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SEROTONERGIC INNERVATION AND MODULATION OF THE STOMATOGASTRIC GANGLION OF THREE DECAPOD CRUSTACEANS (*PANULIRUS INTERRUPTUS, HOMARUS AMERICANUS* AND *CANCER IRRORATUS*)

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SUMMARY

The serotonergic innervation of the stomatogastric ganglion (STG) of three decapod crustacean species, *Panulirus interruptus, Homarus americanus* and *Cancer irroratus*, was studied. Immunohistochemical techniques were used to study the distribution of serotonin-like staining in regions of the stomatogastric system in the three species. In *C. irroratus* and *H. americanus*, but not in *P. interruptus*, serotonin-like staining was found in fibres in the stomatogastric nerve and in neuropil regions of the STG. High performance liquid chromatography confirmed the presence of serotonin in STG of *C. irroratus* and *H. americanus*, but serotonin was not found in STG of *P. interruptus*. Electrophysiological experiments showed that the pyloric motor output of the STG of all three species was influenced by bath applications of serotonin. The STG of *P. interruptus* responded to serotonin concentrations as low as 10^{-9} M; however the STG of the other two species did not respond until serotonin concentrations in excess of 10^{-6} M were applied. We conclude that serotonin may play a hormonal role in the control of the STG of *P. interruptus*, but is likely to be a neurotransmitter released by inputs to the STG of *H. americanus* and *C. irroratus*.

INTRODUCTION

A growing literature attests to the importance of serotonin as a neurotransmitter and/or neuromodulator in crustacean nervous systems (Kravitz, Glusman, Livingstone & Harris-Warrick, 1981; Cooke & Sullivan, 1982). Most of the original studies took advantage of the accessibility and simplicity of peripheral tissues for biochemical and electrophysiological studies of the mechanisms of action of serotonin. These studies showed that serotonin is synthesized by neuronal tissue in a number of neurosecretory

Key words: Serotonin, stomatogastric ganglion, decapod crustaceans, neuromodulation.

organs and is released into the haemolymph (Evans, Kravitz & Talamo, 1976; Sullivan, Friend & Barker, 1977; Sullivan, 1978; Livingstone, Schaeffer & Kravitz, 1981; Cooke & Sullivan, 1982). Moreover, low concentrations of serotonin have a variety of physiological effects on peripheral neuromuscular junctions, as well as on tissues such as the heart and the haemolymph (Florey & Florey, 1954; Dudel, 1965; Cooke, 1966; Sullivan & Barker, 1975; Florey & Rathmayer, 1978; Battelle & Kravitz, 1978; Kravitz *et al.* 1980; Lingle, 1979, 1980; Cooke & Sullivan, 1982; Glusman & Kravitz, 1982; Fischer & Florey, 1983). Although the mechanisms of action of serotonin are fairly well understood at these sites, the behavioural significance of the neurohormonal actions of serotonin are not clear.

The behavioural roles of serotonin in crustaceans are of interest; experiments have shown that exogenously-applied serotonin can act within neuronal ganglia to modulate posture (Livingstone, Harris-Warrick & Kravitz, 1980; Harris-Warrick, Livingstone & Kravitz, 1980) and cardiac activity (Maynard & Welsh, 1959; Cooke, 1966; Cooke & Hartline, 1975; Lemos & Berlind, 1980). However, the targets of serotonin action, as well as the cellular mechanisms of action of serotonin during the modulation of behaviour are not well understood. A recent study by Beltz & Kravitz (1983) has made a step towards understanding the central actions of serotonin by providing for the first time a relatively complete picture of the serotonin system in a crustacean. They identified approximately 100 neurones in the nervous system of the lobster *Homarus americanus* which showed serotonin-like immunoreactivity, and reported a fibre showing serotonin-like immunoreactivity in the superior oesophageal nerve (SON), one of the inputs to the stomatogastric ganglion (STG). This raised the possibility of a serotonergic input to the STG, that could function to modulate the motor outputs generated by the ganglion.

