Dopamine and Histamine in the Developing Stomatogastric System of the Lobster Homarus americanus

STEFAN R. PULVER, VATSALA THIRUMALAI, KATHRYN S. RICHARDS, AND EVE MARDER

Volen Center and Biology Department, Brandeis University, Waltham, Massachusetts 02454

ABSTRACT

Dopamine and histamine are neuromodulators found in the adult stomatogastric nervous system (STNS) of several crustacean species. We used antibodies against tyrosine hydroxylase (TH) and histamine to map the distribution and developmental acquisition of the dopamine and histamine neurons in the STNS of the lobster, Homarus americanus. Embryos, larvae, juvenile and adult animals were studied. TH labeling was present in the STNS as early as E80–85 (80–85% of embryonic development). A subset of preparations in embryos, larvae, juveniles, and adults contained 1–5 labeled somata in the stomatogastric ganglion. Histamine staining appeared in the STNS as early as E50. The distribution of both TH and histamine staining remained relatively constant through development. Electrophysiological recordings demonstrated that receptors for both amines are present in the embryo. Bath application of dopamine increased the frequency of the pyloric rhythm in embryos, and evidence for dopaminergic activation of peripherally initiated spiking in motor axons was seen. In embryos and adults, histamine inhibited the motor patterns produced by the stomatogastric ganglion (STG). These data suggest that the dopaminergic and histaminergic systems in H. americanus appear relatively early in development and that the effects of each are largely maintained through development. J. Comp. Neurol. 462:400–414, 2003. © 2003 Wiley-Liss, Inc.

Indexing terms: amine neuromodulators; embryonic motor patterns; stomatogastric ganglion; crustaceans; peripheral spike initiation

The stomatogastric nervous system (STNS) has become one of the best preparations for understanding how neuromodulators reconfigure neuronal networks (Marder and Calabrese, 1996; Marder and Bucher, 2001; Nusbaum et al., 2001; Nusbaum and Beenhakker, 2002). Over the past 30 years numerous anatomical, biochemical, and electrophysiological studies have demonstrated that more than 20 different substances are present in the adult stomatogastric nervous system, many of them in identified descending projection neurons whose physiological roles are becoming characterized (Nusbaum et al., 2001; Nusbaum and Beenhakker, 2002; Meyrand et al., 2000). We are interested in understanding how neuromodulatory systems develop and the potential roles of neuromodulators in the development of motor systems. Many electrophysiological studies of neuromodulator action of the stomatogastric ganglion (STG) were done in the lobster Panulirus interruptus and the crab Cancer borealis. Despite the advantages of these species for studies of adult modulation, in these species it has not yet been possible to obtain embryos and larvae to study the early development of the STG. In contrast, studies of modulation of the adult STG in Homarus americanus and Homa-
rus gammarus have lagged behind those in P. interruptus and C. borealis, but in H. americanus and H. gammarus developmental studies are feasible (Casasnovas and Meyrand, 1995), and it is possible to study the actions of neuromodulators as early as 50% of embryonic development (Le Feuvre et al., 1999; Richards et al., 1999, 2003; Richards and Marder, 2000). Previous work has shown that some neuromodulators are already present early in embryonic development, whereas others appear only during larval times (Cournil et al., 1995; Fenelon et al., 1998, 1999; Kilman et al., 1999; Li et al., 2002). We now characterize the time course of appearance of the dopaminergic and histaminergic systems in H. americanus and compare the physiological actions of these substances in the embryo to those produced in the adult.

Dopamine was one of the first neuromodulators to be found in the STNS of several decapod species (Kushner and Maynard, 1977; Barker et al., 1979; Kushner and Barker, 1983; Cournil et al., 1994, 1995). In P. interruptus, dopamine alters the STG motor patterns (Eisen and Marder, 1984; Marder and Eisen, 1985; Flamm and Harris-Warrick, 1986a, b) by modulating synaptic strength (Johnson and Harris-Warrick, 1990; Johnson et al., 1993, 1995; Ayali et al., 1998) and one or more of the voltage-dependent currents in STG neurons (Harris-Warrick et al., 1995a, b). However, recent work (Bucher et al., submitted) indicates that the physiological actions of dopamine in H. americanus are quite different from those in P. interruptus, and therefore we compare the data on embryonic H. americanus with those recently described by Bucher et al. (2003) on adults.

