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RESEARCH ARTICLE

Anatomical localization of SER and certain peptides in the developing gastrointestinal tract of the axolotl *Ambystoma mexicanum*

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List of abbreviations

BMSU, Biomedical services unit; **BSA**, Bovine serum albumin; **CGRP,** Calcitonin gene-related peptide; **EC,** Endocrine cells; **FITC**, Fluorescein-isothiocyanate; **GAS/CCK** Gastrin/cholecystokinin; **GINCS** Gastrointestinal neuroendocrine control system; GIT, Gastrointestinal tract; **IHC**, Immunohistochemical; **IN,** Intestine; **IR**, Immunoreactive; **LIN,** Large intestine; **NF,** Nerve fibres; **NT,** Neurotensin; **PACAP**, Pituitary adenylate cyclase-activating polypeptide; **PBS**, Phosphate buffer saline; **SER,** Serotonin; **SIN,** Small intestine; **SP,** Substance P; **SS,** Somatostatin; **ST,** Stomach; **VIP** Vasoactive intestinal polypeptide.

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Abstract

The developing gastrointestinal tract (GIT) of the axolotl, *Ambystoma mexicanum*, was investigated, by the use of standard immunohistochemical techniques, for the localization of immunoreactivity to serotonin (SER) and five regulatory peptides all known to occur in the mammalian GIT. The actual utilized developmental stages started at stage 42 and ended with late juvenile stage. Microwave pre-treatment of the sections was employed to improve the detection of antigens. Entero-endocrine cells reacted specifically with antisera to SER, substance P (SP), somatostatin (SS), gastrin/cholecystokinin (GAS/CCK) and neurotensin (NT). Enteric nerve fibers, on the other hand, reacted specifically with antisera to SER, SP, NT and calcitonin gene-related peptide (CGRP) starting at the first juvenile stage. Based on its nature, the present study represents the second investigation which emerged from the same laboratory and principally yields two new findings. Firstly, neither SER- nor any of the investigated regulatory peptides-immunoreactivity was detected at stages earlier than the pre-hatching stage (42). Secondly, GAS/CCK-immunoreactivity was detected chronologically earlier (at stage 42) and this is consistent with the previously known trophic effect for both hormones during the early development of the GIT. The study provides a description of the qualitative distribution and a semi-quantitative account of the relative abundance of the mentioned neuroendocrine substances within the developing GIT.

INTRODUCTION

For survival, the developing gastrointestinal tract (GIT) with its neuroendocrine control system (GINCS) must be functional by the time of external feeding. The distribution and relative frequency of the gastrointestinal endocrine cells and nerve fibres, which constitute the GINCS, vary among different vertebrates but their general frame is quite similar (Holmgren 1989). These neuroendocrine elements characteristically secrete peptides which control different activities of the GIT and are thus known collectively as regulatory peptides. The latter are built of amino acids and show remarkable structural similarity among different vertebrate species with the biologically active part is usually the most conserved (Holmgren and Jensen 2001). As a consequence, it is believed that this similarity is retained by

natural selection in order to enable these peptides to maintain their vital functions (Gibbins 1989). Owing to its diverse activities, the GIT is the richest source of these biologically active peptides, outside the brain.

A relatively few reports have demonstrated the presence of individual regulatory peptides within the GIT of non-mammalian species (Maake et al. 2001; Pederzoli, et al. 2004; **Çinar et al. 2006**). Immunohistochemical (IHC) studies on the amphibian GIT to investigate its GINCS are also remarkably few. The first study was that of Buchan (1986) who carried out a general investigation utilizing seven different adult amphibian species. In his study, Buchan (1986) demonstrated the presence of immunoreactivity for gastrin, enteroglucagon, vasoactive intestinal polypeptide (VIP), neurotensin (NT), somatostatin (SS), motilin and substance P (SP) in the GIT of neotenic adult axolotl. Subsequently, Gabriel et al. (1992) concentrated on the myenteric plexus of the axolotl stomach and demonstrated the presence of immunoreactivity for some regulatory peptides and serotonin (SER). The other two latest studies, like the present investigation, emerged from the same laboratory (Maake et al. 1999, 2001). The study by Maake et al. (1999) was concerned with examining the IHC localization of some regulatory peptides and SER in the GIT of neotenic and artificially metamorphosed adult axolotl. The latter study revealed an increase in the immunoreactivity of the enteric nerve fibres for VIP, SER and SP after metamorphosis, which was attributed by the authors to the possible post-metamorphic refinement of the GINCS. The latest study of Maake et al. (2001) has dealt with the ontogenetic appearance of the same set of neuroendocrine substances employed in their study of (1999).

Because of the large structural and functional changes which occur during development, it is anticipated that the GINCS is in a state of continuous change to meet the altered ontogenetic demands (Maake et al. 1999). These developmental changes make the study of hormones of particular interest and challenge because the endocrinology of the animals under investigation is continually changing. Therefore, certain endocrine cell types may appear in a particular region of the GIT but later disappear or their number is significantly reduced. Alison (1989, 1990), for instance, detected NT and pancreatic polypeptide- immunoreactive (IR) endocrine cells in the caecum and pyloric stomach of the chick during early development but not in the later stages. Accordingly, one of the main problems regarding our understanding of the ontogeny of the GINCS in different vertebrates is that most of the studies present data on a limited number of developmental stages.

The close correlation between the availability of these regulatory peptides and the activity of the GIT has been disclosed as it was found that the presence of food in the GIT stimulates their release (Jensen 2001; Secord et al. 2001). The latter study provided physiological evidence for the direct effect of these regulatory peptides upon the feeding process by examining the post-feeding changes in the plasma and tissue concentrations of a number of GI neurohormones in the python, *Python molurus*. Interestingly, there was rapid production of these substances after feeding and consequently a functional role in digestion. The mucosal endocrine cells of the GIT can be of either the open or the closed type. The open type is primitive and therefore can be found in the GIT of invertebrates and lower vertebrates. It extends from the basal lamina to the gastrointestinal lumen and thus delivers the peptides directly to the lumen (Fujita and Kobayashi 1977). By luminal contact these cells can receive chemical or mechanical stimuli from the lumen or secrete directly into it. The closed type, however, either delivers its secretion directly to the bloodstream, or exerts a paracrine type of action via the release of the peptide to influence neighboring mucosal epithelial cells. The majority of these gastrointestinal regulatory peptides are expressed in both endocrine cells and nerve fibres of all vertebrate species studied to date, often with a large general similarity in distribution patterns among tetrapods (Johnson et al. 1994). The distribution of a number of regulatory peptides in representatives of adult vertebrate species have received considerable attention and yielded a number of studies. As a result, a general account of the topographic distribution of numerous regulatory peptides as well as their roles during development and in controlling different GIT activities like motility, gastric acid secretion and regional blood flow has been accumulated (Jensen and Holmgren 1994; Reinecke et al. 1997; Olsson and Holmgren 2001).

