Ontogenetic Alteration in Peptidergic Expression Within a Stable Neuronal Population in Lobster Stomatogastric Nervous System

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ABSTRACT
In the adult lobster, Homarus gammarus, the stomatogastric ganglion (STG) contains two well-defined motor pattern generating networks that receive numerous modulatory peptidergic inputs from anterior ganglia. We are studying the appearance of extrinsic peptidergic inputs to these networks during ontogenesis. Neuron counts indicate that as early as 20% of development (E20) the STG neuronal population is quantitatively established. By using immunocytochemical detection of 5-bromo-2′-deoxyuridine incorporation, we found no immunopositive cells in the STG by E70. We concluded that the STG neuronal population remains quantitatively stable from mid-embryonic life until adulthood.

We then investigated the ontogeny of FLRFamide- and proctolin-like peptides in the stomatogastric nervous system, from their first appearance until adulthood by using whole mount immunocytochemistry. Numerous FLRFamide-like-immunoreactive STG neuropilar ramifications were observable as early as E45 and remain thereafter. From E50 to the first larval stage, one to three STG somata stained, while somatic staining was not observed in larval stage II and subsequent stages. From E50 and thereafter, the STG neuropilar area was immunopositive for proctolin. One to two proctolinergic somata were detected in the STG of the three larval stages but were not seen in embryos, the post-larval stage or in adults. Thus, peptidergic inputs to the STG are present from mid-embryonic life. Moreover, whereas in the adult, STG neurons only contain glutamate or acetylcholine, some neurons transiently express peptidergic phenotypes during development. Although this system expresses an ontogenetic peptidergic plasticity, the STG neurons produce a single stable embryonic-larval motor output (Casasnovas and Meyrand [1995] J. Neurosci. 15: 5703–5718). J. Comp. Neurol. 399:289–305, 1998.

Indexing terms: Homarus gammarus; stomatogastric ganglion; FLRFamide; proctolin; bromodeoxyuridine

Nervous systems of both vertebrates and invertebrates require a high degree of plasticity during embryonic and larval development to permit to cope with dramatic behavioral changes. In this context, studies of the ontogeny of rhythmic motor behaviors have provided considerable insight into principles which govern the developmental plasticity of the underlying central neural networks (Weeks and Jacobs, 1987; Waldrop and Levine, 1989; Jacobs and Weeks, 1990; Truman, 1992).

From such studies, two principles of neural network maturation have been postulated. One hypothesis is that the neural networks generating stage-specific motor tasks are built and used at a specific time and then dismantled or discarded when the behavior is no longer expressed. In this case, the neuronal population is usually subjected to massive or partial neuronal cell death which is generally associated with a late neurogenesis enabling the construction of new neural networks involved in new motor tasks. This first principle has been well described in holometabolous insects. In Manduca, for instance, the proleg with-
For this purpose, we performed neuronal cell counts on each neuronal inputs in the developmental maturation of number changes and of developmental acquisition of extrinsic network changes taking place in development. Thus, one can wonder whether extrinsic inputs to STG also play a developmental role in the maturation of the adult STG neuronal phenotypes (Thoby and Simmers, 1998). To test the relative importance of these two phenomena in the developmental maturation of adult neural networks, one needs to use a system with few identified neurons and well defined neuronal inputs. The stomatogastric ganglion (STG) of decapod Crustacea which governs the motion of the foregut is well suited to such investigations. The adult STG consists of a small and well defined neuronal population (about 30 neurons) that has been well described in terms of cellular and synaptic properties. Moreover, the expression of adult motor patterns by the STG is dramatically influenced by more than 20 different electrophysiologically identified neuronal inputs from more rostrally located ganglia (Robertson and Moulin, 1981, 1984; Simmers and Moulin, 1988; Nusbaum and Marder, 1989a,b; Cazalet et al., 1990a,b; Cournil et al., 1990; Harris-Warrick et al., 1992; Combes et al., 1994). Moreover, the STG is morphologically recognizable at early embryonic stages (Casasnovas and Meyrand, 1995) and, at all developmental stages, receives inputs from the anterior ganglia through a single afferent nerve.

In the adult decapod crustacea, it has been shown that neuromodulatory substances play two major roles in the expression of STG networks. On one hand, they can have short-term effects that reconfigure target networks by modifying the expression of the intrinsic properties of individual neurons and/or their synaptic strengths (Dickinson et al., 1990; Meyrand et al., 1991, 1994). On the other hand, central inputs to the STG have been recently shown to play a crucial role in the long-term maintenance of the STG neuronal phenotypes (Thoby and Simmers, 1998). Thus, one can wonder whether extrinsic inputs to STG also play a developmental role in the maturation of the adult STG networks. To this end, useful clues about the developmental role of extrinsic inputs might be gathered from the comparison of their timing of appearance with functional network changes taking place in development.

To determine the relative importance of neuronal cell number changes and of developmental acquisition of extrinsic neuronal inputs in the developmental maturation of adult neural networks, in a first step, we have quantified the neuronal population of the STG through developmental stages of the European lobster, Homarus gammarus. For this purpose, we performed neuronal cell counts on paraffin sections of embryonic and adult STG and monitored proliferative cells by in vivo labeling with the thymidine analogue, bromodeoxyuridine, which is incorporated into DNA of dividing cells during the S-phase (Gratzner, 1982). By using this methodology, we have been able to demonstrate that neither massive neuronal cell death nor late neurogenesis takes place in the STG from mid-embryonic life through adulthood. Thus, the segregation of a single embryonic network into two distinct adult neural networks in the STG (Casasnovas and Meyrand, 1995) occurs without any quantitative changes in the STG neuronal population.

