

Sequential Developmental Acquisition of Cotransmitters in Identified Sensory Neurons of the Stomatogastric Nervous System of the Lobsters, *Homarus americanus* and *Homarus gammarus*

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ABSTRACT

We studied the developmental acquisition of three of the cotransmitters found in the gastropyloric receptor (GPR) neurons of the stomatogastric nervous systems of the lobsters *Homarus americanus* and *Homarus gammarus*. By using wholemount immunocytochemistry and confocal microscopy, we examined the distribution of serotonin-like, allatostatin-like, and FLRF_{NH2}-like immunoreactivities within the stomatogastric nervous system of embryonic, larval, juvenile, and adult animals. The GPR neurons are peripheral sensory neurons that send proprioceptive information to the stomatogastric and commissural ganglia. In *H. americanus*, GPR neurons of the adult contain serotonin-like, allatostatin-like, and Phe-Leu-Arg-Phe-amide (FLRF_{NH2})-like immunoreactivities. In the stomatogastric ganglion (STG) of the adult *H. americanus* and *H. gammarus*, all of the serotonin-like and allatostatin-like immunoreactivity colocalizes in neuropil processes that are derived exclusively from ramifications of the GPR neurons. In both species, FLRF_{NH2}-like immunoreactivity was detected in the STG neuropil by 50% of embryonic development (E50). Allatostatin-like immunoreactivity was visible first in the STG at approximately E70–E80. In contrast, serotonin staining was not clearly visible until larval stage I (LI) in *H. gammarus* and until LII or LIII in *H. americanus*. These data indicate that there is a sequential acquisition of the cotransmitters of the GPR neurons. J. Comp. Neurol. 408:318–334, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: allatostatin; serotonin; FLRF_{NH2}; colocalization of transmitters; crustaceans; neuropeptides

Rhythmic movements are produced by central pattern generating circuits that, in the adult animal, are often richly modulated by amines and neuropeptides that shape their output to the needs of the animal (Marder and Calabrese, 1996). Many central pattern generating circuits are active during developmental stages while the animal is changing shape and growing. In such cases, the output of the central pattern generating circuit must be altered to suit the neuromuscular apparatus it is driving to produce age-appropriate behaviors. In principle, these alterations could be made by changing the number and kinds of synaptic connections or numbers of neurons in the circuits. Alternatively, stage-appropriate changes in motor

patterns could be produced by alterations in the modulatory control of the circuit. Furthermore, stage-appropriate modulatory substances could play an important role in the

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formation and stabilization of synaptic connections. For these reasons, it is instructive to study the developmental acquisition of the neuromodulatory inputs to a central pattern-generating circuit during the period when it is altering its outputs to adapt to changing body plans and feeding behaviors.

The adult lobster stomatogastric ganglion (STG) consists of approximately 30 neurons, most of which are motor neurons that innervate the muscles of the stomach. The central pattern-generating circuitry consists of interactions among these motor neurons, several interneurons, and terminals of some of the modulatory projection neurons that bring inputs to the STG from more anterior ganglia (Coleman et al., 1992; Harris-Warrick et al., 1992; Maynard, 1972; Mulloney and Selverston, 1974a,b; Nussbaum et al., 1992). The neuropil processes of the modulatory projection neurons contain a large number of amines and neuropeptides (Christie et al., 1997; Marder et al., 1995) that alter the motor patterns produced by the STG (Harris-Warrick et al., 1992; Marder and Weimann, 1992). The STG is formed early in embryonic development, and its full complement of neurons is present before 40% of embryonic development (E40; Casasnovas and Meyrand, 1995; Fénelon et al., 1998; Garzino and Reichert, 1994). The motor patterns produced during late embryonic and larval times are distinct from the adult stomatogastric ganglion motor patterns that appear only after the development of the gastric mill apparatus in postlarval stage IV (LIV; Casasnovas and Meyrand, 1995). Therefore, we wished to determine whether the modulatory substances that are present in the adult were present during embryonic, larval, and juvenile time periods.

In this study, we used wholemount immunocytochemistry followed by laser-scanning confocal microscopy to study the developmental expression of serotonin (5HT)-like, Phe-Leu-Arg-Phe-amide (FLRF_{NH2})-like, and allatostatin-like immunoreactivities in the stomatogastric nervous system. These substances are found colocalized in the sensory gastropyloric receptor (GPR) neurons that project into the stomatogastric ganglion and to anterior regions of the nervous system. The GPR neurons respond to stretch of several of the stomach muscles and provide both phasic and modulatory inputs to the STG (Katz and Harris-Warrick, 1989; Katz et al., 1989; Kiehn and Harris-Warrick, 1992). In the accompanying paper (Fénelon et al., 1999), we describe the developmental expression of three additional neuromodulatory substances, red pigment-concentrating hormone (RPCH), proctolin, and a tachykinin-like peptide.

The previously published description of the development of the stomatogastric motor patterns was obtained by using the European lobster, *Homarus gammarus* (Casasnovas and Meyrand, 1995). However, much of the anatomic work on modulator distribution was done previously with the closely related species, *Homarus americanus* (Beltz et al., 1984; Goldberg et al., 1988; Kushner and Maynard, 1977; Marder et al., 1986; Mortin and Marder, 1991; Mulloney and Hall, 1991; Turrigiano and Selverston, 1991). Therefore, a secondary aim of this work was to determine whether the modulator distribution seen in embryonic, larval, juvenile, and adult animals of these two closely related species shows significant species differences either in the adult or in the timing of their developmental acquisition.

MATERIALS AND METHODS

Animals and dissection

Experiments were performed on embryonic ($n = 24$), larval ($n = 53$), juvenile ($n = 16$), and adult ($n = 12$) *H. americanus* and on embryonic ($n = 9$), larval ($n = 19$), and adult ($n = 7$) *H. gammarus*. For *H. americanus*, embryos and larvae were obtained from a lobster-rearing facility located at the New England Aquarium (Boston, MA). For *H. gammarus*, embryos were collected from egg-bearing female lobsters obtained from a local fishery supply in Arcachon, France, and were kept in large tanks of circulating and aerated 15°C seawater. After hatching, the larvae were transferred into small individual rearing cups flushed with circulating aerated seawater at 15°C and were fed once or twice daily with frozen *Artemia*.

In both species, the percent staging system for lobster embryos (Helluy and Beltz, 1991), based on eye index (EI; Perkins, 1972), was used to determine the age of each embryo. The length and width of the eyes of the animals were measured through the transparent eggshell with an ocular micrometer on a binocular microscope prior to dissection. Each value of EI can be converted into a percentage of the embryonic development (Helluy and Beltz, 1991). *H. americanus* is smaller at hatching than *H. gammarus*; 100% of embryonic development occurs at an EI of 580 for *H. americanus* and at an EI of about 780 for *H. gammarus*. Larval stages were determined by noting the external morphologic features of animals as described in Herrick (1895). The basic features used for larval staging are 1) first stage larva (LI), no swimmerets, trapezoidal telson; 2) second stage larva (LII), external swimmerets now formed; 3) third stage larva (LIII), uropods present; 4) fourth stage postlarval (LIV), quadrangular telson. Juveniles were sized by measuring the carapace length (CL) from the anterior point of the rostrum to the posterior edge of the thorax. This is approximately half the total length of the animal's body (without claws extended).

The eggshells of embryos were cut open, and embryos were removed from the yolk in physiological saline. At all developmental stages, the limbs, antennae, and abdomen were removed. The foregut was dissected, split open along the midline (except in embryos smaller than E70), laid flat, and pinned down onto a Sylgard-lined Petri dish. In older larvae, juveniles, and adults, the stomatogastric ganglion and the proximal ends of its motor nerves were then dissected free from the muscles. In smaller larvae and embryos, the nerves were left in place attached to the stomodeum.

Fixation

The tissues were fixed in a solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C. Preparations from juveniles and adults were fixed in the Petri dish in which they were dissected. Embryos and larvae with the stomodeum attached were fixed by pinning the preparation to the Sylgard, cutting out a small square of Sylgard with the preparation attached, and immersing the Sylgard square and preparation in fixative. This method provided support and reduced damage to the fragile tissues.

Immunocytochemical staining

Indirect immunofluorescence staining of tissues was carried out according to the protocol of Beltz and Kravitz (1983). Briefly, after fixation, the tissues were washed in

0.3% Triton X-100 and 0.1% sodium azide in 0.1 M sodium phosphate buffer, pH 7.4 (PT), five or six times for one hour per wash. The tissues were then incubated overnight in a primary antiserum (described below) or in a mixture of two antisera for double labeling that was diluted in PTA plus 10% goat normal serum (PTA-NGS) to reduce nonspecific staining. The tissues were then washed in PTA as described above and immersed in a secondary antiserum (also diluted in PTA-NGS) that was specific to the species in which the primary antiserum was generated. Finally, the tissues were washed five or six times for one hour per wash in 0.1 M sodium phosphate buffer, pH 7.4. Stained tissues were mounted on glass slides with a solution of 80% glycerol in 0.02 M sodium phosphate buffer, pH 7.4. Tissues were maintained at 4°C throughout staining and washing, and slides were stored at 4°C wrapped in aluminum foil.

Immunologic reagents

The polyclonal serum used to detect allatostatin (AST)-like immunoreactivity was raised in rabbit against allatostatin B₂ (Pratt et al., 1991). The serum (a gift of R. Feyereisen; University of Arizona, Tucson, AZ) was used at a dilution of 1:300. Preincubation of the diluted serum with 10⁻⁶ M AST-3 for one hour at room temperature completely abolished staining in the adult *H. americanus* stomatogastric nervous system.

5HT was detected with two reagents. The first, a polyclonal antiserum generated in rabbit, was purchased from Eugene Tech (Eugene, OR) and was used at a dilution of 1:300. The second, a monoclonal antibody generated in rat, was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY) and was used at a 1:50–1:100 dilution. The staining patterns with these two reagents did not differ, except that nonspecific background staining was lower with the monoclonal antibody. We used preparations stained with the monoclonal antibody for almost all of the data presented in this paper but included three preparations stained with the rabbit antiserum. The distribution during development of FLRF_{NH2}-like immunoreactivity in *H. gammarus* has been described previously (Fénelon et al., 1998).

