

Differentiations of 5-HT and GAS cells in the digestive canals of *Rana chensinensis* tadpoles

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Abstract: In the current study, 5-hydroxytryptamine (5-HT) and gastrin (GAS) cells in the digestive canals of *Rana chensinensis* tadpoles at different developmental stages were investigated by immunohistochemistry. Results showed that the 5-HT cells were only detected in the duodenum before metamorphosis began, and were extensively distributed in the stomach, duodenum, small intestine, and rectum thereafter, with the highest counts found in the duodenum and rectum when metamorphosis was completed. The GAS cells were only distributed in the stomach and duodenum, and only rarely detected in the duodenum before metamorphosis began, but increased in the stomach during metamorphosis and showed zonal distribution in the gastric mucosa when metamorphosis was completed. Metamorphosis is a critical period for amphibians, during which structural and functional physiological adaptations are required to transition from aquatic to terrestrial environments. During metamorphosis, the differentiations of 5-HT cells in the gastrointestinal canals of tadpoles could facilitate mucus secretion regulation, improve digestive canal lubrication, and help water-shortage food digestion in terrestrial environments. Conversely, GAS cell differentiations during metamorphosis might contribute to the digestive and absorptive function transition from herbivore to omnivore.

Keywords: *Rana chensinensis* tadpole; Digestive canal; 5-hydroxytryptamine (5-HT) cell; Gastrin (GAS) cell; Ontogeny

The digestive canal is not only involved in digestion and absorption functions, but is the largest endocrine organ in the animal body. The digestive canal is comprised of various endocrine cells that play important roles in digestion, absorption and many other physiological activities. Species-specific cell types and local distributions in different areas of the digestive canal are found in different animals (Solcia et al, 2000; Rehfeld, 2004).

In general, the argentaffin or argyrophilia gastrointestinal endocrine cells, such as 5-hydroxytryptamine (5-HT) cells and gastrin (GAS) cells, are individually spread among epithelial cells. As a monoamine neuron transmitter, 5-HT can induce contractions in smooth muscles by acting on cholinergic excitatory neurons and thus adjust gastrointestinal motility and secretory function (Grundy, 2008; French et al, 2014). GAS stimulates secretions of gastric acid and pepsin, promotes growth of gastrointestinal mucosa, and facilitates gastrointestinal development (Baldwin et al, 2010; Duritis et al, 2013).

The distribution of endocrine cells in digestive canals is closely correlated with the habitats and food choices of animals, e.g. water content in food and external environments (Reinecke et al, 1999). In amphibians and reptiles, the distribution of 5-HT is affected by feeding habits (Gibson et al, 1976). Most amphibians experience metamorphosis, during which animals undergo habitat (aquatic to terrestrial) and feeding habit (herbivorous to carnivorous) transitions. However, studies on the differentiations of 5-HT and GAS cells in the digestive canals of tadpoles during metamorphosis are rare. The morphological and structural changes in the digestive canals of tadpoles, feeding habit adaptations, as well as the differentiations and roles of 5-HT and GAS cells during postembryonic development need to be

Received: 12 November 2013; Accepted: 20 January 2014

Foundation items: This study was supported by the Natural Science Foundation of Shaanxi Province (SJ08C101) and the Fundamental Research Funds for the Central Universities (GK200902039).

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elucidated.

Earlier studies found that the digestive canal wall in *Rana chensinensis* tadpoles thickened during metamorphosis as a likely adaptation to changes in their feeding habits (Li & Zhang, 2008). In the present study, immunohistochemistry was applied to detect the differentiations and distribution patterns, as well as roles in metamorphosis and adaptation, of 5-HT and GAS cells in the digestive canals of tadpoles.