In a second step, we have studied the distribution and appearance in the stomatogastric nervous system (STNS) of embryonic, larval, and adult lobsters H. gammarus of two neuropeptide immunoreactivities, FLRFamide- and proctolin-related peptides known to modulate the adult STNS motor patterns (Hooper and Marder, 1984; Marder et al., 1986; Marder, 1987). We show that FLRFamidergic and proctolinergic inputs to the STG are present from mid-embryonic life onwards. Furthermore, we demonstrate an unexpected plasticity in the expression of proctolin and FLRFamide phenotypes within the STG neuronal population. This neuromodulator plasticity occurs at a different time from the functional partitioning of the single embryonic-larval network into the two distinct neural networks of the adult (Casasnovas and Meyrand, 1995). Some of these data have been presented in abstract form (Casasnovas et al., 1997).

MATERIALS AND METHODS

Animals and dissection

All experiments were performed on embryos (n=99), larvae (n=39), post-larvae (n=10), and adults (n=18) of H. gammarus. Embryos were collected from egg-bearing female lobsters obtained from a local fishery supply and kept in large tanks of circulating and aerated 15°C seawater. The percent staging system for lobster embryos (Helluy and Beltz, 1991), based upon 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 with an ocular micrometer on a binocular microscope through the transparent eggshell prior to dissection. Each value of EI can be converted into a percentage of the embryonic development by dividing the measured EI at a given time by EI at hatching. Thus, hatching corresponds to 100% of the embryonic development (E100); E50 to halfway through embryogenesis, and E0 to fertilization. After hatching the larvae were transferred into small individual rearing cupules flushed with circulating aerated sea-water at 15°C and were fed twice daily with frozen Artemia. Larval stages were determined by noting the external morphological features of animals as described in Herrick (1895). The basic features used for larval staging are 1) first stage larva: no swimmerets, telson trapezoidal; 2) second stage larva: external swimmerets now formed; 3) third stage larva: uropods present; and 4) fourth stage or post-larva: telson quadrangular.

Eggsells of embryos were cut open and embryos were removed from the yolk. For immunocytochemistry, subsequent dissection was performed as previously reported (Casasnovas and Meyrand, 1995 for embryos and larvae; Selverston and Moulin, 1987 for adults) in aerated superfused physiological saline (479.12 mM NaCl, 12.74 mM...
KCl, 13.2 mM CaCl$_2$, 10 mM MgSO$_4$, 3.9 mM Na$_2$SO$_4$, and 5 mM HEPES, pH 7.45).

**Fixation and tissue processing for standard paraffin embedding**

For paraffin embedding, embryos and adult STG were immersed at room temperature for 5 hours in a fixative containing 28% paraformaldehyde, 25% saturated picric acid in water, and 5% acetic acid. After fixation, animals or specimen were rinsed for 15 minutes in 0.1 M Na phosphate (pH 7.4) and then dehydrated through graded alcohols (30, 50, 70, 95, and 100% ethanol, 10 minutes each). After xylene treatment (two times for 90 minutes each at room temperature and one rinse at 63°C), animals were transferred to pure paraffin for embedding. Serial 5 to 10 µm longitudinal sections were cut on a microtome and saved on glass vecabond-coated slides. After staining (for neuronal cell body counts) or after immunocytochemistry (for 5-bromo-2′-deoxyuridine [BrdU] labeling) sections were dehydrated with short steps of 70%, 95%, and absolute ethanol, treated with xylene, mounted in Entellan (Merck, Nogent-sur-Marne, France), coverslipped, and then observed through a Lietz orthoplan microscope.

**Neuronal cell body counts**

Twenty-nine embryos at different stages of embryonic development and five dissected adult STG were processed for paraffin embedding. Blocks of E20–40, E50–100, and adult STG were sectioned, respectively, 5, 6.3, and 10 µm thick. All sections were saved in serial order, deparaffinized in xylene, passed through 100%, 95%, and 70% ethanol, and stained in 1% cresyl violet acetate in distilled water. After the treatment described above, for each section of the STG, the neuronal cell bodies and other landmarks were traced by using a drawing tube, and each neuronal soma was identified in all drawings in which it appeared by marking it with a single number. Neuronal somata were identified by the position (periphery of the fine neuropile), the size (large oval outline), the granular purple blue cytoplasm, and the large pale nucleus with nucleoli. One experimenter made all the neuron counts on coded slides, and some sections were also checked by a second experimenter. For each developmental time point and adults, the number of STG cells was expressed as mean ± SD. Statistical comparisons among groups were assessed by analysis of variance (ANOVA).

**BrdU labeling**

Proliferation of cells was monitored by in vivo labeling with the thymidine analogue BrdU. BrdU is incorporated into DNA of dividing cells during the S-phase and is detected by using a specific monoclonal antibody (Gratzner, 1982). Embryos removed from eggshells at different stages of embryonic development and intact larvae were exposed to BrdU (Sigma, Saint Quentin Fallavier, France; 10$^{-4}$ M diluted in physiological saline) at 13–15°C for 24 hours. All animals survived during the labeling period. The heart beat frequencies were not impaired in any embryos and no signs of major impairment of locomotor activity were noted in any larvae. Embryos and larvae were then fixed after a quick rinse in phosphate buffered saline (PBS, pH 7.4). As commercially available anti-BrdU antibody binds to single stranded DNA, DNA denaturation was performed by incubating animals in 1 N HCl, PBS, 0.3% Triton X-100 for 45 minutes at room temperature and then rinsing in 0.1 M borate buffer (pH 8.5) for 15 minutes. Animals were then processed for paraffin embedding and serial sections saved as described above. Sections were placed in 10% normal goat serum (NGS) for 2 hours before overnight incubation at 4°C in undiluted monodonal mouse anti-BrdU antiserum (Amersham, Les Ulis, France). Sections were rinsed 1 hour in PBS with 0.3% Triton X-100 (PBST) and placed in goat anti-mouse (1:20; Sigma) immunoglobulins for 2 hours at room temperature followed, after 1 hour rinse in PBST, by incubation in mouse peroxidase-anti-peroxidase complex (1:50; Sigma) for 2 hours at room temperature. Immunoreactivity was visualized by using 3,3’-diaminobenzidine tetrahydrochloride (DAB). All immunolabels were diluted in PBS containing 1% NGS and 0.3% Triton X-100. Specificity of anti-BrdU antibody for marine crustaceans has been previously established (Harzsch and Dawirs, 1994). Further controls included omission of anti-BrdU antibody, incubation in anti-BrdU antibody followed by inappropriate secondary or tertiary antibody or processing of sections from animals with no exposure to BrdU. All control experiments resulted in an absence of specific staining.