The actions of histamine have been characterized in P. interruptus (Claiborne and Selverston, 1984a). In P. interruptus, histamine inhibits ongoing pyloric rhythms by activating a Cl⁻ channel in many of the neurons of the STG (Claiborne and Selverston, 1984a). These actions are responsible for many of the effects of the inferior ventricular (IV) neurons in P. interruptus (Dando and Selverston, 1972; Sigvardt and Mulloney, 1982). Histamine actions in the adult H. americanus have not been previously reported. Therefore, we include here a first characterization of the effects of histamine on the adult pyloric rhythm in H. americanus, to facilitate the comparison with the earlier developmental stages. The presence of histamine in the adult STNS of H. americanus has been previously described (Mulloney and Hall, 1991). We now add a developmental description of the acquisition of histamine-like immunoreactivity and compare the patterns of histamine early in development to those seen in adults.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on adult (n = 28), juvenile (thorax length [TL] = 1–2 cm; n = 7), larval (n = 27), and embryonic (n = 52) H. americanus. Adults were purchased from Yankee Lobster (Boston, MA) and kept in aerated seawater tanks at 10–13°C. Juveniles and larvae were either obtained from the New England Aquarium (Boston, MA) or raised from embryos at Brandeis University. Bernardino females carrying developing embryos were obtained from the Massachusetts State Lobster Hatchery (Martha’s Vineyard, MA), and embryos at different stages were removed from the females just prior to use. Juveniles, larvae, and embryos were held in seawater tanks at 12–15°C. Larvae and embryos were staged according to Helluy and Beltz (1991). Embryos are referred to as their percent of embryonic development (e.g., E50 is 50% of embryonic development). All animals were anesthetized for 20 minutes in an ice bath prior to dissection.

**Immunocytochemistry**

We used an antibody against tyrosine hydroxylase (TH), the enzyme that catalyzes the rate-limiting step in dopamine biosynthesis, to identify dopamine-synthesizing neurons in the STNS. Previous studies have shown that anti-TH antibodies stain dopaminergic neurons in many invertebrates, including insects (Nässel and Elekes, 1992; Mesce et al., 2001; Crisp et al., 2002) and mollusks (Croll et al., 1999; Hernadi and Elekes, 1999; Voronezhskaya et al., 1999). Most importantly, in H. americanus, the presence of dopamine in the TH-stained L-cell was confirmed by biochemical methods (Siwicki et al., 1987). Moreover, in the closely related lobster H. gammarus, Cournil et al. (1994, 1995) showed that nearly all cells stained with an anti-dopamine antibody also stained for TH.

Preparations that were processed for TH immunocytochemistry were dissected in saline containing (in mM) 479.12 NaCl, 12.74 KCl, 13.67 CaCl₂, 20 MgSO₄, 3.91 Na₂SO₄, 5 HEPES, pH 7.4–7.5. In adult and juvenile animals, the nerves of the STNS were dissected free from the stomach in chilled saline and pinned flat on a Sylgard (Dow Corning, Midland, MI) lined Petri dish (Mulloney and Selverston, 1974, Harris-Warrick et al., 1992). In larvae and embryos, the foregut was removed, split open along the midline, and pinned to a small Sylgard cube (Kilman et al., 1999). The nerves of the STNS were uncovered but not removed from the stomach musculature. After dissection, tissues were fixed for 1–2 hours in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and then left overnight in 0.1 M phosphate buffer, pH 7.4. The next day the preparations were washed 4–5 times over the course of 7–8 hours in PTA (0.1 M sodium phosphate buffer, 0.3% Triton X-100, and 0.1% NaN₃, pH 7.4). Tissues were then incubated for 2 days in a mouse monoclonal antibody raised against TH (Immunostar, Hudson,
WI; catalog # 22941) at a dilution of 1:200 with 5% goat normal serum (GNS) in PTA. Preparations were then washed as above in PTA and incubated overnight in 1:400 anti-mouse IgG coupled to Alexa Fluor® 488 (Molecular Probes, Eugene, OR) with 5% GNS in PTA. After secondary incubation, the preparations were washed 5–6 times in 0.1 M phosphate buffer and mounted on glass slides in 80% glycerol and 20% 20 mM Na₂CO₃, pH 9.5. All processing was carried out at 4°C.

