Comparative neurochemical features of the innervation patterns of the gut of the basal actinopterygian, *Lepisosteus oculatus*, and the euteleost, *Clarias batrachus*

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Abstract


The structure and physiology of enteric system are very similar in all classes of vertebrates, although they have been investigated only occasionally in non-mammalian vertebrates. Very little is known about the distribution of the neurotransmitters in the gut of actinopterygian fishes. Anatomical and physiological studies of enteric nervous systems in the spotted gar (*Lepisosteus oculatus*) and airbreathing catfish (*Clarias batrachus*), a non-teleost and teleost actinopterygian, respectively, have not been undertaken. This study provides the first comprehensive characterization of the range of neurochemical coding in the enteric nervous system of these two species, including the chemical diversity of the mucosal endocrine cells in the pyloric stomach of *Clarias*. Autonomic innervation of the secretory glands is also described and reported herein for the first time for fishes.

We also report splanchnic (spinal) innervation of the stomach, submucosal ganglia (that also colocalize with nNOS) and caudal intestine of *Clarias*. In both fish species, numerous 5HT, ChAT, nNOS and TH-positive nerve fibres have been observed. These discoveries demonstrate that much more physiological and pharmacological data are needed before a comprehensive model of enteric nervous system control in vertebrates can be developed.

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Introduction

The typical actinopterygian enteric nervous system (ENS) consists of nerve cell bodies in the myenteric plexus with axons protruding into the muscle layers as well as reaching blood vessels, endocrine cells and glands in the submucosa and mucosa. In the majority of teleosts examined, there are few ganglia that contain a small number of cell bodies in submucosal tissue (Olsson 2009, 2011). The vagus nerve innervates the oesophagus and stomach, and the proximal part of the intestine. It contains fibres of both cranial and spinal origin (Olsson and Holmgren 2010). In general, the neurons of the myenteric plexus in the vertebrate intestine are involved in the regulation of the activity of the smooth muscle, whereas neurons in the submucosal plexus regulate mucosal secretion and blood flow. Submucosal ganglia are rare or absent in the oesophagus and stomach of many species (Gibbins 1994). Thus, in the stomach of these species, myenteric neurons control both the external muscle and the mucosa.

The distribution of individual neurotransmitters and their contribution to gut motility of vertebrates were recently reviewed by Olsson (2011). Most of these transmitters are hypothesized as also having a key role in the control of gut secretion, as 5HT containing neurons reported previously in teleostean gut (Anderson 1983). As it is the case for neurotransmitters present in the central nervous system or ENS of...
tetrapods, the neurotransmitter architecture resulting from the coexistence of variable neuroactive substances may also define the chemical coding of ENS in actinopterygian fishes. Encoding in nerve cells varies considerably between species (Gibbins 1995; Sang and Young 1996). Thus, a target cell can be innervated by different types of neurons, which express the same neuroactive substances or functionally related neurons can express different neuropeptides (Gibbins 1989).

With the exception of Olsson’s (2011) and Uyttebroek et al. (2010), study of larval and adult zebrafish (Danio rerio), very few studies have resolved coding of neurons innervating specific target tissues in the ray-finned fishes (Actinopterygii). Thus, very little is known concerning the microanatomical and functional complexity of the gut nervous system in nearly all other ray-finned fishes.

The focus of this study is to broaden our knowledge on the chemical coding of the actinopterygian enteric nervous system based on data gathered from two different species, the spotted gar (Lepisosteus oculatus), a holostean preteleost and the airbreathing higher teleost, Clarias brachus using confocal immunofluorescence. To this end, the immunohistochemical localization of several transmitters was determined. The expression of these transmitters is also statistically determined in certain populations of submucosal neurons, but not in the myenteric cell bodies observed.

**Materials and Methods**

**Animals and tissue preparation**

Thirteen individual specimens of Lepisosteus oculatus of both sexes ranging from 13 to 15 cm in length were used in this study. Specimens were obtained from local suppliers, kept in aerated, recirculating tap water at 12°C and maintained in a 12-h light/dark cycle. Ten specimens of Clarias brachus, 15–20 cm total length, were procured from a fish market in Allahabad, India and transferred to the Department of Zoology of the University of Allahabad, India, where they were maintained in circulating aquaria at 28°C. After anaesthesia in 0.1% solution of tricaine (MS 222, Sigma-Aldrich, Milano, Italy), the gastrointestinal tract was removed and washed in ice-cold phosphate-buffered saline solution (PBS). Biopsies were removed from the oesophagus, cardiac and pyloric stomach, proximal, middle and distal intestine, and rectum from all specimens, and fixed by immersion in 4% paraformaldehyde in PBS at 4°C for 4–6 h. Tissues were subsequently washed in PBS, dehydrated, embedded in paraplast, and processed into 5- to 10-µm-thick histological sections with preliminary haematoxylin/eosin and triple staining methods.

**Confocal Immunofluorescence, microscopy and image processing**

Histological sections were incubated in primary antibodies (used individually and in double-label experiments, Table 1) that were diluted in a permeabilizing solution (PBS, 0.2% Triton X-100, 0.1% sodium azide) according to the optimal dilutions (detailed in Table 1) and placed on the slides at room temperature in a moist chamber. Sections were then treated with fluorescently labelled secondary antibodies diluted in PBS as follows: 1: 50 goat-anti-rabbit secondary antibodies conjugated with fluorescein isothiocyanate and 1: 100 goat-anti-mouse secondary antibodies conjugated with the red fluorophore, Alexa 568. Sections were then left to incubate in the dark at room temperature for 2 h. After washing, the sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent photobleaching and then cover-slipped. Tissue preparations for control experiments were deactivated by excluding primary antibodies or overnight incubation with 50 µM of the complementary antigen (Table 2). The results of the control experiments were negligible non-specific immunolabelling of the nerve structures. Images were acquired using a Carl Zeiss LSM 700 confocal microscope equipped with solid-state laser lines (wavelengths of 405, 488 and 639 nm) and a spectral variable secondary dichroic beamsplitter.

