., 2003; Lu et al., 2010], but not in the raphe nuclei of mice, guinea pigs, and cats [Leonard et al., 1995; Léger et al., 1998]. Conversely, a previous double-labeling study in amphibians demonstrated that only the cells in the caudal part of the raphe column also contain NOS [López et al., 2005]. Thus, the presence of NOS in the raphe nuclei seems to be another variable feature that requires precise experimental confirmation in many vertebrate groups.

In the caudal rhombencephalic alar plate abundant and intensely NOS-ir cells are located in the Nsol of gars, which form a separated cell population from the numerous TH-ir, CB-ir, CR-ir, and ChAT-ir cells closely located within this area. Similar data have been reported only in cladistian fishes [López et al., 2016], whereas NADPH-d-reactive cells in the Nsol were generally absent in teleosts, with the exception of the tench [Arévalo et al., 1995]. However, this is a characteristic present in most amphibians, with the exception found in *H. septentrionalis* [Pinelli et al., 2014], in all species of birds studied, and in almost all species of mammals, with the exception of the cat [Mizukawa et al., 1989]. In reptiles this trait is more

variable since the Nsol contains nitrergic cells in the turtle and lizard [Brüning et al., 1994b; Smeets et al., 1997], but not in chameleons and snakes [Bennis et al., 1996; Jiang and Terashima, 1996]. In addition, the absence of colocalization of NOS and TH in cells of the Nsol has been confirmed in amphibians and reptiles [Smeets et al., 1997; López et al., 2005]. Of note, the presence of nitrergic cells in the Nsol led to the suggestion that NO may be related to the modulation of visceral sensitive afferents, the control of blood pressure, and the regulation of respiratory frequency [Lawrence et al., 1998; Wu et al., 2002; Granjeiro and Machado, 2009; Kong et al., 2009].

Spinal Cord

A nitrergic cell population has been observed throughout the spinal cord of gars, mainly located in the dorsal horn of the spinal cord, but cells are also found in the intermediate and ventral spinal gray, and around the medial longitudinal fascicle. Of note, the large cholinergic somatomotor neurons do not colocalize NOS. The occurrence of spinal nitrergic cells mainly located in the dorsal horn is an ancient and highly conserved feature of vertebrates, from lampreys to mammals (Table 2). Comparatively, nitrergic cells have been observed in the intermediate and ventral spinal gray of teleosts [Arévalo et al., 1995; Brüning et al., 1995; Giráldez-Pérez et al., 2008], amphibians [Crowe et al., 1995; Muñoz et al., 1996, 2000; González et al., 1996, 2002b; Huynh and Boyd, 2007], reptiles [Jiang and Terashima, 1996; Radmilovich et al., 1997; Smeets et al., 1997; Morona et al., 2006, 2007], birds [Atoji et al., 2001; Necker, 2004, 2005], and mammals [Dun et al., 1992, 1993; Terenghi et al., 1993; Vizzard et al., 1994, 1997; Doone et al., 1999; Kluchová et al., 2002; Lukácová et al., 2002; Bombardi et al., 2013]. However, colocalization of NOS with ChAT in the spinal motoneurons is an unusual feature that has been described only in some species of teleosts [Pushchina et al., 2007; Giráldez-Pérez et al., 2009] and mammals [Dun et al., 1992; Terenghi et al., 1993; Wetts and Vaughn, 1994]. However, the effect of NO in the regulation of the locomotor activity has been demonstrated in the lamprey spinal cord [Kyriakatos et al., 2009], and other functions for NO in the spinal cord are inhibition of the nociceptive information and pain control [Hervera et al., 2010; Chu et al., 2011; Cury et al., 2011; Jin et al., 2011, 2012].