**Whole-mount immunocytochemistry and confocal microscopy**

Embryonic and larval neuronal system-muscle preparations pinned on Sylgard cubes as well as free-floating adult entirely dissected STNS were processed for immunodetection of FLRFamide- and proctolin-like peptides by using indirect immunofluorescence techniques. After fixation (overnight at 4°C with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4–7.5), preparations were rinsed five times over approximately 5 hours in PBST and incubated with either a rabbit anti-FMRFamide polyclonal antibody (1:800; INCSTAR, Stillwater, MN) or a rabbit anti-proctolin polyclonal antibody (1:1,500; gift of Dr. Agricola, Friedrich Schiller University, Jena, Germany) for 24–48 hours. Tissues were again rinsed five times over approximately 5 hours in PBST and then incubated overnight in goat anti-rabbit rhodamine-conjugated immunoglobulins (1:100; Sigma). Preparations were then thoroughly rinsed in PBS (at least five times over 5 hours), mounted on glass slides, and coverslipped by using a solution of 80% glycerol, 20% 20 mM sodium carbonate, pH 9.5. All immunolabels were diluted in 10% NGS PBST and all incubations were performed at 4°C. Controls included omission of primary antibody and incubation in primary antibody followed by an inappropriate secondary antibody. All control experiments resulted in an absence of specific staining. Previous work has shown that the STNS of Homarus americanus and Cancer borealis contains SDRN- and TNRN-FLRFamide but contains no FMR-Famidem peptides (Trimmer et al., 1987; Weimann et al., 1993). Preincubation of the FMRFamide serum with 10$^{-4}$ M TNRN-FLRFamide overnight at 4°C completely blocked staining in the adult H. gammarus STNS (n = 3). In contrast, preincubation of the FMRFamide serum with 10$^{-4}$ M proctolin or cholecystokinin1–8 peptides overnight at 4°C did not diminish the immunostaining in the adult H. gammarus STNS (n = 3). Thus, we assumed that the FMRFamide serum binds to native FLRFamide peptides.

All preparations were viewed with a LEICA TCS 4D laser scanning confocal microscope equipped with a krypton/argon mixed gas laser. In general, for embryos and larvae, 30 to 50 optical sections of the STG, 1 to 1.5 µm
apart, with a Leica 40× oil immersion lens and 30 to 40 optical sections of cerebral ganglia, commissural ganglia, or the oesophageal ganglion, 1.5 to 2.5 µm apart with a Leica 25× oil immersion lens were taken from single whole-mount preparations. In adults, all acquisitions were made using a Leica 10× lens, and 40–60 optical sections of each ganglion were taken from a single whole-mount preparation. All images were made by using the maximum projection program provided in the Scanware software. All figures were produced with Photopaint and Corel DRAW software and printed on an Epson Stylus color printer.

RESULTS

Stomatogastric nervous system

In adult decapod crustacea, the stomatogastric ganglion (STG) contains mostly motor neurons that are organized in two distinct neural networks producing two different rhythmic motor patterns governing the motion of the distal part of the foregut. All the adult STG neurons are identified and well characterized in terms of cellular properties and synaptic connectivity (Harris-Warrick et al., 1992). Almost all STG motor neurons send their axon through the dorsal ventricular nerve (dvn), which bifurcates into the lateral ventricular nerves (lvn) and then branches again to innervate specific and precisely identified muscles of the stomach (Maynard and Dando, 1974). All inputs that control the STG networks travel via a unique afferent nerve, the stomatogastric nerve (stn), and arise from the paired commissural ganglia (CoGs) and the oesophageal ganglion (OG; Fig. 1A). The OG is linked to the two CoGs via the superior oesophageal nerves (son) and the inferior oesophageal nerves (ion; Fig. 1A). Casasnovas and Meyrand (1995) have shown that the embryonic STG is detectable very early in the development of the lobster H. gammarus, presents a similar neural organization to that of the adult and contains motoneurons innervating well-defined embryonic stomodeal muscles. Anterior ganglia are also present in embryos and larvae with a similar connectivity except that the CoGs are fused to the brain through all developmental stages (Fig. 1B), while in adult they are linked to the brain by a long connective belonging to the ventral nerve cord.

Establishment of the neuronal population within the STG throughout development

As the motor outputs produced by the STG networks are different in embryos and adults (Casasnovas and Meyrand, 1995) one can wonder whether these different motor patterns are produced by the same neuronal population or by a renewed population. Indeed numerous studies have shown that neuronal cell death and late neurogenesis are common features in the development of invertebrate nervous systems (Williams and Herrup, 1988; Levine et al., 1992). Almost all STG motor neurons send their axon through the dorsal ventricular nerve (dvn), which bifurcates into the lateral ventricular nerves (lvn) and then branches again to innervate specific and precisely identified muscles of the stomach (Maynard and Dando, 1974). All inputs that control the STG networks travel via a unique afferent nerve, the stomatogastric nerve (stn), and arise from the paired commissural ganglia (CoGs) and the oesophageal ganglion (OG; Fig. 1A). The OG is linked to the two CoGs via the superior oesophageal nerves (son) and the inferior oesophageal nerves (ion; Fig. 1A). Casasnovas and Meyrand (1995) have shown that the embryonic STG is detectable very early in the development of the lobster H. gammarus, presents a similar neural organization to that of the adult and contains motoneurons innervating well-defined embryonic stomodeal muscles. Anterior ganglia are also present in embryos and larvae with a similar connectivity except that the CoGs are fused to the brain through all developmental stages (Fig. 1B), while in adult they are linked to the brain by a long connective belonging to the ventral nerve cord.

