The distribution of cells containing FMRFamide- and 5-HT-related molecules in the embryonic development of *Viviparus ater* (Mollusca, Gastropoda)

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The timing and spatial distribution of cells containing FMRFamide- and 5-HT-related molecules in the embryonic development of the mollusc *Viviparus ater* are examined using immunohistochemistry. FMRFamide-like molecules emerge in the early stage E8 (8% of embryonic development) before the 5-HT immunoreactivity, and they are not only found during nervous system ontogeny. As the parts of the digestive tract differentiated, the pattern of the diffuse gut endocrine cells, present in adults, start to be established (E20-E30), and both open and closed cell types are immunoreactive to anti-FMRFamide antibody. From their appearance (E20), cells with a 5-HT-like phenotype are distributed in the central nervous ganglia and progressively assembled during embryonic development. The early occurrence of both these molecules in V. ater embryos reinforces the growing view that neurotransmitters play a regulatory role in embryogenic processes. In particular, the very early presence of FMRFamide-related factors suggests an involvement of these molecules in the regulation of basic, not only neuronal, cell behaviours, while 5-HT seems to be a more specific neural development signal.

Key words: Mollusc; Viviparus ater; Embryogenesis; FMRFamide; 5-HT; Immunocytochemistry

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Embryonic development is known to be driven by a variety of soluble factors. In addition to the most widely studied classical growth factors, evidence across animal phyla focuses on the presence in embryos from several species of invertebrates and vertebrates of neurochemicals prior to organ development (Buznikov, 1990). The involvement of neurotransmitters in the regulation of basic development processes, i.e. cell proliferation, migration, differentiation, morphogenesis and gene expression, has been reported (Lauder, 1993; Whitaker-Azmitia*et al.,* 1996; Buznikov*et al.,* 2001). With respect to invertebrates, and in particular gastropod molluscs, neuropeptides such as Phe-Met-Arg-Phe-NH2 (FMRFamide) and biogenic amines such as serotonin (5-HT), which are known to function widely as neuroactive compounds in the mature nervous system (Walker, 1986; Hernadi*et al.,* 1998), have also been seen in early embryonic stages (Marois and Croll, 1992; Voronezhskaya and Elekes, 1993; Croll and Voronezhskaya, 1995; 1996; Marois and Carew, 1997a; Diefenback*et al.,* 1998; Dickinson*et al.,* 2000; Buznikov*et al.,* 2003; Dickinson and Croll, 2003). 5-HT is involved in mollusc oocyte maturation, fertilization, cleavage divisions (Krantic*et al.,* 1993; Abdelwajid*et al.,* 1994; Leclerc*et al.,* 2000; Buznikov*et al.,* 2003) and participates in many aspects of neural development by regulating neurite outgrowth and the synaptogenesis of specific neurons (Haydon*et al.,* 1984; Goldberg and Kater, 1989; Goldberg, 1998). In addition, pharmacological studies suggest that 5-HT has auto-regulatory functions on the growth of serotonin-containing neuron (Diefenbach*et al.,* 1995). The available results on the timing of the appearance of cells containing FMRFamide-related molecules in molluscan embryos mainly refer to nervous system ontogenesis. Neurobiological development studies have demonstrated the early presence of FMRFamide immunoreactive cells, while these are among the

very first to develop in the gastropod nervous system and a role of these molecules in the process of morphogenesis and neurogenesis has been suggested (Croll and Voronezhskaya, 1995; Voronezhskaya and Elekes, 1996; 2003; Dickinson *et al.,* 1999; 2000; Dickinson and Croll, 2003). Given that most studies are limited to neural embryogenesis, here the temporal and spatial distribution of FMRFamide- and 5-HT-like molecules are examined in neural and non-neural embryonic development in *Viviparus ater*. This prosobranch constitutes an interesting embryo model system to investigate molecular signals involved in gastropod development as previously reported (Franchini, 2002). Indeed V. ater is gonochoristic and viviparous with progeny leaving the parent as juvenile snails; this allows comparison with other gastropods with a direct, fully intracapsular, or indirect, with free living larvae, developments.