The extended FLRF_{NH2}-like peptides were detected with a 1:200 dilution of a polyclonal antiserum (20091; anticardioexcitatory peptide; INCSTAR, Stillwater, MN) raised in rabbit against Phe-Met-Arg-Phe-amide (FMRF_{NH2}). Previous work has shown that the predominant FMRF_{NH2}-like peptides in *H. americanus* are Ser-Asp-Arg-Asn (SDRN)- and Thr-Asn-Arg-Asn (TNRN)-FLRF_{NH2} (Trimmer et al., 1987). Because preincubation of this serum with 10⁻⁶ M TNRN-FLRF_{NH2} for one hour at room temperature completely blocked staining in the adult *H. americanus* stomatogastric nervous system, we assume that this serum binds to native FLRF_{NH2} peptides. In addition, we used two other well-characterized antisera to FLRF_{NH2}-like peptides on a limited number of preparations for comparison with the staining obtained with the INCSTAR antiserum. Both 231 (O'Donohue et al., 1984) and 671 (Marder et al., 1987) showed staining patterns similar to that obtained with the commercially available serum. However, in addition, the INCSTAR antiserum stained somata in the STG of some *H. americanus* adults and varicosities in the sheath of the anterior connecting nerves (see Fig. 1). We discuss the somata in this paper, whereas the sheath staining is under further investigation.

We used several different secondary antibodies: rhodamine isothiocyanate (RITC)-labeled goat anti-rabbit (Boehringer-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled

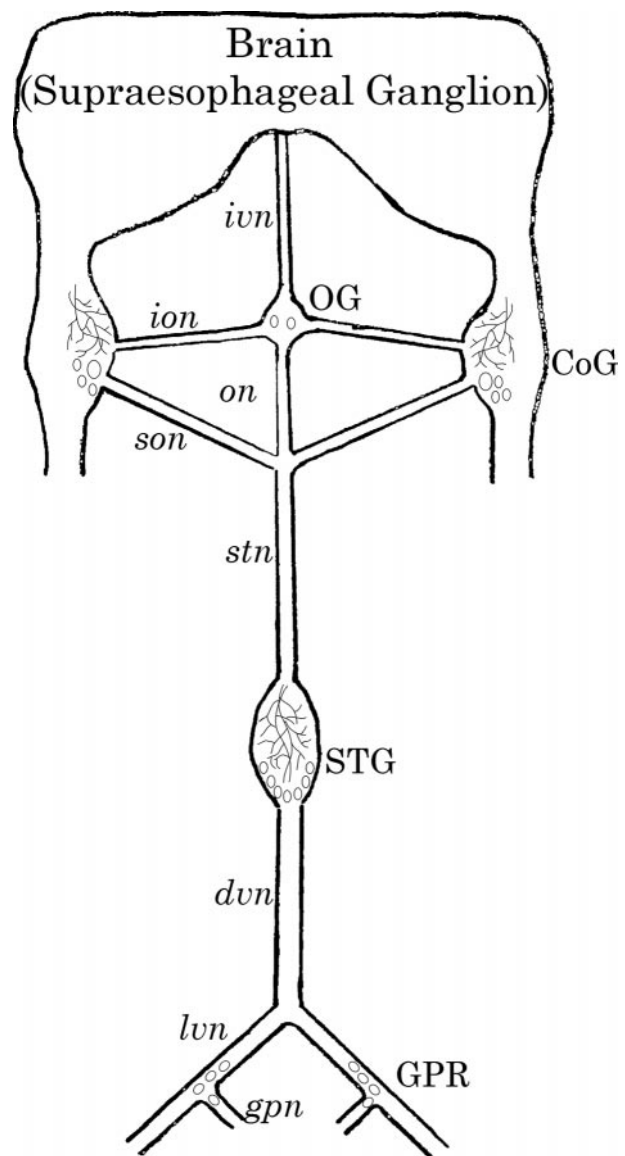


Fig. 1. Schematic diagram of the stomatogastric nervous system (STNS) early in development. The neurons of the stomatogastric ganglion (STG) receive modulatory inputs from more central neurons in the commissural ganglia (CoG) and the esophageal ganglia (OG) and from sensory neurons in the periphery, including the gastropyloric receptor (GPR) neurons. The connecting nerves run between the ganglia and to and from the muscles. The brain, or supraesophageal ganglion, is relatively much larger and closer to the STG early in development than in adulthood. For other comparable summary diagrams, only the adult stomatogastric nervous system is labeled with the names of the ganglia and the nerves; ganglia and cells (s) are labeled on the right half of the diagram, whereas fibers (f) are labeled on the left. dvn, dorsal ventricular nerve; gpn, gastropyloric nerve; ion, inferior esophageal nerve; ivn, inferior ventricular nerve; lvn, lateral ventricular nerve; on, esophageal nerve; son, superior esophageal nerve; stn, stomatogastric nerve.

goat anti-rabbit or anti-rat (Boehringer-Mannheim), and Texas Red-labeled goat anti-rabbit (Accurate Chemical and Scientific Corporation) or anti-rat (Molecular Probes, Eugene, OR) immunoglobulin G (IgG). All secondaries were used at dilutions of 1:75–1:100. Incubation of the tissue with the secondary antiserum without prior incubation

in a primary serum or with a secondary to a species inappropriate for the primary serum resulted in no specific staining.

Imaging

The data in this paper were collected in two laboratories using two laser-scanning confocal microscopes. Most of the figures shown here were viewed and imaged on a Bio-Rad MRC-600 laser-scanning confocal microscope (Cambridge, MA) through $\times 10$ and $\times 20$ air interface objective lenses and $\times 40$ and $\times 100$ oil-immersion objective lenses. The filter blocks used for double labeling were optimized for the separation of Texas Red and FITC. Optical sections were taken approximately every 1–2 μm for embryos and larvae, and every 5 μm for adults and for all low-magnification ($\times 10$) images. These images were compiled into maximum projection "z series." Images were processed with Confocal Assistant (BioRad) and Adobe Photoshop 4.0 software (Adobe Systems, Mountain View, CA) and were printed on a Codonics NP-1600 printer (Codonics Inc, Middleburg Heights, OH). Some data collection and image processing were done by using a Leica TCS 4D (Heidelberg, Germany), as described fully by F  nelon et al. (1998).

RESULTS

A schematic diagram of the larval stomatogastric nervous system is shown in Figure 1.

The STG is connected to more anterior centers through the single stomatogastric nerve (stn). The stn enters the esophageal ganglion (OG), which is connected to the paired commissural ganglia (CoGs) by the inferior esophageal nerves (ions) and the superior esophageal nerves (sons). Many of the modulatory projection neurons that influence the motor patterns of the STG have their somata in the OG and CoGs (Coleman et al., 1992; Dickinson and Nagy, 1983; Nagy and Dickinson, 1983; Nagy et al., 1994; Norris et al., 1996; Nusbaum and Marder, 1989). Another source of modulatory inputs to the STG is found in the GPR neurons (Katz et al., 1989). In *H. americanus*, there are four pairs of these found bilaterally in the peripheral nerves (Katz and Tazaki, 1992).

Developmental distribution of FLRF_{NH2}-like immunoreactivity in *H. americanus*

Figure 2 shows images of wholemount preparations of the STG of embryonic, larval, and adult *H. americanus* stained for FLRF_{NH2}-like immunoreactivity imaged at the same magnification to illustrate the change in size of the STG as the animal grows from embryonic to adult size. The size of the whole STG at E80 is approximately the same as that of a single adult soma. FLRF_{NH2}-like staining is seen in the STG already at E50, the earliest time studied (not shown). Figure 2A shows the FLRF_{NH2}-like staining in an E80 embryo. At this time, the STG neuropil is stained intensely, and fibers are visible in the stn. In addition, there appear to be trailing fibers in the dorsal ventricular nerve (dvn). Figure 2B shows a stained LI preparation. FLRF_{NH2}-like immunoreactivity is present in the neuropil of the STG and in the stn and the motor nerves exiting the STG. Figure 2C shows a stained LII preparation. Although the STG neuropil itself has not changed much in size, the nerves are elongated, and staining is seen not only in the dvn fibers themselves but in the sheath around the nerve. This becomes even more pronounced in the LIII preparation (Fig. 2D). Figure 2E shows the distribution of FLRF_{NH2}-like immunoreactivity in the adult. The sheath staining is

no longer found close to the STG, and there is a dense ramification of stained fibers within the neuropil.

The stained neuropil in the STG could be derived from projections of cells in anterior ganglia or from the GPR neurons. The adult OG shows three to four FLRF_{NH2}-stained somata. Figure 3A shows a wholemount preparation of an OG from an LIII animal. One intensely stained FLRF_{NH2}-like OG soma is clearly visible. Two dimly stained somata also are present. Stained OG neurons were seen as early as E60. The adult CoGs show numerous FLRF_{NH2}-like stained somata. Stained somata and dense neuropil in the CoGs were seen at all times of development examined. An example of this is seen in Figure 3B, in which the dense neuropil is brightly stained, and a cluster of stained somata can be visualized. The GPR sensory neurons in the adult animal show FLRF_{NH2}-like immunoreactivity, and we saw FLRF_{NH2}-like immunoreactivity in the GPR neurons from late embryo (E60) through adult ($n = 16$) stages. The GPR neurons in the embryos and larvae were defined as somata in the lateral ventricular nerves posterior to the extrinsic gastric mill 3 (gm3) muscles. Figure 3C shows three stained GPR neurons in an LIII animal. The GPR neurons in late embryos and early larval stages of *H. gammarus* also show FLRF_{NH2}-like immunoreactivity (P. Meyrand, S. Faumont, and V. F  nelon, unpublished observations).