**Image data and colocalization analysis**

Where populations appeared by visual inspection of colocalize for both tissue antigens, quantitative colocalization analysis of ganglia was performed using the Fiji image-processing package (http://fiji.sc/wiki/index.php/Fiji). To reduce noise signals, backgrounds for red and green split-channel

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**Table 1** Primary antibodies used in this study

<table>
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<th>Source</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Animal source</th>
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<tr>
<td>Calbindin D28K (H-50)</td>
<td>Santa Cruz Biotechnology</td>
<td>1 : 100</td>
<td>Rabbit</td>
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<td>5-Hydroxytryptamine (5HT)</td>
<td>Dako Denmark Glostrup</td>
<td>1 : 50</td>
<td>Mouse</td>
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<tr>
<td>1-Methyl-4-phenylpyridinium</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
<td>1 : 100</td>
<td>Mouse</td>
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<tr>
<td>Neuronal nitric oxide synthase (nNOS)</td>
<td>Bachem, Weil and Rhein, Germany</td>
<td>1 : 100</td>
<td>Rabbit</td>
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<tr>
<td>Choline</td>
<td>Sigma-Aldrich St. Louis, MO, USA</td>
<td>1 : 100</td>
<td>Mouse</td>
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**Table 2** Chemicals used for immunohistochemistry

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<td>Mouse 5-Hydroxytryptamine (H209)</td>
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<td>Human tyrosine hydroxylase-internal sequence (400-500)</td>
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<tr>
<td>Mouse choline acetyltransferase c-terminal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Human nNOS c-terminal (1995-1289)</td>
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images were corrected via background removal. Pearson’s correlation coefficient (PC) and Manders (M) overlap coefficient were recorded. Values for PC coefficient range between −1.0 and 1.0, where 0 indicates no significant correlation and 1.0 indicates total colocalization. M coefficient values range from 0 to 1.0; where values of 0 and 1 indicate no and total overlap of channel-positive pixels in the image, respectively. We considered combined PC values above 0.5 and M values above 0.9 as significant indicators of colocalization.

Results

Gross morphology and microstructure of the gut

Lepisosteus oculatus. The oesophagus is long and thick-walled. The oesophageal mucosa shows longitudinal folds and appears formed by columnar epithelial and mucous-secreting cells. In addition, numerous tubular glands are present in the wall of the oesophagus and in the oesophagus-stomach transition. A sharp turn marks the boundary between the oesophagus and the stomach. This boundary is marked internally by the disappearance of the thick mucosal folds. The stomach is a short tubular chamber that meets the intestine at an acute angle. This boundary is marked externally by the presence of a deep constriction and, internally, by the presence of a pyloric valve. Intestinal (i.e. pyloric) caeca are associated with the pyloric valve. Epithelial cells in the gastro-oesophageal mucosa are presumably oxyntico-epithelial cells like those described in all the major non-mammalian tetrapod groups (Smit, 1968). These were not differentiated into oxyntic and peptic cells as was performed in previous studies of other taxa. After the pylorus, the intestine shows a smaller diameter than that of the first part of the gut and follows a tortuous course. It first descends, then makes an U-turn and ascends in parallel with the first part, then traces another ‘U-turn’ and descends, again in parallel, to end into the anus (the most distal part of the intestine was misinterpreted as a spiral valve in the early literature).

Clarias batrachus. The oesophagus of C. batrachus is relatively long and thick-walled, serving as a passage to the stomach. The oesophageal mucosa consists of stratified and columnar epithelial cells and mucous-secreting cells. The stomach is pouch-like and divisible into cardiac and pyloric regions, according to the mucosal folds and distributions of the underlying gastric glands. The anterior part of the intestine, the duodenum, is wide, and the internal lining has a characteristic honeycomb structure organized into small compartments filled with partially digested food materials. The duodenum forms a loop over the dorsal aspect of the stomach and then curves posteriorly. The remainder of the intestine is more or less straight, with the rectum slightly wider than the anterior sections of the intestine (see: Stroband and Kroon 1981).

Immunohistochemical observations

Lepisosteus oculatus.

Calbindin and 5HT. Calbindin-immunopositive nerve cell bodies and fibres were present in the myenteric plexus of the oesophagus. Calbindin-immunopositive nerve plexuses were also observed at the external surface. Double labelling for calbindin and 5HT in the oesophagus revealed the presence of numerous submucosal 5HT-positive nerve cell bodies that were often located near the tubular glands; these cells did not co-express calbindin. 5HT-immunopositive cell bodies were often surrounded by varicose calbindin-immunoreactive fibres. We observed calbindin-positive nerve fibres running between the gland cells, but these cells did not co-express 5HT (Fig. 1A). A type of calbindin-immunopositive nerve ending with flattened laminar appearance was observed that was present in the myenteric plexus of caudal intestine, a structure that resembles the intraganglionic laminar endings of proximal gut (Olsson et al. 2004). But the origin of these endings are unknown.

