Sequential Developmental Acquisition of Neuromodulatory Inputs to a Central Pattern-Generating Network

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ABSTRACT
The activity of the adult stomatogastric ganglion (STG) depends on a large number of amnergic and peptidergic modulatory inputs. Our aim is to understand the role of these modulatory inputs in the development of the central pattern-generating networks of the STG. Therefore, we analyze the developmental and adult expressions of three neuropeptides in the stomatogastric nervous system of the lobsters Homarus americanus and Homarus gammarus by using wholemount immunocytochemistry and confocal microscopy. In adults, red pigment-concentrating hormone (RPCH)-like, proctolin-like, and a tachykinin-like immunoreactivity are present in axonal projections to the STG. At 50% of embryonic development (E50), all three peptides stain the commissural ganglia and brain, but only RPCH- and proctolin-like immunoreactivities stain axonal arbors in the STG. Tachykinin-like immunoreactivity is not apparent in the STG until larval stage II (LII). The RPCH-immunoreactive projection to the STG consists of two pairs of fibers. One pair stains for RPCH immunoreactivity at E50; the second RPCH-immunoreactive pair does not stain until about LII. One pair of the RPCH fibers double labels for tachykinin-like immunoreactivity. The adult complement of neuromodulatory inputs is not fully expressed until close to the developmental time at which major changes in the STG motor patterns occur, suggesting that neuromodulators play a role in the tuning of the central pattern generators during development. J. Comp. Neurol. 408:335–351, 1999.
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Indexing terms: red pigment-concentrating hormone; tachykinin; proctolin; stomatogastric ganglion; Homarus gammarus; Homarus americanus

The large number of different neuropeptides found in the nervous system as well as their complicated patterns of colocalization suggest that they could provide delicate and precise timing cues during development. Therefore, we were interested in examining the detailed chronology of neuropeptide acquisition during the development of a relatively well-understood central pattern-generating circuit that is richly modulated in the adult. The stomatogastric nervous system of decapod crustaceans provides just this opportunity. There are at least 15 different substances that provide neuromodulatory input to the stomatogastric nervous system in one or more species of decapod crustaceans (Beltz et al., 1984; Blitz et al., 1995; Christie et al., 1994, 1995a,b, 1997; Cournil et al., 1990, 1995; Goldberg et al., 1988; Kushner and Barker, 1983; Kushner and Maynard, 1977; Marder, 1987; Marder et al., 1986, 1987; Morton and Marder, 1991; Mulloney and Hall, 1991; Nusbaum and Marder, 1988; Turrigiano and Selverston, 1991; Welmann et al., 1993, 1997).

Many of these substances are found in descending modulatory neurons that project into the neuropil of the stomatogastric ganglion (STG). Activation of identified modulatory projection neurons reconfigures the networks found in the adult STG, so that the same group of neurons

Grant sponsor: NIH; Grant number: NS17813; Grant sponsor: The McKnight Foundation; Grant sponsor: The Human Frontiers Science Program Organization; Grant sponsor: NATO; Grant number: CRG 9710073; Grant sponsor: The W.M. Keck Foundation.
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Received 3 August 1998; Revised 23 December 1998; Accepted 29 December 1998

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can produce a wide variety of different motor patterns (Coleman et al., 1995; Dickinson and Nagy, 1983; Dickinson et al., 1988, 1990; Meyrand et al., 1991, 1994; Nagy and Dickinson, 1983; Norris et al., 1994, 1996; Nusbaum and Marder, 1989a,b). These reconfigurations occur as neuromodulatory substances alter intrinsic and synaptic properties in the STG network (Bal et al., 1994; Cazalets et al., 1987; Elson and Selverston, 1992; Harris-Warrick et al., 1995a,b; Hooper and Marder, 1987; Johnson and Harris-Warrick, 1990; Johnson et al., 1995; Kiehn and Harris-Warrick, 1992a,b; Weimann et al., 1997). Consequently, the full range of behavioral flexibility found in the adult requires these extensive modulatory mechanisms, and changes in the modulators found during development could alter the patterns of activity in the networks during development.