Retina

In the retina of the gars studied, a numerous population of small nitrergic cells was observed in the inner nuclear layer with short processes ramifying within the in-

ner plexiform layer. This nitrergic cells most likely represent a population of amacrine cells that is distinct from the TH-ir, 5-HT-ir, ChAT-ir, and CB-ir amacrine cells, whereas some NOS-ir cells also contain CR. Retinal NO might modulate the neuronal communication between bipolar and ganglion cells, like the transient conversion of synaptic inhibition to excitation [Hoffpauir et al., 2006]. Amacrine cells, either NADPH-d reactive or NOS-ir, have been thoroughly reported in the retina of teleosts [Ostholm et al., 1994; Pottek et al., 1997; Haamedi and Djamgoz, 2002], amphibians [Nöll et al., 1994; Kurenni et al., 1995; Blom et al., 2009], reptiles [Haverkamp et al., 2000], birds [Fischer and Stell, 1999; Wilson et al., 2011; Tekmen-Clark and Gleason, 2013], and mammals [Vielma et al., 2012], representing a feature shared in all osteichthyes studied. Of note, holosteans, like cladistians, lack other nitrergic cells in the retina [López et al., 2016; present results]. In particular, the lack of nitrergic labeling in the ganglion cell layer is in line with the lack of labeling in the extensive retinal central projections [Northcutt and Butler, 1976].

A greater diversity of nitrergic retinal cell types has been described in other vertebrate groups, probably related with new functions of retinal NO, such as light-adaptive changes [Greenstreet and Djamgoz, 1994; Djamgoz et al., 2000; Angotzi et al., 2002; Sato et al., 2011] or circadian effect in the photoreceptor activity [Ko et al., 2013]. Thus, nitrergic ganglion cells and photoreceptors have been reported in the retina of teleosts [Ostholm et al., 1994; Pottek et al., 1997; Ota et al., 1999; Haamedi and Djamgoz, 2002; Gaikwad et al., 2009], amphibians [Nöll et al., 1994; Kurenni et al., 1995; Blom et al., 2009], reptiles [Blute et al., 1997], birds [Fischer and Stell, 1999; Wilson et al., 2011; Tekmen-Clark and Gleason, 2013], and mammals [Blom et al., 2012], where protoreceptor cells always lack nitrergic labeling. Furthermore, nitrergic bipolar neurons have been described in the retina of chondrichthyes [Shiells and Falk, 1992], amphibians [Nöll et al., 1994; Kurenni et al., 1995; Blom et al., 2009], and reptiles [Cao and Eldred, 2001], whereas horizontal nitrergic cells have been reported only in teleosts [Ota et al., 1999; Haamedi and Djamgoz, 2002] and reptiles [Haverkamp and Eldred, 1998; Cuenca et al., 2000].

Concluding Remarks

This study represents the first detailed neuroanatomical description of the distribution of the nitrergic cells and fibers in the CNS of holostean fishes. The current results,

jointly with those recently reported for the brain of cladistians, show a pattern of organization of the nitrergic structures that is essentially similar in these nonteleost actinopterygian fishes, especially in the subpallial telen-cephalon, hypothalamus, midbrain, rhombencephalon, spinal cord, and retina. The comparison with other groups of vertebrates identifies several features of the nitrergic system that are highly conserved in the nervous system, such as the presence of nitrergic cells in pallial/cortical areas, striatum, amygdaloid complex, basal hypothalamus, mesencephalic optic tectum, torus semicircularis and tegmentum, the rhombencephalic reticular formation, the dorsal horn of spinal cord, and the amacrine subpopulation in the retina. This highly conserved pattern of distribution of the nitrergic cells and fibers suggests that NO might have been involved from early stages in the evolution of vertebrates in functions such as motor control, emotion/behavior control, the regulation of the hypothalamic functions, and the modulation of sensitive information in midbrain areas, the spinal cord or the retina. On the contrary, the presence of nitrergic cells in the olfactory bulb, thalamus, pretectum, and cerebellum, or its colocalization with cholinergic motoneurons of cranial nerves/spinal cord and the serotonergic cells in the raphe nuclei, are highly variable features. Furthermore, particular characteristics of the nitrergic system of gars include the absence of nitrergic labeling in certain subpallial areas, like the Vn and VL, the coexpression of NOS and TH in most of the suprachiasmatic neurons, the prominent nitrergic cell population located exclusively in the nucleus of the lateral recess of the large inferior hypothalamic lobes, and the absence of localization of NOS in the important cholinergic nuclei of the rhombencephalon.

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