Therefore, in an attempt to understand the cellular mechanisms by which serotonin affects simple behavioural activities, we have studied the serotonergic innervation of the STG of decapod crustaceans. The STG of a number of species of decapods have been extensively studied over the past 15 years. In all species studied the STG contains only about 30 neurones which control the movements of the foregut (Maynard, 1972; Selverston, King, Russell & Miller, 1976). Additionally, in each species investigated thus far, about half of the neurones of the STG participate in the generation of the pyloric pattern which is responsible for the rhythmic peristaltic movement of foregut muscles. In the spiny lobster, *Panulirus interruptus*, a great deal is known about the synaptic connectivity among the pyloric neurones (Maynard & Selverston, 1975; Eisen & Marder, 1982), their membrane properties (Gola & Selverston, 1981; Russell & Hartline, 1978, 1982), the neurotransmitters involved (Marder, 1976; Lingle, 1980; Eisen & Marder, 1982; Marder & Eisen, 1984) and the generation of the final output pattern by these neurones (Hartline, 1979; Selverston & Miller, 1980; Miller & Selverston, 1982*a,b*). Although the STG in *P. interruptus* can generate the pyloric rhythm in the absence of input from other centres, it is strongly influenced by modulatory input from the paired commissural ganglia (Russell, 1979; Russell & Hartline, 1978, 1982). However, in some species of decapods the STG is less capable of generating the normal pyloric rhythm in the absence of input from other ganglia (Moulins & Cournil, 1982).

A variety of neurones in the oesophageal and commissural ganglia have also been

demonstrated to have important roles in activating or modulating the pyloric system motor output (Nagy, Dickinson & Moulins, 1981; Nagy & Dickinson, 1983; Dickinson & Nagy, 1983; Robertson & Moulins, 1981*a*,*b*). Thus, it seemed interesting to compare the possible role of serotonin as a modulator of pyloric output in several species that differ in the extent to which the pyloric rhythm is dependent on input from other ganglia. To this end, we chose to investigate three different crustacean species: the California spiny lobster, *P. interruptus*, which has the most intensively studied stomatogastric system in which the pyloric rhythm can operate in the absence of extra-ganglionic inputs (Selverston *et al.* 1976), the Maine lobster, *H. americanus*, in which pyloric activity is extremely labile in the absence of central inputs and the crab, *Cancer irroratus*, in which pyloric output continues robustly for several hours without extra-ganglionic inputs.

This paper represents the first step in characterizing the serotonergic innervation of the stomatogastric system of decapod crustaceans. We show by immunocytochemical and biochemical techniques that serotonin is present in the neuropil of the STG of the lobster, *H. americanus*, and the crab, *C. irroratus*, but is not detectable in the STG of the spiny lobster, *P. interruptus*. Electrophysiological recordings show that bath application of exogenous serotonin produces changes in the pyloric motor pattern in all three species and that the overall effects are qualitatively similar in all three species. However, in *P. interruptus* serotonin is active at concentrations as low as 10^{-9} M, while in *C. irroratus* and *H. americanus* serotonin is active only at concentrations in excess of 10^{-6} M. These data demonstrate a significant difference in the characteristics of neuronal and hormonal inputs to the STG of these three species, and are consistent with the role of serotonin as a neuronally-released neurotransmitter or neuromodulator in *H. americanus* and *C. irroratus*, while in *P. interruptus*, serotonin may act as a haemolymph-borne neurohormone, as previously suggested (Sullivan *et al.* 1977; Sullivan, 1978). Preliminary reports of some of these data have appeared in abstract form (Beltz *et al.* 1983).

METHODS

Animals

Panulirus interruptus were purchased from Pacific Biomarine (Venice, CA) and *Cancer irroratus* and *Homarus americanus* from local (Boston, MA) suppliers. The animals were held in instant Ocean aquaria at 5–12 °C until used. Both male and female animals were used.