Previous work on the development of the STG motor patterns of the lobster, Homarus gammarus (Casasnovas and Meyrand, 1995), showed that, during embryonic development, the STG produces a single rhythm. When the animals reach postlarval stage IV (LIV), the adult pyloric and gastric mill rhythms begin to appear. These important developmental modifications in the motor patterns produced by the STG occur after the full complement of STG neurons is present and without any changes in neuronal number in the STG (Fénelon et al., 1998). One possibility is that the changes in motor pattern observed during development are a direct consequence of the acquisition of specific modulatory inputs to the STG. This could occur because of the acute action of specific modulatory substances to tune the networks on the STG or if these neuromodulators act as growth signals or to specify synaptic strength (Baird et al., 1996; Haydon et al., 1984; Lauder, 1993; Mattson et al., 1988; Mccobb and Kater, 1988; Mccobb et al., 1988; Okado et al., 1992). For these reasons, it is important to determine the developmental time course of the appearance of the modulatory inputs to the STG. Moreover, because full understanding of the modulation of the adult system will require understanding the full complement of cotransmitters in identified modulatory inputs, it is important to characterize not just the development of one or several of the input modulators but as many as possible, with attention to the cotransmitter patterns seen.

In the accompanying paper (Kilman et al., 1999) we described the sequential acquisition during embryonic and larval development in both H. americanus and H. gammarus of three of the neuromodulators found in the stomatogastric nervous system, one of the sensory and modulatory inputs to the STG. In this paper, we describe the adult distribution, developmental acquisition, and colocalization patterns of proctolin, red pigment-concentrating hormone (RPCH), and a peptide of the tachykinin family in both H. americanus and H. gammarus. We studied these peptides because they are robust modulators of the stomatogastric motor patterns in several crustacean species.

RPCH is an octapeptide originally isolated and sequenced in shrimp (Fernlund and Ossendorf, 1972) and has been characterized since in other crustacean species (Klein et al., 1995; Linck et al., 1993). RPCH strongly modifies the pyloric, gastric mill, and cardiac sac rhythms (Dickinson and Marder, 1989; Dickinson et al., 1990, 1993, 1997; Nusbaum and Marder, 1988; Richards et al., 1996). The pentapeptide proctolin originally was isolated from cockroach (Brown and Starratt, 1975) and subsequently was isolated and localized in the adult stomatogastric nervous system of H. americanus (Marder et al., 1986) and during development in H. gammarus (Fénelon et al., 1998). Proctolin strongly modulates the pyloric and gastric mill rhythms in several crustacean species (Golowasch and Marder, 1992; Heinzl, 1988; Heinzl and Selverston, 1988; Hooper and Marder, 1984, 1987; Marder et al., 1986), and there are several identified modulatory projection neurons that use proctolin as one of their neurotransmitters (Coleman et al., 1995; Nusbaum and Marder, 1989a,b). Tachykinin-like immunoreactivity was found in the stomatogastric nervous system of adult Cancer borealis, H. americanus, and Panulirus interruptus (Blitz et al., 1995; Goldberg et al., 1988). Subsequently, an endogenous tachykinin-like peptide was purified from C. borealis and named CabTRP (Christie et al., 1997). CabTRP strongly excites the pyloric rhythm and appears to be closely related to the endogenous peptide that is labelled with the antibody used previously and in this study. These papers show that modulators appear with a characteristic developmental profile and that the acquisition of neuromodulators of the stomatogastric nervous system occurs over a protracted time period, with some neuromodulator immunoreactivities not fully present until close to the developmental time at which major circuit alterations occur (Casasnovas and Meyrand, 1995).

MATERIALS AND METHODS

Animals and dissection

Embryonic (n = 19), larval (n = 47), and juvenile (n = 14) H. americanus were obtained from the New England Aquarium lobster rearing facility (Boston, MA). Adult H. americanus (n = 16) were purchased from Commercial Lobster (Boston, MA) or from Shaw’s Supermarket (Waltham, MA). Adult H. gammarus (n = 7) were obtained from a fishery supply in Arcachon, France. Fertilized eggs from some females were removed and raised in individual containers to obtain embryonic (n = 6) and larval (n = 10) H. gammarus. The methods for animal care, staging, dissection, fixation, immunocytochemical processing, and confocal imaging are described in the accompanying paper (Kilman et al., 1999).

The antisera used to localize immunoreactivity to RPCH was a gift of R. Elde (University of Minnesota). We used this rabbit polyclonal antisera at a dilution of 1:250. The antisera used to localize proctolin was a gift of H. Agricola (Jena, Germany). This rabbit polyclonal serum was used at a dilution of 1:1,000. Tachykinin immunoreactivity was localized with a rat monoclonal antibody (Accurate Chemicals, Westbury, NY) generated against substance P. We used this antibody at a dilution of 1:200. Experiments in the crab C. borealis show that the native tachykinin recognized in that species by this same monoclonal antibody is not substance P but the tachykinin CabTRP (Christie et al., 1997).