The differences between the developmental staining patterns in the two species are described below. In *H. gammarus*, several of the STG somata transiently display FLRF_{NH2}-like immunoreactivity during late embryonic and early larval stages (F  nelon et al., 1998). Therefore, we examined carefully the somata of the *H. americanus* STG neurons during embryonic, larval, and juvenile, and adult stages. Figure 4 shows an example of the method we used to visualize and count stained STG somata. Figure 4A is a top view maximum projection of 23 optical sections of a STG from a postlarval LIV *H. americanus*. In this image, the neuropil processes and stained fibers are clearly visible. These sections were taken with the sensitivity range of the confocal microscope set to optimize collection from the brightly stained neuropil to avoid overexposure and loss of resolution because of saturation. At these settings, stained somata are only faintly visible in some sections. To visualize the somata more clearly, a second set of sections was collected at higher gain. Figure 4B shows a projection of six sections from the center of the stack. Note that, with these collection conditions, the oversaturated neuropil appears blotchy, and the boundaries of adjacent varicosities are blurred. Nonetheless, the somata are now clearly visible. Figure 4C shows four sections farther toward the bottom of the stack. Here, a third soma is seen. Figure 4D shows the five bottom sections of the stack, with two additional stained somata. From the analysis of these sections, we conclude that there are five somata in this preparation that show FLRF_{NH2}-like immunoreactivity. This method was used to examine the preparations at all stages from E50 to adults, and the results of this analysis are shown in Table 1.

Even with the careful counts made possible with the confocal microscope, at many stages there was significant variability in the number of cells stained (Table 1). In *H. gammarus*, the staining in somata disappeared by LII (F  nelon et al., 1998). However, in *H. americanus* (Fig. 4, Table 1), the staining persists to much later developmental times and does not disappear until the animals have reached carapace lengths of >25 mm. Surprisingly, in

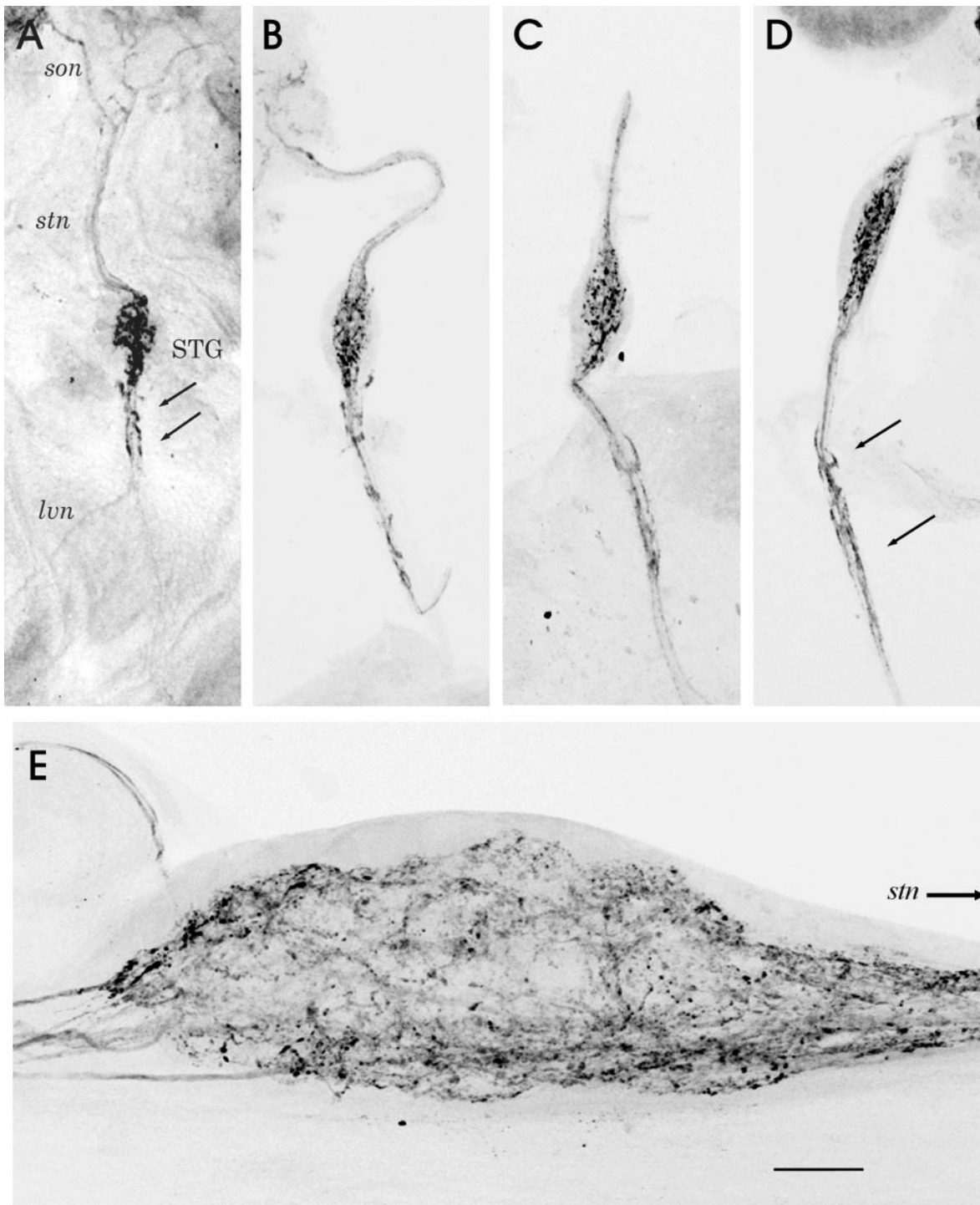


Fig. 2. FLRF_{NH2}-like immunoreactivity in the STG through development and adulthood. In A and D, the arrows indicate a structure staining in the sheath surrounding the dvn. **A:** STG and connecting nerves at 80% of embryonic development (E80). **B:** Larval stage I (LI). **C:** LII. **D:** LIII. **E:** Adult. Scale bar = 100 μ m in E (also applies to A–D).

H. americanus, we found that, although five of five juvenile animals with carapace lengths between 25 mm and 30 mm showed no STG somata staining, and another juvenile with a carapace length of 52 mm also showed no stained somata, approximately half of the adults we examined

did show stained STG somata. With other antisera to FLRF_{NH2}-like immunoreactivity, no STG somata were stained in adults (Marder, 1987), and no STG somata were stained for FLRF_{NH2}-like immunoreactivity in adult *H. gammarus* using the INCSTAR antiserum (Fénelon et al., 1998).

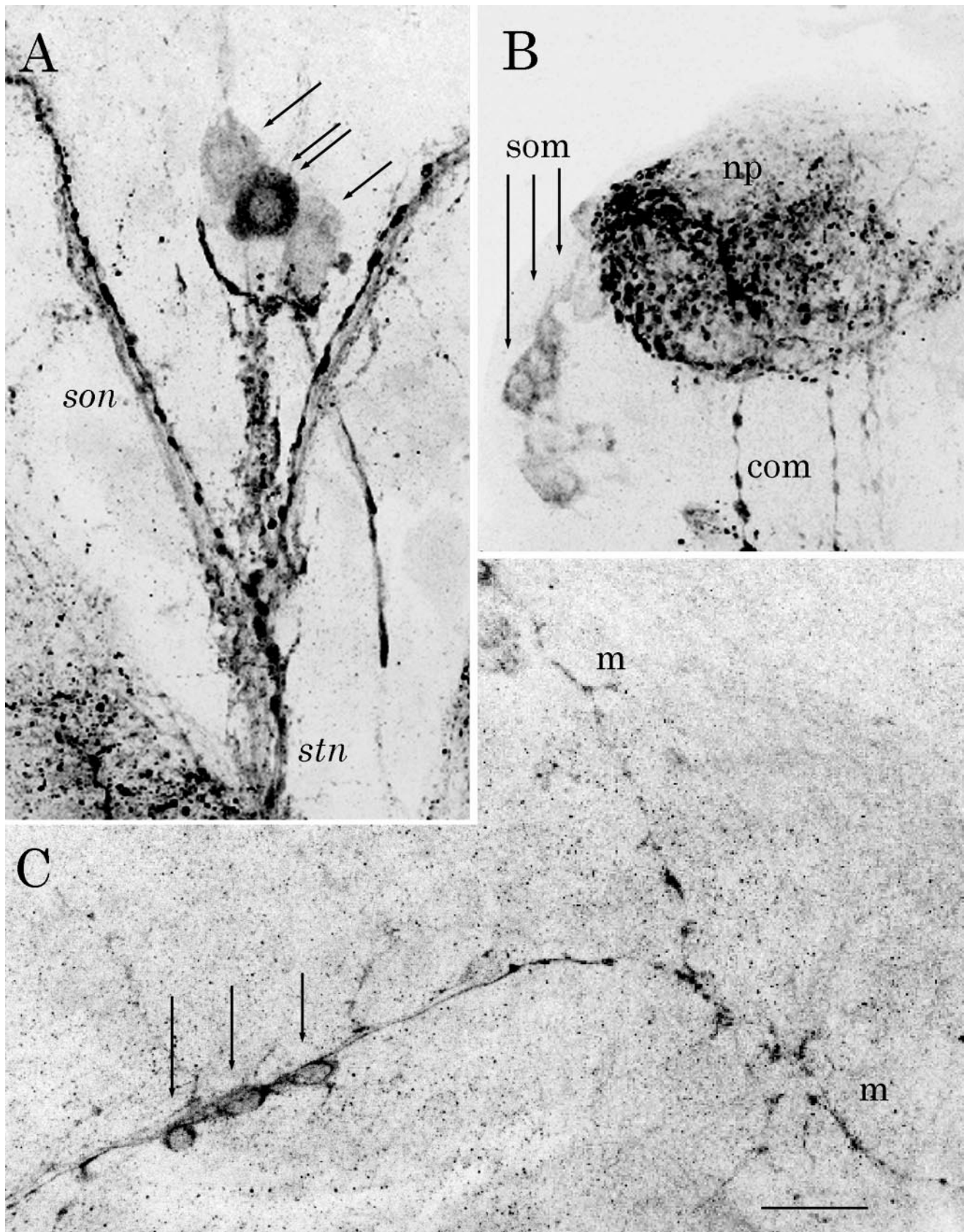


Fig. 3. Potential neuronal sources of STG neuropil FLRF_{NH2}-like immunoreactivity early in development. **A:** LIII OG showing two lightly labeled cells (single arrows) and one darkly labeled cell (double arrows). **B:** LI CoG showing several labeled somata (som), neuropil

(np), and fibers in the commissure (com). **C:** E60 GPR neurons (arrows), including their arborization on pyloric stomach muscles (m). Scale bar = 50 μ m in C (also applies to A,B).