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We counted the neuronal cell bodies in the STG by using a method (see Materials and Methods) that has already been successfully used in adult decapod crustacean STG (H. americanus: Maynard, 1971; Panulirus interruptus: King, 1976; Cancer borealis: Kilman and Marder, 1996). In the adult, two STG had 26 neurons and the remaining STG had 28, 29, or 30 neurons (Fig. 2 gives the mean number). In embryos the number of neuronal cell bodies counted in serial sections varied from 22 to 34. The age of the STG did not affect the number of neuronal somata within the STG (P > 0.1 with a Kruskal-Wallis non parametric ANOVA test; Fig. 2). Furthermore, in none of the observed cresyl violet-stained sections of the 29 embryonic STG or 19 OG was a figure of mitosis observed. The CoGs were more difficult to consistently and precisely locate on embryonic serial sections. However, in eight embryos, the CoGs were unambiguously recognizable and no figure of dividing cells
was found. In contrast, numerous large diameter dividing cells (10–16 µm) at different stages of mitosis were consistently found on all embryonic sections of the cerebral ganglia and of the ventral nerve cord ganglia (not shown). Together these data suggest that the neuronal population within the STG is quantitatively established very early during the embryonic development.

To test the hypothesis that a rapid turnover of the neuronal population of the STG during the embryonic and larval period could lead to an apparent constant number of neurons, we used bromodeoxyuridine (BrdU). This thymidine analogue, which is incorporated into replicating DNA, was subsequently immunocytochemically detected by specific anti-BrdU monoclonal antibody on paraffin sections of 48 embryos at different stages of embryonic development and of 12 larvae. All embryos and larvae exposed to 24 hours BrdU displayed numerous stained cell nuclei within the cerebral ganglia (Fig. 3A). Among these stained cells, two types could be unambiguously distinguished according to their morphology and their distribution (Harzsch and Dawirs, 1994). One type characterized by a round and regularly shaped stained nucleus and often found in clusters was the neuroblasts and/or their progeny. In contrast, stained cell displaying a long and irregularly shaped nucleus and generally isolated were the glial cells. Observation at higher magnification shown that numerous clusters consisted of one large (>10 µm) and one (<7 µm; Fig. 3A) or more smaller stained cells. Although this kind of cluster profile was never observed in the STG, we found one (n = 7) to two (n = 7) small isolated and round stained cell(s) in only 14 out of the 48 embryonic STG (Fig. 3B). These isolated stained STG cells had a similar diameter to the one of the surrounding unstained neuronal cells (Fig. 3B) and were only found during the first half of embryonic development (Fig. 4). All E35–E45 STG showed BrdU immunostaining with one (n = 2) or two (n = 6) round isolated immunoreactive cells. In contrast, only 1 out of 13 (less than 8%) E55–E65 STG displayed BrdU immunostaining and all STG by E70 and throughout the larval stages were devoid of BrdU immunostaining (n = 26; Figs. 3C, 4). Furthermore it is worth noting that the percentage of BrdU immuno-positive cells among the neuronal population of the STG was always very small, never more than 7% and decreased rapidly throughout embryonic development becoming zero by E70. Thus, these results demonstrate that the neuronal population of the STG is quantitatively established by E70 and remains stable throughout embryonic and larval development until adulthood.

**FLRFamide- and proctolin-like immunoreactivity in the adult STNS**

In a previous study, using electrophysiological techniques, Casasnovas and Meyrand (1995) found that the embryonic STG neuronal population produces a single rhythmic motor output. Furthermore, during the subsequent stages of development, this single activity is progressively restructured to give rise to two distinct rhythmic motor patterns characteristic of the adult STG. Thus, from the results of the preceding section, the same neuronal population appears able to generate a single embryonic and then two distinct adult motor patterns. One can wonder what sort of mechanisms may be responsible for this maturation. In the decapod Crustacea, it is well known that neuromodulators play a major role in the expression of adult neural networks with short or long term effects (Dickinson et al., 1990; Meyrand et al., 1991, 1994; Thoby and Simmers, 1998). More than 20 different neuromodulatory neurons have been electrophysiologically identified in the anterior ganglia (Robertson and Moulins, 1981, 1984; Simmers and Moulins, 1988; Nusbbaum et al., 1989a,b; Cazalets et al., 1990a,b; Courtil et al., 1990; for review, see Harris-Warrick et al., 1992; Combes et al., 1994). In the adult H. gammarus, although these extrinsic inputs have been extensively studied using electrophysiological approaches (see above), immunocytochemical data are still lacking. Thus, we performed a comparative immunohistochemical detection of FLRFamide related peptides and proctolin within the STNS of H. gammarus throughout development as well as in the adult.

Eight whole-mount preparations of the adult H. gammarus STNS were treated with the FMRFamide antiserum. In all preparations, dense FLRFamide-like immunostaining was seen in processes throughout the central neuropil region (Fig. 5A). In all preparations, none of the STG somata were stained (Fig. 5A). Clear staining was seen in at least eight fibers in the stn and many processes appear to terminate in, or give rise to, large stained varicose structures. In the single efferent motor nerve, dvn, at least three fibers were consistently stained (not shown). The two anterior ganglia CoGs had dense neuropile staining close to the ion. In addition, approximately ten large-medium sized (30–60 µm) somata and some small (5–10 µm) cells were immunostained (Fig. 5B1). Stained fibers were seen in the son (a bundle of six to twelve fibers depending on the proximity to the CoGs, Fig. 5B1) and in the ion (at least ten fibers close to the CoGs [Fig. 5B1] and then three to four close to the OG [Fig. 5C1]). In the OG, three somata were
Fig. 3. 5-bromo-2’-deoxyuridine (BrdU) staining of dividing cells in the embryonic nervous system of the lobster. The dividing cells are visualized with a BrdU antibody on serial paraffin sections. **A:** Section through the cerebral ganglion illustrating two neighboring BrdU-positive cells in an E57 embryo. Note that the staining is specifically limited to the cell nucleus. Also note that one stained nucleus (large star) is larger (10 µm) than the other (small star; 6 µm). **B:** A unique cell nucleus (star) is immunopositive in the stomatogastric ganglion (STG) from an E55 embryo. **C:** From the second half of embryonic development, no dividing cells are revealed in the STG (example shown, E98), whereas dividing cells are stained in surrounding tissues. Br, cerebral ganglion; St, stomodeum. Scale bars = 10 µm.
immunopositive (Fig. 5C1). In addition, two immunopositive cells were found in the oesophageal nerve that links the OG to the brain. Two to three FLRFamide-like immunoreactive cells were present as well as at least two fibers (data not shown).