MATERIALS AND METHODS

Experimental animals

Rana chensinensis zygotes were collected from water bodies surrounding Dayu Reserve, Changan district, Xi'an, Shaanxi Province, China, and were hatched in the laboratory setting. The stomach, duodenum, intestine (jejunum and ileum) and rectum were sampled from the tadpoles at G28, G35, G36, G38, G39, G41, G42, G45 and G46 stages (Gosner, 1960) and were then fixed in improved Bouin's solution. Regular paraffin sections (6 μm) were prepared for immunohistochemistry examination.

Experimental methods

StreptAvidin-Biotin complex (SABC) was used for immunohistochemistry staining. After regular dewaxing and rehydration, paraffin sections were incubated in 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. Sections were washed three times for 5 min each with distilled water. Sections were then soaked in 0.01 M citrate buffer (pH 6.0), and were heated to boiling point (above 95 $^\circ\text{C}$) with a microwave oven. This heating process was repeated twice with a 10-min interval. Sections were washed twice for 5 min each with PBS buffer. After adding normal goat serum (blocking solution), the sections were incubated at room temperature for 20 min. After adding properly diluted primary antibodies of 5-HT and GAS (polyclonal, rabbit-anti-human, 1:100, Boster), sections were incubated at 4 $^\circ\text{C}$ overnight. Sections were washed three times for 2 min each with PBS buffer. After adding properly diluted biotinylated secondary goat-anti-rabbit antibody, sections were incubated at 37 $^\circ\text{C}$ for 30 min and were then washed three times for 2 min each with PBS. SABC (Boster) was added to the sections, which were then incubated at 20–37 $^\circ\text{C}$ for 30 min and then were washed four times for 5 min each with PBS. Sections were stained with DAB (Boster) chromogenic reagent and were incubated at room temperature for 5–30 min and were then washed with distilled water. The

sections were then dried by baking, were put on a drop of mounting medium and were sealed with a cover slide for observation under a microscope. Negative control paraffin sections were treated with PBS instead of primary antibody.

Data collection and statistical analysis

Five inconsecutive sections were selected for each part of the tadpole digestive canal at the G28, G36, G39, G42 and G46 stages, and each of the sections were chosen from five serial sections, respectively. Under a microscope (Olympus) ($\times 400$), four fields of vision were picked randomly and the immunopositive cells within were counted and the relative cell densities were obtained ($n=20$). The densities of the immunopositive cells (mean \pm SD) in a specific part of the digestive canal at different developmental stages were analyzed by one-way ANOVA via SPSS13.0. Differences among various groups were determined by Duncan test. $P<0.05$ and $P<0.01$ was set as statistical differences and significant statistical differences, respectively.

RESULTS

5-HT cells

No immunopositive 5-HT cells were found in the stomach walls of tadpoles at G28 to G38 stages, but they were detected in the gastric intrinsic membranes at G39 (Figure 1A), the gastric mucosa at G42 (Figure 1B) and the gastric gland at G46 (Figure 1C). The 5-HT cells with weak immunoreactions were extensively distributed in the duodenum (Figure 1D). At G36, the 5-HT cells were bottle-shaped (Figure 1E), with their cellular processes pointing toward the intestinal cavity. Remarkable differentiations were observed in cell locations, which were interspersed among the mucous epithelium. The 5-HT cells at G42 were round (Figure 1F). At G46, the immunopositive 5-HT cell counts in the enteric epithelium were increased and interspersed among the intestinal villus epithelium (Figure 1G).

In the intestine, the immunopositive 5-HT cells were detected from G39 and were found in the crypts (Figure 1H) at G42, and the mucous epithelium and intestinal lamina propria at G46 (Figure 1H, I). In the rectum, 5-HT cells were not detected from G28 to G41 (Figure 1J), but were randomly observed in the epithelium at G42 (Figure 1K). These immunopositive 5-HT cells were bottle-shaped and distributed along the basal area of the epithelium with their cellular processes