Immunohistochemistry

All tissues were processed for immunohistochemistry as whole mounts using an indirect immunofluorescence technique (Hökfelt, Fuxe & Goldstein 1975; Beltz & Kravitz, 1983). The stomatogastric systems of mature crabs (*C. irroratus*) and lobsters (*P. interruptus* and *H. americanus*) (shown schematically in Fig. 1) were dissected in cold saline. Dissected tissues were fixed for 12–36 h in 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4). Subsequent processing with antibodies followed the whole mount technique described in Beltz & Kravitz (1983), using commercially prepared anti-serotonin antibodies (Immunonuclear Corporation).

Additionally, several preparations were processed using anti-serotonin antibodies from Immunotech, Inc., Chapel Hill, N.C. No differences were seen in the results with the two antibodies; all figures in this paper come from preparations processed with the Immunonuclear antibody. Stomatogastric ganglia of *P. interruptus* were, in addition, sectioned prior to immunohistochemical processing and treated as described in Beltz & Kravitz (1983).

Absorption controls (Immunonuclear antibody) were conducted to test the specificity of immunoreactivity for serotonin. The anti-serotonin antibody, at the working dilution, was pre-incubated with (1) serotonin creatinine sulphate (0·5 mg ml^{-1}), (2) formaldehyde cross-linked serotonin-BSA (the antigen against which the antibodies were raised) at a BSA concentration of 0·3 mg ml^{-1}, with a BSA: serotonin ratio of approximately 10: 1 (w/w), or (3) BSA (0·5 mg ml^{-1}). The serotonin-BSA conjugate was supplied by Immunonuclear Corporation, and the concentrations determined from their analysis of the compound. The antigen/anti-serotonin antibody mixture was incubated at 4 °C for 16–24 h, centrifuged at 100 000 ***g*** for 20 min, and the supernatant (pre-absorbed serum) collected. STG from *C. irroratus* were incubated with either anti-serotonin antibody, serum pre-absorbed with the serotonin-BSA conjugate, or serum pre-absorbed with BSA alone. The results of these three experiments were then compared.

Tests conducted by Immunonuclear Corporation indicate that there is no cross-reactivity of their anti-serotonin antibodies with norepinephrine, epinephrine or dopamine. Additionally, it has also been demonstrated that the immunofluorescence is unaffected by preabsorption with octopamine (Beltz & Kravitz, 1983).

Chemical assay for serotonin by high performance liquid chromatography with electrochemical detection

STG were dissected with one ganglion-length of nerve at each end, transferred to 50 µl of 0·1 M perchloric acid, 0·37 mM sodium bisulphite, pH 1·1, and frozen on dry ice. Ganglia were either assayed immediately or stored at −20 °C until assayed. The tissues were freeze-thawed 6–10 times on dry ice, then homogenized with a ground glass micro-homogenizer at 0·5 °C. The homogenate was centrifuged at 10 000 ***g*** for 1–2 min to remove particulate debris, and the supernatant was assayed for serotonin by high performance liquid chromatography with electrochemical detection. Separations were carried out using a Biophase ODS reverse phase column (25 cm, 5 µm C-18-bonded silica, Bio-Analytical Systems, West Lafayette, Ind.) at 24 150 kPa (3500 psi) with 0·15 M monochloroacetic acid: 18% methanol: 2·5 mM EDTA: 0·1 mM sodium octyl sulphate, pH 3·0 as the mobile phase. Serotonin was identified by electrochemical detection at +0·65 V. The identity of the tissue serotonin peak was confirmed in two ways. First, the tissue serotonin peak co-eluted with authentic serotonin in monochloroacetic acid buffer with both 9% and 18% methanol. Second, the oxidation voltage was varied from +0·55 to +0·65 V; the apparent oxidation potential of tissue serotonin was identical to that of authentic serotonin, as seen by parallel changes in the peak amplitude of the oxidation current. Standard curves of peak current *vs* authentic serotonin were made to measure the endogenous serotonin content; these curves were linear over the range 15–10 000 fmol serotonin.