However, knowledge of the distribution of these regulatory peptides in the GIT during ontogeny is scarce and mostly restricted to mammals and birds (Larsson et al. 1987; Flatt et al. 1991; Ciccotosto and Shulkes 1996; Salvi et al. 1995, 1998 & 2000; Kuramoto et al, 2007, Machado-Santos et al, 2009). As a result, the need has arisen for exploring the ontogenetic aspects of the GINCS of lower vertebrates in order to gain reliable insight into its phylogenetic development and thus establish possible evolutionary links, if any. The latter target necessitates the availability of comparative studies throughout the different classes of vertebrates. Moreover, this expansion could lead to greater awareness of the physiological mechanisms in vertebrates, including mammals, because current phylogenetic generalizations are based on studies of minute fractions of different vertebrate species. Consequently, a number of ontogenetic studies utilizing both fish and amphibians have been conducted (Reinecke et al. 1997; Chiba

1998; Kurokawa et al. 2000; Kamisaka et al. 2001; Maake et al. 2001; Holmberg et al. 2001; Al-tikriti et al. 2012). Investigating the anatomical localization of these regulatory peptides in the developing GIT of different vertebrate species is anticipated to contribute to the understanding of the physiological functions of these peptides *in vivo*. The present study, thus, aimed to gain more insight into the GINCS of the axolotl and constitutes a detailed description of the qualitative distribution and a semi-quantitative account of the relative abundance of SER and five regulatory peptides, namely, SP, SS, GAS/CCK, NT and CGRP in further developmental stages during the ontogenetic development of the GIT.

MATERIALS AND METHODS

Animals and staging

Priciples of animal care and use were carefully followed during the present investigation. All developmental stages of the axolotl, *Ambystoma mexicanum*, utilized in this study were obtained from the Biomedical services unit (BMSU), School of Biosciences, Birmingham, UK. Juvenile stages were kept under normal feeding condition before being sacrificed and their manipulation was performed according to the BMSU guidelines. Very early developmental stages i.e. 38, 39, 40 and 41 (according to the criteria of Bordzilovskaya et al. 1989); early stages 42, 43, and 44; post-hatching stages i.e. 3, 6, 9 and 12 days after hatching and three successive juvenile stages aged 47, 87, 147 days after hatching were used. The de-jellying of the embryonic stages was performed in urodelen saline (0.8% NaCl) following the procedure of Asashima et al. (1989). Some larvae started to hatch early at stage 41, however, the majority hatched at stage 44 where the mouth opening was complete. Consequently, stage 44 was considered as the hatching stage during sampling. Larvae were fed on Daphnia after 4-6 days from hatching while juveniles were fed on fresh chopped and intact Tubifex.

Histological preparations

All specimens were fixed in Bouin's solution without acetic acid, which has commonly been employed for gut IHC investigations owing to its remarkable ability to preserve the antigenic sites. Embryonic and early larval stages were fixed by whole body immersion for 3-5 h at room temperature according to the specimen size while the whole GIT of the late larval as well as juvenile stages was excised and its different parts were fixed using the same procedure as for the early stages. Thereafter, specimens were washed in 70% ethanol, dehydrated in an ascending series of ethanol and embedded in Paraplast (tissue embedding medium, St. Louis, MO). Serial sections were cut at 4 µm and mounted on gelatine-coated slides.

Immunohistochemical protocol

Prior to processing for the IHC technique, sections were deparafinized and hydrated. The slides were then transferred to a glass staining jar filled with citrate buffer solution (pH 6) and processed in a microwave oven with the power set at 350 W for 10 min started at the boiling point. After microwaving, sections were allowed to cool at room temperature for 30 min and rinsed afterwards in distilled water followed by phosphate buffered saline (PBS, pH 7.4). They were then subjected to an indirect IHC method for the demonstration of SER, SP, SS, GAS/CCK, NT and CGRP. To reduce unspecific binding, sections were treated with PBS containing 2% bovine serum albumin for 30 min at room temperature. They were then incubated with one of the primary antisera for 12 h in a moist chamber at 4ºC and washed three times in PBS (10 min each). The primary rabbit antisera were detected using streptavidin fluorescein-isothiocyanate (FITC) conjugated to anti-rabbit 1gG (F0205, Dako, 1:50) whereas the primary rat antisera were detected with biotinylated goat anti-rat 1gG (Bioscience product, Emmenbrücke, Switzerland, 1:100) for 30 min at room temperature in the dark. Thereafter, sections treated with biotinylated goat anti-rat were washed in PBS and incubated with streptavidin FITC (Bioscience products, 1:100) for an additional 30 min at room temperature followed by three washes in PBS. However, sections treated with streptavidin FITC conjugated to anti-rabbit were directly washed three times in PBS. Sections were finally mounted in carbonate-buffered glycerol prior to examination.

A semi-quantitative grading of the peptides-IR entero-endocrine cells and enteric nerve fibres was carried out in order to estimate the topographic distribution of SER- and regulatory peptides-immunoreactivities. This has been done by observing their expression per unit area (0.002 mm^2). Thus, (-) corresponds to no IR- endocrine cells or IR-nerve fibres visible per visual field, (±) corresponds to low density of IR-endocrine cells or IR-nerve fibres visible per visual field, (+) corresponds to a moderate density of IR-endocrine cells or IR-nerve fibres visible per visual field and (++) corresponds to high density of IR-endocrine cells or IR-nerve fibres visible per visual field. The visual field was constantly seen through objective 10x.

The specificity of the antisera used was tested by pre-absorption of each antiserum by its antigen (40 µg, 400 µg peptide/ml diluted antiserum) and replacement of the primary antiserum with non-immune serum. As positive

control, sections of adult axolotl shown to contain SER and the investigated regulatory peptides (Maake et al. 1999) were also processed in each incubation series of the 3rd juvenile stage. The 3rd juvenile stage sections were then used as a positive control for all other stages. The use of control sections indicated the specifity of the obtained reactions. Photomicrographs were taken with a Zeiss Axiophot (Zeiss, Zürich, Switzerland). For examination and photographing, the fluorochromes were visualised with fluorescence modules specific for FITC (BP 450-490 nm excitation filter, FT 510, LP 515-565 nm emission filter). Black and white Kodak TMAX 400 films, rated at 400 ASA were used for photographing.

RESULTS

The present study started with the embryonic stage 38 onwards but showed negative results up to stage 41. Based on that, stage 42, which displayed immunoreactivity to SP and GAS/CCK, was regarded as the first developmental stage for the GINCS to appear and develop afterwards. The immunoreactivity of stage 43 was evidently similar to that of stage 42. It should be mentioned that the microwave pre-treatment reflected positively on the detection of antigens probably by overcoming the masking effect of formalin fixative as without microwave oven heating, the sections exhibited weak or even no immunostaining. In contrast, sections exposed to the microwave heating exhibited a strong staining pattern compared to the non-treated sections processed in the same incubations in parallel.

The chronological appearance of SER- and the investigated regulatory peptides- immunoreactivities alongside the semi-quantitatively grading of their relative frequency in the axolotl GIT during ontogeny is given in Table 1. As can be seen from the table, the immunoreactivity of the investigated substances increased progressively through the developmental stages and the distribution of the IR elements may be varied according to the analyzed area of the GIT. The density of SER- and regulatory peptides-immunoreactivities stabilized in the 3rd juvenile stage, where it displayed the adult distribution pattern.