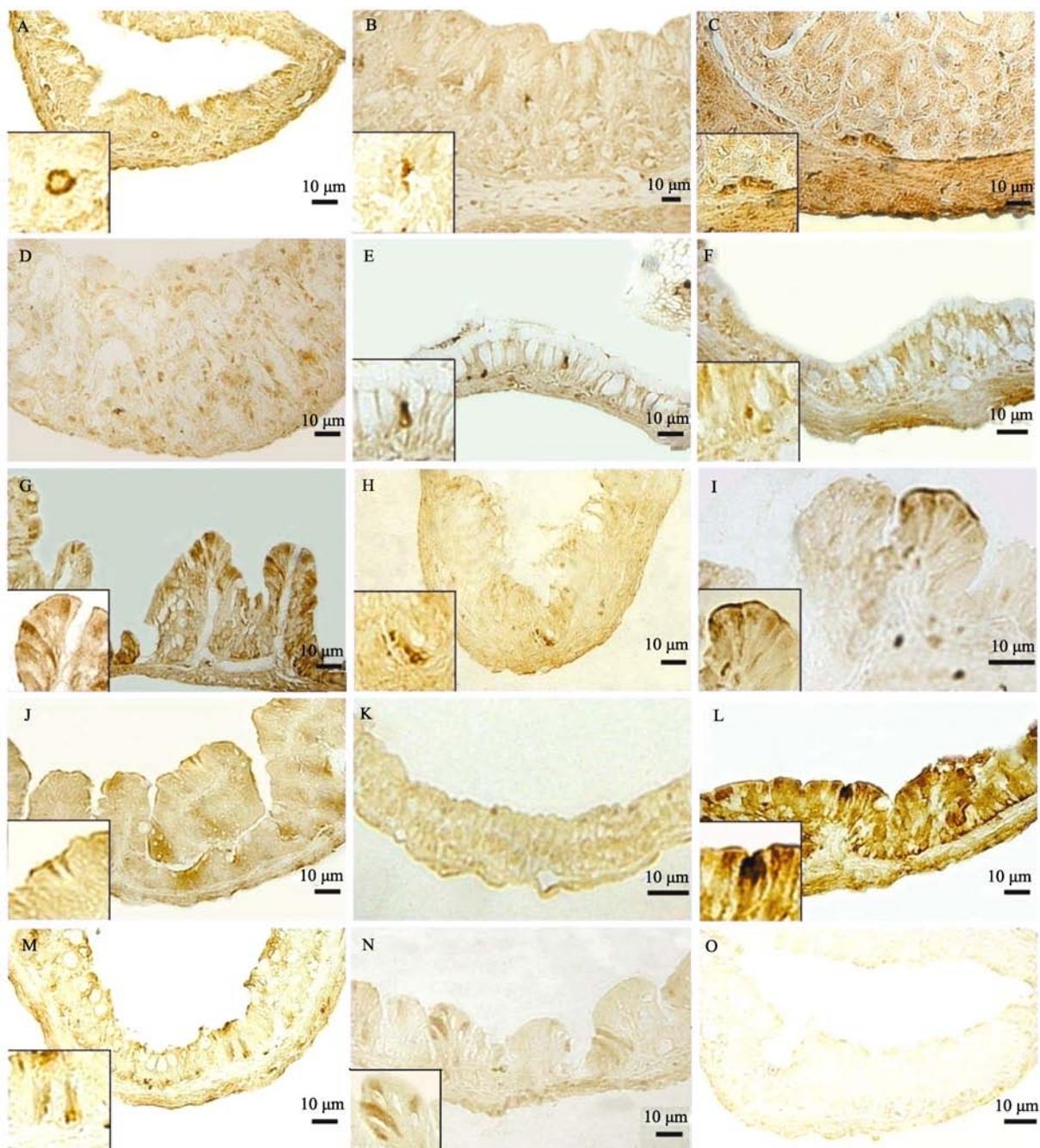


Figure 1 5-HT cells in the digestive tract of *R. chensinensis* tadpoles

A–C: 5-HT cells in stomach walls (A: G39; B: G42; C: G46); D–G: 5-HT cells in duodenum (D: G28; E: G36; F: G42; G: G46); H–J: 5-HT cells in small intestine (H: G42; I: G46; J: G46); K–N: 5-HT cells in rectum (K: G28; L: G41; M: G42; N: G46); O: negative control. The bottom left corners are enlargements of positive cells ($\times 400$).

pointing toward the intestinal cavity (Figure 1L). At G46, these 5-HT cells were mainly located in the mucous epithelium folds (Figure 1M).