Estimate of ganglion volume

Ganglion dimensions were measured prior to fixation using the ocular micrometer eyepiece in the dissecting microscope. Approximate volumes were calculated assuming the tissue was an oblong of the dimensions measured, and are therefore only estimates of actual volume. These measurements reveal an order of magnitude difference in size between the smallest (*C. irroratus*) and largest (*P. interruptus*) of the three ganglia (Table 1).

Electrophysiology

STG were prepared for electrophysiological recordings as previously described (Selverston *et al.* 1976; Mulloney & Selverston, 1974), using conventional methods and equipment for extracellular and intracellular recordings of STG activity (Eisen & Marder, 1982). Extracellular recordings from nerves were made either with monopolar or bipolar stainless steel pin electrodes insulated with Vaseline, or with bipolar suction electrodes. Data were taken on an FM tape recorder, and then played back onto either a pen recorder or an oscilloscope, from which they were filmed with a Grass kymograph camera. All experiments were done at 10–14 °C with saline superfusing over the ganglia at 4–12 ml min^{-1}. Solutions were changed by means of switching ports in the inflowing perfusion line. Solutions containing serotonin (serotonin creatinine sulphate, Sigma Chemical Co.) were freshly prepared in physiological saline no more than 5 min before application.

Physiological salines

P. interruptus saline (mM): NaCl, 479; KCl, 12·8; $CaCl_2$, 13·7; Na_2SO_4, 3·9; $MgSO_4$, 10; Trizma Base [Tris (hydroxy-methyl) amino methane], 11; maleic acid, 4·8; pH 7·4–7·6.

C. irroratus saline (mM): NaCl, 440; KCl, 11; $MgCl_2$, 26; $CaCl_2$, 13; Trizma Base, 11; maleic acid, 5·0; pH 7·7–7·6.

H. americanus saline (mM): NaCl, 462; KCl, 16; $CaCl_2$, 26; $MgCl_2$, 8; glucose, 11·1; Tris Base, 10; maleic acid, 10; pH 7·4.

RESULTS

The stomatogastric nervous system

The complete stomatogastric nervous system of decapod crustaceans consists of the stomatogastric ganglion (STG), the oesophageal ganglion (OG), the paired commissural ganglia (CG) and the nerves that connect them (Fig. 1). The STG contains the somata of about thirty neurones, most of which are excitatory motor neurones which send axons out to innervate the muscles of the stomach (Maynard & Dando, 1974). The neuropil of the STG is the site of synaptic interaction among the STG neurones themselves (King, 1976*a*,*b*) and between the STG neurones and many of the approximately 120 (*P. interruptus*, King, 1976*a*) to 240 (*H. americanus*, Maynard, 1971) fibres which travel in the stomatogastric nerve (STN). The OG contains 11–14 neurones and has two integrating regions, one at the junction of the STN with