SER-immunoreactivity was expressed in both entero-endocrine cells and enteric nerve fibres. The first SER-IR endocrine cells appeared at hatching (stage 44) in both stomach and small intestine anlage. At day 3 after hatching, SER-IR endocrine cells were few along the GIT and continued to have the same distribution in other early stages up to 12 days after hatching. Thereafter, they exhibited an increase in number towards the 3rd juvenile stage and were mostly of the open type (Table 1 & Fig. 1 A-F). SER-IR nerve fibres were absent from not only the early stages but also the 1st juvenile stage and were rare within the layers of the GIT of the 2nd and 3rd juvenile stages but were more expressed in the myenteric plexus, where a small number of nerve fibres was found (Fig. 1 E&F). No SER-IR perikarya were detected in different parts of the GIT.

SP-immunoreactivity was detected in both entero-endocrine cells and enteric nerve fibres. SP-IR endocrine cells were first observed in the stomach and intestine anlage at the pre-hatching stage (42). At hatching and 3 days later, they were also confined to the stomach and small intestine but were seen afterwards in few numbers along the whole GIT of the remaining early stages. During the juvenile stages, they were more numerous in the intestine than in the stomach, with their number tending to gradually increase towards the distal end of the GIT (Table 1). They were thus showing particular density in both small and large intestine (Table 1& Fig. 2 B& C); however, this density was high along the GIT of the 3rd juvenile stage especially at its end. SP-IR endocrine cells observed in the different developmental stages were collectively of the open type. SP-IR nerve fibres were absent from all early developmental stages studied. They were first detected in the developing muscle layer of the stomach (Fig. 2A) and small intestine at the 1st juvenile stage, increased progressively in number and stabilized in the 3rd juvenile stage where they showed the adult distribution pattern but with no obvious difference in the innervation patterns between different portions of the GIT (Table 1 & Fig. 2 D) with moderate density in the muscle layer, low density in the myenteric plexus and submucosa. However, no SP-IR perikarya were frequently detected throughout the whole GIT in any of the investigated developmental stages.

SS-immunoreactivity was restricted to the entero-endocrine cells, which were mostly of the open type. These cells were first detected at hatching (stage 44) in the small intestine anlage. Three days after hatching, SS-IR endocrine cells remained few and restricted to the small intestine. They appeared in the stomach afterwards where they gradually exhibited their highest density towards the 3rd juvenile stage (Fig. 3 A-C). SS-IR endocrine cells also had tendency to increase in number with further development. Meanwhile, there was gradual decrease in the number of SS-IR endocrine cells towards the distal end of the GIT with no frequently detectable IR-endocrine cells in the large intestine.

GAS/CCK-immunoreactivity was first detected at the pre-hatching stage (42) (Fig. 3D) like that of SP. The GAS/CCK-IR endocrine cells, which were located in the small intestine anlage, were few in number and weakly stained. Since they first appeared at stage (42) and throughout the more advanced developmental stages studied, GAS/CCK-IR endocrine cells were confined to the small intestine and were completely absent from the rest of the GIT (Table 1). GAS/CCK-IR endocrine cells were collectively of the open type and their number remained low in the early stages. However, they increased progressively in the juvenile stages where they were numerous at both the 2nd and 3rd juvenile stages (Fig. 3 E&F).

NT-immunoreactivity occurred in both entero-endocrine cells and enteric nerve fibres. The first NT-IR endocrine cells appeared at hatching (stage 44) in the small intestine anlage (Table 1). They remained few and were restricted to the small intestine until 12 days after hatching. At the 1st juvenile stage, NT-IR endocrine cells were moderate in number with no obvious difference between different portions of the GIT (Table 1 & Fig. 4A) but became numerous in both the small and large intestine of the 2nd and 3rd juvenile stages (Fig. 4 B-D) while their number in the stomach remained moderate (Table 1). NT-IR endocrine cells observed were mostly of the open type. NT-IR nerve fibres could not be detected until after the 1st juvenile stage. They were few along the whole GIT of the 2nd and 3rd juvenile stages (Table 1) and were always associated with blood vessels (Fig. 4 C&D). No NT-IR perikarya were detected in different parts of the GIT.

CGRP-immunoreactivity had the most limited distribution among the investigated peptides. It was undetectable within the GIT of early stages. Furthermore, CGRP-immunoreactivity was restricted to the nerve fibres, which were seen in a very low numbers and were associated with the blood vessels at the 1st juvenile stage (Table 1). Thereafter, their number increased slightly with age but were always few (Table 1 and Fig. 4E). As a result of the scarcity of CGRP-IR nerve fibres detected along the GIT, a gradation of their density, if present, would be difficult to recognize. No CGRP-IR perikarya were detected along the GIT.

Table 1: Distribution and relative frequency of SER- and peptide-immunoreactive endocrine cells and nerve fibers in the axolotl gastrointestinal tract during development.

Developmental stage	Age*	GITP	SER	SP	SS	GAS/CCK	NT	CGRP
Pre-hatching (42)	13	**ST** EC	-	±	-	-	-	-
		IN EC	-	±	-	±	-	-
Hatching (44)**	17	**ST** EC	±	±	-	-	-	-
		SIN EC	±	±	±	±	±	-
3 days post-hatching	20	**ST** EC	±	±	-	-	-	-
		SIN EC	±	±	±	±	±	-
		LIN EC	±	-	-	-	-	-
6 days post-hatching	23	**ST** EC	±	±	±	-	-	-
		SIN EC	±	±	±	±	±	-
		LIN EC	±	±	-	-	-	-
9 days post-hatching	26	**ST** EC	±	±	±	-	-	-
		SIN EC	±	±	±	±	±	-
		LIN EC	±	±	-	-	-	-
12 days post-hatching	29	**ST** EC	±	±	±	-	-	-
		SIN EC	±	±	±	±	±	-
		LIN EC	±	±	-	-	-	-
first juvenile***	64	**ST** EC	±	+	+	-	+	-
		NF	-	±	-	-	-	±
		SIN EC	+	+	+	+	+	-
		NF	-	±	-	-	-	±
		LIN EC	++	++	-	-	+	-
		NF	-	-	-	-	-	±
second juvenile	104	**ST** EC	++	+	+	-	+	-
		NF	±	+	-	-	±	±
		SIN EC	++	++	+	++	++	-
		NF	±	+	-	-	±	±
		LIN EC	++	++	-	-	++	-
		NF	±	±	-	-	±	±
third juvenile	164	**ST** EC	++	++	++	-	+	-
		NF	±	+	-	-	±	±
		SIN EC	++	++	+	++	++	-
		NF	±	+	-	-	±	±
		LIN EC	++	++	-	-	++	-
		NF	±	+	-	-	±	±

* Age is given in days.

** No immunoreactive sites were observed in the large intestine.

* ** No immunoreactive nerve fibres were detected before this stage.

