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 FLRF12-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 (FLRF12) 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, FLRF12-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 (L1) 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; FLRF12; 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; Nusbaum et al., 1982). The neuropil processes of the modulatory projection neurons contain a large number of amacrine 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, 1995).

The STG is formed early in embryonic development, and its full complement of neurons is present before 40% of embryonic development (E40; Cassasqvas and Meyrand, 1995; Feenol et al., 1998; Grazino and Reichert, 1994). The motor patterns produced during late embryonic and larval stages 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; Cassasqvas and Meyrand, 1965). Therefore, we wished to determine whether the modulatory substances that are present in the adult are 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 (FLRPN)-like, and allatostatin-like immunoreactivities in the stomatogastric nervous system. These substances are found colocalized in the sensory gastropharylic 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 presumably function as sensory and modulatory inputs to the STG (Katz and Harris-Warrick, 1998; Katz et al., 1989; Kiehn and Harris-Warrick, 1992). In the accompanying paper (Feenol 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 (Cassasqvas and Meyrand, 1965). 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., 1986; 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 Bessemer Aquatorium (Bosto, MA). For H. gammarus, embryos were collected from several areas of the Maritimes and the French Atlantic area. Larvae and adult female lobsters obtained from a local fishery supply in Anacostia, 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 postlarval stage for the system to cover embryos (Helfay and Belts, 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 (Helfay and Belts, 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 (1885). The basic features used for larval staging are 1) first larval stage (L1), no swimmerets, trapezoidal telsaon; 2) second stage larva (LII), external swimmerets now formed; 3) third stage larva (LIII), uropods present; 4) fourth stage postlarval (LIV), quadrangular telsaon. Juvenile stages were determined by 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 forogut 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 length and width of the motor neurons 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 removing 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 antisemur (described below) or in a mixture of two antiseria 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 antisemur (also diluted in PTA-NGS) that was specific to the species in which the primary antisemur 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 50% 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 B 5 (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. americana stomatogastric nervous system.

5HT was detected with two reagents. The first, a polyclonal antisemur 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 (Weehawken, N.Y.) 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 preplurions stained with the monoclonal antibody for almost all of the data presented in this paper but included three preparations stained with the rabbit antisemur. The distribution during development of FLRFamide-like immunoreactivity in H. gammarus has been described previously (Fenelon et al., 1989).

The extended FLRFamide-like peptides were detected with a 1:200 dilution of a polyclonal antisemur (2009;1; anticardioexcitatory peptide; INCSF, Stullwater, MN) raised in rabbit against Phe-Met-Arg-Phe-amide (FMRFamide). Previ-
ous work has shown that the predominant FMRFamide-like peptides in H. americana are Ser-Asp-Arg-Am (SDDR)- and Thr-Asp-Arg-Am (TNR)-FLRFamide (Timmer et al., 1987). Because preincubation of this serum with 10^-6 M TNKR-FLRFamide for one hour at room temperature completely blocked staining in the adult H. americana stoma-
togastic nervous system, we assume that this serum binds to native FLRFamide peptides. In addition, we used two other well-characterized antisera to FLRFamide-like peptides on a limited number of preparations for comparison with the staining obtained with the INCSF antisemur. Both 291 (ODonohue et al., 1984) and 671 (Marder et al., 1987) showed staining patterns similar to that obtained with the commer-
cially available serum. However, in addition, the INCSF antisemur stained somata in the STG of some H. americana 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: rhoda-
mine isothiocyanate (FITC)-labeled goat anti-rabbit (Boehr-
inger-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or anti-rat (Boehr-
inger-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or anti-rat (Boehringer-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or anti-rat (Boehringer-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or anti-rat (Boehringer-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL), fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit or anti-rat (Boehringer-Mannheim, Indianapolis, IN) or anti-rat (Pierce, Rockford IL).
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 x10 and x20 objectives and x40 and x100 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 (×10) images. These images were compiled into maximum projection "z-series." Images were processed with Confocal Assistant (BiRad) and Adobe Photoshop 4.0 software (Adobe Systems, Mountain View, CA) and were printed on a Codonics NP-1600 printer (Codenics Inc, Middleburg Heights, OH). Some data collection and image processing were done by using a Leica TCS 4D (Heidelberg, Germany), as described fully by Fénelon 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 pair of commissural ganglia (CoGs) by the inferior esophageal nerves (ions) and the superior esophageal nerves (se). 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., 1989; 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 FLRFamide-like immunoreactivity in H. americanus