RESULTS

Distribution of RPCH-like immunoreactivity in the adult stomatogastric nervous system

The distribution of RPCH-like immunoreactivity had not been studied previously in either H. americanus or H.
Therefore, as part of our study of the developmental acquisition of this peptide, it was necessary to study the distribution of RPCH-like immunoreactivity in the complete stomatogastric nervous system of the adult, consisting of the STG, esophageal ganglia (OG), commissural ganglia (CoGs), and their connecting and motor nerves. The stomatogastric nervous systems of adult *H. americanus* (*n* = 6) and *H. gammarus* (*n* = 4) were immunoactive for RPCH. Figure 1A shows RPCH-like immunoreactivity in the neuropil of the STG of *H. americanus*. Figure 1B shows that preincubation of the antibody with $10^{-4}$ M RPCH eliminates specific staining in the nervous system. An RPCH-stained STG of *H. gammarus* is shown in Figure 1C.

In these preparations, several axons can be seen entering from the stomatogastric nerve (stn), and these branch to form a dense neuropil of thick fibers and punctate varicosities in the STG. This neuropil is the terminal arborization of four fibers, two of which travel through each superior esophageal nerve (son) before entering the stn. These fibers presumably originate from two of the four or five stained somata in each CoG. Some or all of these axons also branch in a neuropil-like structure at the junction of each son with the stn, and it is likely that all four contribute to the profuse RPCH-stained neuropil in the CoGs as well. There are no RPCH-stained somata or fibers in the OG or the inferior esophageal nerve (ion). The STG motor axons do not stain for RPCH, although a portion of the dorsal ventricular nerve (dvn) sheath may occasionally show weak staining (see below). The RPCH-like staining patterns are identical in the two species, and this pattern is summarized schematically in Figure 6.

**Tachykinin-like immunoreactivity in the adult stomatogastric nervous system**

In adults, the stomatogastric nervous systems of both species also contain tachykinin-like immunoreactivity (*n* = 9). The adult distribution of tachykinin-like immunoreactivity in *H. americanus* has been described previously by Goldberg et al. (1988), who used the same monoclonal antibody we used here along with conventional fluorescence microscopy. Goldberg et al. described a tachykinin-like-immunoreactive neuropil in the STG, the CoGs, and at the stn-son junction as well as a dense, club-shaped structure in the CoG neuropil lying next to the ions. The entire tachykinin-like-immunoreactive STG neuropil arose from only two fibers in the stn. Several somata were stained in the CoGs as well as one bipolar soma in the OG with a single projection in each ion. There was also one fiber in each son, but it was unclear whether these fibers arose from the single midline OG neuron through the ions and CoGs or whether all the son fibers originated in the...
CoG somata. We were able to answer this question with the double-label experiments described below.

Figure 2A shows an STG from *H. americanus* that was stained with the anti-tachykinin antibody. Figure 2B is an STG that was stained after preincubation of this antibody with $10^{-4}$ M CabTRP. All specific staining was abolished in the stomatogastric nervous system subsequent to CabTRP preabsorption. Figure 2C shows the STG of an adult *H. gammarus* stained with the anti-tachykinin antibody. The staining pattern in the complete adult stomatogastric nervous system of the two species was similar and is summarized in the schematic in Figure 8.