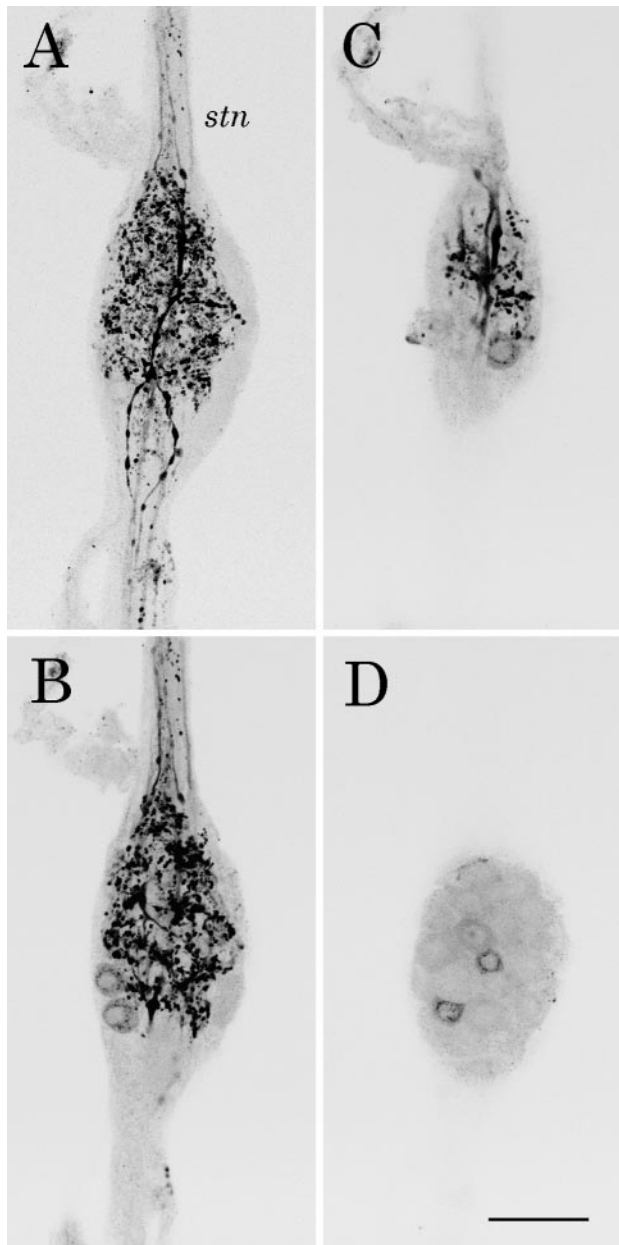


Fig. 4. FLRF_{NH2}-like immunoreactive neuronal somata in a postlarval stage IV (LIV) STG of *Homarus americanus*. Neuronal somata in the STG generally stain much more lightly than the neuropil (see text). Ganglia were imaged twice on the confocal microscope: once to collect cell body number and a second time with lower gain to collect information about the neuropil arborization. **A:** LIV STG imaged for optimum resolution of the neuropil. **B–D:** The same LIV STG imaged for cell body counts. Each of the images in B–D is a summation of four to six optical sections taken about 2 μ m apart. Scale bar = 50 μ m.

Figure 5 summarizes the distribution of FLRF_{NH2}-like immunoreactivity in embryonic, larval, and juvenile *H. americanus* stomatogastric nervous systems. Many of the features of this distribution appear relatively constant throughout development. The stained OG, CoG, and GPR neurons are present already by middle embryonic stages, and the stained neuropil regions in the STG and elsewhere also are present quite early. In contrast, in the embryo, one STG soma begins to stain in late embryonic life, and several

TABLE 1. FLRF_{NH2}-Like Stomatogastric Ganglion Somata During Development of *Homarus americanus*

Stage of development ¹	Number of FLRF-like STG somata
E50–E60	0, 0, 1
E80–E100	0, 1, 1, 1, 1, 3
L I	0, 1, 1, 2, 3
L II	0, 4, 4, 4
L III	0, 3, 3, 5
L IV	3, 3, 5
Juveniles (CL 10–22 mm)	0, 4, 4, 4
Juveniles (CL 25–30 mm)	0, 0, 0, 0, 0
Adults	0, 0, 0, 0, 3, 3, 3, 4, 4

¹STG, stomatogastric ganglion; CL, carapace length; E, embryonic stage of development (e.g., E50 = 50% of embryonic development); L, larval stage.

somata stain in most larval animals and in small juvenile animals. Several somata also stain in 50% of adult animals.

Developmental distribution of AST-like immunoreactivity in *H. americanus* and *H. gammarus*

The distribution of AST-like immunoreactivity in the adult stomatogastric nervous system of the crab, *Cancer borealis* (Skiebe and Schneider, 1994), and other crustaceans, including *H. americanus* (Skiebe, 1998), has been determined previously. AST-like immunoreactivity is found in the GPR neurons in all species studied thus far. The distribution of AST-like immunoreactivity in the adult *H. gammarus* is essentially the same as that seen with *H. americanus*.

We studied the pattern of AST-like immunoreactivity in animals from E50 and later in both *H. americanus* and *H. gammarus*. In contrast to FLRF_{NH2}-like immunoreactivity in the STG, AST-like immunoreactivity was not visible in the STG at E50 (not shown), although AST-like staining was clearly visible in the brain at E50 (not shown). Figure 6A shows that, by E80, the neuropil of the STG is brightly stained for AST-like immunoreactivity. Figure 6B shows the STG neuropil of an LII animal. Figure 6C shows the AST-like staining in an adult *H. gammarus* STG, and Figure 6D shows the CoG from the same animal.

Two neurons stain for AST in the OG of animals from E50 and later. The CoGs show stained somata and neuropil early in development that resemble qualitatively that seen in the adult. AST-like immunoreactivity was present in the GPR cells as early as E80. In some embryonic and early larval preparations, more than four peripheral neurons on each side showed AST-like immunoreactivity. These additional somata may constitute other, unidentified peripheral sensory neurons.

We saw no essential difference between the distribution of AST-like immunoreactivity during development in *H. americanus* and *H. gammarus*. Figure 7 provides a schematic overview of the distribution of AST-like immunoreactivity at E50, LII, and in juveniles (CL = 32 mm). This shows that, in contrast to FLRF_{NH2}-like staining, the AST-like staining in the stomatogastric nervous system is not clearly visible until late embryonic life.

Developmental distribution of 5HT-like immunoreactivity in *H. americanus* and *H. gammarus*

5HT is found in the GPR neurons (Beltz et al., 1984; Katz et al., 1989; Katz and Tazaki, 1992), and 5HT-like immunoreactivity appears in the brain and nerve cord of lobsters in midembryonic development (Beltz et al., 1990).

FLRF_{NH2}-Immunoreactivity

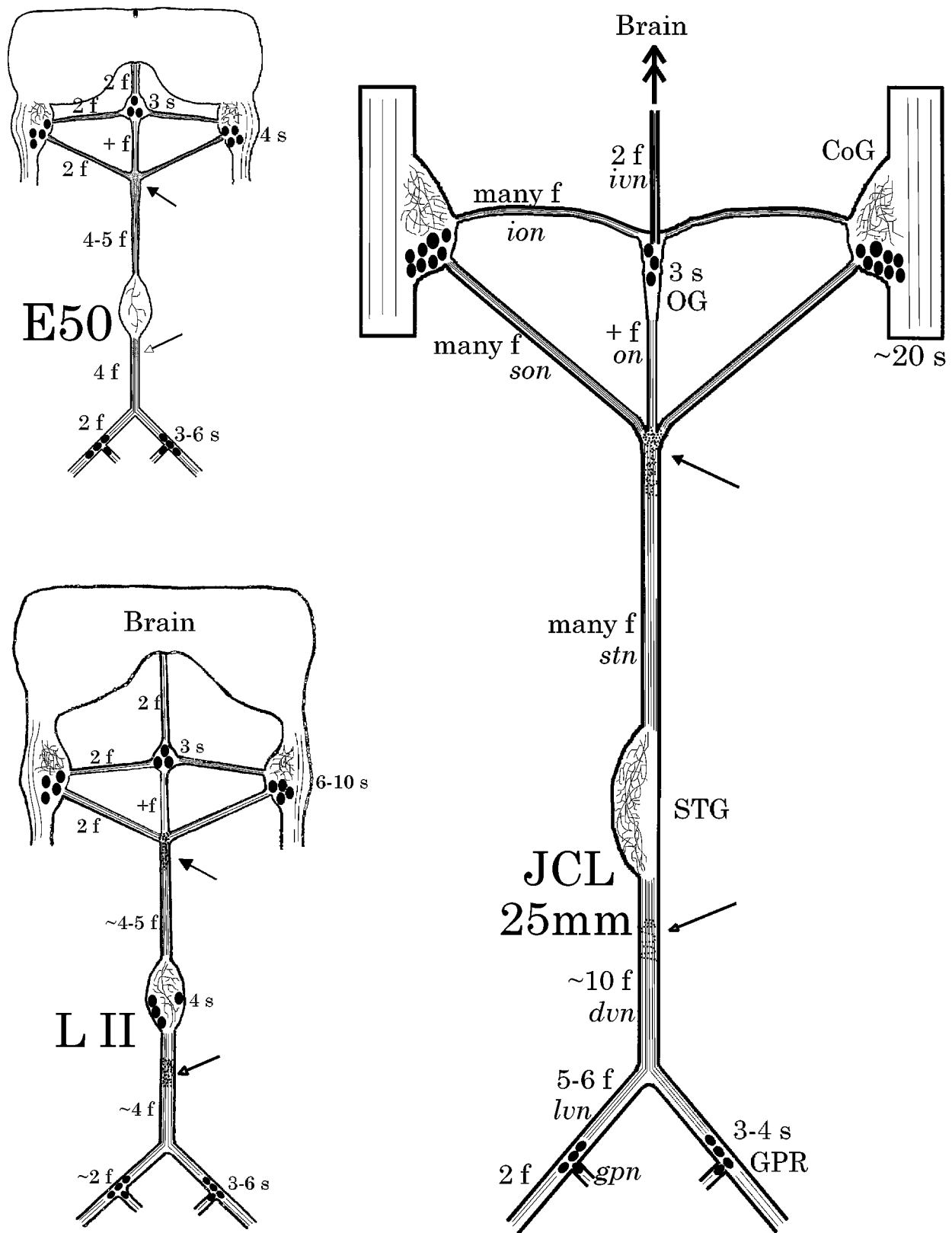


Fig. 5. Schematic summary diagrams of FLRF_{NH2}-like immunoreactivity in E50, LII, and juvenile *H. americanus*. For all ages and in all summary diagrams, somata numbers (s) are indicated on the right half of the diagram, whereas fiber numbers (f) are indicated on the left half. Open arrows indicate staining in the sheath surrounding the