In the stomach, 5HT-positive nerve fibres run parallel to the longitudinal muscle and also lie against the inner surface of the longitudinal muscle. A network of 5HT- and calbindin-immunopositive nerve fibres is present in the circular muscle. Vagal branches lie outside the muscle layers and intermingle to form ganglionated plexus possessing 5HT immunoreactivity. 5HT-positive unipolar or multipolar neurons and nerve fibres are present in submucosal localizations. 5HT-positive neurons receive synaptic contact by both 5HT- and calbindin-positive nerve-fibre varicosities. In addition, calbindin-immunopositive nerve fibres are found surrounding the gastric glands or are in close vicinity of gastric-gland cells (presumably oxynticopeptic cells) in the mucosa. Calbindin and 5HT immunoreactivity were also found in the enteroendocrine cells of both closed and open types of the mucosa in the oesophagus and stomach. Enteroendocrine cells of the open type are also located between the oxyntic cells and co-express both calbindin and 5HT (Fig 1B). Densities of nerve cell bodies were observed in the myenteric plexus of the pyloric caeca. These cells make up several ganglia comprised of a majority of 5HT-positive nerve cell bodies that also co-express calbindin (Fig. 2). 5HT-immunoreactive nerve fibres innervate the longitudinal muscle and circular muscle where nerve 5HT fibres intermingle with calbindin-positive fibres. We also observed calbindin and 5HT immunoreactivity in the enteroendocrine cells of the mucosa. Elsewhere in the submucosa of the stomach, no ganglia were observed that colocalized for calbindin and 5HT (Fig. 2).

A network of nerve fibres and neurons both co-expressing calbindin and 5HT lie in the myenteric plexus of the middle intestine. 5HT nerve fibres run within the longitudinal muscle. The circular muscle within this region was supplied by a majority of calbindin-positive nerve fibres. Endocrine cells of both closed and open morphology are
Fig. 1—Colocalization preparations with antibodies against Calbindin and 5HT, ChAT and nNOS, and TH-nNOS in the gastrointestinal tract of the spotted gar (*Lepisosteus oculatus*). Calbindin–5HT. — A. Calbindin-immunoreactive nerve fibres run in large bundles (red arrows) in the gland cells (asterisk) in oesophagus. Calbindin branching endings (arrowheads) are also seen. Enteroendocrine cell (EC) in basal epithelium shows colocalization of calbindin and 5HT. — B. Enteroendocrine cells (arrows) are present in the gland oxyntic cells in the stomach. — C. 5HT-positive nerve cell bodies in compact myenteric ganglia (G) of the distal intestine. nNOS immunoreactivity is found in a few nerve cell bodies and nerves running in the circular muscle (C) and submucosal layer (S). ChAT-nNOS. — D. nNOS immunoreactivity in nerve bundles (N) in submucosal layer near to oesophageal glands (arrows) and in the mucosal cells (asterisk). — E. Shows ChAT and nNOS immunoreactivity in the nerves running in the myenteric plexus (arrow) and circular muscle (C) of oesophagus. F. ChAT-positive nerve cell in the myenteric plexus of the stomach (arrow). nNOS-immunoreactive nerve fibres innervating the circular muscle (C). LM longitudinal muscle. — G. ChAT and nNOS-positive (arrows) nerve cell bodies are present in the submucosal layer of the stomach. TH-nNOS. — H. Overlay of TH and nNOS immunoreactivity in nerve running in the subserous muscle coat. CM, circular muscle. — I. Overlay of TH and nNOS is found in the submucosal nerve cell bodies (arrow) and nerve fibres in the circular muscle (CM) of the oesophagus. — J. TH-positive nerve cell bodies in the myenteric ganglia (G) of distal intestine. TH immunoreactivity is also noticed in nerve running the longitudinal muscle (LM). nNOS-immunoreactive nerve bundles (arrows) are found in the myenteric plexus in close association with ganglia, and the submucosal layer (S). Scale bars 20 μm in A, B, D, E, F, G, I, J, 50 μm in C, E, H.
present in the mucosa. The closed type cells contain calbindin immunoreactivity and the open type cells co-express both calbindin and 5HT.

In the distal intestine, the submucosa is thicker and has many isolated muscle fibres coursing into the intestinal villi. The muscular coats of the distal intestine are very well developed. The longitudinal muscle is supplied by 5HT-positive nerve fibres oriented parallel to the muscle cells.

In the myenteric plexus of the distal intestine, there are groups of ganglia with a majority of 5HT-positive (Fig. 1C) nerve cell bodies; however, some cells in the intestine colocalize for calbindin and 5HT (Fig. 2). A network of nerve fibres with immunoreactivity for both calbindin and 5HT lies against the inner surface of longitudinal muscle, a structure probably emanating from the nerve bodies in the ganglia; however, confirmation of this input will require retrograde labelling experiments.

The circular muscle layer is thicker and supplied by calbindin- and 5HT-positive nerve fibres. These fibres course through the circumferance of the circular muscle, oriented along its transverse plane. In addition to separate, overlapping calbindin- and 5HT-positive nerve fibres innervating the circular muscle, some fibres colocalize for both the neurotransmitters.

The mucosa is composed of folds with rounded edges that were arranged in parallel. Enteroendocrine cells were observed among the intestinal cells. Some of these enteroendocrine cells are densely packed within the basal lamina and were immunoreactive for calbindin. Other cells reach the intestinal lumen by a long process and co-express both calbindin and 5HT.

**ChAT and nNOS.** ChAT-positive fibres are found in more or less irregular bundles within the muscle fibres. ChAT immunoreactivity is found in the inner muscular coat consisting of a thick layer of longitudinally oriented fibres (Fig. 1E). In the myenteric plexus, the two antisera labelled two different nerve fibre populations of ChAT and nNOS positivity (Fig. 1E).

The cytoplasm of the columnar epithelial cells lining the lumen of the oesophagus contained nNOS immunoreactivity. Scattered through the connective tissue between the epithelium and the inner muscular coat in the submucosal layers, we observed nNOS immunoreactivity in nerve fibres and nerve bundles that have a flattened appearance (Fig. 1D). Nerve varicosities with nNOS immunoreactivity were observed in close proximity to the numerous tubular glands within the submucosa (Fig. 1D).