Materials and Methods

Adult female specimens of *Viviparus* ater were collected in the spring in canals near Modena (Italy). Embryos at various stages were removed from the different parts of the developmental pouch and immediately fixed in Bouin's fluid and 2% p-formaldehyde in 25 mmol/l phosphate buffer pH 7.3. Embryonic development was defined by measuring the embryo's major diameter and the stages were expressed as a percentage of total embryonic development whereby 0% (embryonic stage E0) corresponds to the fertilized egg and 100% (embryonic stage E100) to hatching. The earliest embryo stage collected was E5, corresponding to an early trochophore stage. The immunocytochemical procedure was performed on 7 μm-thick paraffin serial sections of embryos at various stages using the following antibodies: rabbit anti-FMRFamide (Peninsula Laboratories, USA) and anti- 5HT (Biogenesis, UK) polyclonal antibodies. Sections were incubated overnight at 4°C in primary antibodies, diluted 1:1000, and immunoreactivity was visualized by an immunoperoxidase technique using avidin-biotin peroxidase complex (Hsu*et al.,* 1981).

Controls of immunocytochemical reactions were performed by substituting primary antibodies with non-immune sera and/or absorbing the antibodies with homologous antigens. Nuclei were counterstained with hematoxylin.

Results

Examination of V.ater embryos demonstrated the appearance of FMRFamide-like molecules in initial stages of development. At stage E8, (8% of embryonic development), undifferentiated structures constituted by cells associated to thin fibres were observed in a dorsal and anterior region of the embryos near the primitive intestine where cerebral ganglia will later be seen. In these primordial ganglionic organizations, a pair of immunoreactive (IR) cells, resembling nerve cells, was found associated to reactive fibres (Figure 1A, 1B). Sections from the E10 developmental stage, showed another positive neuron-like cell, in which pedal ganglion will form, and a cell was located under the covering epithelial layer in the organizing foot. An IR cell in the heart primordium that was forming as a thickening of the pericardial cavity wall (Figure 1C, 1D), and positive fibres in a primordial cell organization located under this cavity (Figure 1E) were also seen. No positive cells were present in the various parts of gastro-intestinal tract. As development proceeded, the foot marked the ventral side by stage E15-20 and and some isolated or grouped IR cells and fibres were present in the main ganglia of the central nervous system, distinguished as morphologically organized structures, i.e. the cerebral, pedal and ganglia of the visceral mass (Figure 1F, 1G). One basal intraepithelial neuron-like cell was located at the base of tentacle near the eye (Figue 1H), positive fibres innervated tentacle epithelium and IR cells and fibers were still observed in the developing heart (Figure 2A). At stages E20-E30, positive cells appeared in different parts of the intestine, but not in related glands, i.e. salivary and digestive glands. Single cells, which were either triangular in shape, located near the epithelium basal lamina and similar to closed-type endocrine cells, or elongated, opening into the lumen and comparable to open endocrine cells, were present. These were interspersed in the esophagus, stomach and intestine (Figure 2B) epithelial walls. Moreover, positive fibres were in close contact with unreactive gut epithelial cells. Intraepithelial IR cells and fibres were present in the developing chemosensory organ, the osphradium. With further development, the immunoreactivity increased in the neuron cell bodies and neuropile fibres of all embryo ganglia (Figure 2C) and in fibres running to the peripheral tissues, i.e. tentacle, foot, heart, kidney and mantle