For histamine immunocytochemistry, we dissected animals in low calcium saline ([in mM] 479.12 NaCl, 12.74 KCl, 3 CaCl₂, 12.74 MgCl₂, 10 MnCl₂, 5 HEPES, pH 7.4–7.5) to reduce histamine release at synaptic sites in the STNS. After dissection, tissues were fixed for 30–45 minutes in 4% 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride in the low calcium saline used for dissection. The preparations were washed as above in PTA and then incubated overnight in a rabbit polyclonal antibody raised against histamine (Accurate Chemical, Westbury NY, catalog # B80-1) at a dilution of 1:500–1:1,000 in PTA with 10% GNS. The tissues were then washed as above in PTA and incubated overnight in 1:400 anti-rabbit IgG coupled to Alexa Fluor 488 in PTA with 10% GNS. After secondary incubation, preparations were mounted as above.

Imaging

Mounted preparations were viewed with the appropriate filters on a Leica TCS confocal microscope fitted with an argon (458/488 nm) and krypton lasers (568 nm). Optical sections were taken every 1–2 μm in embryonic and larval preparations and every 3–4 μm in juvenile and adult preparations. The resulting images were compiled into maximum projections in the 3-dimensional reconstruction program Amira (TGS, San Diego, CA) and then processed with Photoshop 6.0 (Adobe Systems, Mountain View, CA) and Canvas 8 (Deneba, Miami, FL).

Electrophysiology

Embryos between E71 and E99 and adults (approximately 85 mm TL) were used in electrophysiological experiments. In embryos, the foregut was removed, and a lateral incision made down the length of the stomach. The lateral incision preserved the ventral pyloric dilator (PD) innervated muscles (pdm) and the muscles along one side of the stomach. The STNS was uncovered, but not separated from the stomatogastric musculature. The entire stomach was pinned in a Sylgard-lined Petri dish and superfused with 12°C saline. Pyloric motor output was monitored by recording extracellularly in intracellular recordings by their common temporal relationship to spikes on the pdm and lpn.

Dopamine (Sigma, St. Louis, MO) was applied to embryonic preparations in which the descending inputs from anterior ganglia were intact (n = 8), and in preparations in which the nerve containing descending inputs (stomatogastric nerve [stn]) had been cut (n = 9) and in preparations in which the STG itself had been removed (n = 4). Histamine (Sigma) was applied only to preparations with anterior ganglia intact (embryos: n = 13; adults: n = 19).

Both substances were dissolved in H. americanus saline and bath-applied at concentrations ranging from 10⁻⁴ to 10⁻² M using a switching port.

Data were acquired using a Digidata 1200A board and pClamp 8.0 suite of software (Axon Instruments, Union City, CA). Spikes and bursts were extracted using Spike 2 (Cambridge Electronic Design, Cambridge, UK) and analyzed with Microsoft Excel. Results were plotted and statistical tests calculated using SigmaPlot and SigmaStat (SPSS, San Rafael, CA).

RESULTS

The stomatogastric nervous system

The STNS consists of four ganglia, the STG, the paired commissural ganglia (CoGs), and the single esophageal ganglion (OG) as well as their connecting nerves (Fig. 1). The STG contains approximately 30 neurons, including the motor neurons that innervate the pyloric and gastric mill muscles of the foregut. The CoGs and OG contain the cell bodies of modulatory projection neurons that send fibers through the inferior esophageal nerves (ions), superior esophageal nerves (sons), and stn to the STG neuropil. The paired pyloric suppressor (PS) modulatory projection neurons are found in the inferior ventricular nerve (ivn) and project to the STG via the sons, ions, and stn. Previous work has shown that the ganglia and nerves of the STNS are present before 50% of embryonic development (Fénelon et al., 1996; Le Feuvre et al., 2001). Although the anatomy of the STNS is well developed in the embryo, the full complement of STG neuromodulators is not present until late in larval life (Fénelon et al., 1999; Kilman et al., 1999; Li et al., 2002).