dvn, whereas closed arrows indicate a neuropil-like staining in the nerve at the junction of the stn and sons. A plus sign indicates that, although fibers or somata were stained clearly, a satisfactory count could not be obtained.

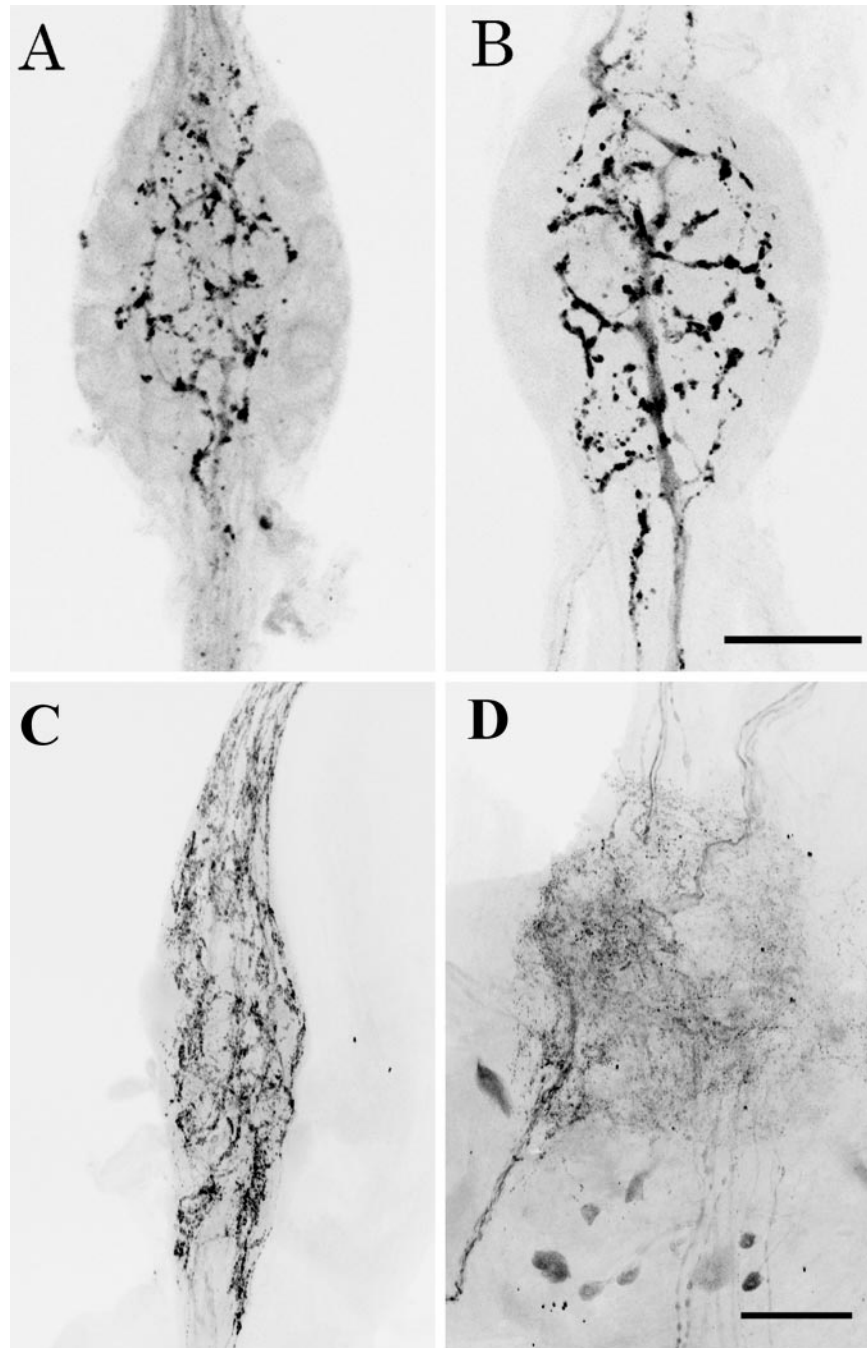


Fig. 6. Allatostatin (AST)-like immunoreactivity in the STG through development. Images of the entire STG of *H. americanus* at E80 (A) and LII (B) and in adult *H. gammarus* STG (C) and CoG (D). Scale bars = 50 μ m in B (also applies to A), 250 μ m in D (also applies to C).

We compared the time course of the appearance of 5HT-like immunoreactivity in the stomatogastric nervous system with the time courses of FLRF_{NH2}-like and AST-like immunoreactivities that are found in the GPR neurons.

5HT-like staining is not clearly present in neuropilar processes in the STG until part way through larval development in both species, although it is detectable earlier in *H. gammarus* than in *H. americanus*. Figure 8A shows the STG of an E80 *H. americanus* stained with the antisero-tonin antibody. Note that there is no obvious staining seen

($n = 8$ of 9), although, at the same time of development, the brain (Fig. 8B1) and the commissural ganglia (Fig. 8B2) are brightly stained. In *H. americanus*, convincing 5HT-like immunoreactivity is present first in the STG neuropil at LII (Fig. 8C; $n = 7$ of 8). However, in *H. gammarus*, the STG neuropil begins to stain by LI ($n = 3$; Fig. 8D). In both species, the staining increases in intensity dramatically by LIII and is bright by LIV (Fig. 8E; *H. americanus*). The staining undergoes no obvious change from this point through adulthood (Fig. 8F; *H. gammarus*) in both species.

AST-Immunoreactivity

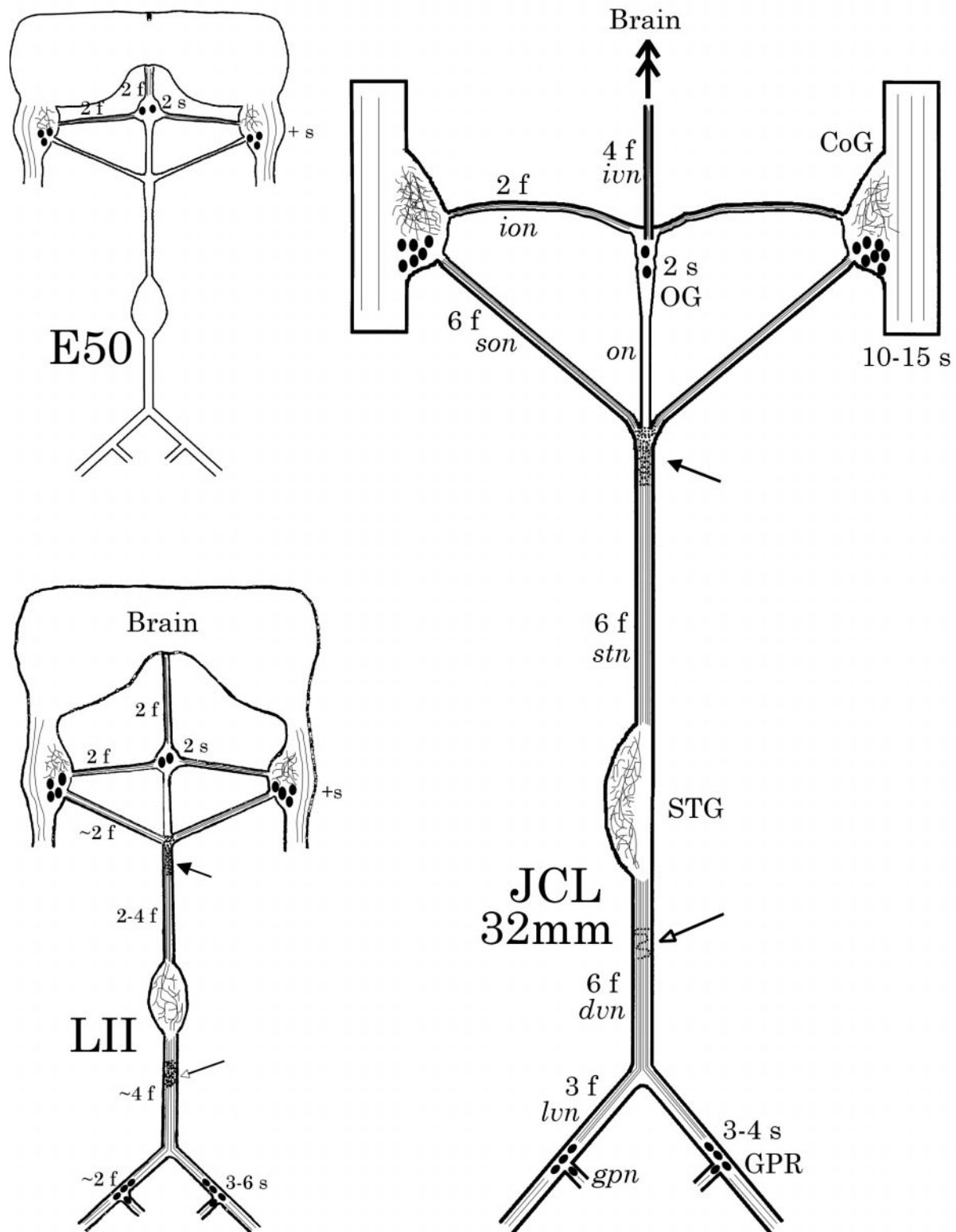


Fig. 7. Summary diagram of AST-like immunoreactivity in E50, LII, and juvenile lobsters. AST-like immunoreactivity is present in the anterior portion of the STNS at E50, but staining is absent in the STG and nerves connected to it. Beginning in late embryonic life and

continuing into larval life, staining appears and grows in intensity and extent through the posterior STNS. The staining in the juvenile STNS is indistinguishable from the adult.