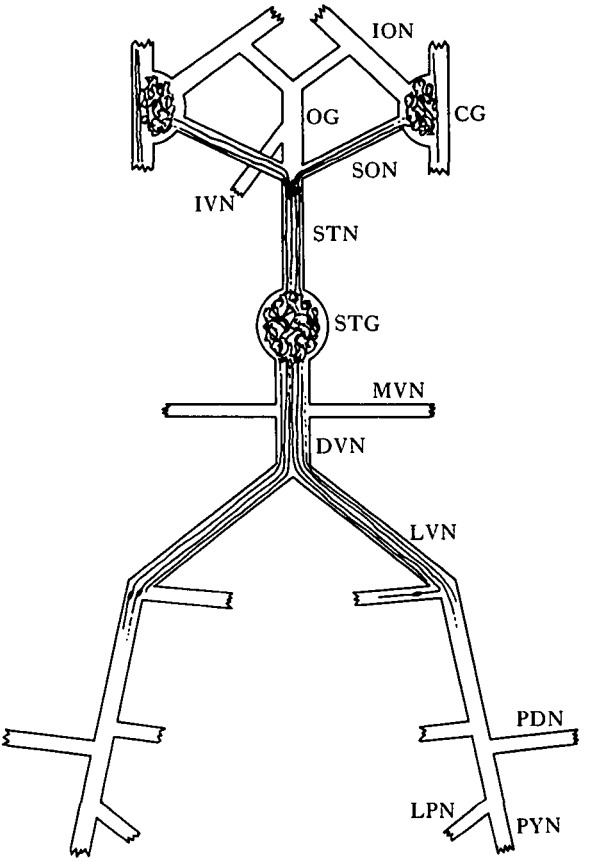
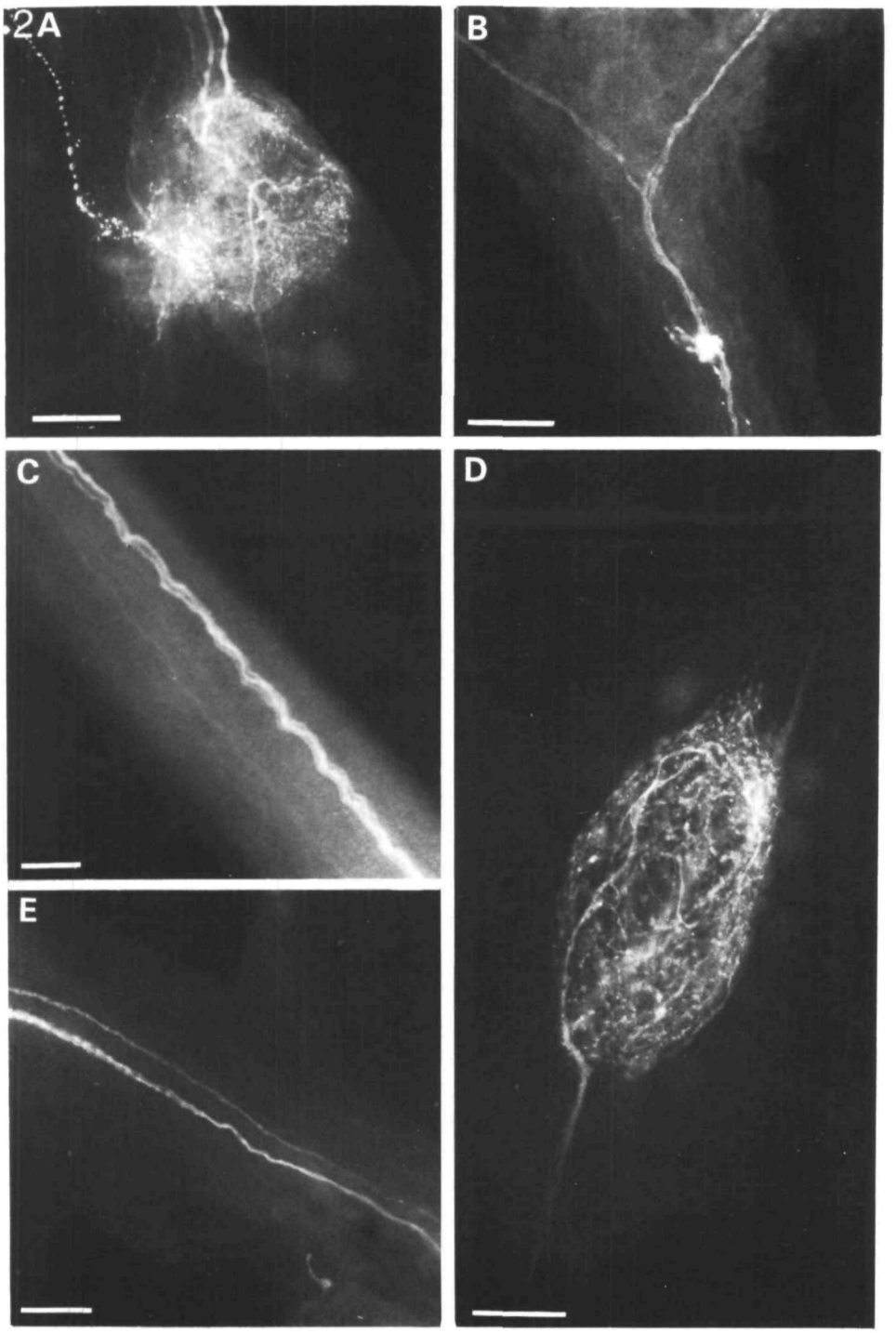