Tyrosine hydroxylase immunoreactivity in the embryonic, larval, and adult STG

Figure 2 shows whole mounts of STGs at four developmental stages stained with the TH antibody. Figure 2A shows an E67 STG; no immunoreactivity was present in either the neuropil or cell bodies of the STG. Five of five embryos between E40 and E70 also lacked staining in the STG. However, TH-labeled cells in the CoGs and other parts of the nervous system were visible in these preparations (data not shown). Between E80 and E85, TH staining became visible in the STG. Figure 2B shows TH immunoreactivity in an E89 STG. The STG neuropil and varicosities in the stn (arrowhead) were heavily stained, and two cells were lightly stained (arrows). In three of six preparations between E80 and E90, TH-labeled cell bodies were present in the STG. The number of cell bodies ranged from 1 to 5 (Table 1). Also visible in Figure 2B are varicosities in the dvn (asterisk). Stained trailing fibers in the dvn were present in two of six preparations between E80 and E90. However, the TH antibody did not stain any other peripheral motor nerves in embryonic preparations.

After hatching, TH staining persisted in the STG throughout larval life. Figure 2C shows TH immunoreactivity in a larval stage 3 (L3) STG. One large TH-stained process (arrowhead) was visible entering and ramifying in
the STG. A single lightly stained cell body was also present in the STG (arrow). In four of four larval stage 4 (L4) preparations, no cell bodies were visible in the STG. However, TH-stained cell bodies were present in four of nine other larval preparations (Table 1). Most larvae did not show staining posterior to the STG; however, in one L2 and one L3, two lightly stained TH fibers were visible in the dvn. No TH staining was visible in other peripheral motor nerves in larvae.

The same essential features of the TH distribution were seen in adult preparations (Fig. 2D, n = 5). Two large, densely labeled fibers (filled arrowheads) projected down the stn and branched extensively in the STG neuropil. A TH-stained cell body (arrow) was present in the STG, and a smaller TH fiber was present in the stn (open arrowhead). In two of five adult preparations, a lightly stained TH cell body was visible in the STG.

In all adult preparations, a single TH fiber looped into the dvn (Fig. 2D, double asterisk) and then returned to the STG. However, no TH staining was ever visible in the posterior portions of the dvn or in any peripheral motor nerves. In juvenile (TL 1–2 cm) STGs, we saw staining patterns similar to those in adult STGs (n = 4; Table 1).

TH immunoreactivity in the CoGs and anterior nerves of the STNS

TH immunoreactivity was present in CoGs as early as E35 of embryonic development (Pulver and Marder, 2002).
Figure 3A shows TH staining in the CoG of an E81 animal. A large densely stained cell body (L-cell) sent a projection into the CoG, back into the brain, and then posteriorly down the commissure (Co). This cell is the L-cell, previously shown to contain dopamine in *H. americanus* (Siwicki et al., 1987) and homologous to the dopaminergic L-cell described in *H. gammarus* (Cournil et al., 1994) and other crustacean species (Cooke and Goldstone, 1970; Goldstone and Cooke, 1971). Figure 3D is a tracing of the morphology of the L-cell stained in Figure 3A. During embryonic times, the Co is shortened and the CoG is fused to the base of the brain. At this time, the L-cell soma lies not in the CoG itself, but in the posterior portion of the brain. TH staining was also present in a smaller cell in the CoG (asterisk), the CoG neuropil itself, and in Co fibers.

Figure 3B shows the CoG of a L2 animal stained for TH. The L-cell soma (arrow) was densely stained but remained closer to the brain than the CoG neuropil (Fig. 3E). Two other TH cells were also visible in the Co between the brain and CoG (asterisks). Figure 3C shows TH immunoreactivity in the CoG of
a juvenile animal (TL 1.5 cm). The L-cell soma was within the CoG (arrow) and sent a projection to the brain and then back posteriorly through the Co (Fig. 3F). Three smaller TH-immunoreactive cell bodies were also present in the CoG (asterisks). A single fiber projected out of the CoG into the sn; in favorable preparations, this fiber could be traced to one of the smaller TH-stained cell bodies. Results similar to those shown in Figure 3 were obtained in 6 embryonic, 13 larval, 4 juvenile, and 5 adult preparations.

Figure 3G–I shows TH labeling in the sn and stn through development. A single fiber in each son projected into the stn in embryos (Fig. 3G), larvae (Fig. 3H), and adults (Fig. 3I). No TH-containing cell bodies were visible in the OG, ion, or ivn. These staining patterns were conserved through larval and adult development (larvae: n = 13; juvenile: n = 4; adult: n = 5).