Cell densities were analyzed by quantifying the immunopositive 5-HT cell counts in tadpoles at different

developmental stages (Table 1). In the stomach walls, immunopositive 5-HT cells were detectable from G39, and density significantly increased at G42 ($P < 0.05$) but not at G46 ($P > 0.05$). In the duodenum and intestine, no significant differences in the immunopositive 5-HT cell

densities were found among G36, G39 and G42 ($P>0.05$), but a dramatic increase was observed in the duodenum at G46 ($P<0.01$). No significant differences in immunopositive 5-HT cell densities were found in the rectum at G39 and G42 ($P>0.05$), though a dramatic increase was observed at G46 ($P<0.01$).

GAS cells

Individual immunopositive GAS cells near the

Table 1 Densities of 5-HT cells in the digestive canal of *R. chensinensis* tadpoles at different developmental stages

Gosner stage	Stomach	Duodenum	Small intestine	Rectum
G28	0 ^a	0 ^a	0 ^a	0 ^a
G36	0 ^a	0.8±0.20 ^b	0 ^a	0 ^a
G39	0.6±0.22 ^a	1.1±0.23 ^b	0.7±0.21 ^b	0.4±0.16 ^{a,b}
G42	1.4±0.31 ^b	1.3±0.30 ^b	0.8±0.29 ^b	0.9±0.23 ^b
G46	1.5±0.27 ^b	4.2±0.39 ^c	1±0.21 ^b	3.6±0.37 ^c

Data were based on microscope (×400) observations. Different superscript letters in the same column represent significant differences.

cavosurface were detected in the stomach wall at G39, but not at G28 or G36 (Figure 2A). Immunopositive dark GAS cell groups were found in the lamina propria at G42 (Figure 2B), and were significantly increased in the gastric mucosa epithelium and submucous layer at G46 (Figure 2C, D).

In the duodenum, no immunopositive GAS cells were found at G28 and G36, but they were randomly observed in the epithelium at G38 (Figure 2E). Immunopositive GAS cells were interspersed in the epitheliums and lamina propria at G39 and G42 (Figure 2F, G), which increased at G46 (Figure 2H).

Cell densities were analyzed by quantifying the immunopositive GAS cell counts in tadpoles at different developmental stages (Table 2). In the stomach wall, no difference in the counts of immunopositive GAS cells were found between G39 and G42 ($P>0.05$), though a dramatic increasing was observed at G46 ($P<0.01$). In the duodenum, GAS densities exhibited no significant differences among G39, G42 and G46 ($P>0.05$).