5HT staining in the GPR neurons

Because all of the 5HT staining in the STG is thought to arise from the GPR neurons (Katz et al., 1989), and because it appears considerably later than the AST-like and FLRF_{NH2}-like staining, we did a series of double-label experiments to stain for both AST and 5HT ($n = 6$). An examination of the double labeling in STG neuropilar processes shows that all of the AST-like staining in the neuropil of the STG is found in processes that also contain 5-HT. Figure 9A shows an adult *H. americanus* stained for 5HT (visualized in red), and the same preparation stained for AST (visualized in green) is shown in Figure 9B. Colocalization is shown in yellow in Figure 9C. Note that almost every neuropilar process is yellow and that there are no processes that consistently show only 5HT or AST labeling. This indicates that, if all of the 5HT staining in the neuropil of the STG arises from the GPR neurons, then all of the AST-like staining in the STG neuropil also must arise from the GPR neurons.

Figure 9D–F shows the colocalization of AST and 5HT in the GPR neurons of a juvenile (CL = 32 mm) *H. americanus*. Each of the three GPR neurons shown here contains both 5HT- and AST-like staining, although the relative staining intensities of the two cotransmitters in the three neurons appears slightly different.

To rule out the possibility that some of the double-labeled STG neuropil processes could arise from modulatory projection neurons in the CoGs that also colocalize 5HT and AST, we examined double-labeled CoGs for the existence of somata that colocalize these substances. Figure 9G,H shows adult double-labeled *H. americanus* CoGs. The white arrows (Fig. 9G,H) indicate the double-labeled projection from the son that is the anterior projection of the GPR neurons in the CoGs. The white asterisks (Fig. 9G,H) show the presence of somata that are single labeled for 5HT (red). There also are clearly visible somata showing only single-labeled AST-like-immunoreactive somata (green), but no double-labeled somata were seen in the CoGs. Figure 9I shows a juvenile CoG that, again, shows no double-labeled CoG somata. There are no 5HT-staining processes in the ion or 5HT-stained neurons in the OG. Therefore, there are no candidate projection neurons other than the GPR neurons that could contribute double-labeled AST- and 5HT-immunoreactive processes to the neuropil of the STG. Figure 10 summarizes the distribution of 5HT staining at E50, LI/LII, and in juvenile animals.

The changes in the distribution of the three GPR-derived cotransmitter immunoreactivities through development are summarized in Figure 11. The cotransmitters are staggered in time of first appearance both in the STG neuropil and in the GPR somata.

DISCUSSION

Many neurons contain several cotransmitters, often including small molecules, such as acetylcholine (ACh), amines such as 5HT, and one or several neuropeptides (Kupfermann, 1991; Marder et al., 1995). Except for the case of neuropeptides synthesized from the same peptide precursor, these cotransmitters are produced in development by turning on several different genes. Therefore, it is interesting to ask how the synthesis of the different cotransmitters used in adult neurons is regulated during development. One could imagine the scenario that each cotransmitter is regulated separately to appear at a spe-

cific time in development or that the biosynthesis for all of the cotransmitters is coordinately activated.

Sequential developmental acquisition of cotransmitters

Our data argue that the cotransmitters of the GPR neurons are acquired sequentially during development. The GPR neurons in adult *Homarus* are thought to contain ACh, 5HT (Beltz et al., 1984; Katz et al., 1989), AST-like peptides (Skiebe, 1999), FLRF_{NH2}-like peptides (Katz and Tazaki, 1992), and cholecystokinin-like peptides (Turrigiano and Selverston, 1991). We studied the developmental acquisition of three of these cotransmitter substances, 5HT, the AST-like peptides, and the FLRF_{NH2}-like peptides, in both *H. americanus* and *H. gammarus*. In both species, FLRF_{NH2}-like immunoreactivity is present in the STG by E50 (Fig. 5; Fénelon et al., 1998), whereas AST-like immunoreactivity is not present until approximately E80. In the STG, 5HT-like immunoreactivity is delayed considerably, and it is not present robustly until LI or LII in *H. gammarus* or until LIII or LIV in *H. americanus*. It takes approximately two months for animals to progress from E50 to E80, and each larval stage takes approximately two weeks. Therefore, these differences in stage of appearance represent months of the developing animal's life.

There are considerable data from other systems regarding acquisition of transmitter phenotypes. The time at which a neuron is competent to use a given transmitter varies widely between systems. For example, in some cells, the ability to express a transmitter is present before the neuron is born. Rat sympathoadrenal precursor cells can synthesize both catecholamines and ACh at E14.5, before the final cell division resulting in nondividing neurons (Vandenbergh et al., 1991). For other cells, transmitter determination occurs long after the neuron is born and has reached its target. In the ferret basalocortical pathway, neurons in the basal ganglia project to cortex in the neonate but do not express their mature cholinergic phenotype until choline acetyltransferase is expressed 6 weeks postnatally (Henderson, 1991). Therefore, it is not without precedent that we find GPR neurons do not express their serotonergic phenotype until weeks or months after they are already in place over their muscle targets and arborize in the STG neuropil.

Studies of cotransmitter acquisition in vertebrates often rely on changing percentages of double-labeled cells through development as evidence of a changing cotransmitter complement. The difficulty with interpreting these studies is that, in systems with unidentified neurons, changing percentages of double-labeled cells may reflect a changing population of neurons due to neuronal overproduction and cell death during development rather than a change in cotransmitter expression. Therefore, without evidence of whether neurons in these areas still are dividing or are undergoing programmed cell death, this type of evidence is ambiguous. With this caveat in mind, data from several systems suggest that cotransmitters in a neuron may be expressed at different developmental times (Burnstock, 1995; Forloni et al., 1989; Ni and Jonakait, 1989). The data presented here on the GPR neurons of the stomatogastric nervous system provide a clear case of sequential acquisition of several cotransmitters in a given neuron *in vivo*.

Beltz and coworkers (Beltz and Kravitz, 1987; Beltz et al., 1990) have previously studied the time course of the appearance of 5HT and the peptide proctolin in other

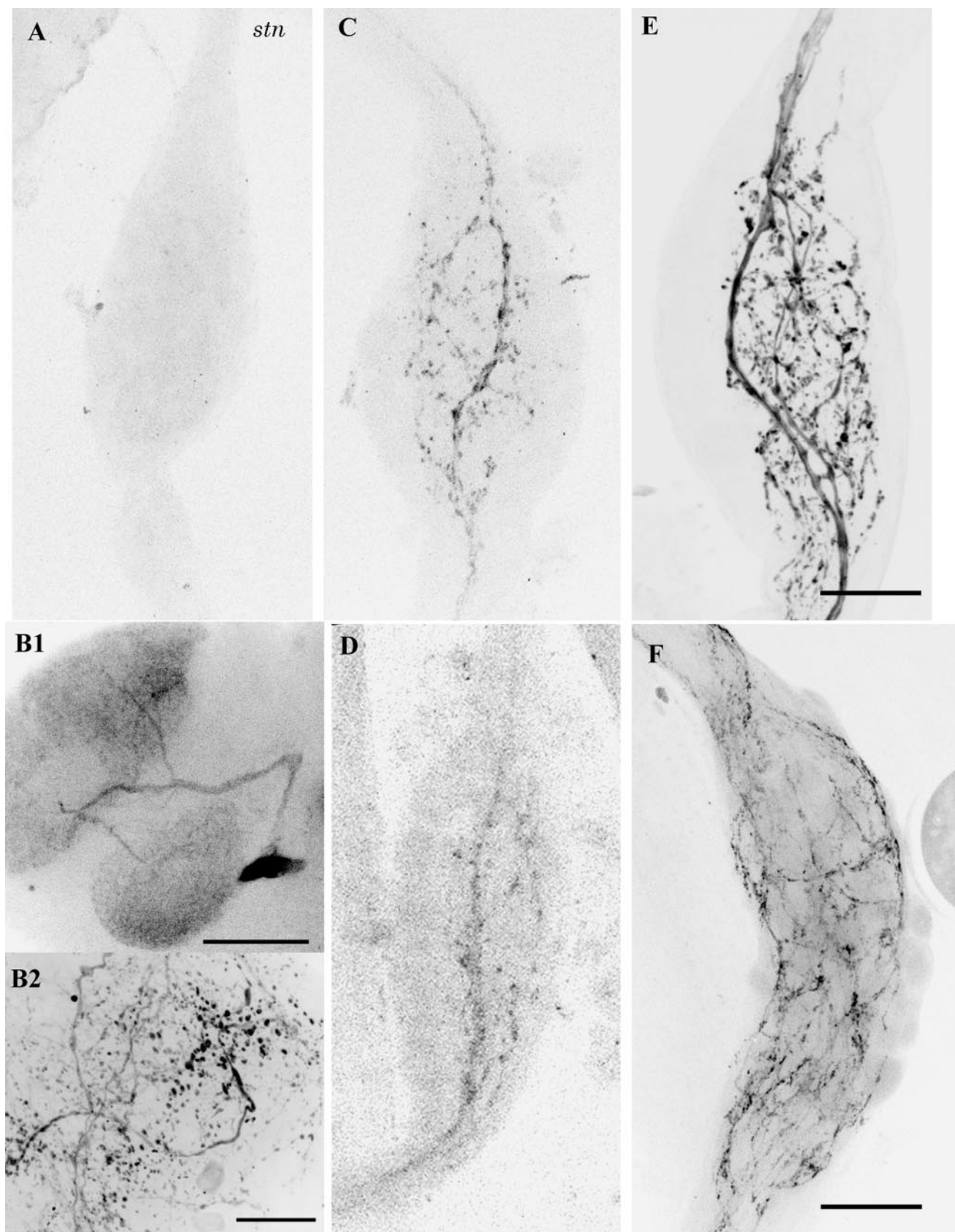


Fig. 8. Serotonin immunoreactivity through development. **A:** E100 STG showing no staining for serotonin. **B1:** E80 brain showing the stained deutocerebral giant neuron and its arbors in the olfactory and accessory lobes. **B2:** L1 CoG showing bright staining for serotonin. **C:** LII *H. americanus* STG showing faint serotonin staining.