Figure 4 summarizes the distribution of TH immunoreactivity at different developmental stages in H. americanus. The number of fibers (f) and number of stained cell bodies are noted where appropriate.

Histamine immunoreactivity in the embryonic, larval, and adult STG
Histamine labeling was present in the STG as early as 50% of embryonic development. Figure 5A shows the STG of an E84 animal stained for histamine. The single input nerve to the STG, the stn, contained one histamine fiber (arrowhead). Dense varicosities were present in the STG neuropil and in the anteriormost regions of the dvn (asterisk). No histamine-immunoreactive cell bodies were present in the STG. Similar labeling patterns were seen in seven embryos from E50 to E90. The same essential fea-
tures of the histamine distribution were seen in L1 and L2 animals (n = 6) (Fig. 5B, L1). By L3, two stained fibers were visible entering the STG neuropil from the stn (Fig. 5C, arrowheads). Similar results were obtained in 4 L3 and 4 L4 animals. The pattern of staining in late larval STGs was similar to that seen in juvenile (n = 3) and adult (n = 4) animals (Fig. 5D, adult).

**Histamine immunoreactivity in the CoGs and anterior nerves of the STNS**

Histamine immunoreactivity was also present in the CoG neuropil as early as 50% of embryonic development.

In embryos, one to two lightly stained cell bodies were present surrounding the heavily stained CoG neuropil regions (n = 7; data not shown). This same pattern per-
sisted throughout larval life \( (n = 14) \). Figure 6A shows a L3 CoG stained for histamine. Dense varicosities filled the CoG neuropil, and fibers were visible in the son, ion, and Co. A single large cell body was present outside the neuropil area in the Co (arrow). In the same preparation, histamine fibers were present in all the anterior nerves of the STNS (Fig. 6B). Small, varicose fibers were visible in the stn and son, whereas larger, ribbon-like fibers were present in the OG and ions. No cell bodies were present in the OG; however, histamine labeling was clearly visible in the ivn.

The ivn in *H. gammarus* and *H. americanus* contains the PS neuron somata. The PS neurons are homologous to the IV neurons in *P. interruptus* (Dando and Selverston, 1972; Claiborne and Selverston, 1984b) and *C. borealis* (Christie, 1995). The PS cells contain histamine in adult *H. americanus* (Mulloney and Hall, 1991) (Fig. 7B). Figure 7A shows histamine-immunoreactive fibers in the anterior nerves and PS cells of an E70 animal. Two large cell bodies (arrows) sent histamine-containing fibers into the OG and out the ions and on. Similar results were obtained in five of five embryos between E50 and E89 dissected.

Fig. 8. Schematics of histamine distribution in the STNS of *H. americanus* through development. The number of fibers (f) and number of stained cell bodies are noted where appropriate.
specifically to reveal the PS cells. PS cells labeled for histamine as early as E55 (n = 2) and contained histamine throughout larval (n = 5), juvenile (n = 2), and adult (n = 3) life.

Figure 8 is a summary of the developmental acquisition of histamine in *H. americanus*. The developmental stage as well as relevant nerves and ganglia are noted where appropriate. The only features of the histamine staining that changed as the animals matured were increases in the number of fibers in the son and cell bodies in the CoGs.

**Physiological effects of dopamine on the embryonic pyloric motor pattern**

In adult animals the motor patterns generated by the STG are monitored by placing extracellular electrodes on the motor nerves and intracellular electrodes in the somata of the motor neurons. However, in the embryos and smaller larvae, this is technically challenging. Therefore, in these small animals, it has become customary to monitor the motor patterns produced by the STG by recording from the muscles innervated by the motor neurons of the STG (Casasnovas and Meyrand, 1995; Le Feuvre et al., 1999, 2001; Richards et al., 1999; Richards and Marder, 2000). We chose to monitor dopamine’s effects on the output of the pyloric network in embryos by recording from either the pdm or lpm.

Figure 9 shows an intracellular recording from an E84 pdm with all descending modulatory inputs to the STNS intact. In control conditions (Fig. 9A), well-defined bursts of excitatory junctional potentials (EJPs) occurred every 1.5–2.5 seconds. In the presence of 10^-5 M dopamine (Fig. 9B), the pdm burst frequency increased, and large muscle action potentials were visible during every burst (asterisks). Both effects of dopamine reversed after the preparation was washed with control saline (Fig. 9C). In five of eight preparations (E71–E91) dopamine induced muscle action potentials. In all preparations the pdm burst frequency significantly increased from 0.45 ± 0.04 Hz in control conditions to 0.64 ± 0.04 Hz in 10^-5 M dopamine (P < 0.001, paired t-test). After washing, the EJP burst frequency returned to 0.47 ± 0.04 Hz.