Figure 2 shows images of whole-mount preparations of the STG of embryonic, larval, and adult H. americanus stained for FLRFamide-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. FLRFamide-like staining is seen in the STG already at E50, the earliest time studied (not shown). Figure 2A shows the FLRFamide-like staining in an E50 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 LiI preparation. FLRFamide-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 LiI preparation (Fig. 2D). Figure 2E shows the distribution of FLRFamide-like immunoreactivity in the adult. The sheath staining is no longer found close to the STG, and there is a dense ramifications 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 stage shows three or four FLRFamide-stained somata. Figure 3A shows a whole-mount preparation of an OG from a LiI animal. One intensely stained FLRFamide-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 FLRFamide-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 FLRFamide-like immunoreactivity, and we saw FLRFamide-like immunoreactivity in the GPR neurons from late embryo (E90) 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 mg3) muscles. Figure 3C shows three stained GPR neurons in an LiI animal. The GPR neurons in late embryos and early larval stages of H. gomma- rhus also show FLRFamide-like immunoreactivity (R. Meyrand, S. Faumont, and V. Fénelon, unpublished observations).

The differences between the developmental staining patterns in the two species are described below. In H. gommarus, several of the STG somata transiently display FLRFamide-like immunoreactivity during late embryonic and larval stages (Fénelon 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 FLRFamide-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. gommarus, the staining in somata disappeared by LiI (Fénelon et al., 1995). 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
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 FLRFamide-like immunoreactivity, no STG somata were stained in adults (Marder, 1987), and no STG somata were stained for FLRFamide-like immunoreactivity in adult H. armatus using the INCSTAR antiserum (Fenelon et al., 1998).
Fig. 3. Potential neuronal sources of STG neuropil FLRβ-actin-like immunoreactivity early in development. A: Li31-495 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 GPB neurons (arrows), including their arborization on pyloric stomach muscles (m).
Scale bar = 50 μm in C (also applies to A,B).
Table 1. FLRF<sub>NH2</sub>-like Stomatogastric Ganglion Somata During Development of Homarus americanus

<table>
<thead>
<tr>
<th>Stage of development&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of FLRF-like STG somata</th>
</tr>
</thead>
<tbody>
<tr>
<td>E50-E590</td>
<td>0, 8, 1</td>
</tr>
<tr>
<td>E40-E100</td>
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</tr>
<tr>
<td>LI</td>
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</tr>
<tr>
<td>LIi</td>
<td>0, 4, 4</td>
</tr>
<tr>
<td>LIIV</td>
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</tr>
<tr>
<td>Juvenile (CL = 10-22 mm)</td>
<td>3, 3, 5</td>
</tr>
<tr>
<td>Juvenile (CL = 23-30 mm)</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Adulc</td>
<td>0, 0, 0, 0, 0, 0, 3, 3, 3, 4, 4</td>
</tr>
</tbody>
</table>

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

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 crustacea, 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<sub>NH2</sub>-like immunoreactivity in the STG, AST-like immunoreactivity was not visible in the STG at E50 (not shown), although A5-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 L1I 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 CoG showed 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, LI1, and in juveniles (CL = 32 mm). This shows that, in contrast to FLRF<sub>NH2</sub>-like staining, the AST-like staining in the stomatogastric nervous system is not clearly visible until late embryonic life.
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 ssn. A plus sign indicates that, although fibers or somata were stained clearly, a satisfactory count could not be obtained.
We compared the time course of the appearance of SHT-like immunoreactivity in the stomatogastric nervous system with the time courses of FLRFamide-like and AST-like immunoreactivities that are found in the GPR neurons.

SHT-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 antisero트onin 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 SHT-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 L1 (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.
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-like staining, double-labeling experiments to stain for both AST and 5HT (n = 6). An examination of the double labeling in STG neuropil processes shows that all of the AST-like staining in the neuropil of the STG is found in processes that are 5HT-positive. Figure 9A demonstrates 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 neuropil 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, 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 neuronal 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 an adult double-labeled CoG. 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 soma that is single labeled for 5HT (red). There also are clearly visible somata showing only single-labeled AST-like-immunoreactive somata (gray) and double-labeled somata in the CoGs. Figure 9I shows that, as in Figure 9A, there is no double-labeled CoG somata. There are no 5HT-staining processes in the ion or 5HT-stained neurons in the CoG. Therefore, there are no putative 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, LI1, 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 turnover on 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 specific 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 (Skrobe, 1998; FLRF-like peptides (Katz and Tazaki, 1992), and cholecystokinin-like peptides (Turrigino and Selverston, 1991). We studied the developmental acquisition of these cotransmitter substances, 5HT, the AST-like peptides, and the FLRF-like peptides, in both H. americanus and H. gammarus. In both species, FLRF-like-immunoreactivity is present in the STG by E50 (Fig. 6; Penedon et al., 1998), whereas AST-like immunoreactivity is not present until approximately E80. In the STG, 5HT-immunoreactivity is delayed considerably, and it is not present robustly until LI or LI1 in H. gammarus or until LI1 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 sympathetic repressor 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 expression occurs long after the neuron is born and has reached its target. In the fat body basal compartment, 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 (Burnstock et al., 1989). In the fat body, the transmitters are expressed over their muscle targets and arborize in the developing 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; Forlani et al., 1999; N. and Jonakait, 1988). 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: E300 brain showing the stained dextrorotary giant neuron and its arbors in the olfactory and accessory lobes. B2: Li CSG showing bright staining for serotonin. C: LII H. americanus STG showing faint serotonin staining.