### Tachykinin-like and RPCH-like immunoreactivities are colocalized in adults

The tachykinin-like-immunoreactive neuropil in the STG differs from those visualized with most other neuromodulator stains. Figure 2A shows that the branched fibers in the STG end in thick fibers with bulbous varicosities, and there are relatively few punctate varicosities compared with what is seen with antibodies for other neuromodulators (Beltz et al., 1984; Marder, 1987; Marder et al., 1986, 1987; Turrigiano and Selverston, 1991). However, the thick fibers with bulbous varicosities also appear in the RPCH-immunoreactive neuropil of the STG in addition to the more typical fine fibers with punctate varicosities. To investigate these structures, we performed double immunolabelling with the anti-tachykinin and anti-RPCH antibodies in both *H. americanus* ($n = 4$) and *H. gammarus* ($n = 2$). We found that the pair of tachykinin-immunoreactive fibers projecting to the STG is one of the two pairs of RPCH-immunoreactive fibers. Figure 3 shows an adult STG that has been double labeled with both the anti-tachykinin antibody (Fig. 3A) and the RPCH antiserum (Fig. 3B). All of the tachykinin-like-immunoreactive fibers also show RPCH-like immunoreactivity. Figure 3B shows that, in addition to those fibers that were double labeled with tachykinin-like immunoreactivity, there are also two fibers (Fig. 3B, large arrows) and many groups of punctate varicosities (Fig. 3B, small arrows) that contain only RPCH immunoreactivity. Because all tachykinin-like immunoreactivity is colocalized with RPCH, and because the single midline OG neuron does not stain for RPCH, we can rule out the possibility that this neuron is the source of the tachykinin-like immunoreactivity in the STG neuropil. Double-label immunostaining with the anti-tachykinin and anti-proctolin antibodies showed that these immunoreactivities are not colocalized ($n = 2$; data not shown).

### RPCH-like immunoreactivity in development

RPCH-like immunoreactivity was present in the neuropil of the STG of both *H. americanus* and *H. gammarus* at E50, the earliest developmental time examined (Fig. 4; 12 of 13 embryos between E50 and E90 showed STG neuropil staining). The neuropil staining at this point was relatively simple and was devoid of the punctate varicosities found in the adult STG. This staining appears to arise from only two fibers in the stn. Although there was a stained neuropil in the CoGs, we were unable to count accurately the number of stained CoG somata. As the animal progresses through the larval stages, the stained STG neuropil becomes more complex and dense, with punctate varicosities beginning to appear at about LII ($n = 4$) and becoming prominent by LIII ($n = 4$) (Fig. 4B).

About half of the preparations (6 of 12) we examined from *H. americanus* in LI–LIII also showed RPCH-like immunoreactivity in most of the somata of the STG. It is difficult to visualize the somata staining in z-series images of the entire ganglion because of the overlying neuropil. However, when the optical sections from the neuropil are...
summed separately (Fig. 4C) from the sections of the cell body region (Fig. 4D), both regions become more clear. The punctate nature of the neuropil varicosities is visible more clearly in Figure 4C, and the discrete spots of cytoplasmic staining in many STG neurons are revealed in Figure 4D. We saw no STG somata that stained this way in H. gammarus (n = 6).

Another feature of RPCH-like immunoreactivity that changes over time is the staining in the dvn sheath (Fig. 5A, H. americanus; Fig. 5B, H. gammarus). This densely immunoreactive structure is stained only within the peripheral sheath surrounding the nerves, whereas there are a few faintly stained fibers running underneath in the nerve itself. These faint fibers may be the source of the branching

Fig. 3. Double labeling of tachykinin and red pigment concentrating hormone (RPCH) immunoreactivity in the stomatogastric ganglion (STG) of adult H. americanus. A: Tachykinin-like staining (visualized with fluorescein secondary antibody) labels two fibers but few punctate varicosities. B: The same preparation shown in A stained for RPCH (visualized with Texas Red secondary antibody) labeled four fibers, two of which also show tachykinin-like staining. The two large fibers labeled only by RPCH (large arrows) make many punctate varicosities (small arrows). Scale bar = 100 µm.
Fig. 4. Red pigment concentrating hormone (RPCH)-immunoreactivity in the stomatogastric ganglion (STG) of embryos and larvae. 

**A:** In embryos at 50% of embryonic development (E50), RPCH immunoreactivity in the STG is limited to a simple, sparsely branched fiber with no varicosities. 

**B:** By larval stage III (LIII), RPCH immunoreactivity is found in a more branched varicose neuropil as well as in discrete spots in most neuronal somata. Optical sections from this ganglion were summed separately from two regions: 

**C:** The first region, from the center of the ganglion, shows the stained neuropil. 

**D:** The second region, from the cell body area, shows the discrete spots of staining in the cell bodies. Scale bar = 50 µm.
sheath structure, because they never stain beyond it into the lower dvn and lvns. In early embryos, this staining is immediately adjacent to the STG but appears progressively more posteriorly in older animals, until, at LIV, it is found one-third to half of the way along the dvn to the split of the lvns. This structure, which also stains for other neuropeptides (Kilman et al., 1999), is found in adults but stains more faintly than in developing animals. At all stages, there is a branching neuropil at the junction of the stn with the sons. Figure 6 presents a summary of RPCH-like immunoreactivity at three developmental time points: E50, LII, and adulthood.