Because dopamine affected the stomatogastric motor rhythm generated with the descending modulatory inputs intact, we were curious to see what effect dopamine would have on PD neuron motor patterns when the modulatory inputs to the STG were removed. After cutting the stn, and thereby removing modulatory inputs to the STG, there were either no EJPs in the pdm (n = 4) or very slow and irregular EJPs at < 0.08 Hz (n = 5). Figure 10A shows recordings from a preparation in which the pdm recording was silent after the stn was cut. Dopamine at 10^-5 M induced slow bursting as well as small single EJPs (Fig. 10B). At 10^-4 M dopamine, the pdm burst frequency and single EJP frequency increased (Fig. 10C). These effects reversed upon wash with normal saline (Fig. 10D). Similar results were obtained in all stn cut experiments regardless of whether there was initial activity in control saline. In the presence of 10^-5 M and 10^-4 M dopamine, respectively, the EJP burst frequencies in pdm were 0.13 ± 0.02 Hz and 0.34 ± 0.04 Hz (n = 9).

In a recent study, Bucher et al. (2003) found that dopamine activated peripheral spike initiation in the motor axons of the adult PD neurons in *H. americanus*. To test whether the single EJPs observed in the pdm in the embryonic stn cut preparations were caused by spikes in the motor axon that arose peripherally in response to dopamine, we recorded from the pdm after cutting the dvm. Figure 11A shows that after removal of the STG, no EJPs were seen in the pdm in control saline. In the presence of 10^-4 M dopamine (Fig. 11B, C), a large number of EJPs were visible, indicating that the motor nerve itself is sensitive to dopamine. Few of these events remained in wash conditions (Fig. 11D). Similar results were obtained in four preparations.

**Physiological effects of histamine on the embryonic pyloric motor pattern**

The effects of histamine on the embryonic pyloric motor pattern were monitored by recording from either the lpm or the pdm. In three experiments (E87–E99), we recorded from lpm and pdm simultaneously. Figure 12 shows simultaneous intracellular recordings from lpm and pdm in an E99 animal with all anterior nerves intact. Figure 12A shows a slow time base recording of the effects of application of 10^-4 M histamine (arrow). Figure 12B–F shows expanded time scale snapshots of lpm and pdm activity at different times. In control conditions (Fig. 12A, left of arrow; Fig. 12B) the lpm and pdm received bursts of EJPs.
at a frequency of 0.56 Hz. After 2 minutes in histamine (Fig. 12A,C), EJPs in lpm became erratic, whereas regular bursts of EJPs continued in the pdm. After 6 minutes in histamine (Fig. 12D), the lpm was completely silent, and the pdm received bursts of EJPs once every 5–10 seconds. After 10 minutes in histamine, both lpm and pdm were completely silent (Fig. 12E). Subsequent to approximately 10 minutes of washing with control saline, EJPs returned to both the lpm and pdm (Fig. 12F). Similar results were obtained in five lpm preparations (control: 0.55 ± 0.07 Hz; wash: 0.5 ± 0.09 Hz) and six pdm preparations (control: 0.50 ± 0.06 Hz; wash: 0.45 ± 0.05 Hz), in embryos ranging from E70 to E99.

**Physiological effects of histamine on the adult pyloric motor pattern**

The previous section demonstrates that histamine receptors are present in the STNS during embryonic times. To see whether the effects of histamine on pyloric motor patterns were similar to those in adult animals, we applied histamine to the adult *H. americanus* STNS and monitored the output of the PD, LP, and pyloric (PY) motor neurons. In 10^{-7} M and 10^{-6} M histamine, the pyloric rhythm was not significantly affected (n = 4 for each). Figure 13A shows intracellular recordings from the PD and LP neurons and an extracellular recording from the pyn in control saline. Bath application of 10^{-5} M histamine (Fig. 13B) did not affect PD neuron burst frequency significantly. However, 10^{-5} M histamine significantly reduced the number of spikes in the PD neurons from 18.4 ± 2.7 in control saline to 4 ± 2.4 in 10^{-5} M histamine (n = 5; P < 0.05). The number of LP neuron spikes/burst was also decreased from 7.9 ± 2.7 in control saline to 4.0 ± 2.4 in 10^{-5} M histamine (n = 5; P < 0.05). Bath application of 10^{-4} M histamine completely silenced the PD and LP neurons (n = 6; P < 0.001; Fig. 13C). The PY neurons produced long bursts (Fig. 13C).