D: *H. gammarus* STG showing faint staining for serotonin by L1. **E:** LIV STG showing robust staining (*H. americanus*). **F:** *H. gammarus* adult stained for serotonin. Scale bars = 100 μ m in B1, 50 μ m in B2 and in E (also applies to A,C,D), 250 μ m in F.

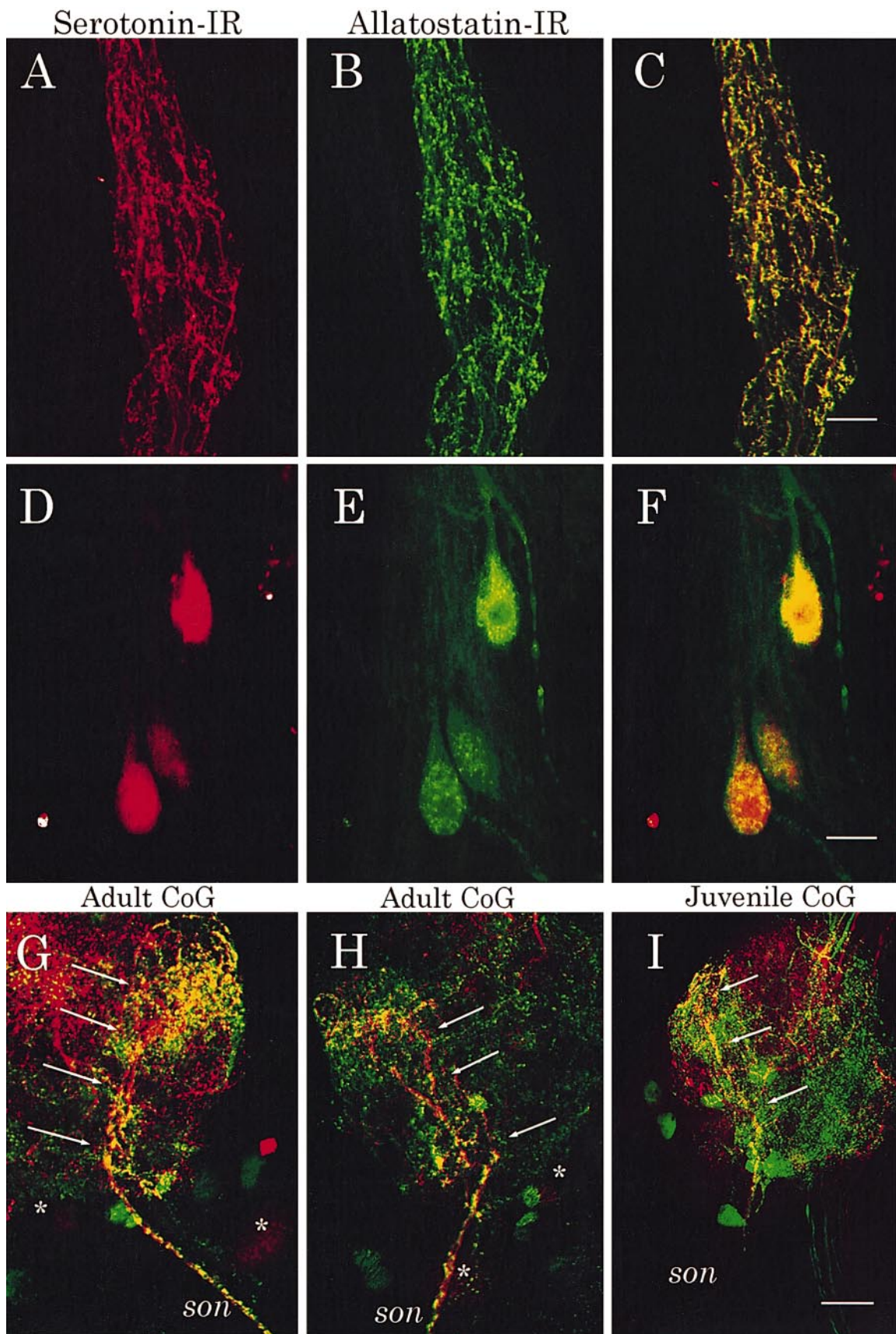


Fig. 9. Double labeling of serotonin- and AST-like immunoreactivities (IR). **A:** Serotonin staining is shown in red. **B:** The same adult STG labeled for AST-IR (shown in green). **C:** Yellow indicates double labeling of fibers and varicosities. All neuropil processes are double labeled. **D:** GPR somata from a juvenile labeled for serotonin. **E:** AST-like immunoreactivity. **F:** Double labeling in the same neurons shown in E. Note that the most intense double labeling is seen in small compartments in the cytoplasm that also show the most intense

labeling for AST-like immunoreactivity. **G,H:** Adult CoG. **I:** Juvenile CoG double labeled for AST- and serotonin-like IR. In G–I, the double-labeled son projection into the neuropil is labeled with arrows. Two serotonin-labeled somata are marked by asterisks in G and H, and several AST-labeled somata are visible in each image, but no double-labeled somata are present. Scale bars = 100 μ m in C (also applies to A,B) and I (also applies to G,H), 25 μ m in F (also applies to D,E).

Serotonin-Immunoreactivity

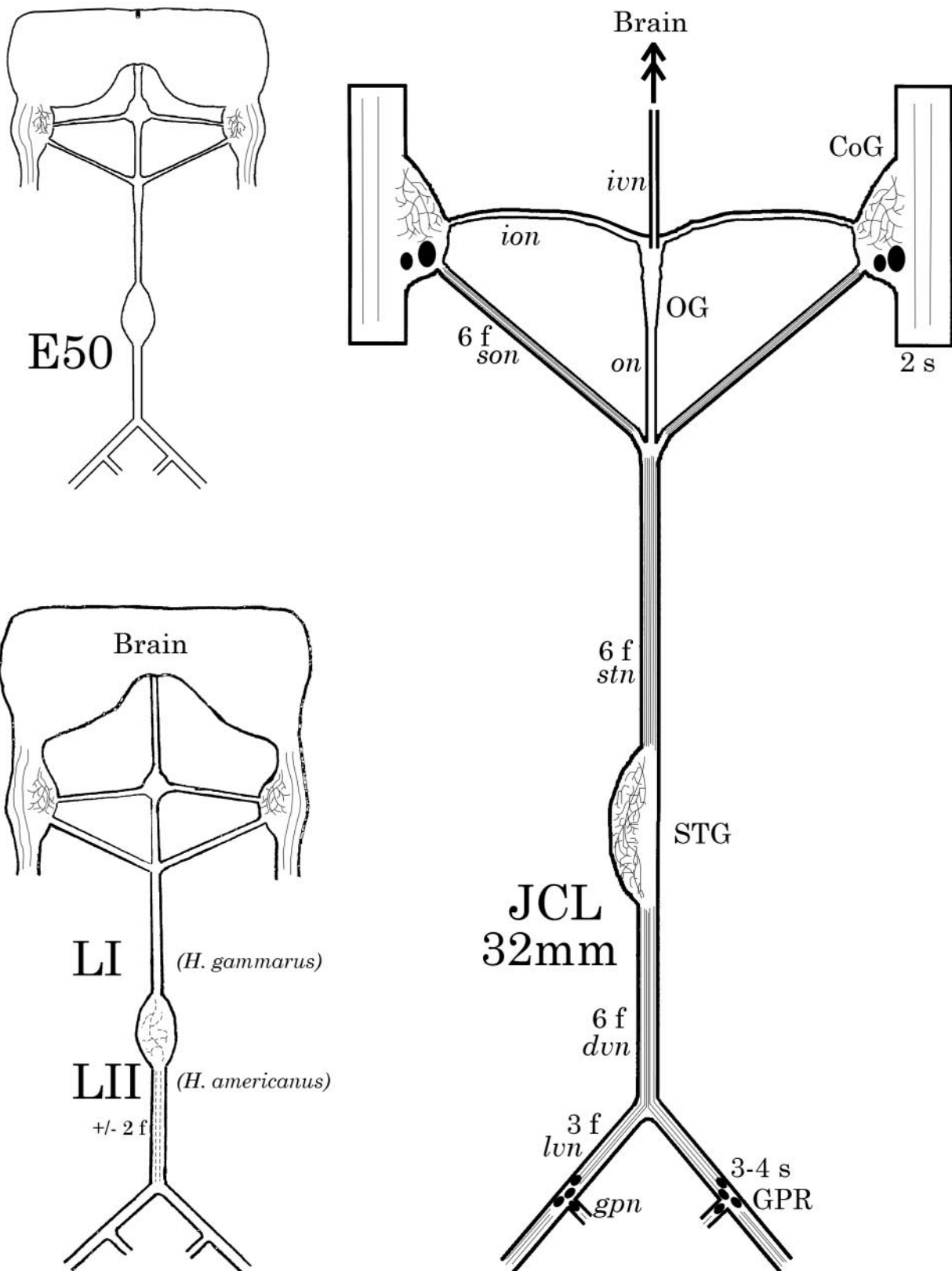


Fig. 10. Summary diagram of serotonin immunoreactivity in E50, LI/LII, and juvenile *H. americanus* and *H. gammarus*. E50 animals do not show serotonin immunoreactivity in the stomatogastric nervous system, except in the CoG. By LI in *H. gammarus* and by LII in

H. americanus, faint staining begins in the STG neuropil, but it is faint or absent in the fibers and GPR neurons. Staining in the juvenile stomatogastric nervous system is indistinguishable from that seen in the adult.