Nearer the transitional region between the oesophagus and stomach and within the stomach itself, the mucosal folds are not so deeply indented. In the cytoplasm of shorter, cylindrical epithelial cells of the pyloric stomach,
ChAT immunoreactivity was observed. In the underlying tubular gastric glands and submucosa, nNOS-positive nerves course among the glands, the dense band of connective tissue layers, and the nerve cell bodies that are found in submucosa. We also observed (Fig. 1G) nerve cell bodies of mixed expression: fibres either co-expressing ChAT and nNOS (Fig. 2) and, in some cases, fibres expressing ChAT immunoreactivity. In addition, nNOS-immunoreactive varicosities were observed in close proximity to nerve cell bodies.

ChAT-positive nerve cell bodies were observed in the myenteric plexus of the tubular stomach and the nerves running in the longitudinal muscle (Fig. 1H). In addition, nNO-positive varicose nerve fibres also run in the dense connective tissue and dense bundles of the thicker circular muscle (Fig. 1E,F).

In the myenteric plexus of distal intestine, we observed ChAT-immunoreactive ganglionic nerve cell bodies. We observed that nNOS-positive nerve varicosities did not innervate circularly arranged muscle fibres of the inner muscular coat; however, we did observe nNOS-positive innervation of the muscularis mucosa in the distal intestine. In additional, we did not observe populations of nerve cell bodies colocalizing ChAT and nNOS at significant levels in the myenteric plexus of the distal intestine (Fig. 2).

**TH and nNOS.** TH-positive nerve bundles are distributed throughout the muscle coat of the oesophagus (Fig. 1H), stomach, pyloric caeca and distal intestine. These bundles were also observed in the myenteric plexus and submucosal layers. Some of these fibres colocalized both TH and nNOS. Many submucosal neurons were both nNOS- and TH-immunoreactive as shown by coincidence of the red and green labels appearing in orange in the oesophagus (Figs. 1I and 2) and cardiac stomach (Fig. 2). TH nerves arose extrinsic to the stomach, pyloric caeca and distal intestine. These bundles innervated the circular muscle, some co-expressing 5HT. 5HT-positive neurons and nerves were present also among the gastric glands of the submucosa (Fig. 2A). In the stomach, a significant level of colocalization of calbindin and 5HT was observed only in populations within the myenteric plexus of the pyloric caeca (Fig. 3).

In the duodenum, 5HT-immunoreactive nerve fibres were seen in the longitudinal muscle. Numerous calbindin-immunoreactive nerve bundles were observed abutting the outer surface of the circular muscle. Dense 5HT varicosities were scattered throughout the rest of this muscle. Clustered 5HT-immunoreactive nerve cell bodies were observed against the inner surface of the circular muscle. Calbindin-immunoreactive nerve cell bodies and 5HT-positive ganglia were both observed in the myenteric plexus. Both 5HT- and nNOS at significant levels (Fig. 2). The ganglia were innervated by nNOS-positive and TH-positive nerve varicosities (Fig. 1J). Spinal postganglionic sympathetic, nitricergic nerves innervating the distal intestine and the stomach were previously reported also including nitric oxide (Luckensmeyer and Keast1995; Olsson et al. 2004; Nilsson 2011). Distribution of neurotransmitters in the gut neurons and fibres is shown in Table 3.

**Calbindin and 5HT.** 5HT and calbindin immunoreactivity was detected in nerve fibres in the muscle coat of the oesophagus. 5HT-positive neurons lie in the myenteric plexus. Varicose nerve fibres co-expressing calbindin and 5HT innervate the circular muscle, and 5HT-immunopositive neurons also lie between the muscle cells. Oesophageal glands are present in the submucosa between the circular muscle and the muscularis mucosae. Both calbindin- and 5HT-positive nerves were observed in close vicinity of the oesophageal glands cells, indicating innervation. We observed no submucosal populations with significant colocalization for 5HT and calbindin (Fig. 3).

Bundles of 5HT-immunoreactive nerve fibres were observed in the muscle layers of the stomach. The myenteric plexus of the stomach contained multipolar neurons with calbindin immunoreactivity. Calbindin-immunopositive nerves were observed in the circular muscle, some co-expressing 5HT. 5HT-positive neurons and nerves were present also among the gastric glands of the submucosa (Fig. 2A). In the stomach, a significant level of colocalization of calbindin and 5HT was observed only in populations within the myenteric plexus of the pyloric caeca (Fig. 3).

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| Table 3 Neurotransmitter distribution in the gut of the spotted gar, Lepisosteus oculatus |
| ----------------- | ----------------- | ------------ | ------------- | ------------ | ------------ | ------------ |
|                  | Oesophagus        | Stomach       | Endocrines   | Mid intestine | Distal intestine | Caudal intestine |
| Calbindin        | nc, nf            | nf            | +            | nc, nf       | nc, nf        |               |
| 5HT              | nc                | nc, nf        | +            | nc, nf       | nf            |               |
| ChAT             | nf                | nc, nf        | +            | nc, nf       |               | nc           |
| TH               | nf                | nf            |              | nc, nf       | nf            | nf           |
| nNOS             | nf                | nc            |              | nf           |               |               |

nc, Nerve cell bodies including ones gathered in ganglia in submucosal layers and myenteric plexa; nf, nerve fibres in myenteric plexa, longitudinal and circular muscle, submucosa and gland tissues; +, calbindin and 5HT, immunoreactivity in the mucosal endocrine cells (oesophagus, stomach, pyloric caeca, middle intestine and distal intestine).
calbindin-reactive nerve cell bodies were present in several submucosal ganglia in the duodenum (Fig. 4B); however, we did not observe significant colocalization (Fig. 3). Numerous endocrine cells of the open type co-expressing both calbindin and 5HT were observed reaching the epithelial surface in the mucosa. 5HT-positive nerves were observed coursing through the longitudinal muscle of the mid-intestine. Calbindin-positive nerve bundles were found in the circular muscle. Myenteric ganglia with 5HT-positive nerve cell bodies were also observed (Fig. 4C). Submucosal ganglia were comprised of 5HT-positive nerve cell bodies. Both submucosal and myenteric ganglia of the mid-intestine showed significant levels of colocalization for calbindin and 5HT (Fig. 3). Nerve varicosities with calbindin and 5HT immunoreactivity as well as 5HT nerve cell bodies were observed in close vicinity of the tubulo-alveolar gland cells.