Figure 14 shows dose-response curves of PD and LP neuron responses to histamine. At concentrations of less than 10^{-5} M, histamine had no obvious effect on PD neuron burst frequency (Fig. 14A), the number of PD neuron spikes/burst (Fig. 14B), or the number of spikes/burst produced by the LP neuron (Fig. 14C). Between 10^{-5} M and 10^{-4} M, these values fell steeply, to reach zero at 10^{-4} M. Asterisks mark values significantly different from control values (repeated measures ANOVA).

To determine whether histamine acted directly on the STG or by activating neurons in the anterior ganglia, we separately applied histamine to the STG or the anterior ganglia. These experiments (data not shown) indicated that the histamine actions shown in Figures 13 and 14 were due to actions directly on the STG (n = 2).
Discussion

In adult nervous systems a large number of amines and neuropeptides function as neuromodulatory substances. As neuromodulators may also have developmental roles (Lauder, 1993), it is interesting to ask when during development the neuromodulatory systems that are present in the adult first appear. In some systems there are changes in neurotransmitter action or expression over the course of an animal’s life (Furshpan et al., 1976; Tutblitz and Sylwester, 1990; Kotak et al., 1998), and some neuromodulators may be present early in developmental time, only to disappear later (Ekstrom, 1994; Barale et al., 1996; Voronezhskaya and Elekes, 1996; Voronezhskaya et al., 1999).

Previous work on the developmental acquisition of neuromodulators in the STNS of the lobsters *H. americanus* and *H. gammarus* showed that some substances are...
present in the projections to the STG by midway through embryonic development, whereas others appear later (Cournil et al., 1995; Fénelon et al., 1998, 1999; Kilman et al., 1999). In this paper we demonstrate that histamine falls in the former category, whereas TH labeling falls in the latter category, appearing later in embryonic development. Both dopamine and histamine are physiologically active in the embryo, and both have actions that resemble those that will be seen later in the lifetime of the animal.

Once staining is clearly present in the STNS, the distributions of both TH and histamine immunoreactivity show relatively few changes at different developmental stages. The one major exception to this is that the number of STG neurons that showed clear TH staining was higher in embryos. This is reminiscent of work on some other modulators that are also present in STG somata in young animals (Fénelon et al., 1998, 1999; Kilman et al., 1999). There are now a number of reports of STG somata stained for one or another neuromodulator (Kilman et al., 1999; Skiebe, 1999; Li et al., 2002; Skiebe et al., 2002). In these studies, as in this paper, STG somatic staining is relatively weak compared with either neuropil staining or staining of somata elsewhere in the nervous system. In these other studies, the number of clearly stained STG somata also varied. As these stained somata have not been identified, it is not yet clear whether the immunoreactivities described here fluctuate in specific neurons, and what the physiological consequences of this for neurotransmitter release might be.

Fig. 13. Modulation of the adult pyloric motor pattern by histamine. A: Intracellular recording from the PD and LP neurons and extracellular recording from the pyn in control saline. Resting potentials: PD, −64 mV; LP, −60 mV. B: Bath application of $10^{-5}$ M histamine reduced the number of spikes in both PD and LP. Resting potentials: PD, −56 mV; LP, −61 mV. C: Bath application of $10^{-4}$ M histamine completely silenced the pyloric rhythm. Resting potentials: PD, −62 mV; LP, −61 mV. D: Wash. Resting potentials: PD, −64 mV; LP, −63 mV.

DOPAMINE AND HISTAMINE IN DEVELOPING LOBSTER

411
The distribution of TH in the *H. americanus* STNS is similar to TH distribution in the closely related *H. gammarus*. Two large TH-immunoreactive fibers project to the STG via the sons and stn. The only differences in reported staining between the two species are the presence of TH-immunoreactive somata in *H. americanus* (none have been reported in *H. gammarus*) and the presence of a third TH-immunoreactive fiber in the *H. americanus* stn (only two fibers are present in the *H. gammarus* stn).