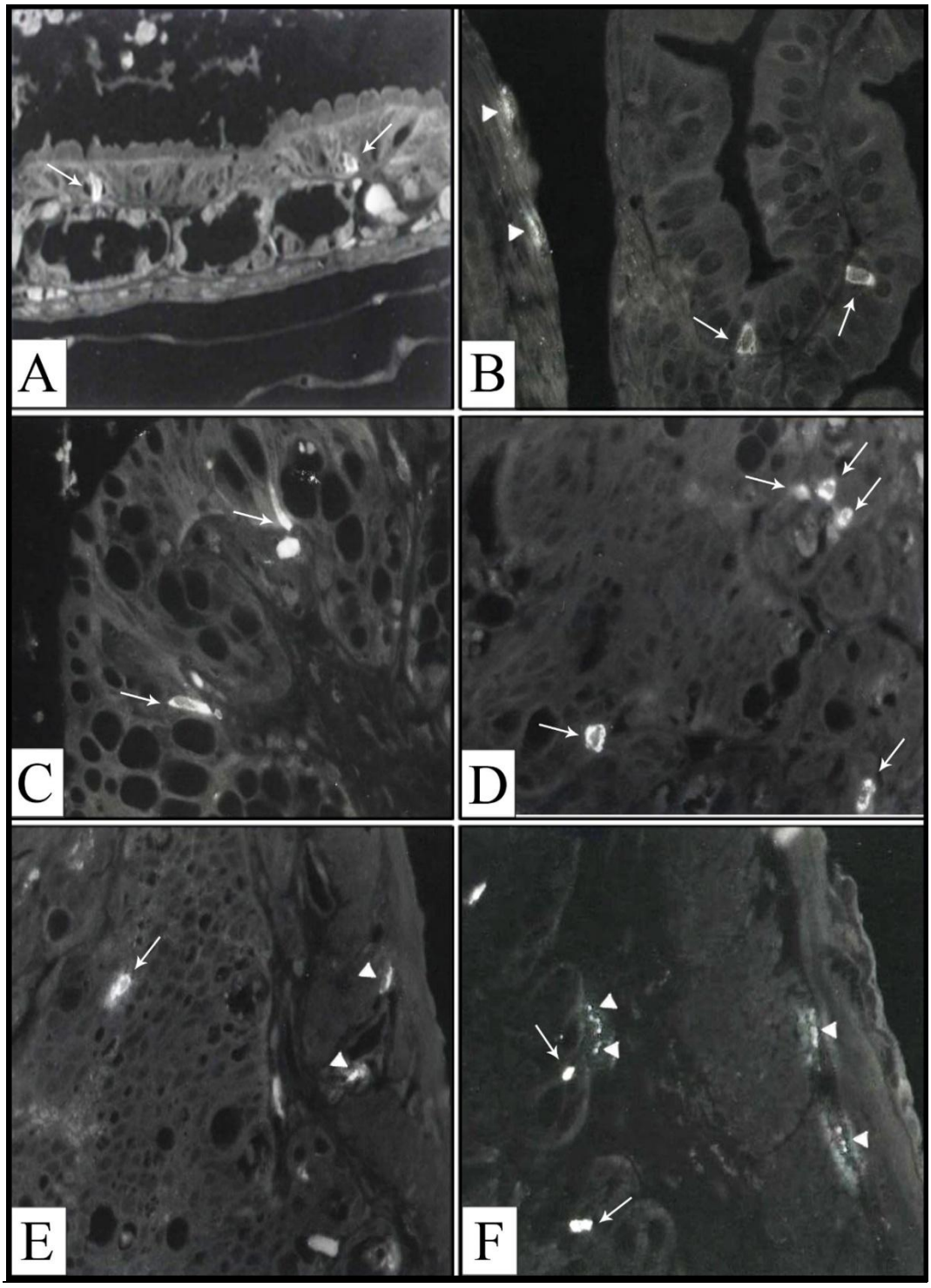

FIG. 1: Immunofluorescence photomicrographs for transverse sections showing SER immunoreactivity at different developmental stages of the axolotl GIT. **(A)** stomach of the 1st juvenile stage. x500 **(B)** small intestine of the 1st juvenile stage. x500 **(C)** large intestine of the 3rd juvenile stage. x500 **(D)** large intestine of the 2nd juvenile stage. x500 **(E&F)** large intestine of the 3rd juvenile stage. X340. Arrows point to entero-endocrine cells whereas arrowheads to enteric nerve fibres.