Five adult STNS were treated with the proctolin antibody. In all STG preparations, the pattern of proctolinergic immunostaining was similar to the FLRFamideergic one. Indeed, proctolin-like immunostaining was found in processes throughout the central neuropil (Fig. 5A2). Furthermore, all preparations, none of the STG neurons showed proctolin-like immunoreactivity (Fig. 5A2). Two proctolin immunoreactive fibers were found in the stn and ramified in the neuropile of the STG. In contrast, the distribution of proctolinergic immunostaining in the anterior ganglia of the STNS differs from the one found for FLRFamide-like peptides. Indeed, in each CoG a fine proctolinergic neuropil was detected as well as one medium sized immunoreactive cell close to the emergence of the ion (arrowhead in Fig. 5B2). Only one immunoreactive fiber was found in each son, the nerve linking each CoG to the stn, whereas a bundle of six to eight immunopositive fibers was found in the ion. OG and its connective nerves (on, ion and ivn; Fig. 5C2) as well as the principal stomodeal motor nerves (dvn and ivn) were always devoid of staining.

**FLRFamide-like immunoreactivity in the STNS throughout development**

In this section we describe the distribution of FLRFamide-like immunoreactivity in the STNS of 11 embryos and 21 larvae. Faint FLRFamide immunostained punctate neuropile appeared in the STG as early as E45 and remained throughout subsequent developmental embryonic and larval stages (Fig. 8A–F). At all studied developmental stages, one to two immunostained fibers were detected in the stn. As in the adult, the general immunostaining in the anterior ganglia remained unchanged during development (Fig. 8B and compare with Fig. 5B2). The immunostaining in the CoGs consisted of one soma (arrowhead in Fig. 9B), some fibers and a sparse neuropil area (Fig. 9B). In some preparations one faintly stained fiber was found in each son. The OG, ion, on, and dvn were always devoid of immunostaining as in the adult. Figure 9A presents a typical pattern of proctolinergic immunostaining in the brain as observed as early as E20.

Again, the most dramatic change in the distribution of proctolinergic staining throughout development was found in the STG. One to two FLRFamide-like immunopositive neuronal somata were found in embryonic STG as early as E50 (an E75 is shown in Fig. 6B), while only one soma stained in first larval stage (Fig. 6C). In contrast, no immunostained somata in STG of LII, LIII, and LIV stages (Fig. 6D–F) nor in the adult (Fig. 5A1) were found. The time course of the evolution of the number of FLRFamide-like immunopositive neuronal cell bodies in the STG is summarized in graph of Figure 10 (dark dots).

**Proctolin-like immunoreactivity in the STNS throughout development**

In this section we describe the distribution of proctolin-like immunoreactivity in the STNS of 11 embryos and 21 larvae. Faint proctolinergic immunostained punctate neuropile appeared in the STG as early as E45 and remained throughout embryonic and larval development (Fig. 6A–F). At all studied developmental stages, two to five fibers were stained in the stn and two intensely stained fibers with varicosities were observed in the dvn with one entering in each Ivn (Fig. 7D). In most preparations (even in post-larva: LIV) intense, globular, and neuropilar-like staining was observed downstream from the STG in the dvn (for example, see arrow in Fig. 6C) and in some preparations two immunostained fibers were seen to leave the dvn at this level. Moreover, whatever the developmental stage, the general pattern of staining in the anterior ganglia is similar to the adult one. Indeed, two (n = 6) to three (n = 13) somata were immunopositive in the OG (Fig. 7C and compare with Fig. 5C1). In embryos and larvae as well as in the adults the CoG labeling is typified by a dense neuropile and a cluster of immunopositive cells at the emergence of the son (Fig. 7B and compare with Fig. 5B1). Figure 7A presents an overview of the typical pattern of immunostaining observed in the brain of embryos as well as larvae.

The most dramatic difference in the distribution of FLRFamide-like staining throughout development was found in the STG. Indeed, two to three FLRFamide-like immunopositive neuronal somata were found in embryonic STG as early as E50 (an E75 is shown in Fig. 6B), while only one soma stained in first larval stage (Fig. 6C). In contrast, no immunostained somata in STG of LII, LIII, and LIV stages (Fig. 6D–F) nor in the adult (Fig. 5A1) were found. The time course of the evolution of the number of FLRFamide-like immunopositive neuronal cell bodies in the STG is summarized in graph of Figure 10 (dark dots).
Fig. 5. Immunohistochemical localization of FLRFamide- and proctolin-like peptides in the adult stomatogastric nervous system (STNS). The stomatogastric ganglion (STG) contains numerous immunoreactive fibers and neuropilar processes for FLRFamide, whereas none of the STG somata are stained (A1). Several clusters of immunoreactive cells with one more important cluster near the son emergence are found in each commissural ganglia (CoGs) and a densely stained neuropile (n) near the ion nerve. Close to the CoG, immunoreactive bundles contain 10 and 6–12 neuronal processes in, respectively, the ion and son (B1). Three strongly immunoreactive oesophageal ganglion (OG) somata are labeled in the OG and, in addition, two faint fusiform cell bodies are localized within the on nerve (arrowheads, C1). The proctolinergic antibody stains a dense neuropile within the STG, but none of the STG cell bodies (A2). Proctolinergic immunoreactivity in the CoGs consists of a brightly stained neuropile (n) and only one medium size cell body close to the ion nerve (arrowhead, B2). The OG of H. gammarus does not display proctolinergic-like immunoreactivity (C2). dvn, dorsal ventricular nerve; ion, inferior oesophageal nerve; ivn, inferior ventricular nerve; lvn, lateral ventricular nerve; on, oesophageal nerve; son, superior oesophageal nerve; stn, stomatogastric nerve. Scale bars = 100 µm.
STNS. This comparison summarizes the slight developmental differences found in the FLRFamidergic and proctolinergic staining in the anterior ganglia and points out the striking difference observed in the STG with the expression of a transitory FLRFamidergic and proctolinergic phenotype in several STG somata.