In the myenteric plexus of the rectum, numerous nerve cell bodies and ganglia with 5HT immunoreactivity were observed. In the thicker circular muscle, numerous nerve bundles contained calbindin and 5HT immunoreactivity. Mucosal endocrine cells of closed and open morphology co-expressed both calbindin and 5HT (Fig. 4D).

**ChAT and nNOS.** Double immunolabelling with antibodies for ChAT and nNOS revealed that nNOS consistently labelled nerve varicosities in the submucosa. A subset of these enteric nerves were observed in close vicinity to mucous cells in the epithelium. These nerves were also seen in close proximity to tubular oesophageal glands and between gland cells (Fig. 4E). Nerve varicosities with nNOS immunoreactivity near the gland cells often colocalized with ChAT antibodies (Fig. 4E). Rarely were nerve bundles observed with ChAT immunoreactivity; however, ChAT immunoreactivity was observed in many other structures including nerve cell bodies in the small ganglia near the glands, and in some spindle cells in the mucosa, presumably endocrine cells.

ChAT-immunopositive nerve fibres were observed running in the muscle layers of the oesophagus with some fibres running in the myenteric plexus where nNOS-positive nerves were also observed. Thick, completely circular muscle fibres of the inner muscle coat were primarily innervated by nNOS-positive nerve bundles.

ChAT-immunoreactive neurons and nNOS-positive nerves were observed in the myenteric plexus of the cardiac stomach. nNOS-positive nerve fibres were observed running in the circular muscle. A subset of ChAT-positive cells with closely associated nNOS-positive nerves were also present in the intramuscular layers. These cells are structurally different from those recently described in the muscular coats of the intestine in zebrafish (Ball et al. 2012) or comprising networks of cells scattered throughout the myenteric plexa and circular muscle layers in mammals (Gibbins 2012). The ChAT immunoreactivity of nerve cell bodies in the

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**Fig. 3**—Results of nerve cell bodies colocalization analysis in FIJI for *Clarias batrachus*. Bar heights and whiskers represent sample means (for \( n > 1 \)) and standard deviation, respectively.
Fig. 4—Colocalization preparations with antibodies against calbindin and 5HT and ChAT and nNOS in the gastrointestinal tract of the Indian catfish, Clarias batrachus. Calbindin-5HT. — A. 5HT-positive nerve cell clusters (arrows) in submucosal localization close to gastric gland (G). This image shows a close association of calbindin-positive nerve fibres (arrows) with the gland cells. — B. Labelling with antibodies against 5HT and calbindin shows the occurrence of nerve cells (arrows) that are immunoreactive to 5HT and gathered in submucosal ganglia in the duodenum. — C. 5HT-positive nerve cell bodies in a ganglion (G) in the myenteric plexus in the mid-intestine. LM longitudinal muscle. — D. Calbindin-5HT-positive enteroendocrine cells (EC) in the basal epithelium of the rectum.

ChAT-nNOS. — E. A network of nNOS-positive nerve fibres (NF) surrounding the oesopharyngeal glands (G). — F. Large nNOS-positive nerve varicosities (arrows) are scattered throughout the glands of cardiac stomach (asterisks). ChAT-nNOS-positive nerve cell bodies are present in close vicinity to the gland cells (arrows) — G. ChAT-positive enteroendocrine cells (EC) in the pyloric stomach. They extend the full width of the gastric epithelium. — H. Numerous ChAT-positive nerve cell bodies (arrows) are distributed in the submucosa of the pyloric stomach few of which showing overlay of ChAT and nNOS. They are seen in loosely arranged clusters and along nNOS-positive large nerve bundles (arrow). — I. Clusters of ChAT-positive neurons in the myenteric plexus of mid-intestine. nNOS nerve fibres (arrows) are seen in the myenteric plexus. — J. Loosely arranged ChAT-nNOS nerve cell bodies (arrows) in the submucous layer of mid-intestine. A network of nNOS-positive nerve fibres (arrows) also reveals a concentric organization of nerve fibres surrounding the mucous glands (G). Scale bars: 20 μm in A,B,D,F,I; 50 μm in C,E,G,H,J.
submucosal ganglia of the cardiac stomach colocalized with nNOS immunoreactivity (Figs. 4F and 3).

A close association of extensive bundles of nNOS-positive nerve fibres was present in close vicinity of the gastric-gland cells. These fibres gave rise to nerve endings that surrounded the gastric glands (Fig. 4F). In the pyloric stomach, extensive nerve bundles with nNOS immunoreactivity were observed in the muscularis mucosa and around the blood vessels. These nerves were also observed in close contact or embracing several ganglia (Fig. 4H). These nerve cells in the ganglia demonstrated ChAT immunoreactivity or colocalization for both enzymes (Figs. 4H and 3).

Two types of ChAT-positive endocrine cells residing in the pyloric mucosa, one projecting as a fairly long cytoplasmic processes in both luminal and basal directions and another occurring on the surface of the epithelium (Fig. 4G). This suggests a correspondence between cell types and hormones contained in them. ChAT neurons in the submucosal ganglia of the pyloric stomach should be motor neurons supplying these types of endocrine cells (Furness 2000).

In the submucosa of mid-intestine, the tubulo-alveolar mucous glands received a rich supply of nNOS-positive nerve fibres. In the enteric plexus of the duodenum and distal intestine, ChAT-positive neurons were observed in nNOS-immunoreactive nerve fibres (Fig. 4I). nNOS-immunoreactive nerve bundles were observed mainly in the inner part of circular muscle.