Tachykinin immunoreactivity in development

Although tachykinin-like staining was present in the brain and commissural ganglia at E50, the STG and the interganglionic nerves in the stomatogastric nervous system were not immunoreactive until substantially later in both species. At LII, when the STG neuropil begins to show tachykinin-like staining, the pattern is similar to that seen in the adult but is very faint. Figure 7A shows an H. americanus LI STG before the neuropil begins to stain. By LII, most preparations have a faintly stained neuropil (n = 6 of 7; Fig. 7B). This is also the case for H. gammarus. Figure 7C shows the STG from an LI H. gammarus that shows no stained fibers in the STG neuropil, and Figure 7D shows that fibers begin to stain at LII in H. gammarus. The fibers in the sons and the stn are visible in favorable preparations; however, in many cases, they are too faint to visualize until LII or LIV. The structure of the neuropil is similar to that of the adult. The thick fibers do not have many fine branches and sometimes end in bulbous varicosities. The intensity of staining increases between LII and LIV, when it stains as brightly as the adult STG neuropil. Fewer CoG somata were stained at LII than in the adult, and the number of stained somata increased gradually through larval and juvenile development. Figure 8 shows a summary of the tachykinin-like staining at three stages of development: E50, LII, and adulthood.

Proctolin-like immunoreactivity in development

Proctolin-like staining in the adult H. americanus has been described previously (Marder et al., 1986). Fénelon et al. (1998) reported that proctolin-like staining is present in the stomatogastric nervous system at E50 in H. gammarus in essentially the same distribution as in the adult stomatogastric nervous system, with a few exceptions, and we corroborate those essential findings here for H. americanus. There are immunoreactive neuropils in both the CoGs and the STG (Fig. 9A) at E50. All of the STG immunoreactivity arises from two fibers, each of which travels from one CoG through the son to reach the stn and the STG. Each son contains one bright fiber and one faint fiber. Some fibers leave the CoG through the ion, but they exit the ion through the labral nerve before reaching the OG. These features of proctolin-like staining do not change through embryonic, larval, and juvenile life and are present unchanged in the adult (Fig. 10).

Fénelon et al. (1998) found that, during development in H. gammarus, some STG somata are immunoreactive for proctolin transiently during development. In H. americanus, animals from LII until late juvenile stages show one
or two stained STG somata. Figure 9B shows an STG from an LII *H. americanus* in which one soma stains for proctolin. The intensity of immunolabelling of somata varied between preparations and was not obviously correlated with the intensity of staining of the neuropil. Figure 9C shows an STG (juvenile; carapace length, 20 mm) in which the labeled soma is strongly labeled, and Figure 9D shows two more faintly stained somata in another STG (juvenile; carapace length, 35 mm). Table 1 lists the number of proctolin-stained somata found in preparations.
Fig. 7. Tachykinin-immunoreactivity in the stomatogastric ganglion (STG) of larval Homarus. A: LI H. americanus shows no specific staining. B: Larval stage II (LII) H. americanus neuropil fibers show staining. C: LI H. gammarus does not show tachykinin-like staining. D: LII H. gammarus does show tachykinin-like staining. Scale bars = 50 µm (B also applies to A).
of H. americanus at different stages of development. We did not examine juveniles larger than a carapace length of 43 mm, but somata still stained consistently in these relatively mature animals. No somata were stained in the STG of large adult lobsters (n = 4). Figure 10 shows a summary of proctolin immunoreactivity at three stages of development: E50, LII, and adulthood. For conventions and abbreviations, see Figure 6.