Fig. 1. Diagrammatic representation of the distribution of serotonin-like immunoreactivity in the stomatogastric nervous system of *Cancer irroratus*. Sketched within the outlines of the drawing are staining somata, neuropil areas and fibres. Abbreviations are as follows: STG, stomatogastric ganglion; STN, stomatogastric nerve; ION, inferior oesophageal nerve; SON, superior oesophageal nerve; OG, oesophageal ganglion; CG, commissural ganglion; IVN, inferior ventricular nerve; MVN, medial ventricular nerve; DVN, dorsal ventricular nerve; LVN, lateral ventricular nerve; PDN, pyloric dilator nerve; LPN, lateral pyloric nerve; PYN, pyloric nerve. The STN is the only input nerve into the STG; the DVN is the major output nerve and contains axons of all of the pyloric motor neurones.

the superior oesophageal nerves (SONs), and the other at the junction of the inferior oesophageal nerves (IONs) near the majority of the OG somata. The two CGs each contain approximately 400 neurones, and are known to contain neurones important for the physiological activity of the motor pattern generator of the STG (Selverston *et al.* 1976; Russell, 1976, 1979; Miller & Selverston, 1982*a,b*; Eisen & Marder, 1982; Russell & Hartline, 1982).

Fig. 2. Serotonin-like immunoreactivity in *Cancer irroratus*. (A) CG showing a dense neuropil and several immunoreactive fibres. Stained somata are not seen in this plane of focus. Calibration bar, 200 μm. (B) Immunoreactive fibres at branch of SONs and STN. Fibres seen in each SON and in STN. Small neuropil just posterior to the junction of SONs and STN. Calibration bar, 100 μm. (C) Immunoreactive fibre bundle in STN containing 3–4 neuronal processes. Calibration bar, 50 μm. (D) STG containing a very dense, compact immunoreactive neuropil. Fibres in the STN and DVN are continuous with this neuropil. Calibration bar, 100 μm. (E) Two bundles of immunoreactive processes containing approximately three fibres each are found in the DVN. Calibration bar, 100 μm.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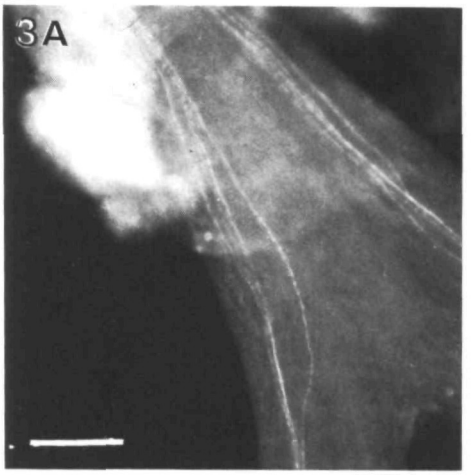
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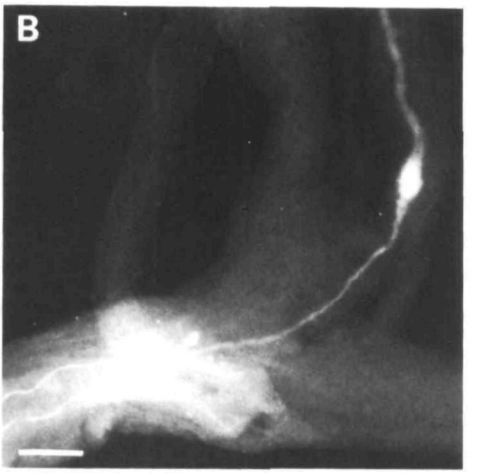