In merged images, most of the immunoreactive nerve cell bodies in the submucosal ganglia in the duodenum, middle and distal intestine were ChAT positive. Colocalization for ChAT and nNOS was observed only in few nerve cells (Figs. 4J and 3).

**TH and nNOS.** We observed numerous nerve bundles in the myenteric plexus of the oesophagus with nNOS immunoreactivity, some surrounding blood vessels. A large majority of glands in the oesophagus and stomach were innervated by nNOS-positive nerve varicosities; however, a subset of nerves show a different chemical encoding consisting of adrenergic, TH-positive nerve fibres that were in close proximity to gland cells or that formed varicosities near the gland units (Fig. 5A). These findings are in agreement with patterns of gastrointestinal innervation reported by Nilsson (2011).

Adrenergic-nerve fibres were also observed running in the longitudinal muscle of the stomach, while many nNOS-positive nerve fibres were identified in the myenteric plexus. In the duodenum, the myenteric plexus contained few nNOS-positive neurons. Some clustered neurons were also distributed throughout the plexus, along with nNOS- and TH-positive nerve bundles that are both TH and nNOS-immunoreactive (Fig. 5B); however, colocalization analysis revealed only weak colocalization (Fig. 3).

In the duodenum, middle and caudal intestine, numerous neurons were present in the submucosa and gathered in loosely arranged clusters. TH consistently labelled the nerve cell bodies; however, some cells colocalized with nNOS (Figs 5C,D and 3).

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**Fig. 5**—Colocalization preparations with antibodies against TH and nNOS in the gastrointestinal tract of the Indian catfish, *Clarias batrachus*. — **A.** Oxyntic gland (G) region of the stomach is strong innervated by TH-positive nerve varicosities (arrows) surrounding the oxyntic cells (arrows). In upper image are seen TH and nNOS varicosities that are interspersed among the gland cells. — **B.** Clusters of TH-nNOS-positive nerve cells (arrows) in the myenteric plexus of the duodenum. — **C.** Numerous TH-positive clusters of nerve cells gathered in ganglia (G) in the submucosa of the crypts (C) of the duodenum. — **D.** TH-nNOS-positive nerve cells (arrows) arranged in compact ganglia (G) in the submucosa of caudal intestine. BV, blood vessel. Scale bars: 20 μm in A,B,D; 50 μm in C.
peptides have been cytochemically identified in enteric neurons; however, various amines and various neurotransmitters, including 5HT, enkephalins, substance P, somatostatin, VIP, NPY and galanin (see Fujita et al. 1988). Costa et al. (1996) presented the most thorough description of enteric chemical coding to date in their study of the guinea pig ileum. Costa et al. (1996) described at least 14 distinct classes of enteric neurons. In general, the excitatory neurons of most vertebrates, including actinopterygian fishes, contain tachykinins and acetylcholine, while inhibitory motor neurons contain VIP/PACAP and nitric oxide (Olsson 2009, 2011). In the present work, a dense innervation of the muscle by 5HT-immunoreactive nerve fibres was observed. This contrasts with mammals in which 5HT nerve terminals supply enteric ganglia, but not the muscle. 5HT causes contraction of the muscle (Olsson and Holmgren 2010). The results suggest that excitatory muscle motor neurons in fish utilize both ACh and 5HT as transmitters.

Most of the work on the fish enteric nervous systems has focused on teleosts species (Olsson 2011, 2011). These studies revealed that teleost enteric systems contain neuronal populations comprised of calbindin-positive nerve cell bodies and other subpopulations that express 5HT and ChAT. Comparative studies of both the structure of the ANS and autonomic control in fishes are scant with the majority focusing on teleosts and none devoted to preteleost actinopterygians (for a review, see Olsson and Holmgren 2010).

The present study is the first to provide comprehensive information of the chemical coding of the nerves innervating secretory glands (oesophageal, gastric glands and mucous glands) and compact ganglia in the gut of fishes. In the two species studied, the ENS contains a diversity of neuron types, including motor neurons supplying muscles and interneurons. Although previous immunohistochemistry studies have demonstrated the existence of diverse classes of neurons in one region of the gut, pharmacological and physiological data have been lacking, information essential in elucidating chemical coding and determining function. Ours is the first study to report a multitude of neurotransmitters in myenteric neurons, including cell bodies in the ganglionated plexuses of the duodenum in Clarias, and the caudal intestine and the myenteric compact ganglia in the spiral valve intestine of the gar species.

According to Furness’ (2000, 2012) classification, three types of extrinsic motor neurons innervate the gut: vagal motor neurons that innervate the striated muscle of the oesophagus, noradrenergic (sympathetic) neurons that innervate enteric neurons and the muscle of sphincter regions, and noradrenergic vasoconstrictor neurons that innervate arteries within the gut wall. A more nuanced assessment reveals that extrinsic innervation of the fish gut is mainly vagal in the proximal part (i.e. stomach and proximal intestine), while the distal intestine and rectum are innervated by spinal nerves (Burnstock 1959; Olsson 2009; Nilsson 2011). However, the exact role of the extrinsic innervation has received little attention (Olsson 2009). Nilsson (1983) proposed a modified terminology, one based upon Langley, where the parasympathetic pathways running in the cranial nerves are described as