**DISCUSSION**

Central pattern-generating networks are richly modulated in adult animals by a variety of descending and sensory pathways (Marder and Calabrese, 1996). One could imagine that the neurons and circuits of central pattern-generating networks develop coordinately with the development of the modulatory inputs. Alternatively,
Fig. 9. Proctolin immunoreactivity in the stomatogastric ganglion (STG) of larval and juvenile H. americanus. A: An E50 STG shows a dense neuropil stained for proctolin. B: A larval stage II (LII) STG shows one faint soma (arrow) in addition to neuropil staining. C: A juvenile (carapace length [CL] 20 mm) shows one bright soma and neuropil staining. D: A juvenile (CL 35 mm) shows two faint somata and neuropil staining. For image clarity, B and D are projections of only the optical sections containing these somata. Scale bar = 50 µm in A,B, 100 µm in C,D.
the backbone of the central pattern-generating networks could be formed first, and the modulatory control could be added later in development. The STG generates a rhythmic motor pattern (Casasnovas, 1996; Casasnovas and Meyrand, 1995), before many of the modulatory inputs are present (this paper; Fénelon et al., 1998; Kilman et al., 1999). This suggests that the backbone wiring of the network may not depend on modulatory control but that modulatory control may be important for fine tuning of the embryonic network into its adult configuration. The long period of time over which the modulatory inputs to the STG appear is interesting and suggests that there may be specific stages of these fine-tuning processes that may benefit from the addition of specific, incoming modulators.

This general strategy may be common for other developing central pattern generators, because it has been sug-
suggested that the formation of the basic locomotor pattern-generating circuitry in the developing spinal cord is independent of supraspinal inputs both in the chick (Bradley and Bickoff, 1992) and the rat (Okado et al., 1992). It is noteworthy that, similar to the data reported in these studies, the descending inputs to the chick and rat spinal cord also show a protracted developmental time course. In chicks, descending serotonergic fibers first appear in the spinal cord on embryonic day 6, and their maximal density was attained at 1 week posthatching (Okado et al., 1992). In rat, the first corticospinal fibers arrive in the spinal cord immediately after birth, and the adult pattern is established at the end of the third week (Schreyer and Jones, 1987). Serotonergic fibers start to invade the rat spinal cord and make synapses by embryonic day 17, but the adult pattern is seen only by postnatal day 21 (Rajaofetra et al., 1989).

**Ambiguities associated with modulator immunoreactivity**

In these studies, we used immunoreactivity to follow the presence of amines and neuropeptides. In some cases, the identity of the antigen is fairly certain. For example, proctolin is not thought to be a member of a peptide family, but, instead, the same pentapeptide has been found in numerous arthropod species, including H. americanus (Marder et al., 1986; Schwarz et al., 1984; Siwicki et al., 1987); therefore, we believe that it is highly likely that the proctolin-like immunoreactivity we have characterized is authentic proctolin. Although RPCH is closely related to the insect adipokinetic hormones, the structure of RPCH in different crustacean species appears to be highly conserved (Klein et al., 1995; Linck et al., 1993), and, although the identity of the RPCH-like peptide in Homarus is not known, it is likely that it is closely related to authentic shrimp RPCH. In contrast, determination of the exact structure of the peptides that give rise to the allatostatin-like staining is likely to require biochemical purification and cloning of the precursor gene(s), if the case in Homarus is anything like that in Carcinus maenas, in which 20 different allatostatin-like peptide sequences are found encoded by the precursor gene (Duve et al., 1997). At present, we know that the antibody staining is blocked by insect Diploptera-allatostatin 3 (D-AST-3) preincubations and that D-AST-3 is active physiologically in the embryonic, larval, and adult stomatogastric nervous systems (Richards et al., 1996; K.S. Richards and E. Marder, personal communication). When using an antibody that recognizes several members of a peptide family (e.g., those used here to study the Phe-Leu-Arg-Phe-amide [FLRF-NH2], AST, and tachykinin-like peptides) it is very likely that different members of that family are found in different neurons, and it is possible that different members of the family may be expressed at different times in development.

**Neuromodulator staining in the STG through development**

Figure 11 summarizes the work in these papers and previously published information (Cournil et al., 1995; Fénelon et al., 1998; Scholz et al., 1998) on the developmental appearance of eight neuromodulators in the STG of H. gammarus and H. americanus. The six modulators reported in these two papers were studied in both species, whereas the development of dopamine immunoreactivity was only studied in H. gammarus (Cournil et al., 1995), and the distribution of nitric oxide-sensitive cyclic GMP staining was only studied in H. americanus (Scholz et al., 1998).

The first important finding is that the stomatogastric ganglion neuropil is immunoreactive for proctolin, RPCH, and FLRF-NH2 by halfway through embryonic development (Fig. 11). These projections appear to be a portion of the adult projections and remain in place throughout subsequent development. Preliminary data on H. gammarus indicate that proctolin and FLRF-NH2-like immunoreactivity first appear sometime between E20 and E40 (V. Fénelon and P. Meyrand, personal communication), but we do not know the exact time of appearance of any of these early-appearing modulators in the STG of either species.