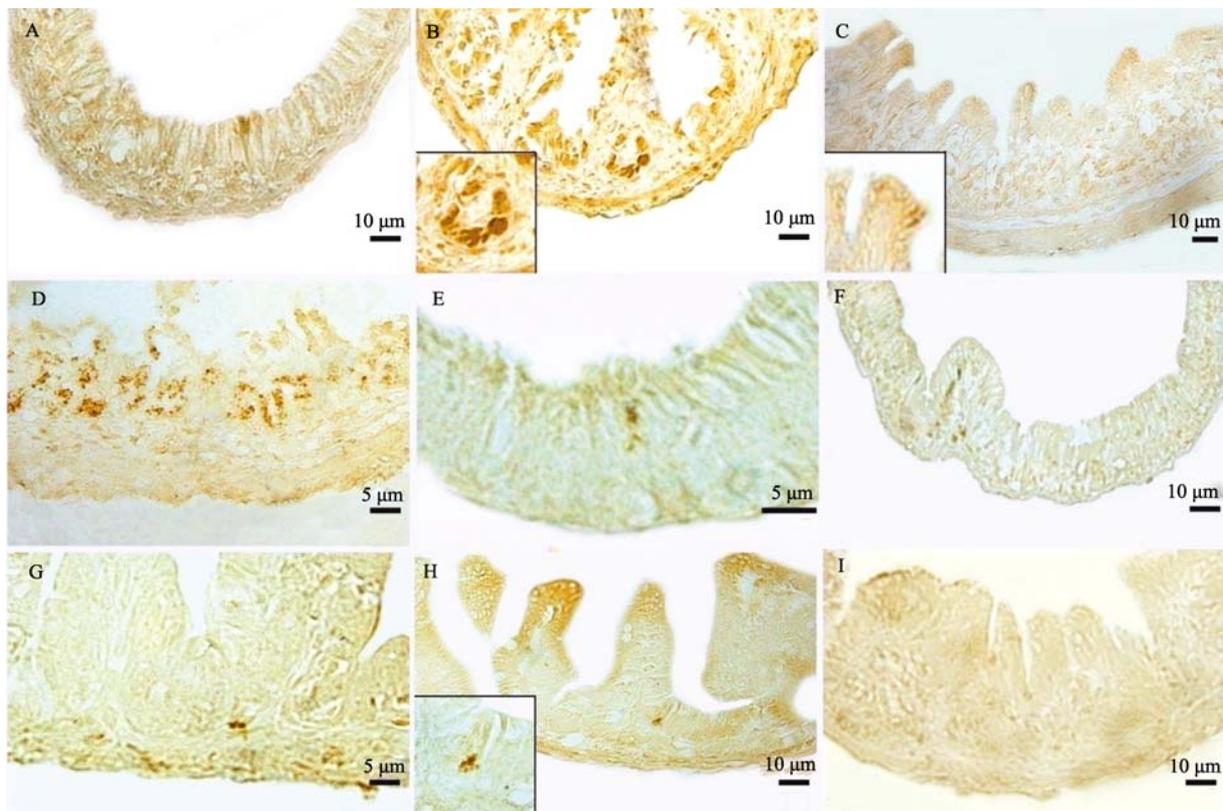


Figure 2 GAS cells in the stomach and duodenum of *Rana chensinensis* tadpoles

A–D: GAS cells in stomach walls (A: G39; B: G42; C: G45); E–H: GAS cells in the duodenum (E: G38; F: G39; G: G42; H: G46); I: negative control. Bottom left corners are enlargements of positive cells (×400).

Table 2 Densities of GAS cells in the stomach and duodenum of *R. chensinensis* tadpoles at different developmental stages

Gosner stage	Stomach	Duodenum
G28	0 ^a	0 ^a
G36	0 ^a	0 ^a
G39	0.4±0.16 ^b	0.5±0.17 ^b
G42	0.8±0.25 ^b	0.6±0.22 ^b
G46	3.7±0.40 ^c	0.8±0.20 ^b

Data were based on microscope (×400) observations. Different superscript letters in the same column represent significant differences.

DISCUSSION

Differentiations of 5-HT cells in the digestive canal of tadpoles and their adaptations to terrestrial environments

Studies found that differentiations in 5-HT cells began at an early stage of the evolution of vertebrate animals (El-salhy *et al.*, 1985). These cells are distributed along the digestive canal and inhibit gastric acid secretion, promote the autonomic and coordinated stomach wriggle, and adjust animal feeding and vasodilatation (Ormsbee & Fondacaro, 1985; Grundy, 2008; French *et al.*, 2014). In *Paralichthys olivaceus*, no 5-HT cells were found in 3- to 9-day-old larvae and only a scattering were found in 15-day-old larvae, whereas, a similar 5-HT cell distribution as found in adults was observed in 50-day-old larvae (Shi *et al.*, 2006). In *Xenopus laevis* adults, 5-HT cells were found to be distributed along the duodenum, intestine and rectum at relatively low densities (Zhen *et al.*, 2007). In *R. temporaria* tadpoles, immunopositive 5-HT cells were detected in the stomach and fore-intestine at G26-G35 (Villaro *et al.*, 2001). In this study, relatively few 5-HT cells were found in the stomach of *R. chensinensis* tadpoles at G36. Therefore, differentiations in 5-HT cells would appear to be species-specific.