**DISCUSSION**

In the present study we have investigated the developmental acquisition of two peptidergic systems in the STNS of the lobster. We have found that 1) very early during development the STG neuronal population is quantitatively established and remains stable throughout adulthood, 2) FLRFamide- and proctolin-like immunoreactivities are present in the neuropile of the STG by mid-embryonic life, and 3) some STG neurons express transient FLRFamidergic and proctolinergic phenotypes during embryonic and larval life.

A quantitative stable neuronal population in the STG throughout development

The analysis, based upon cell counts on sections during the development of *H. gammarus*, showed an early estab-
establishment of the STG neuronal population. Regardless of the embryonic stage, from E20 to hatching, the mean number of STG neurons was not statistically different from that of the adult. In addition, no figure of dying neurons as described by Truman and Schwartz (1982) or of mitosis was detected in any cresyl violet stained paraffin STG sections of 52 specimens (embryos, larvae, and post-larvae). This strongly suggests that this ganglion does not display any massive neuronal cell death nor late neurogenesis. This is in contrast with numerous other systems.

Fig. 7. FLRF-like immunoreactivity in the stomatogastric nervous system (STNS) in embryonic, larval and post-larval stages. A: A low magnification view of the whole cerebral ganglion (Br) showing numerous brightly stained fibers and clusters, cells, and neuropil zones in a first larval stage (LI). B: FLRFamide-like immunoreactivity in a commissural ganglia (CoG) of a third larval stage (LIII) is similar to that of the adult (compare with Fig. 5B1). C: FLRFamide-like immunoreactivity in a oesophageal ganglion (OG) of a third larval stage (LIII) is similar to that of the adult with three immunoreactive somata (arrowheads). However, the two faintly stained somata found in the oesophageal nerve of the adult (arrowheads, Fig. 5C1) are not immunoreactive in embryos and larvae. D: By embryonic stages (example shown, E100) at least two neuronal processes are immunolabeled in the dvn and one immunoreactive fiber is found in each lvn (arrows). dvn, dorsal ventricular nerve; ion, inferior oesophageal nerve; ivn, inferior ventricular nerve; lvn, lateral ventricular nerve; son, superior oesophageal nerve; STG, stomatogastric ganglion; stn, stomatogastric nerve. Scale bars = 100 µm.
Neuronal cell death appears as a nearly universal phenomenon in invertebrates as well as vertebrates during development (Truman, 1983; Williams and Herrup, 1988; Oppenheim, 1991; Raff et al., 1993). Late neurogenesis is also a common feature. It takes place during development of invertebrates (Booker and Truman, 1987; Truman and Bate, 1988; Cash and Carew, 1989; Ito and Hotta, 1992; Truman, 1992; Harzsch and Dawirs, 1994, 1996; Levine et al., 1995; Harzsch et al., 1997) as well as in adulthood of invertebrates (Technau, 1984; Cayre et al., 1994) and vertebrates (Goldman and Nottebohm, 1983; Bayer, 1980, 1982).

To confirm our neuronal counts, we performed in vivo labeling by using BrdU (Gratzner, 1982). This technique has been extensively used in both vertebrates and invertebrates nervous systems and has also been validated in decapod crustaceans (spider crab: Harzsch and Dawirs, 1994, 1996; American lobster: Harzsch et al., 1997). By using this technique we found in the embryonic brain and in the ventral nerve cord ganglia of H. gammarus immunostained clusters of one large cell accompanied by one or more smaller cells, in agreement with Harzsch and Dawirs (1994, 1996). In contrast to this classical pattern of immunopositive cell clusters, we have found rare (one or two) isolated BrdU immunostained cells in the H. gammarus...
STG only during the first half of the embryonic life. From E70 onwards no more BrdU immunolabeled cells were detected. Recently, Harzsch et al., (1997) also showed that BrdU immunostaining slowed down at about E70 in the developing H. americanus brain and ventral nerve cord ganglia. Thus, our study clearly demonstrates that the neuronal STG population remains stable from mid-embryonic life throughout adulthood and rules out the possibility of late neurogenesis. However, the nature of the rare isolated BrdU immunopositive cells we found in the STG remains unclear. From our observations, we can however exclude several hypothesis. Firstly, it appears that these rare isolated small cells cannot be neuroblasts. Indeed, in the brain and ventral nerve cord ganglia (present study; Harzsch and Dawirs, 1994, 1997) neuroblasts could be identified according to their large sized nucleus (close to 12 µm), which was generally accompanied by smaller stained cells. In contrast, in the present study, in the STG, we noticed that the diameter of the isolated stained cell was not different from the one of the surrounding unstained neuronal cells within the STG. Secondly, we can rule out the possibility that the rare immunopositive cells taken into account in the first embryonic period might be glial cells. According to morphological criteria, it is possible to distinguish between future neurons and dividing glial cells. Indeed, glial cells in Crustacea have a very distinct nucleus (small and flat or irregularly shaped or long-spindle-shaped) that is unambiguously distinguishable from neuronal nuclei (Maynard, 1971; King, 1976; Harzsch and Dawirs, 1994). We have only taken into account the stained cells in the STG that have a round and regular nucleus. Last, the absence of BrdU immunostaining in the STG from E70, strengthened by the absence of mitotic figures in any sections of the 52 STG and the fact that whatever the embryonic stage the mean number of neuronal cells within the STG is not different from the adult one, strongly argue against the hypothesis of the rare BrdU immunopositive cells in the STG being ganglion mother cells postponing their final division. However, until unam-

**Fig. 9.** Proctolinergic immunoreactivity in the rostral ganglia of the nervous system at different stages of development. A: An overview of the proctolinergic immunoreactivity in the brain. As early as E40, the pattern of proctolin-like immunoreactivity consists of fibers which travel throughout the cerebral ganglion. B: Whole mount preparation of a commissural ganglion (CoG) from post larval stage (LIV). During all stages of development, immunostaining in the CoG is unaffected and similar that of the adult (see Fig. 5B2) with a stained neuropile (n) and one immunoreactive cell body (arrowhead). Scale bars = 100 µm.