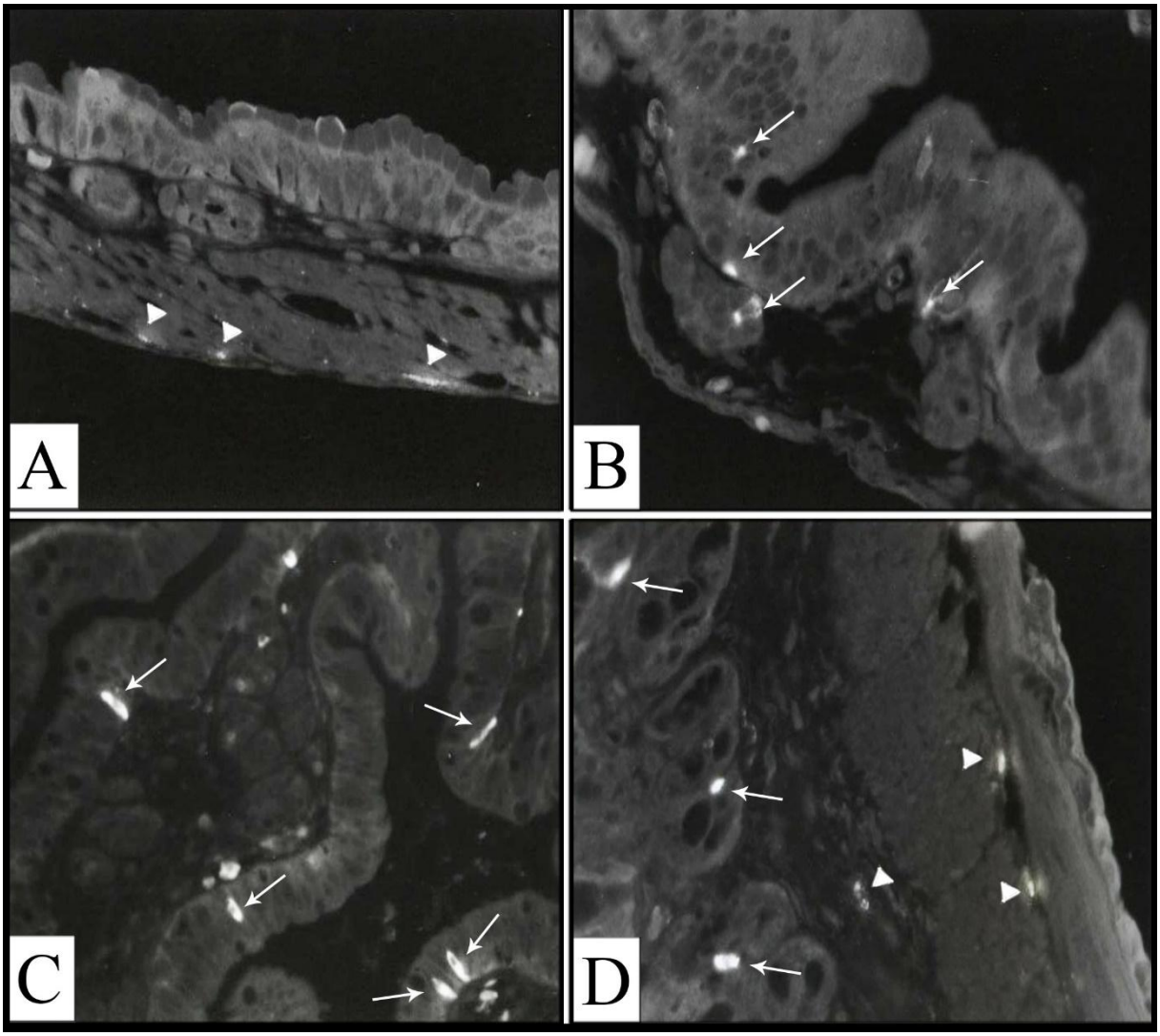

FIG. 2: Immunofluorescence photomicrographs for transverse sections showing SP immunoreactivity at different developmental stages of the axolotl GIT. **(A)** stomach of the 1st juvenile stage. x500 **(B)** large intestine of the 1st juvenile stage. x500 **(C)** small intestine of the 3rd juvenile stage. x500 **(D)** small intestine of the 3rd juvenile stage. x340 Arrows point to entero-endocrine cells whereas arrowheads to enteric nerve fibres.

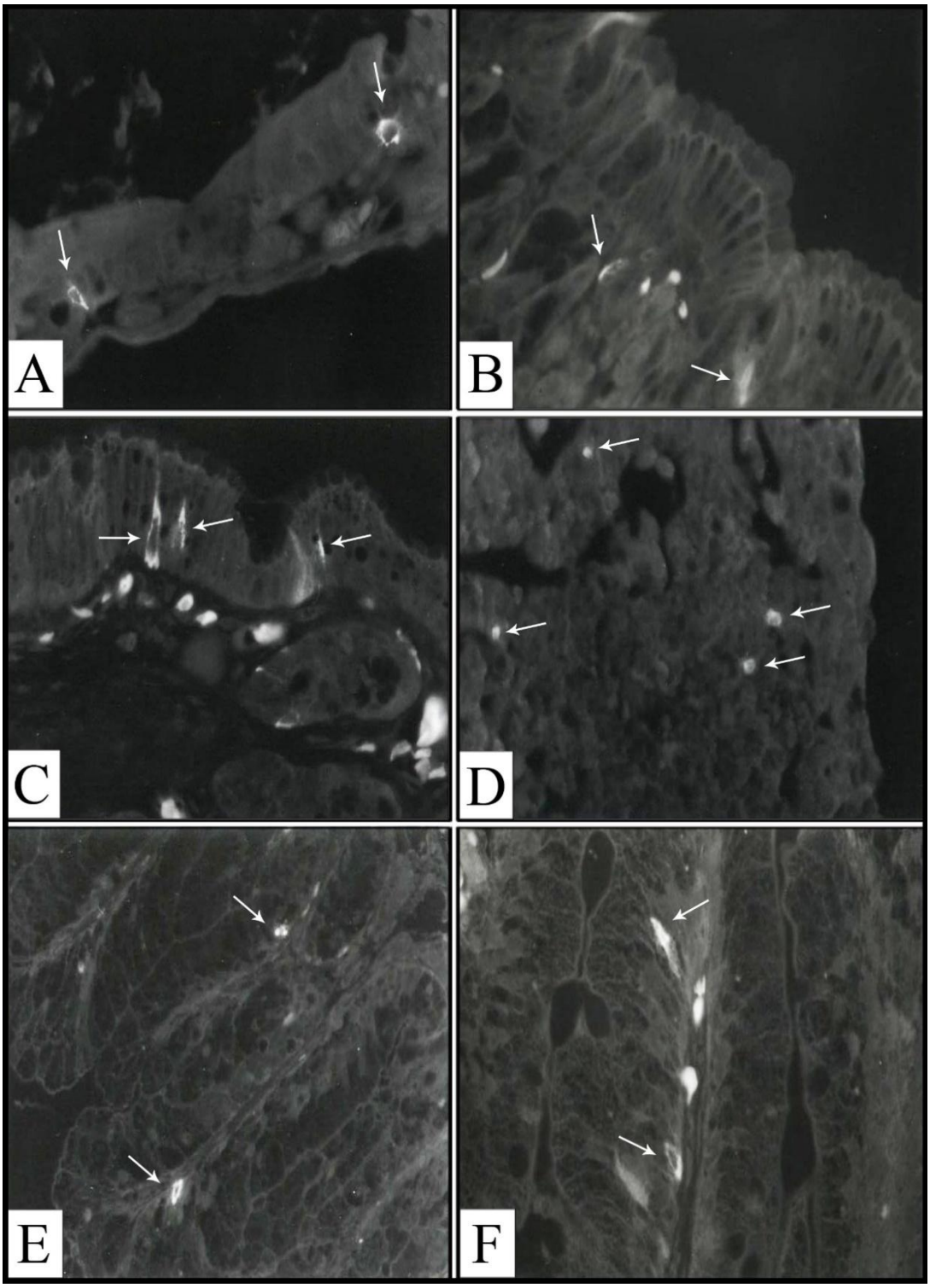

FIG. 3: Immunofluorescence photomicrographs for transverse sections showing SS-**(A-C)** and GAS/CCK- **(D-F)** immunoreactivity at different developmental stages of the axolotl GIT**.** **(A)** stomach of the 1st juvenile stage. **(B)** stomach of the 2nd juvenile stage. X400.**(C)** stomach of the 3rd juvenile stage. **(D)** small intestine of the pre-hatching stage (42). **(E)** small intestine of the 2nd juvenile stage. **(F)** small intestine of the 3rd juvenile stage. Arrows point to entero-endocrine cells. x500