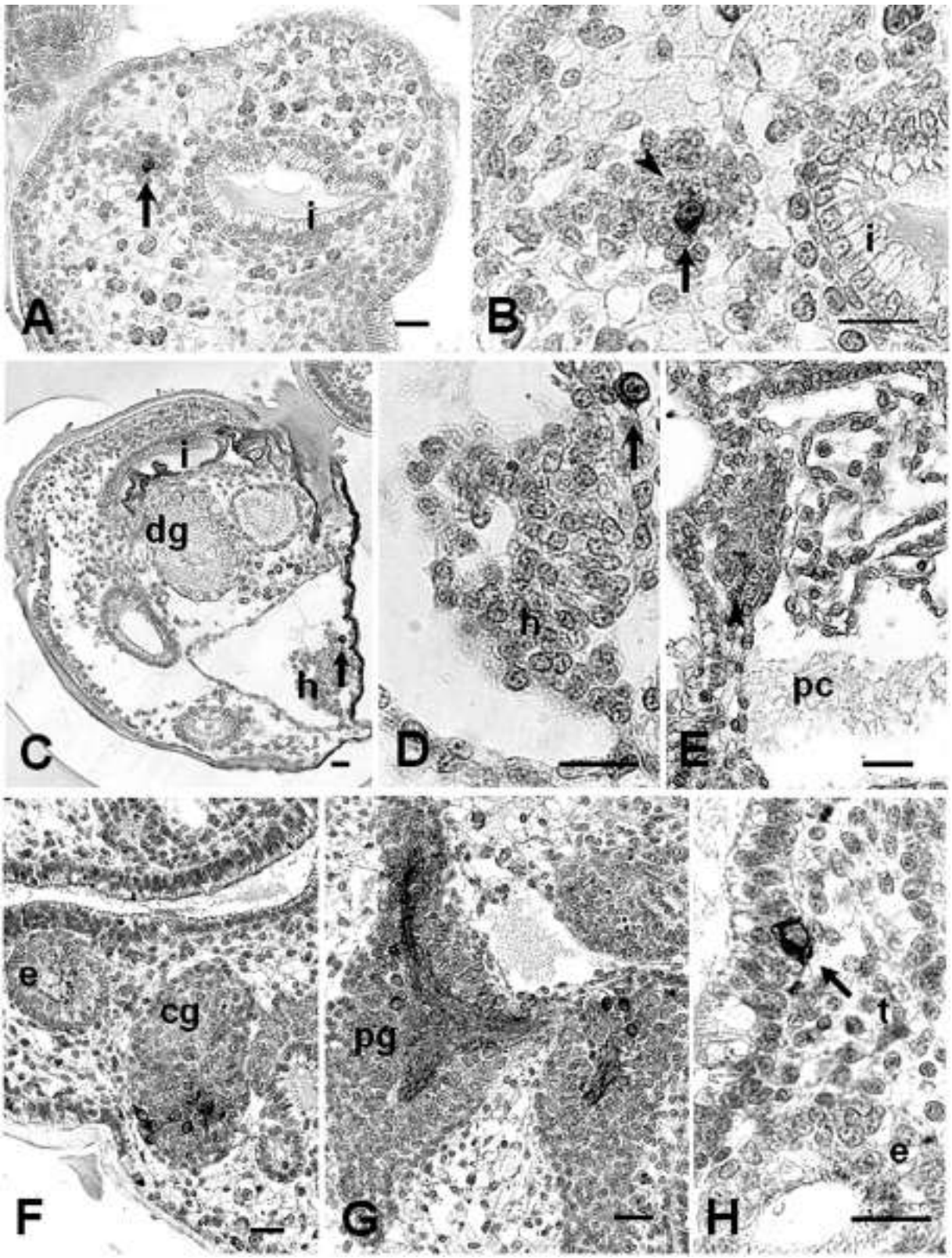

Figure 1. Sections of V. ater embryos (E8: A, B; E10: C-E; E20: F-H) immunostained with anti-FMRFamide antibody and counterstained with hematoxylin. Bar = 20 μm. A positive neuron-like cell (arrow) near the anterior primitive intestine (i) is shown (A) and it is seen associated to IR fibres (arrowhead) at high magnification (B). One cell (arrow) in the heart primordium (h) (C, D) and fibres (arrowhead) under the pericardial cavity (pc) (E) are stained. Isolated and grouped IR neurons are shown in cerebral (cg) (F) and pedal (pg) ganglia (G). Note an intraepithelial IR neuron-like cell (arrow) at the base of tentacle (t), near the eye (e) (H). Digestive gland rudiment (dg).