### Table 4: Neurotransmitter distribution in the gut of the air-breathing fish, Clarias batrachus

<table>
<thead>
<tr>
<th>Transmitter</th>
<th>Oesophagus</th>
<th>Stomach</th>
<th>Endocrines</th>
<th>Mid-intestine</th>
<th>Rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin</td>
<td>nf</td>
<td>nc,nf</td>
<td>+</td>
<td>nc,nf</td>
<td>nc,nf</td>
</tr>
<tr>
<td>5HT</td>
<td>nc,nf</td>
<td>nc,nf</td>
<td>+</td>
<td>nc,nf</td>
<td>nc,nf</td>
</tr>
<tr>
<td>ChAT</td>
<td>nc,nf</td>
<td>nc,nf</td>
<td>+</td>
<td>nc, nf</td>
<td>nc</td>
</tr>
<tr>
<td>TH</td>
<td>nf</td>
<td>nf</td>
<td></td>
<td>nf</td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>nf</td>
<td>nc,nf</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

nc, Nerve cell bodies including ones gathered in ganglia, submucosal layers, myenteric plexa and also in muscle cells; nf, nerve fibres in myenteric plexa, longitudinal and circular muscle, submucosal layers and gland tissues; +, calbindin, 5HT and ChAT immunoreactivities in the mucosal endocrine cells (Esophagus, stomach, pyloric stomach and middle intestine).

Neurotransmitters found in the enteric neurons and nerves are reported in Table 4.

### Discussion

**Enteric nervous system’s general structure and function**

The enteric nervous system (i.e. intramural nervous systems) is located in the wall of the whole of the digestive tract, from the oesophagus to anus in vertebrates (Furness and Costa 1987). It comprises two main assemblies of neurons and their fibres as well as the extensive projections of neurons to the lamina propria mucosae and submucous and muscular layers (Furness et al. 1999). The complexity of the enteric nervous system reflects not only the large number of neurons involved, but also the variety of neuronal cell types according to their characteristic shape, ultrastructure and chemical coding (Furness 2000).

Substantial advances in the knowledge of neural circuits that control intestinal motility, secretion and blood flow have been made in the last two decades (Furness 2012). Functional, pharmacological, neurochemical, morphological and immunohistochemical methods have provided important tools to unravel the neurophysiological role and function of enteric circuits (Furness and Costa 1987; Furness et al., 1988, 2000; Furness et al., 1989). In functional terms, there are five broad types, and many subtypes, of enteric motor neurons: (i) excitatory and (ii) inhibitory neurons controlling gut muscles, (iii) secretory, vasodilatory neurons, (iv) secretomotor neurons that are not vasodilatory and (v) neurons that innervate the enteroenocrine cells (Furness 2000; Furness et al. 2000).

**Immunohistochemistry**

Recent immunocytochemical analysis has greatly increased our knowledge of the chemical diversity of enteric neurons. Two neurotransmitters, acetylcholine and noradrenaline, are well known enteric transmitters; however, various amines and peptides have been cytochemically identified in enteric nerve-
cranial autonomic, and all sympathetic and sacral parasympathetic pathways as spinal autonomic.

In teleosts, the sympathetic chains continue anteriorly into the head, with sympathetic ganglia contacting several of the cranial nerves (Nilsson 2011). The anterior splanchnic (i.e. spinal) nerve is composed of postganglionic neurons and runs from the caeliac ganglion to various viscera (Nilsson 2011). In Clarias, fine branches of TH-positive nerve fibres enter the oesophageal wall and contribute directly to the subepithelial plexus in the antrum and cardiac and pyloric stomach. A population of TH-positive nerve fibres surrounds the glandular cells, and fine varicose nNOS-positive nerve fibres are fairly prominent in the subepithelial plexus. Karila et al. (1997) described adrenergic cells containing NOS in the caeliac ganglion projecting to the stomach in Gadus. In the stomach of Lepisosteus, 5HT fibres emanate from the subepithelial plexus and supply the oesophageal glands, while calbindin fibre varicosities surround the gastric cells in both Lepisosteus and the Clarias. In the Lepisosteus, fine fibres of TH-positive fibres down through the longitudinal coat to join the myenteric plexus in the oesophagus, stomach, pyloric caeca and spiral valve intestine. In addition, we observed that nerve cell bodies in the compact ganglia of the myenteric plexus of the distal intestine in Lepisosteus show TH immunoreactivity. Mucous cells either covering the oesophageal epithelium or present in the mucous glands of the mid-intestine in the catfish (Srivastava 2005) are innervated by nNOS-positive nerve fibres. It appears that autonomic pathways are involved in the control of most secretory cells and glands in both of our study species. Spinal adrenergic pathways may have a direct effect on the control of secretion of gastric acid, especially in the Clarias; however, no studies have described spinal adrenergic control of acidic secretory structures in the enteric system. Vagal parasympathetic control of acid secretion may be mediated by other neurotransmitters such as NO and 5HT, whereas in mammals, the control is cholinergic (Schubert and Peura 2008). This is supported by our observations in Clarias that these substances are expressed by nerves abutting gastric-gland cells and because nNOS-immunopositive nerve fibres directly innervate the mucous cells in the oesophageal epithelium and mid-intestine. In addition, the presence of 5HT endocrine cells in the tubular gastric glands of Lepisosteus demonstrate that 5HT may also act indirectly by a paracrine pathway in the control of the oxytococeptive cells via release from endocrine cells. The hormonal mechanisms, however, are as important in the control of most glands (Holmgren and Olsson 2011) and, in this case, should not be ignored.

The abundant secretion of mucus by the mucous glands in the catfish mid-intestine perhaps forms a barrier against bacteria, virus and toxins, and together with bicarbonate, may prevent acid and lytic enzymes in the fish gut lumen from destroying the underlying mucosal epithelium (Holmgren and Olsson 2011). The extensive nitricergic innervation of the mucous glands suggest that control of mucous secretion is under the vagal influence. However, a cholinergic innervation is absent.