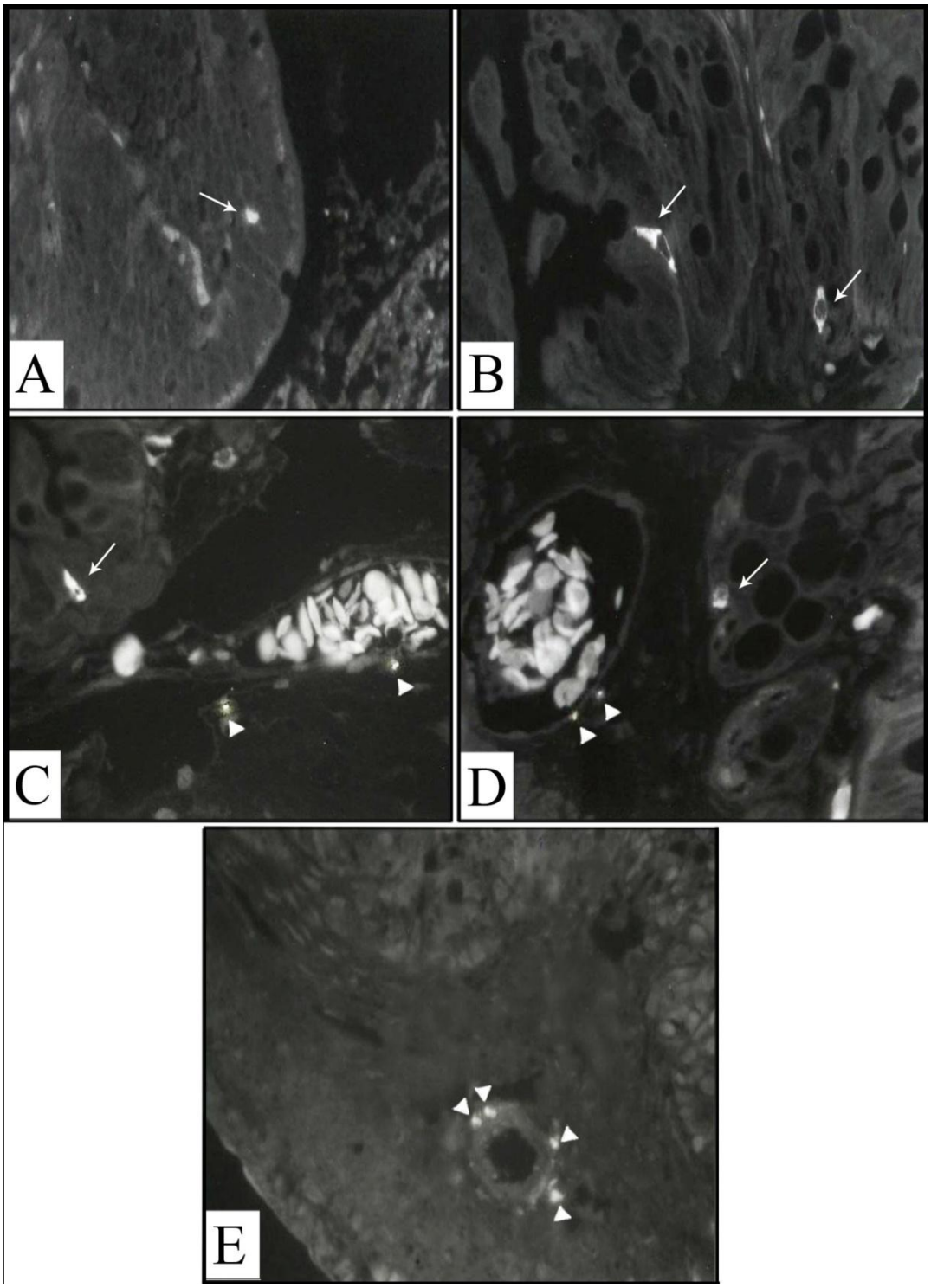

FIG. 4: Immunofluorescence photomicrographs for transverse sections showing NT-**(A-D)** and CGRP-immunoreactivity **(E)** at different developmental stages of the axolotl GIT. **(A)** small intestine of the 1st juvenile stage. x500 **(B)** large intestine of the 2nd juvenile stage. x500 **(C)** large intestine of the 2nd juvenile stage. x600 **(D)** large intestine of the 3rd juvenile stage. x600 **(E)** small intestine of 3rd juvenile. X600 Arrows point to entero-endocrine cells whereas arrowheads to enteric nerve fibres.

DISCUSSION

Histological analysis of the serial sections stained conventionally with haematoxylin and eosin has revealed that until stage 37, the mesodermal cells which form the prospective connective and muscular tissues did not exist in the area occupied by the presumptive GIT wall (unpublished observation). Consequently, embryonic stages up to stage (37) were totally excluded. The present investigation alongside the previous IHC studies on the amphibian GINCS are in agreement that the same regulatory peptides are also expressed in the mammalian GIT. There are, however, marked difference in the distribution of the individual peptide containing mucosal endocrine cells and enteric nerve fibres both among amphibian species and between amphibians and mammals. It can also be concluded from the amphibian studies that there does not appear to be a general pattern regarding the topographic distribution of their GI peptides. Indeed, it has been indicated by Buchan (1986) that although the GINCS of amphibians bears general similarities with its mammalian counterpart, it is not possible to have a generalized statement on the distribution of the peptides even among the amphibians themselves owing to the interspecies variations. Moreover, within a given species the change in the developing GINCS can be expected not only during the broad embryonic, larval, juvenile and adult stages but also within each individual life stage. However, at the healthy adult and late juvenile stages, the neurohormonal regulatory processes are presumably unchanged as the relation between these hormones and their target tissues are well established. However, the significant differences between juvenile and adult stages in the intestinal motility in response to certain regulatory peptides indicated that this is not the case (unpublished observation). Meanwhile, as the estimation of the IHC results of the present investigation were semi-quantitative, it is not possible to conclude that the GINCS of late juvenile and adult stages are similar.

While the present study followed the criteria of Bordzilovskaya et al. (1989) for sampling the early investigated stages, the study of Maake et al. (2001) relied on measuring the body length which, because of the variability in the egg size combined with the expected differences in the rate of development from one spawning to the other, cannot be generalized. While the intervals between the early embryonic stages are short, those of the advanced stages increase gradually to become days starting from stage 37 where development turned to be very slow (Schreckenberg and Jacobson 1975; Bordzilovskaya et al. 1989 and unpublished observation). These studies, which represent the sole source for axolotl staging, showed discrepancy in determining the beginning of hatching and mentioned nothing with regard to the duration by which it terminated. In practice, the hatching of the axolotl larvae begins in a very small scale at stage 41 and progresses until stage 44, with the mouth open, by which time all the larvae abandon the jelly coat. Because stage 44 witnessed the hatching of the greatest number of larvae, it was considered as the hatching stage. According to the present study, stage 42 was the first developmental stage for the ontogenetic appearance of the GINCS.

The outcome of the present investigation supports and expands the results obtained by Maake et al. (1999, 2001). In terms of their findings, the two studies share three basic characteristics. Firstly, with the exception of CGRP, the ontogenetic appearance of the investigated regulatory substances was completed by the time of the onset of external feeding. However, there was disagreement in the time of appearance of SER- and GAS/CCK-immunoreactivities. Secondly, the chronological appearance of the endocrine cells had the priority over the nerve fibres and generally followed the PD direction. Thereafter, the dually distributed neurohormonal peptides appeared afterwards in the nerve fibres. Thirdly, the general topographic distribution of SER and the investigated regulatory peptides showed similar trends in both studies. Moreover, the increase in the density of both endocrine cells and nerve fibres with age is not only in agreement with the study of Maake et al. (2001) but also with other previous ontogenetic studies conducted on bony fish (Reinecke et al. 1997), Xenopus (Holmberg et al. 2001) and mammals (Larsson 1977). For instance, the entero-endocrine cells had priority in appearance during ontogeny over the enteric nerve fibres and the topographic distribution of the investigated substances showed general agreement. Meanwhile, the hatching stage (44), which displayed the beginning of the onset of histological differentiation was the stage at which most investigated neuroendocrine regulatory substances appeared, however, CGRP-IR enteric nerve fibres were undetectable during the whole set of the early stages. The GIT of the three successive juvenile stages exhibited few CGRP-IR nerve fibres along its whole length.