Proctolin, the FLRF-NH2-like peptides, and RPCH have been studied extensively in the adults of several species, including C. borealis and P. interruptus, in which each of them is strongly excitatory (Dickinson and Marder, 1989; Hooper and Marder, 1987; Marder et al., 1986; Nusbaum and Marder, 1988; Weimann et al., 1993). Preliminary data on the adult H. americanus (Richards et al., 1996) indicate that the same is true in H. americanus. Therefore, these modulators may play a role in the early activation of the STG network. We speculate that these early modulators provide excitatory drive to allow activity-dependent processes that are important in tuning synaptic strength and intrinsic properties to occur. For these reasons, it will be crucial to determine whether the receptors for these modulators are present at these early times.

Additional modulators appear as the animals progress through development. Allatostatin-like immunoreactivity appears before hatching but later than the three peptides discussed above (Fig. 11). Cockroach AST-3 is an effective inhibitor of the stomatogastric ganglion motor patterns (Richards et al., 1996; Skiebe and Schneider, 1994) and neuromuscular junctions (Jorge-Rivera and Marder, 1997) in adult C. borealis and H. americanus. This suggests that an inhibitory control system may be present already by late in embryonic development.

Dopamine was found in the stomatogastric ganglion neuropil by late embryonic development (Cournil et al., 1995) in H. gammarus but was not studied in H. americanus (Fig. 11). Although the physiologic actions of dopamine have been examined extensively in P. interruptus (Eisen and Marder, 1984; Flamm and Harris-Warrick, 1986a,b; Harris-Warrick et al., 1995a,b; Johnson and Harris-Warrick, 1990; Johnson et al., 1993, 1995; Marder and Eisen, 1984), we have relatively little information concerning its actions in the STG in the adult of either Homarus species. Preliminary work on H. gammarus embryos indi-

**TABLE 1. Number of Proctolin-Staining Somata in the Stomatogastric Ganglion of Embryonic, Larval, Juvenile, and Adult Homarus americanus**

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Number of proctolin-immunoreactive stomatogastric ganglion somata</th>
</tr>
</thead>
<tbody>
<tr>
<td>E45-E60</td>
<td>0, 0, 1</td>
</tr>
<tr>
<td>E80–E100</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>LI</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>LII</td>
<td>0, 1, 2</td>
</tr>
<tr>
<td>LIII</td>
<td>0, 0, 1, 1</td>
</tr>
<tr>
<td>LIV</td>
<td>1, 2, 2</td>
</tr>
<tr>
<td>J juveniles (CL 10–43 mm)</td>
<td>1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 2</td>
</tr>
</tbody>
</table>

1CL, carapace length; E, stage of embryonic development; L, larval stage.
cates that dopamine is relatively ineffective in modulating the STG motor patterns early in development (Casasnovas, 1996). This suggests the possibility that the dopamine receptors or dopamine-activated signal-transduction pathways may develop relatively late.

Serotonin first appears in the neuropil of the STG in LI animals in H. gammarus, but not until one or two stages later in H. americanus (Kilman et al., 1999; Fig. 11). It is noteworthy that the STG embryonic motor pattern is modulated by serotonin as early as E60, indicating that the receptors for serotonin are present significantly before there is serotonin present in the STG neuropil (Casasnovas, 1996; K.S. Richards and E. Marder, personal communication). These receptors could respond to hormonally released serotonin early in development or could be developed precociously in anticipation of the arrival of the serotonin later in development.

Of all of the peptides studied to date, the tachykinin-like peptide(s) was the last to appear (Fig. 11). This peptide becomes strongly labelled only close to the time at which the embryonic motor patterns start to be modified at the LIV stage (Casasnovas and Meyrand, 1995). The tachykinin-like peptide that appears is colocalized with RPCH in two of the input fibers, and this may constitute another example of the sequential acquisition of differential cotransmitters in the same input fibers. If so, then it is possible that the RPCH-containing fibers could elicit different actions early and late in larval life, as their cotransmitter complement is changing.