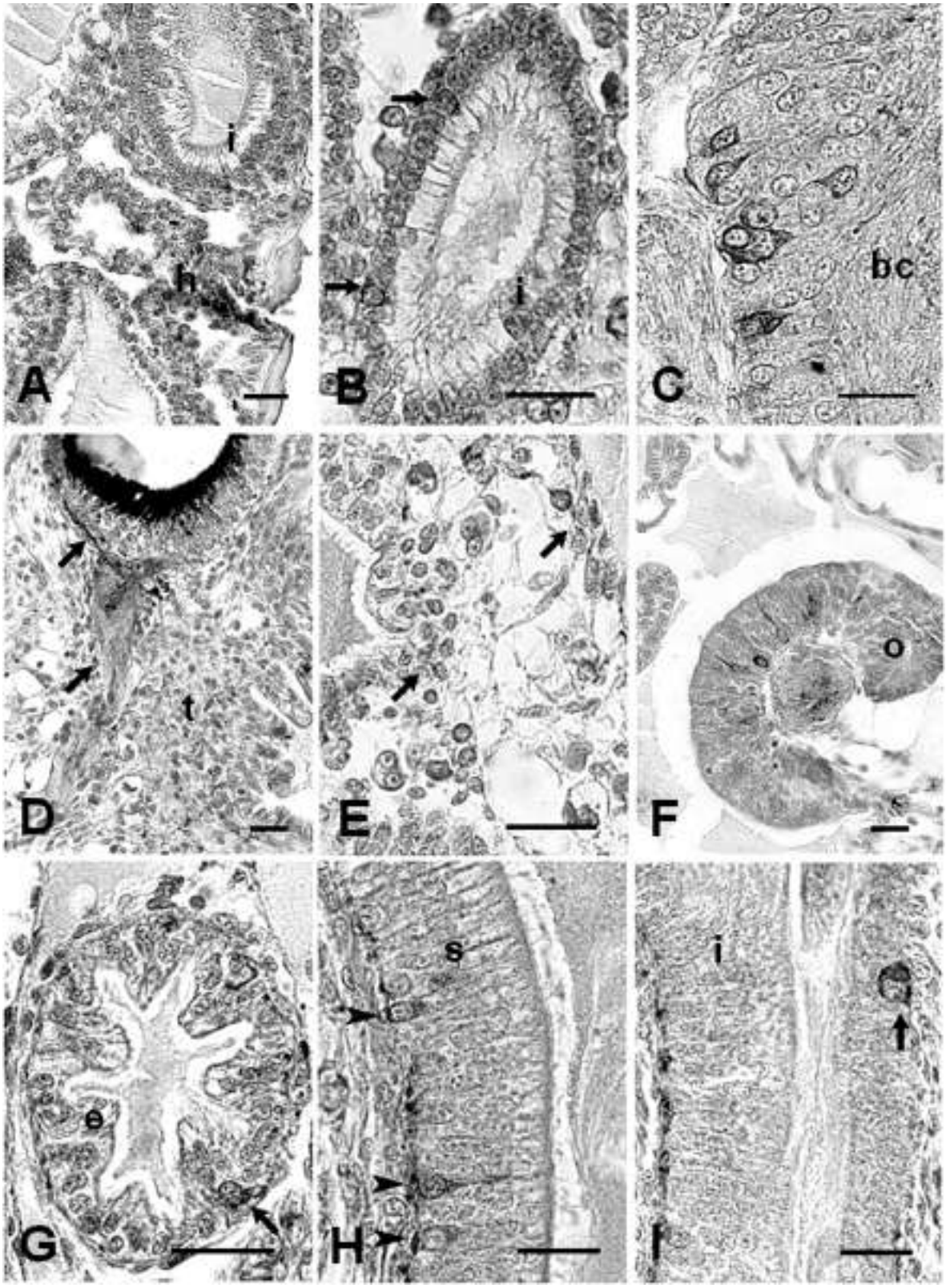

Figure 2. Sections of V. ater embryos (E20: A; E30: B; E50: C-H; E60: I) immunostained with anti-FMRFamide antibody and counterstained with hematoxylin. Bar = 20 μm. Immunoreactivity is found in developing heart (h) (A). Positive gut cells, resembling closed (arrows) and open type endocrine cells (arrowheads), are seen interspersed in the esophagous (e), stomach (s) and intestine (i) epithelia (B, G-I). Positive neurons and fibres in buccal ganglia (bg) (C), peripheral fibres (arrows) in tentacle (t) (D), and mantle (E) are shown. Note the reactivity in the osphradium (o) (F).