During ontogeny, the differentiation of the GI mucosal endocrine cells may depend on several inputs coming from the local mesenchyme. Some of these signals may be neuropeptides that are early secreted by neuroblasts, which have migrated from the neural crest to within the wall of the GIT (Rawdon and Andrew 1993). Meanwhile, the appearance of most investigated regulatory peptides well in advance of the onset of digestive activity may be explained as pre-adaptation to the active feeding which follows hatching. Another possible explanation is that the early differentiation of the regulatory peptide-containing cells is indicative of peptides having functions other than

digestive functions during early embryonic development. For instance, by acting as growth factors, their early appearance is therefore relevant for the development of the gut. Indeed, certain regulatory peptides, which appeared early during the development of the axolotl GIT, are previously known for their trophic effects upon the developing GIT and were implicated in cellular proliferation and growth (Badawy and Reincke, 2003). The biochemical processes regulated by trophic hormones include increased amino acid uptake, stimulation of protein, RNA and DNA synthesis (Zachary et al. 1987).

The onset of exogenous feeding necessitates that physiological mechanisms of the developing GIT such as digestion and absorption are functional and therefore the GINCS ought to be concurrently expressed. The present study confirms that all the investigated regulatory substances appeared before the start of the external feeding, with the exception of CGRP which seems to have no digestive function within the axolotl GIT. This general finding is in accordance with the previous study of Maake et al. (2001) as well as that of Holmberg et al. (2001) on Xenopus. Based on the present data, four different developmental phases of the expression of GINCS can be proposed: (1) phase I which witnessed the first appearance of SP and gastrin/cholecystokinin (GAS/CCK) at the pre-hatching stage (42) where the GIT showed very limited histological differentiation; (2) phase II which witnessed the first appearance of VIP three days after hatching and PACAP three days later (Badawy and Reinecke (2003); (3) phase III which witnessed the first appearance of SER, SS and NT at hatching (stage 44); and (4) phase IV which displayed few nerve fibres immunoreactive to CGRP only at the juvenile stages. According to the present investigation, SP-, GAS/CCK-IR endocrine cells were collectively of the open type, however, very few closed endocrine cells showed immunoreactivity to SER, SS and NT. The common observation of the predominance of the open IR mucosal endocrine cells probably reflects a primitive condition as has previously been reported for other lower vertebrates (Fujita and Kobayashi 1977).

On the basis that it included an additional number of inter-developmental stages, the present study represents a complement to the previous investigations carried out by Maake et al. (1999, 2001) and conducted in the same laboratory. However, there were some minor disagreements with the study of Maake et al. (2001). For instance, GAS/CCK-IR endocrine cells were detected after the onset of exogenous feeding according to Maake et al. (2001), however, the present study revealed that they appeared at stage 42 but their distribution was restricted to the small intestine in both studies. The early detection of GAS/CCK-immunoreactivity in comparison to the study of Maake et al. (2001) may be attributed to the employment of microwave pre-treatment, as the two studies were conducted using the same experimental protocol. It is well known that antigen identification in paraffin sections may be limited by the masking effect of fixatives, such as the formalin-induced cross-linking of proteins. The method of antigen retrieval with microwave pre-treatment used in this investigation was based on the exposure of paraffin sections to high temperatures in citrate buffer solution (pH 6). This in turn improved the immunostaining and thus led to the avoidance of possible false negative results especially at the early stages (Pileri et al. 1997; Maake, personal communication). Generally, the main differences between the two studies is largely located around hatching, which cannot be guaranteed to happen at the same time even within a single spawning, probably because of the un-synchronized development.

The phenomenon of early detection of some specific regulatory peptides in the developing GIT of vertebrates before the onset of its physiological functions has been reported in several previous investigations (Walsh and Dockray 1994). IHC studies on the GIT of elasmobranches showed that most types of endocrine cells and nerve fibres develop during the early embryonic stages, while a few appear around or shortly after hatching (Holmgren 1989). In Xenopus, endocrine cells immunoreactive to CCK were detected before the full differentiation of the GIT and this was attributed to a possible trophic action (Scalise and Vigna 1988). Alison (1989, 1990) also noted that SS, NT, glucagon and pancreatic polypeptide-IR endocrine cells were first seen in the undifferentiated GIT of chicken. These endocrine cells were identified at a stage when the gut lining epithelium is pseudo-stratified or only recently had formed a simple layer, in which, the surrounding epithelial cells were relatively undifferentiated. Based on his results, Alison (1989) concluded that the differentiation of gut endocrine cells in birds does not depend on the interaction with other well-differentiated epithelial cell types. If the final GIT histological differentiation is to be considered, the early detection of the GAS/CCK-IR endocrine cells is consistent with the results of the study of Reinecke et al. (1997) conducted on a bony fish, the turbot, Scophthalmus maximus. According to the present study only SP- and GAS/CCK-immunoreactivities were detected before the onset of histological differentiation. However, SER-, SS- and NT-immunoreactivities appeared during the onset of GIT histogenesis at hatching (stage 44). The early ontogenetic appearance of these regulatory peptides before the starting of the GIT activity, suggests that they may regulate cellular growth and proliferation but thereafter their role changes to the regulation of digestive function

(Larsson 1977; Tagliafierro et al. 1989). The possible role of the regulatory peptides as stimulators for cell proliferation has been reviewed by Zachary et al. (1987). Some studies demonstrated trophic functions for SP (Nilsson et al. 1985; Shih and Bernard 1997), GAS and CCK (Walsh 1990; Håkanson et al. 1991), SER (Choi et al. 1994; Fujimiya et al. 1997) and NT (Reinecke 1985). In mammals, GAS has been shown to stimulate RNA, protein and DNA synthesis in the GI mucosal cells (Johnson 1976) and CCK has been shown to produce similar trophic effects (Håkanson et al. 1991). Thus, the early ontogenetic appearance of these regulatory substances seems to suggest trophic effects upon the GIT of the axolotl during early development.

SER-IR endocrine cells were observed later than reported by Maake et al. (2001). The only likely explanation for this disagreement is the possible staging differences between the experimental animals utilized in the two studies. SER-IR endocrine cells detected varied markedly in shape. The majority of these cells were open, having apical contact with the GIT lumen, and very few of them were of the closed type with a round shape and located near the basement membrane. A similar phenomenon has been reported for the cloudy dogfish (Chiba 1998) and was in agreement with the present study in that the SER-IR endocrine cells appeared in a PD direction during ontogeny. In birds, SER-IR endocrine cells occur throughout the intestine, being particularly abundant in the upper small intestine and the rectum (El-Salhy et al. 1985; Rawdon and Andrew 1994). However, in mammals SER-IR endocrine cells occur throughout the GIT (Sjolund et al. 1983). This clear variability in the distribution of SER-IR endocrine cells within different vertebrate groups possibly reflects the structural diversity of the GIT linked to different feeding habits. The high density of SER-IR entero-endocrine cells and nerve fibres within the GIT of different vertebrate groups confirms its important role in the control of various GIT functions like gastric acid secretion and smooth muscle contraction in the GIT (Guyton, 1988, Martel, 2006). Moreover, it has recently been demonstrated that SER has an important role in feeding and gut contraction in honeybee (French et al. 2014). Meanwhile, the absence of SER-IR perikarya from the whole GIT of the axolotl indicates that SER innervation is completely extrinsic.