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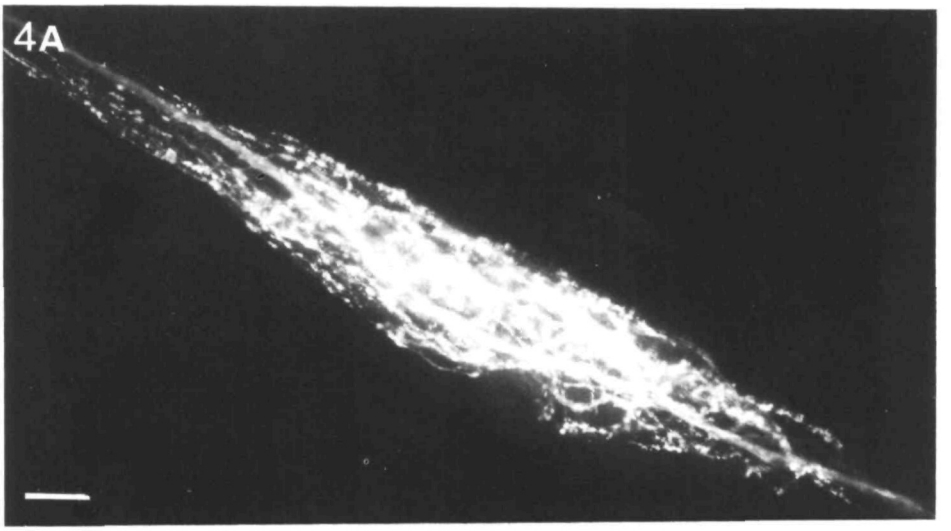
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B

Serotonin-like immunoreactivity in the stomatogastric systems

The stomatogastric system of the crab, *C. irroratus*, showed the most prominent system of immunoreactive cells and fibres when treated with an anti-serotonin antibody. Therefore the pattern and distribution of serotonin-like immunoreactivity will be described in detail for *C. irroratus*, followed by shorter descriptions of the results obtained for *H. americanus* and *P. interruptus*. The overall pattern of serotonin-like immunoreactivity in the stomatogastric system of the crab is diagrammatically represented in Fig. 1.

The serotonin-like immunoreactivity in the CGs of *C. irroratus* is confined to 1–3 cells and a neuropil (Fig. 2A), and resembles that previously reported for *H. americanus* by Beltz & Kravitz (1983). The SONs contain 2–3 immunoreactive fibres (Fig. 2B) which can be traced to a small immunoreactive neuropil at the junction of the SONs and the STN (Fig. 2B). We have not been able to determine whether the serotonin-staining somata in the CGs give rise to these fibres. The SON immunoreactive fibres can be traced into the STN (Fig. 2C) where there appear to be 3–4 immunoreactive fibres. The neuropil region of the STG shows a dense, widely ramifying and branching staining pattern which fills the whole neuropil volume (Fig. 2D). No immunoreactive somata in the STG were observed.

The dorsal ventricular nerve (DVN) contains the axons of most of the STG motor neurones as well as sensory and possibly neuromodulatory fibres. Six immunoreactive fibres in two bundles stain in the DVN (Fig. 2E). Each of these bundles is continuous with a single bundle in each of the lateral ventricular nerves (LVNs), formed when the DVN bifurcates (Fig. 3A). Because of the densely stained neuropil in the STG itself, it is difficult to trace individual fibres from the STN through to the DVN, so it is at present unclear how many of the STN fibres and DVN fibres terminate in the STG and how many go straight through it, or what the branching pattern is of individual axons in the STG.