D: H. gmonaric STG showing faint staining for serotonin by LI. E: LIV STG showing robust staining (H. americanus). F: H. gmonaric adult stained for serotonin. Scale bars = 100 μm in B1, 50 μm in B2 and in E (also applies to A,C,D), 250 μm 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 varicocities. All neuropil processes are double labeled. D: OPR somata from a juvenile labeled for serotonin. E: AST-like immunoreactivity. F: Double labeling in the same neuron 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 som 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 μm in C (also applies to A,B) and I (also applies to G,H). 25 μm in F (also applies to D,E).
Fig. 10. Summary diagram of serotonin immunoreactivity in E50, LI, LII, and juvenile *H. americanus* and *H. gommerus*. E50 animals do not show serotonin immunoreactivity in the stomatogastric nervous system, except in the CoG. By LI in *H. gommerus* 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

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*FLRF<sub>NRE</sub>, immunoreactive somata reported for H. americanus. See text.

Fig. 11. Summary of changes in GPR cotransmitter expression in H. americanus and H. gammarus through development. The GPR somata stain for FLRF<sub>NRE</sub> by E50, but they do not show AST-like staining until E80 and do not show serotonin staining until later in larval development. The neuropil arbors in the STG stained for FLRF<sub>NRE</sub> grows larger through the larval stages, and neuronal somata stain transiently as well. In H. americanus, somata staining for FLRF<sub>NRE</sub> 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 (Foden et al., 1988). The STG neuropil arbor stained for AST appears at E50 and also grows in size and intensity through the larval stages. The STG neuropil arbor stained for serotonin is not present before LII/LIII, but it gains rapidly in intensity and is as intense as AST-like and FLRF<sub>NRE</sub>-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 neuronomodulators in input neurons, as is certainly seen in the GPR neurons and is likely to occur in other inputs? Physiology and anatomy in a growing fabric in other hypotheses that the modulatory effects of two input fibers with a common modulator are distinguished by the cotransmitter.

If this is the case, then responses evoked by modulatory inputs during development when co-transmitter complements are changing must be very different from the adult response. Modulatory neurotransmitters changes shown in the adult forebrain and LIV (Casanovas and Meyrand, 1995) the point at which all of the modulators we examined were present and relatively abundant. Perhaps the changing ratios of cotransmitters, by taking up extracellular glutamate transmitter and modulate striatal glutamate differences similarly. Thus, a change in the neuronal 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 synaptic 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 in growth factor发挥作用 in other systems (Brüning et al., 1996; Diefenbach et al., 1995; Goldberg and Kater, 1988; Hayden and Kater, 1988; Hayden et al., 1984; McCub and Kater, 1988). In H. americanus, depletion of 5HT affects the development of the serotonergic dorsal giant neurons and reduces the size and shape of their target regions, the olfactory and accessory lobes (Bentzon et al., 1991). 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-responsive after taking up extracellular 5HT, probably released by raphe neurons (Lebrard et al., 1996). Thus, these relay neurons may use 5HT as a growth regulatory molecule, as a transient "bowed transmitter," or both. If 5HT acts as a growth regulatory molecule in the striatal motoneuronal system by affecting neuromuscular outgrowth or by other long-term actions, then a delay in the onset of 5HT immunoreactivity and expression of the gastric mil rhythm may reflect the time over which growth and synaptic reorganization take place.

A second (not mutually exclusive) possibility is that 5HT may play a modulatory role in configuring the striatal glutamatergic neural networks into an adult state. 5HT modulates the adult pyloric and gastric mill rhythms in C. borealis, and H. americanus (Beltz et al., 1984; Flamm and Harris-Warrick, 1996; Kitz and Kater, 1989; Kiehn and Harris-Warrick, 1992; Meyrand et al., 1992). Previous work (Casanovas and Meyrand, 1995) in the lobster H. gammarus 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. gammarus 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, 1986; Kitz and Kater, 1989; Kiehn and Harris-Warrick, 1992; Meyrand et al., 1992). The staining we observe at LI in H. gammarus 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 contractions of the stomatogastric muscles (Katz and Harris-Warrick, 1989; Kiehn and Harris-Warrick, 1992). We do not yet know whether the GPR neurons are active early in development, although movements of the stomach muscles are quite vigorous by ES6. If the GPR neurons are activated by these early embryonic movements, then their postganglionic 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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LITERATURE CITED