**STG somata show transient immunoreactivity to several neuromodulators**

Transient expression of amines, small molecule transmitters, and peptides in development occurs in both vertebrates and invertebrates (Cournil et al., 1995; Davis et al., 1993, 1997; Matteoli et al., 1990; O’Brien and Taghert, 1998; Vanhala et al., 1994; Voronezhskaya and Elekes, 1996; Witten and Truman, 1991, 1996). Neuronal cell bodies in the STG show transient immunoreactivity to at least three neuropeptides during development. In H. gammarus (Fénelon et al., 1998), STG cell bodies transiently display FLRFamide-like immunoreactivity in almost all embryonic and larval animals, but this immunoreactivity disappears in later stages. In H. americanus (Table 1) STG somata stain until early juvenile stages. Subsequently, the staining apparently disappears and then reappears in 50%
of the adult H. americanus stained with the INCSTAR antiserum. Other antiseras against these peptides that otherwise give identical staining patterns (including staining embryonic somata) do not label any adult STG somata. This may indicate that the INCSTAR antiserum recognizes an additional FMRFamide-like peptide in some STG somata that is not recognized by the other antibodies. Perhaps in H. americanus, as in H. gammarus, there is an antigen that is seen during development in several STG somata, but this is a different peptide than that seen in the adult. The variability in number of somata staining in the adult H. americanus probably is not molt-cycle related, because the embryonic and larval animals molt more often than adults, but the variability is higher in adults. Although we did not sex the animals, we think it unlikely that the variability is related to sex differences, because the embryos and larvae should have been roughly 50% males and females, but most animals had at least some cells stained. In contrast, most of our adults are male, because the fishermen who collect them return almost all of the females to the water. However, half our adult H. americanus showed STG somata stained.

The proctolinergic STG somata in H. americanus begin to stain at LI1 and continue to do so in large juvenile animals. The entire cytoplasm is immunoreactive, which is typical of stained neurons in the adult that are thought to make proctolin, but we were unable to determine whether the neuritic arbor of this neuron was proctolin-immunoreactive. The staining occurs continuously throughout development. Thus far, we have only discrete spots throughout the cytoplasm of most or all STG somata, but this is a different peptide than that seen in the adult. The variability in number of somata staining in the STG somata is not recognized by the other antibodies. Because the fishermen who collect them return almost all of the females to the water. However, half our adult H. americanus showed STG somata stained.

There are a number of questions that need to be answered with regard to all of the transiently stained STG somata. It will be important to determine whether the same neuron always is stained from preparation to preparation, and if so, whether the same neuron is stained continuously throughout development. Thus far, we have statistical snapshots only of the numbers of stained neurons but no assurance that these are a fixed population. The identification of the neuronal somata that express neuropeptides should allow us to investigate further the physiologic roles of these peptides. Most of the neurons in the STG are motor neurons; therefore, it is possible that this expression could be important for the establishment of the mature neuromuscular junctions. Alternatively, this expression could be important during the maturation of the functional networks of the STG.

There are small developmental species differences in modulator immunoreactivity. Although there are minor species differences in the staining patterns seen with some of the modulators, the overall picture is of a very similar timetable for development of modulator immunoreactivity in these two species. In addition to the species differences in STG soma staining described above, serotonin staining in the STG is slightly delayed in H. americanus compared with that in H. gammarus (Fig. 11).

CONCLUSIONS

All motor systems are richly modulated by descending inputs that are important for activation of rhythmic motor patterns and that also alter the frequency and phase relationships of the central pattern-generating elements (Marder and Calabrese, 1996; Sillar et al., 1997). A striking example of the developmental role of descending control pathways comes from work on locomotor spinal networks. In Xenopus, the ingrowth of the serotonergic raphe-spinal projections appears to transform a simple immature embryonic spinal locomotor network into a more flexible, adult-like circuit (Sillar et al., 1997). Thus, the early circuit is retained and modified by still unknown cellular mechanisms. The wealth of existing anatomic and physiologic data about the role of neuromodulation in the construction of functional circuits within the stomatogastric nervous system makes it an ideal preparation with which to study the role of a single neuromodulator in the development of adult circuits but the role of the developmental acquisition of the full complement of neuro-modulatory control in the alterations in synaptic and intrinsic properties that occur during circuit maturation. We show here that the full range of neuromodulators found in the adult is expressed gradually during a prolonged developmental time course (Fig. 11). Because the time course of appearance of these modulators in the stomatogastric nervous system is sometimes quite different from their appearance elsewhere in the animal (Beltz and Kravitz, 1987; Beltz et al., 1990; Cournil et al., 1995), this suggests that the specific timings of the appearances of these modulatory neurotransmitters perform important stage-specific functions in the development of a central pattern-generating circuit.

LITERATURE CITED