Although the duodenum wall in *R. chensinensis* tadpoles has already differentiated into a four-layer structure at G38, the remaining intestine is characterized by slow pre-metamorphosis development and morphological changes during metamorphosis (Li & Zhang, 2008). In this study, detectable 5-HT cells were found earlier in the duodenum than in the intestine, and at the same developmental stages, the densities of 5-HT cells in the duodenum were also higher than those in the intestine. We propose that the high 5-HT cell densities could result in high levels of 5-HT cells in the duodenum of tadpoles,

and therefore is beneficial in adjusting stomach wriggle.

Zhang *et al.* (2005) found that the densities of 5-HT cells in the intestines of *Bufo melanostictus* and *R. nigromaculata* adults were significantly high, which was likely related to their habitats being far from water but their digestive canals needing hydration. The high counts of the 5-HT cells found in the intestine and rectum of *Takydromus amurensis* also support their role in water retention (Li, 2004). Fang & He (2006) reported that 5-HT cells in the fore-intestine of *Schizothorax davidi* functioned in digestion and 5-HT cells in the hind-intestine stimulated smooth muscle contractions and evacuation. In metamorphosed juvenile *R. chensinensis* (G46), the densities of 5-HT cells in the rectum increased, which aids adaptation to the terrestrial environment by increasing mucus secretion and promoting stomach wriggle. As an important neurotransmitter, the differentiations of 5-HT cells also play roles in improving digestive ability (Grundy, 2008; French *et al.*, 2014).

Differentiations of GAS cells in the digestive canal of tadpoles and their adaptations to feeding habits

Mammalian GAS cells are mainly distributed in the stomach and duodenum, and function to stimulate gastric acid secretion and promote growth and differentiation of the gastrointestinal mucous membrane (El-Salhy *et al.*, 1981; Wang & Shi, 1989; Ryberg *et al.*, 1990). Shi *et al.* (2006) found that in *P. olivaceus*, the GAS and 5-HT cells shared similar differentiation patterns. Fang & He (2006) found that GAS cells in *S. davidi* were distributed along the fore-intestine. In juvenile *R. temporaria*, the GAS cells in the digestive canal already exhibited the ultrastructure of mature cells, but GAS cell counts continued to increase in adults (Villaro *et al.*, 2001). The increased GAS cells from juveniles to adults are considered an adaptation to transit from herbivorous to carnivorous (Shi & He, 2006; Baldwin *et al.*, 2010; Durity *et al.*, 2013).

In *R. chensinensis* tadpoles, GAS cells in the stomach began to increase from G42 and especially at G46 (after metamorphosis). During metamorphosis, individuals transition from herbivorous to omnivorous, and then carnivorous. The GAS cells can promote the development of the gastrointestinal mucous membrane, and therefore can help an individual adapt to novel digestive functions. Although GAS cells were found in the duodenum in the present study, no significant fluctuation in quantity was observed during the development. No

GAS cells were detected in the intestine and the rectum.

During metamorphosis, *R. chensinensis* tadpoles must transition both morphologically and functionally to adapt to the change from aquatic to terrestrial environments. Increased 5-HT cells were found during the metamorphosis, which was likely because they

promote secretions of gastrointestinal mucus to lubricate and protect the digestive canal from low water food in terrestrial habitats. Whereas, increased GAS cells in the stomach wall of *R. chensinensis* tadpoles was correlated with feeding habit transition from herbivore to carnivore.

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