The appearance of TH in the STG neuropil immediately precedes a period of dramatic growth during a transition between the last two embryonic moults. Between E70 and E80, lobster embryos enter a developmental plateau characterized by a lack of growth in either the eye index or cephalothorax length (Helluy and Beltz, 1991). Between E80 and E90, the pace of development quickens, and embryos grow rapidly. In *Drosophila melanogaster*, depletion of dopamine has been shown to retard larval development (Neckameyer, 1996; Neckameyer et al., 2001), so it is possible that the onset of dopamine biosynthesis in the STG is helping to set the stage for a spurt of rapid growth in the lobster stomach. Later in development, changes in behavior also coincide with the developmental acquisition of dopamine in other parts of the lobster nervous system. For example, dopaminergic processes only appear in neurosecretory processes of the sinus gland (a major neurosecretory organ in lobsters) after the animal undergoes metamorphosis at L4 (Cournil et al., 1995).

The absence of TH before E80 does not preclude the possibility that dopamine is present in the STG in early embryonic times. We have recently shown that transporters for another biogenic amine, serotonin, exist in the ganglion before the ganglion is capable of actually synthesizing serotonin (Richards et al., 2003). TH is present in neurosecretory structures as early as E35 (Pulver and Marder, 2002), so dopamine is probably present in the hemolymph in embryos and could be used as a “borrowed transmitter” by the STG before TH is present.

The developmental acquisition of both serotonin and dopamine is delayed relative to histamine (Kilman et al., 1999). Histamine is currently the only amine neuromodulator known to be present in the STG neuropil by midway through embryonic life. Two of the neuropeptide modulators present early in development, orcokinin and FLRFamide, are also contained in the histaminergic PS projection neurons (orcokinin: Li et al., 2002; FLRF-amide: S.R. Pulver, unpublished data). Thus, it appears that the PS projections to the STG contain a relatively adult-like complement of neuromodulators by midway through embryonic life.

In all decapod species thus far studied, the PS/IVN neurons contain histamine (Claiborne and Selverston, 1984; Mulloney and Hall, 1991; Le Feuvre et al., 2001); however, the activation of these neurons has somewhat different actions in different species. Nevertheless, histamine does have inhibitory actions on the pyloric network neurons in all species, and some of the differences caused by stimulation of the PS/IVN neurons could arise from different cotransmitter complement or different distributions of histamine receptors. That said, we have shown here that histamine acts similarly in *H. americanus* early in development and in the adult.

The effects of dopamine in the adult *H. americanus* STG (Bucher et al., submitted) differ considerably from those extensively studied in the STG of *P. interruptus* (Flamm and Harris-Warrick, 1986a,b; Harris-Warrick et al., 1995b; Ayali and Harris-Warrick, 1999). Most dramatically, dopaminergic activation of peripheral spiking is seen in both the embryos and adults of *H. americanus* and is not seen in *P. interruptus*. In contrast, dopamine application to some of the muscles innervated by the PD neu-
rons in adult *P. interruptus* evokes muscle action potential (Lingle, 1981). Our study has demonstrated that dopamine can induce similar regenerative events in the PD-innervated muscles of embryonic *H. americanus*. Thus, it is possible that enhanced muscle excitation in dopamine in the two species is achieved by similar mechanisms even though dopamine modulates STG motor patterns differently in *P. interruptus* and *H. americanus*.

Understanding how nervous systems function requires knowing the full complement of signaling molecules that allow neurons to communicate with each other in circuits. To understand how circuits function in development, it is important to know which of the signaling molecules are available at any given developmental stage. This work now adds to our knowledge of how the decapod crustacean STNS acquires its adult neuromodulator complement. Interestingly, we are now seeing that most neuromodulators make their appearance in the embryo, and only a relatively few are delayed into larval life.

**ACKNOWLEDGMENTS**

We thank Dr. Michael Tlusty of the New England Aquarium and Michael Syslo of the Massachusetts State Lobster Hatchery and Research Station on Martha’s Vineyard for supplying animals. We gratefully acknowledge Dr. Barbara Beltz for the use of her confocal microscope.

**LITERATURE CITED**