**Fig. 10.** Developmental timetable of the mean number of FLRFamide (filled circle) and Proctolin (open circle) immunopositive neuronal somata within the stomatogastric ganglion (STG). FLRFamide STG neurons first appear in early embryonic development and disappear as early as the second larval stage (LII). In contrast, proctolinergic neurons are not present in embryonic STG. Somata staining appears after hatching and is present throughout the post larval stage (LIV). In the adult, none of the STG neurons exhibit FLRFamide or proctolinergic immunoreactivity. The number of analyzed preparations for each stage of development is indicated in parentheses.

Thus, our study clearly demonstrates that the neuronal STG population remains stable from mid-embryonic life throughout adulthood and rules out the possibility of late neurogenesis. However, the nature of the rare isolated BrdU immunopositive cells we found in the STG remains unclear. From our observations, we can however exclude several hypothesis. Firstly, it appears that these rare isolated small cells cannot be neuroblasts. Indeed, in the brain and ventral nerve cord ganglia (present study; Harzsch and Dawirs, 1994, 1997) neuroblasts could be identified according to their large sized nucleus (close to 12 µm), which was generally accompanied by smaller stained cells. In contrast, in the present study, in the STG, we noticed that the diameter of the isolated stained cell was not different from the one of the surrounding unstained neuronal cells within the STG. Secondly, we can rule out the possibility that the rare immunopositive cells taken into account in the first embryonic period might be glial cells. According to morphological criteria, it is possible to distinguish between future neurons and dividing glial cells. Indeed, glial cells in Crustacea have a very distinct nucleus (small and flat or irregularly shaped or long-spindle-shaped) that is unambiguously distinguishable from neuronal nuclei (Maynard, 1971; King, 1976; Harzsch and Dawirs, 1994). We have only taken into account the stained cells in the STG that have a round and regular nucleus. Last, the absence of BrdU immunostaining in the STG from E70, strengthened by the absence of mitotic figures in any sections of the 52 STG and the fact that whatever the embryonic stage the mean number of neuronal cells within the STG is not different from the adult one, strongly argue against the hypothesis of the rare BrdU immunopositive cells in the STG being ganglion mother cells postponing their final division. However, until unam-
biguous determination of the cell type of these early STG-stained cells, this possibility cannot be definitely ruled out.

In conclusion, the neuronal population of this invertebrate ganglion is established very early in development and remains stable, with no neuronal cell death nor late neurogenesis, in a specie undergoing discontinuous larval development ending with a metamorphosis. Nevertheless, this is not unique. For example, in Aplysia californica, Jacob (1984) also found one isolated [³H] thymidine-labeled cell within a ganglion of the CNS. As we did in the present study, she also noticed no large precursor cells nor neuronal cell division nor cell death within the forming ganglia. In addition, no evidence of
motor neuron loss has also been found in zebrafish, dogfish and stingray (Mos and Williamson, 1986; Williams and Herrup, 1988).

**Transient peptidergic phenotype expression in the embryonic and larval STG**

We found that the STG neurons are already present in early embryonic life and do not undergo any quantitative alteration in subsequent developmental stages throughout adulthood. Strikingly, this quantitatively stable neuronal population displays qualitative neurochemical modifications. Indeed, in contrast to adult in which none of the STG somata show detectable staining for FLRFamide- and proctolin-like peptides, FLRFamide positive somata are found in the STG during the second half of the embryonic life and early larval stage as well as proctolin immunostained STG neurons in all larval stages. According to the results shown in the previous paragraph, this implies that some of the STG somata transiently express FLRFamide- and proctolin-like phenotypes. Preliminary studies using antibodies against allatostatin, substance P, RPCH, serotonin (V. Fénelon, V. Kilman, B. Casasnovas, E. Marder, P. Meyrand), and published work on dopamine (Cournil et al., 1995) suggest that transient expression of modulators is not general but a specific feature for FLRFamide and proctolin staining.

Although, in some cases, the regulatory factor involved in the alteration of the transmitter expression has been determined (Witten and Truman, 1996), the physiological role of transient peptidergic expression remains to be ascertained. However, several possibilities can be proposed. Firstly, as Meyrand and Marder (1991) found two to three FLRFamide-like immunopositive STG motor neurons in adult shrimp, the transient expression of two to three FLRFamide immunostained STG neurons in lobster embryos could represent a phylogenetic reminiscence. This is further suggested by evidence that, in all the other large decapod species, adult STG neurons use either only acetylcholine or glutamate as their transmitter (Marder, 1987). Second, it has been established that FLRFamide-like peptides modulate neuromuscular junctions in shrimp, crayfish and lobster (Meyrand and Marder, 1991; Skerret et al., 1995; Worden et al., 1995; Jorg-Verivera and Marder, 1996) and proctolin functions as a cotransmitter enhancing muscle tension in crayfish (Bishop et al., 1987). Thus, if we assume that STG neurons that transiently express FLRFamide and proctolinergic phenotypes are motor neurons, we could speculate that this transient peptidergic expression may reflect unique functional needs of target muscles at specific embryonic and/or larval stages related to trophic, tension or receptor-channel maturation. We could conversely imagine that target-derived factors could turn down peptidergic expression in some STG neurons at some specific developmental stages. Indeed, this specification of transmitter phenotypes has been demonstrated in the vertebrate peripheral nervous system (for review, see Landis, 1990). Experimental manipulations of neuronal targets in vivo have shown that both classical (Stevens and Landis, 1987; Shotzinger and Landis, 1988) and neuropeptide (McMahon and Gibson, 1987; Horgan and Van Der Koy, 1992; Coulombe and Kos, 1997) transmitter phenotypes can be regulated by interactions between neurons and the targets they innervate. Last, peptidergic transient expression within the STG could compensate for a lack of neuromodulation that will be established later during development. Although this possibility can not be definitely ruled out, this hypothesis seems highly improbable according to the results we will discuss in the next section of this discussion.