SP-IR mucosal endocrine cells were detected in all developmental stages starting from stage (42). They were present along the whole GIT with a significant PD increase in numbers which agrees with the studies of Rombout and Reinecke (1984), Holmgren et al. (1985) and Maake et al. (2001). In mammals, both the myenteric and submucosal plexuses contain SP (Costa et al. 1981). However, in non-mammalian vertebrates the distribution of SP-immunoreactivity in the enteric nervous system (ENS) is somewhat different. Among amphibians, SP was found in both the myenteric and submucosal plexus of Rana esculenta L. (Gabriel 1990) and in submucosal and muscle layers of the GIT of the axolotl (Maake et al. 2001 and the present study). However, SP-immunoreactivity in the ENS of different fish species exhibits no general feature but is commonly expressed in the mucosal endocrine cells (Bjenning and Holmgren 1988; Holmgren 1989). The existence of mucosal endocrine cells showing SP-immunoreactivity has been demonstrated in the GIT of both urodele and anuran amphibians (Buchan et al. 1980; Holmgren et al. 1985; Buchan 1986). However, in several species examined by Buchan (1986) no mucosal endocrine cells with SP-immunoreactivity could be detected and this was recognized as a true species difference. In agreement with the investigations of Holmgren et al. (1985) and Gabriel et al. (1992), the density of SP innervation seen in the present study was low in the myenteric plexus. Contradictory to that, (Buchan 1986) reported high density of SP-IR nerve fibres in the myenteric plexus especially around blood vessels and therefore indicated an additional regional vasoregulatory action for SP. The extensive anatomical expression of SP-immunoreactivity within the wall of the GIT of different vertebrate species indicates that SP is important in the control of several regional functions (Bartho and Holzer 1985; Olsson and Holmgren 2001; Turner et al. 2007). In a recent investigation, SP exerted potent excitatory effects upon the intestinal smooth muscle of both juvenile and adult axolotl stages (unpublished observation). The absence of SP-IR nerve fibres in the early stages of the present investigation suggests that SP-IR mucosal endocrine cells are probably the source of SP during early development.

The absence of SS-IR nerve fibres from the axolotl GIT is in agreement with observation reported for other urodeles such as *Necturus maculosus* (Holmgren et al. 1985) and *Salamandra salamandra* (Buchan et al. 1980) which also revealed SS-immunoreactivity solely in the mucosal endocrine cells. This complete absence of SS-IR nerve fibres probably has direct impact upon the possible effect of SS on the intestinal smooth muscle motor activity in the axolotl (unpublished observation). The detected SS-IR endocrine cells were abundant in the pyloric stomach and few to moderate in the small intestine. Moreover, the number of these endocrine cells tended to decrease in a PD direction until they were infrequent in the large intestine. Several studies revealed that the biggest number of SS-IR endocrine cells occurred in the stomach and the smallest number in the large intestine. This is applicable to urodeles (Holmgren et al. 1985); anurans (Trandaburu and Nurnberger 1995; Maake et al. 1998); birds (Rawdon and Andrew 1999); and mammals (Keast et al. 1987; Patel 1999). Differently, El Salhy et al. (1981) reported that the anuran

large intestine lack any SS-IR endocrine cells. As both investigations showed infrequent IR endocrine cells in the large intestine of the axolotl, the present study alongside that of Maake et al. (2001) is in agreement with the study of El-Salhy et al. (1981). Thus, it seems that the varying distribution patterns reported for SS-IR endocrine cells cannot be solely explained by differences of species.

In lower vertebrate species studied to date, GAS/CCK-immunoreactivity was present in the mucosal endocrine cells only (Holmgren et al. 1982; Reinecke et al. 1997). However, GAS/CCK-IR enteric nerve fibres were observed in other studies (Holmgren and Nilsson 1983; Holmgren et al. 1985). In the GIT of fish GAS/CCK-immunoreactivity is mainly present in the endocrine cells but is also seen in nerve fibres (Bjenning and Holmgren 1988; Burkhardt-Holm and Holmgren 1989). Among amphibians, GAS/CCK-IR mucosal endocrine cells have been observed in the stomach of *Salamandra salamandra* and *Rana temporaria* (Buchan et al. 1980; El-Salhy et al. 1981; Buchan 1986). The observed restriction of GAS/CCK-immunoreactivity to the endocrine cells of the small intestine is in line with the study of Maake et al. (2001) despite the disagreement in the timing of appearance.

NT-IR mucosal endocrine cells have been found in representatives of all vertebrate species (Reinecke et al. 1980 a, b). Among mammals, NT-immunoreactivity has been detected in the enteric nerve fibres (Schultzberg et al. 1980; Reinecke et al. 1983). Contrarily to that, some studies revealed NT-immunoreactivity only in the mucosal endocrine cells (Buchan et al. 1978). NT-IR endocrine cells in mammals, in contrast to those of birds, are confined to the small intestine being most numerous in its distal part (Reinecke et al. 1980 a). The distribution patterns for NT-IR endocrine cells in non-mammalian species differ from those found in mammals in that their highest densities are found in the proximal instead of distal small intestine (Reinecke 1985). The latter study showed that, in further contrast to mammals, considerable amount of NT-IR endocrine cells occurs in the colon of birds. According to the present investigation, the early developmental stages displayed few NT-IR endocrine cells only in the small intestine in a quite similar distribution to that reported by Maake et al. (2001). The juvenile stages, however, exhibited NT-IR endocrine cells in all parts of the GIT. The detection of NT-IR nerve fibres close to GI blood vessels of the juvenile stages suggests a regulatory role for NT upon the regional blood flow. However, the absence of NT-IR perikarya from all parts of the GIT indicates that NT innervation is completely extrinsic.

The present study revealed that CGRP-immunoreactivity was detected rather late in development. It was scarce and expressed only in the enteric nerve fibres, which were mostly seen around the blood vessels. This distribution pattern agrees with the observation reported by Maake et al. (2001). The relatively late ontogenetic appearance of CGRP-immunoreactivity was observed in human gut by Larsson et al. (1987) who reported that SP and VIP-IR nerve fibres appeared in the 15th week of gestation, however, those of CGRP appeared one week later and also in Xenopus (Holmberg et al. 2001). The presence of CGRP-IR nerve fibres around the blood vessels in the GIT of several vertebrate species suggests that CGRP probably acts as regional vasoregulator and therefore has a vital role in regulation of intestinal blood flow. It is evident, according to the present findings, that the origin of CGRP innervation is extrinsic like that of NT as no CGRP-IR perikarya were detected.

In conclusion, the data of the present study indicate that the ontogeny of SER and the investigated regulatory peptides in the GIT of the axolotl seems to correlate with the onset of histological differentiation. The first ontogenetic appearance of the GINCS occurred at stage 42. From the onset of histological differentiation at stage 44 up to the juvenile stages, there was gradual developmental progress in the expression of the GINCS probably to meet the necessarily requirements of digestion. The only exception for this generalization was CGRP, which displayed association with the blood vessels and therefore probably acts as regional vasodilator (Brain et al. 1985) within the axolotl GIT. The regulatory peptides, which are known for their trophic effects, had the priority in appearance during ontogeny. This in turn promotes the cellular differentiation and growth of the gastrointestinal wall during development.

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