Only a few studies with a limited taxonomic scope have investigated the importance of extrinsic innervation in actinopterygian fishes (e.g. Funakoshi and Nakano 2007; Olsson 2009; Nilsson 2011). Previous studies over the last decade using retrograde techniques have identified sympathetic preganglionic cell columns in teleosts (Funakoshi and Nakano 2007). The majority of sympathetic postganglionic neurons in the sympathetic ganglia in teleosts are thought to be adrenergic with targets other than the stomach (Karila et al. 1995). The TH-positive nerves supplying the longitudinal muscle of the stomach and supplying the gastric glands in the Clarias are of spinal origin (Burnstock 1959; Nilsson 1983; Olsson 2009). The contribution of the sympathetic system to the innervation of the mid- and caudal intestine in Clarias and spiral valve intestine in Lepisosteus appears to also be important as suggested by the TH-positive innervation of the longitudinal muscle. However, retrograde studies are needed to identify both the preganglionic (spinal) as well as postganglionic (sympathetic) fibres that are involved in the neural control of the caudal intestine of our study species.

Another open question concerning the enteric nervous system of Clarias and Lepisosteus is the types of neurons that populate the myenteric plexuses, ganglia and submucosa. Using confocal microscopy and double-label experiments, we have been able to characterize the shape and distribution of neurotransmitters within the gut of these two taxa. To our knowledge, ours is the first study to report the presence of intrinsic neurons that aggregate to form ganglia and submucosal neurons and the occurrence of neurotransmitter substances and enzymes (such as TH) in these structures. Olsson (2009) contended that the role and function of specific nerve cells in the enteric systems of fishes is poorly understood, especially when this level of knowledge is compared with what is known about mammalian systems. Functional pharmacological experiments will be essential in further characterizing enteric multipolar neurons and microganglia in the myenteric plexuses and muscle layers in Clarias. For instance, we suggest that future experiments should focus on acetylcholine, adrenaline and NO to better characterize the nNOS-positive ganglionic plexuses lying between the stratum compactum and the circular muscle coat in the duodenum. In addition, the neurochemical and morphological properties of the specific cell bodies remain unclear, especially as compared with the autonomic neurons of mammalian systems that use these substances as the primary transmitter (Sang and Young 1998; Timmermans et al. 1997).

In the intestine of our study species, there were a distinct population of spindleshaped, unipolar neurons that were ChAT-5HT- and nNOS-positive. In addition, we observed in Clarias ChAT- and nNOS-positive multipolar neurons with smooth soma lying in the myenteric ganglia of the stomach and duodenum. In Lepisosteus, we observed very small
ChAT- and 5HT-positive neurons in the myenteric ganglia of the distal intestine. Most of the small submucosal neurons in the duodenum and caudal intestine (i.e. the rectum) in *Clarias* contained ChAT and, to a lesser extent, TH. The TH-nNOS myenteric neurons of the duodenum in *Clarias* are very similar in morphology to the types of neurons reported by Furness (2000) in mammalian intestine.

Based on these initial data, the chemical coding of actinopterygian enteric nervous systems may be similar in many respects to that in mammals. However, in the absence of additional data, it is impossible to establish whether both sensory and motor neurons were present in the enteric systems of stem osteichthyans, the ancestors of actinopterygian and sarcopterygian fishes. How deeply within the vertebrate tree of life enteric sensory neurons evolved is a key piece of information if we are to establish when precise control, not only of digestive function itself, but also of water and electrolyte balance, evolved in vertebrates. Although the microanatomy of the enteric plexuses in the various groups of bony fishes is similar to that in amphibians (Gibbins 1994), nerve cell bodies in both *Lepisosteus* and the *Clarias* form more organized ganglia in the myenteric plexus and in the submucosa. Submucosal ganglia in the oesophagus and stomach were not previously reported in the ENS actinopterygian fishes and are very rare or absent in mammals. We suggest that future studies should seek to classify submucosal neurons as cholinergic, secretomotor and vasodilator in function using ACh as the primary neurotransmitter (Furness 2000). It appears that the enteric nervous activity in these fishes is also influenced by sympathetic innervation to the hindgut. These observations suggest that the level of organization of neurons within the enteric systems of the Actinopterygii is much more complex than previously proposed.

**Endocrine cells**

Various types of endocrine cells, identifiable by their distribution, shape and chemical coding, were observed in the gut of our studies species. In *Lepisosteus*, two types of endocrine cells of both closed and open morphology were present in the gut and dispersed in the gastroenteric mucosa. In these cells, calbindin and 5HT colocalized except for a cell population that expressed only calbindin in the spiral valve intestine and caudal intestine. These two types of cells are also found in the duodenal mucosa of *Clarias*; however, in the pyloric stomach mucosa of this species, two main open types of ChAT-positive cells were also recognizable; both had a peculiar tuft of microvilli at their apical end. Gut endocrine cells secrete hormones that are involved in the paracrine regulation of muscular movement, secretion by intestinal and associated glands and microcirculation in local tissues (Fujita et al. 1988; Rehfeld 2004). The gut endocrine cells or paraneurons are regarded as the primary sensory cells for the detection of food chemical information. The significance of both closed and open type in these processes, as well as the function of their neurotransmitter contents, is unknown. While 5HT-positive cells are commonly found in endocrine structures that are present in the gastrointestinal tract of mammalian and submammalian vertebrates, ChAT-positive cells in the pyloric stomach are not and their presence in our study represent a novel discovery in the fish gut mucosa. According to Vigna (1985), *in situ* hormones can be expressed by de novo endocrine cells in a new tissue or organ, resulting in regulation of new physiological function. Thus, the primary control over gastric acid secretion may be exerted by a regulatory substance. This is perhaps the case with ChAT in the stomach of *Clarias* and the presence of 5HT in the endocrine cells of the gastric epithelium of *Lepisosteus*. This encoding model in which transmitters alter expressions of gut hormones may represent the evolutionary pathway for digestive mechanisms in vertebrates.

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