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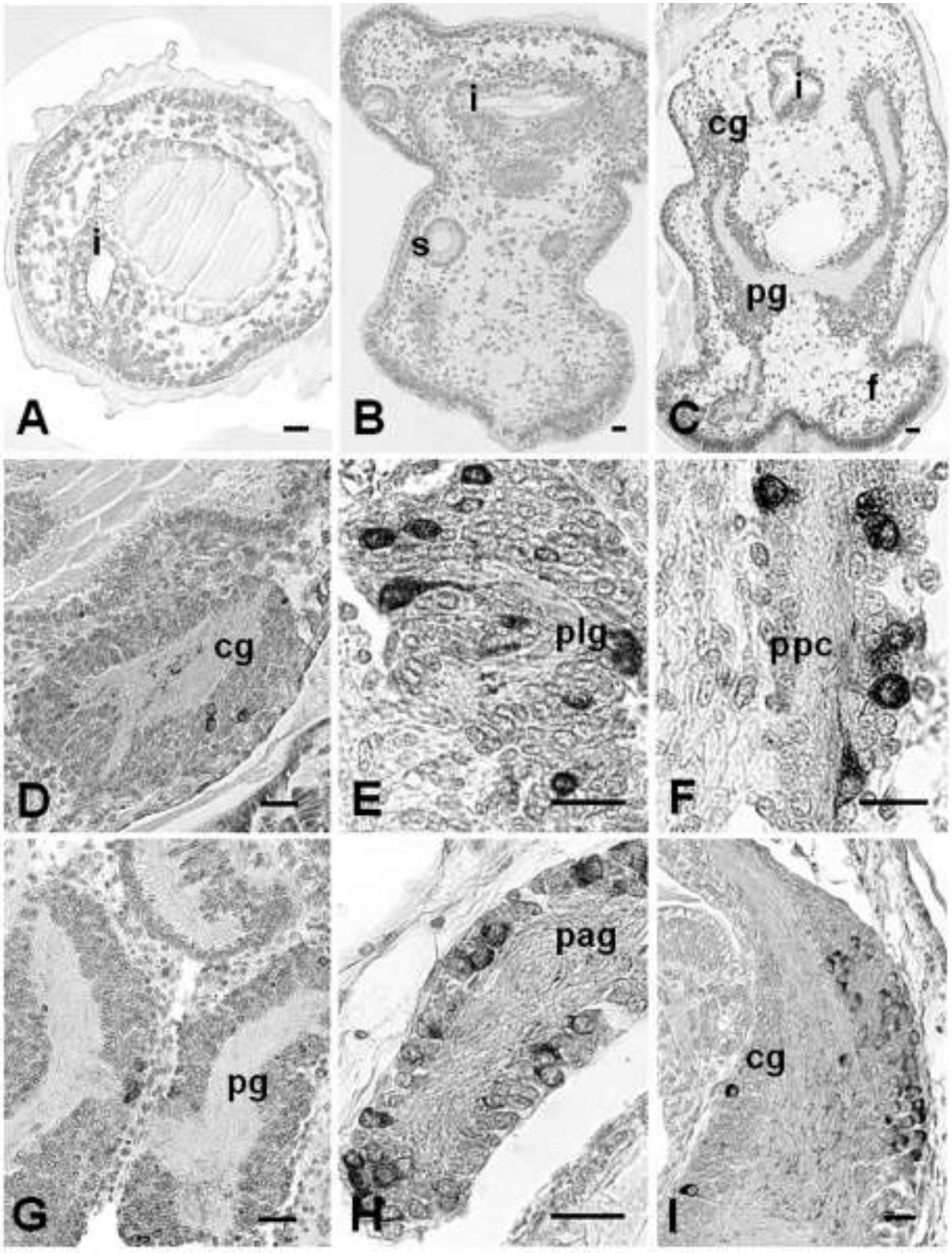

Figure 3. Sections of V. ater embryos (E5: A; E10: B; E15: C; E20: D-F; E30: G; E50: H, I) immunostained with anti-5-HT antibody and counterstained with hematoxylin. Bar = 20 μm. No IR neurons are found till 20% development (A-C) while from stage E20 on, an increasing number of IR neurons is observed in the central ganglia. Sections of cerebral (cg), parietal (pag), pedal (pg), pleural (plg), ganglia, pleuro-parietal nerve cord (ppc) are shown. Foot (f), intestine (i), statocyst (s) .

(Figure 2 D-E). Higher numbers of reactive cells and fibres were also seen in the osphradium (Figure 2F) and gut (Figure 2G-I).

With regards 5-HT-like molecules, no IR cells were observed in the earliest stages of development (Figure 3 A-C), and these were first seen in morphologically organized ganglia of the central nervous system. At stage E20, a few neuron cell bodies and fibres throughout the ganglia began to be immunostained (Figure 3 D-F) and as development proceeded, positive neurons were continuously added (Figure 3 G-I). Before hatching, the number of cells with serotoninergic-like phenotype progressively increased to elaborate the central nervous system. Immunoreactivity was not detected outside the central nervous structures and no peripheral IR neurons were found.