Developmental alterations in FLRFamide-like neuronal immunoreactivity have already been reported in numerous invertebrates (Blackburn et al., 1992; Witten and Truman, 1996; Voronezkaya and Elekes, 1996). It is worth noting that, as in our study, these FLRFamide-like neurons are present in embryonic and some larval stages but are absent in adults. To our knowledge, the only known example of transient expression of a proctolinergic phenotype reported so far in invertebrates is the observation of a single large midline cell stained for proctolin in the second abdominal ganglion of the lobster in embryonic and early larval life (Beltz and Kravitz, 1987). In insects and molluscs, the expression of crustacean-cardioactive-, cardioaccelatory-, bursicon-, enkephalin-, and gastrin/cholecystokinin-like peptides is altered during development (Gesser and Larsson, 1985; Tublitz and Sylwester, 1990; Davis et al., 1993). In vertebrates, regulation of neurotransmitter expression has been extensively documented in the autonomic nervous system during embryonic development (Black and Patterson, 1980) as well as postnatally (Landis and Keefe, 1983; Leblanc and Landis, 1986). Furthermore, developmental regulation of peptide expression also occurs in motor neurons of neonatal rats (Matteoli et al., 1990).

**Developmental study of peptidergic inputs to the STG**

It is now well established that the adult decapod crustacean STG is the target of a large number of neurons (aminergic and peptidergic) most of which have been electrophysiologically identified (Nagy and Dickinson, 1983; Nusbaum and Marder, 1989a,b; Cazalets et al., 1990a; Cournil et al., 1990; Combes et al., 1994). These neurons are known to influence the component neurons of the STG neural networks by altering their intrinsic properties (Nagy and Dickinson, 1983; Harris-Warrick et al., 1989; Kiehn, 1991; Kiehn and Harris-Warrick, 1992) and/or the efficacy of their synapses (Nagy and Dickinson, 1983; Cazalets et al., 1990b; Dickinson et al., 1990; Johnson et al., 1994). It has been demonstrated that these identified modulatory neurons play a major role in the expression of STG neuronal networks, which can even lead to a dynamic neural network reconfiguration (Meyrand et al., 1991, 1994). Thus, it appears that a developmental study of the acquisition of these modulatory inputs is required to investigate the developmental maturation of the STG networks.

During embryonic and larval development we report here that FLRFamide and proctolin immunoreactive processes and/or neurons are present very early (as early as E20-E40) in the brain and in the STNS. Within the STG, FLRFamide- and proctolin-like immunopositive neuromodulatory processes are present from E40-45 throughout adulthood, the overall appearance of staining remaining similar through development except for an increase in staining intensity. This early establishment of the FLRFamide and proctolin in the STNS can be extended to other peptides such as allatostatin- and red-pigment-concentrating-hormone (RPCH)-like peptides (V. Fénelon, V. Kilman, E. Marder, and P. Meyrand, unpublished results). In contrast, amines such as dopamine (Cournil et al., 1995) and 5-HT (Casasnovas, 1996) seem to appear later in the
STNS. Dopamine and 5-HT immunoreactive afferents to the STG are found close to or just after hatching. An ongoing comparative study strengthens this last conclusion as we found the same results in the American lobster, H. americanus (unpublished results) even though Beltz et al. (1990) found that 5-HT appeared earlier than proctolin in the cerebral ganglion and ventral nervous cord of H. americanus. The appearance of peptidergic afferents before aminegic afferents has also been described in other systems. Indeed, within the rat spinal cord, substance P- and enkephalin-like immunoreactivity is detected in the descending bulbo-spinal pathways, in the ventral and lateral funiculi, as early as embryonic day (E) 14–18 (Pickel et al., 1982; Senba et al., 1982). In contrast, the spinal noradrenergic innervation only developed between late fetal and the first half of neonatal life and dopaminergic spinal innervation appeared slowly throughout neonatal life (Commissiong, 1983). However, 5-HT is an exception among the development of aminegic nerves in the spinal cord as 5-HT neurons invade the spinal cord from the anterior and lateral funiculi as early as E14 (Rajaofetra et al., 1989).

FLRFamide and proctolin immunoreactivities have already been reported in the adult central nervous system of several crustacean species such as the spiny lobster and the Maine lobster H. americanus (Marder et al., 1986, 1987; Kobierski et al., 1987; Siwicki et al., 1987), the rock crab (Coleman et al., 1992), and in the freshwater crayfish Procambarus darcii (Siwicki and Bishop, 1986). Comparison of these anatomical maps and our present study in the adult lobster (H. gammarus) demonstrates that, except for slight differences in the number of immunopositive cells and/or fibers in the anterior ganglia and their connective nerves, the overall distribution of FLRFamide and proctolin immunoreactivities is similar in the STNS of the different crustacean species.

In conclusion, we found that the stable STG neuronal population expresses transient peptidergic phenotypes throughout development and receives peptidergic inputs in early embryonic life. In a previous study, we found that the STG neuronal population of embryos and all three stages of larvae expresses a single rhythmic motor output and is organized in a single embryonic neural network. In contrast, the STG neuronal population of post-larva IV and adults produces two distinct rhythmic motor patterns and is organized, at least in the adult, in two distinct neural networks (Casasnovas and Meyrand, 1995). Thus, the present developmental anatomical study demonstrates that during the course of development the segregation of a single embryonic network into two distinct adult neural networks occurs without any quantitative modification of the STG neuronal population. Furthermore, the late emergence of the adult motor patterns does not seem to be related in a simple manner to the first appearance and development of the proctolin- or FLRFamide extrinsic inputs to the STG or to the expression of transient peptidergic phenotypes within the STG.

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LITERATURE CITED