GPR Cotransmitter Immunoreactivity

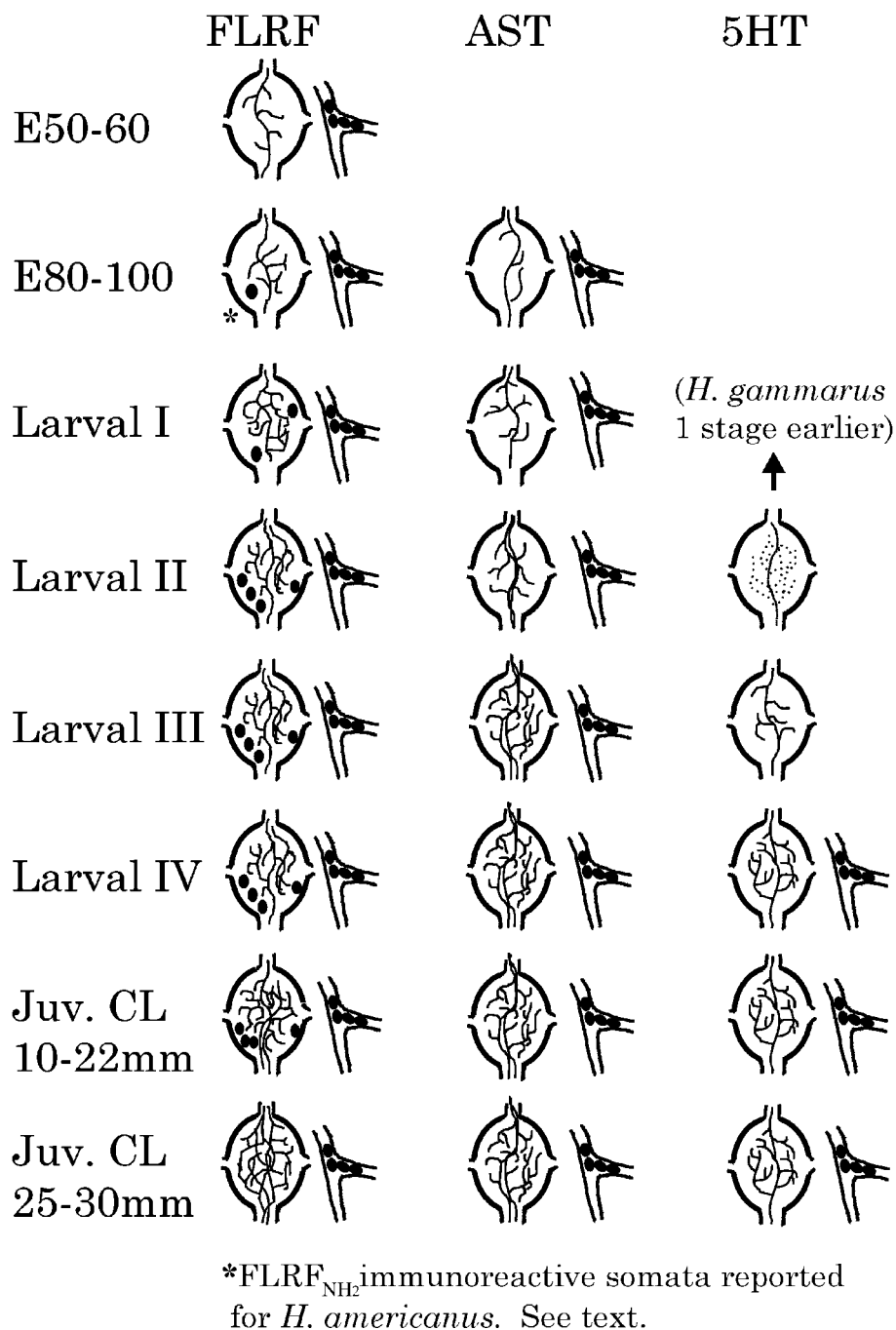


Fig. 11. Summary of changes in GPR cotransmitter expression in *H. americanus* and *H. gammarus* through development. The GPR somata stain for FLRF_{NH₂} by E60, but they do not show AST-like staining until E80 and do not show serotonin staining until later in larval development. The neuropil arbor in the STG stained for FLRF_{NH₂} grows larger through the larval stages, and neuronal somata stain transiently as well. In *H. americanus*, somata staining for FLRF_{NH₂} begins in late embryonic life, rises until it peaks at about

four somata at LII, and remains approximately stable until it achieves a juvenile carapace length (Juv. CL) of 25 mm. Significantly fewer somata stain in *H. gammarus*, and they cease staining by LII (Fénelon et al., 1998). The STG neuropil arbor stained for AST appears at E80 and also grows in size and intensity through the larval stages. The STG neuropil arbor stained for serotonin is not present before L1/LII, but it gains rapidly in intensity and is as intense as AST-like and FLRF_{NH₂}-like immunoreactivity by LIV.

regions of the *H. americanus* nervous system. The T5 and A1 neurons colocalize 5HT and proctolin. In these neurons, 5HT is present by midembryonic life, but proctolin does

not appear until larval stages. In contrast, in the GPR neurons, this order of appearance is reversed, and the peptide cotransmitters appear before the 5HT.

Putative roles of sequential cotransmitter acquisition

Is there a functional purpose for the changing ratio of neuromodulators in input neurons, as is certainly seen in the GPR neurons and is likely to occur in other inputs? Physiology and anatomy in the adult crab have led to the hypothesis that the modulatory effects of two input fibers with a common modulator are distinguished by the cotransmitters that they do not have in common (Christie, 1995). If this is the case, then responses evoked by modulatory inputs during development when cotransmitter complements are changing must be very different from the adult responses. Stomatogastric rhythms do not begin to take on adult form until LIV (Casasnovas and Meyrand, 1995), the point when all of the modulators we examined were present and relatively abundant. Perhaps the changing ratios of cotransmitters allows input neurons to modulate stomatogastric rhythms differently. Thus, a change in the neuromodulatory environment may be responsible for the observed switch at LIV from a single embryonic rhythm to the first indication of the three rhythms produced in the adult by the same neurons.

Possible developmental role of 5HT

In principle, 5HT could influence development of the networks in the STG by two different mechanisms: 1) as a direct modulator of growth and synapse formation and 2) by modulating network activity that then results in long-term changes in synaptic organization and network dynamics. There is a large body of literature that supports the developmental role of 5HT as a growth factor in other systems (Brüning et al., 1997; Diefenbach et al., 1995; Goldberg and Kater, 1989; Haydon and Kater, 1988; Haydon et al., 1984; McCobb et al., 1988; McCobb and Kater, 1988). In *H. americanus*, depletion of 5HT affects the development of the serotonergic dorsal giant neurons and reduces the final size of its target regions, the olfactory and accessory lobes (Benton et al., 1997). In the mouse brain, the 5HT transporter is expressed widely throughout the brain at embryonic stages before synapses have formed; thalamocortical relay neurons do not synthesize 5HT but become 5HT-immunoreactive by taking up extracellular 5HT, probably released by raphe neurons (Lebrand et al., 1996). Thus, these relay neurons may use 5HT as a growth regulatory molecule, as a transient "borrowed transmitter," or both. If 5HT acts as a growth regulatory molecule in the stomatogastric nervous system by affecting neurite outgrowth or by other long-term actions, then a delay between the onset of 5HT immunoreactivity and expression of the gastric mill rhythm may reflect the time over which growth and synaptic reconfiguration take place.

A second (not mutually exclusive) possibility is that 5HT may play a modulatory role in configuring the stomatogastric neural networks into an adult state. 5HT modulates the adult pyloric and gastric mill rhythms in *C. borealis*, *P. interruptus*, and *H. americanus* (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a,b; Katz and Harris-Warrick, 1989; Katz et al., 1989; Kiehn and Harris-Warrick, 1992; Meyrand et al., 1992). Previous work (Casasnovas and Meyrand, 1995) in the lobster *H. gammaurus* showed that embryonic and larval stage animals generate a single rhythmic motor pattern and that the adult gastric mill rhythm begins to emerge from the single embryonic rhythm at about LIV. The expression of 5HT in the STG of the LI *H. gammaurus* precedes the reliable

emergence of the gastric mill rhythm by three larval stages. On the surface, this time delay makes it seem unlikely that the actions of 5HT as a modulator explain the developmental changes in stomatogastric rhythms observed at LIV. However, in the adult, relatively high concentrations of 5HT are necessary to modulate STG rhythms (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a,b; Katz and Harris-Warrick, 1989; Katz et al., 1989; Kiehn and Harris-Warrick, 1992; Meyrand et al., 1992). The staining we observe at LI in *H. gammaurus* and at LII in *H. americanus* was notably less intense than that observed at later stages; in fact, in both species, the subjective brightness of the 5HT staining did not match the adult until LIV. Therefore, increases in the amount of 5HT in the ganglion still may allow for modulatory effects of 5HT to play a role in the emergence of the gastric mill rhythm.

In the adult animal, the GPR neurons are proprioceptors that provide information about the stretch and/or contraction of intrinsic gastric mill muscles (Katz and Harris-Warrick, 1989; Katz et al., 1989). We do not yet know whether the GPR neurons are active early in development, although movements of the stomach muscles are quite vigorous by E50. If the GPR neurons are activated by these early embryonic movements, then their postsynaptic actions on neurons of the STG and the more anterior ganglia will be altered as their cotransmitter complement is successively added during development. Presumably, this will enable the appropriate matching of sensory input to the state of the networks that receive this information as these networks mature.

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