Discussion

The findings of this study show that FMRFamide- and 5-HT-like molecules appear during the embryonic development of *V.ater* at different times and with different expression patterns. FMRFamide-related molecules are detected very early at stage corresponding to 8% development and are first observed in cells morphologically resembling nerve cells in an anterior position where cerebral ganglia will form. Moreover, these cells appear to develop in an anterior to posterior direction, the gradient of neurogenesis, and this sequence differs in other gastropods. This behaviour could be related to different duration and complexity of the embryonic stages. The direct development of V. ater does not include a metamorphic stage and the differentiation of an apical organ which is thought to control the larval behaviours (Dickinson*et al.,* 2000). In *Lymnaea stagnalis*, a direct developing snail with an intracapsular veliger stage, transient neurons IR to FMRFamide were seen at late trochophore/early veliger stage and the first positive cells appeared in the central nervous system only at the beginning of metamorphosis in posterior regions of the embryo, where visceral and parietal ganglia develop. A limited number of neurons expressed these peptides during all intracapsular developmental stages (Croll and Voronezhskaya, 1995; Voronezhskaya and Elekes, 1996; 2003). Posterior neuronal elements, some of which expressed transiently, are also seen in early stages of indirectly developing gastropods such as *Aplysia californica* (Croll and Voronezhskaya, 1995; Dickinson*et al.,* 2000), *Crepidula fornicata* (Dickinson*et al.,* 1999) and *Ilyanassa obsoleta* (Dickinson and Croll, 2003). The appearance of FMRFamide-related molecules in early V. ater neurogenesis may indicate a role in the control of basic development processes, i.e. cell proliferation and migration, and involvement in guiding the subsequent ganglia organization, as a continuous, gradual increase in immunopositivity is observed in all ganglia. The possible function of these molecules as early growth regulatory signals is also supported by their early presence outside the developing nervous system i.e. in the heart primordium. They may be acting in paracrine manner as proposed for other signal molecules that appear in the first stages of embryogenesis (Wall and Hogan, 1994; Ataliotis and Mercola, 1997). In the course of *V. ater* development, as the parts of the digestive tract differentiated, the pattern of the diffuse gut endocrine cells present in adult animals (Franchini*et al.,* 1994) is established, and both open and closed cell types are IR to FMRFamide. No data on the mechanisms controlling the differentiation of these invertebrate cells are available. A previous paper (Franchini, 2002) reported that from early stages of *V.ater* embryogenesis, large mesenchimal cells containing TGF-β1-related molecules surrounded the organizing and differentiating tissue and organs, i.e. the gut regions. These molecules, thought to act as autocrine and paracrine signals of embryonic microenvironment, may together with other growth factors influence gut endocrine cell differentiation. The requirement in gut development of members of the TGF-β superfamily has been demonstrated in *Drosophila* (Reuter*et al.,* 1990).

Regarding 5-HT-like molecules, these emerge later in *V. ater* development than the FMRFamide ones, and are distributed in central ganglia and progressively assembled throughout the embryonic stages. This behaviour is comparable to other gastropod species studied (Goldberg and Kater, 1989; Marois and Croll, 1992; Voronezhskaya and Elekes, 1993) and the differential timing observed in the appearance of the adult 5-HT-like cell pattern reflects the type of development of the examined. In *Aplysia* transient cells are correlated with the differentiation of larval specific organs and are resorbed at metamorphosis when adult serotoninergic system begins to emerge (Marois and Carew, 1997 a; b). The function of 5-HT has been studied

in the neural development of *Helisoma* by *in vitro* and *in vivo* experiments (Goldberg and Kater, 1989; Diefenbach*et al.,* 1995), revealing that this neurotransmitter regulates the development of specific neurons by affecting neurite outgrowth and growth cone motility (Goldberg and Kater, 1989; Goldberg, 1998) and that a neuron may utilize its own transmitter to auto-regulate neurite formation (Diefenbach*et al.,* 1995). The early appearance of FMRF-amide- and 5-HT-like molecules in *V. ater* embryos reinforces the growing evidence of a regulatory role of neurotransmitters in embryogenic processes. In particular, the very early presence of FMRFamide-related factors suggests an involvement of these molecules in the regulation of basic, not only neuronal, cell behaviours, while 5-HT seems to be a more specific neural development signal.

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