Immunoreactive peripheral cell bodies were found in the LVNs or in branches of the LVNs in *C. irroratus*. These cell bodies (Fig. 3B) were 50–60 μm in diameter, and bipolar, with a process travelling centrally in the LVN and another process in the more peripheral motor nerves. They were usually found in the region of the nerve close to stomach muscles, gm8 and gm9 (Maynard & Dando, 1974), but there was variability in the location and the number of these neurones. In one preparation four staining neurones were found, with two in each LVN. In three preparations two neurones were found, and in five preparations only a single neurone was found. Thus far it has not been possible to locate these neurones reliably in unstained preparations.

The specificity of the serotonin-like immunofluorescence was tested by preabsorption of the antibody with serotonin creatinine sulphate, the serotonin-BSA conjugate used as the antigen for raising the antiserum, or BSA. Specific fluorescence

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Fig. 3. (A) DVN of a *Homarus americanus* preparation divides to form the LVNs. One of two immunoreactive fibre bundles in DVN is found in each LVN. Calibration bar, 200 μm. (B) Bipolar neurone in branch off the LVN in *Cancer irroratus*. Calibration bar, 100 μm.

Fig. 4. (A) Brightly staining immunoreactive neuropil is seen in *Homarus americanus* STG. No immunoreactive cell bodies are found. Calibration bar, 100 μm. (B) No serotonin-like immunoreactivity is evident in *Panulirus interruptus* STG. Calibration bar, 100 μm.

in *C. irroratus* STG was eliminated following pre-absorption with serotonin or with the serotonin-BSA conjugate but was unaffected by pre-absorption with BSA.

The pattern of serotonin-like immunoreactivity in the *H. americanus* stomatogastric system was similar to that found in *C. irroratus*. There is a single, immunoreactive cell body in each CG (see also Beltz & Kravitz, 1983), and fluorescent fibres in the SONs and the STN. A large and densely staining neuropil is seen in the STG (Fig. 4A). Unlike *C. irroratus*, no neuropil at the junction of the SONs was evident, nor were any immunoreactive peripheral cells found. However, these negative findings should be viewed with caution, since it is possible that there is a small OG neuropil which might become evident in a preparation with a particularly low background, and it is certainly possible that peripheral cells in *H. americanus* exist, but were missed.

In contrast to the dense, stained neuropil found in the STG of *C. irroratus* and *H. americanus*, we have not been able to demonstrate any specific immunoreactivity in motor nerves, STN, or neuropil of the *P. interruptus* STG using whole mounts (Fig. 4B). However, there was staining in the neuropil of the CGs in *P. interruptus*, indicating that immunohistochemical localization of serotonin can be successfully performed with whole mounts of *P. interruptus*. We also sectioned the STG prior to immunohistochemical processing. 10–20 μm frozen sections were cut with a cryostat and processed, but in all cases sectioned material also showed a complete absence of serotonin-like immunoreactivity (not shown). *P. interruptus* tissues were also pre-incubated in 10^{-5} M tryptophan (Livingstone *et al.* 1981), the precursor of serotonin, for 4 h before immunohistochemical processing in an attempt to raise serotonin levels and thereby amplify any threshold immunohistochemical signal. These preparations also showed no specific immunoreactivity. In total, 12 preparations from *P. interruptus* failed to show any serotonin-like immunoreactive staining in the STG.

Assay of endogenous serotonin levels by HPLC

To verify that the serotonin-like immunoreactivity represents authentic endogenous serotonin, and to determine whether the apparent differences among species seen by immunofluorescence techniques are real, we conducted quantitative HPLC analyses of individual and pooled STG from each of these species. Whole ganglia were dissected, homogenized and analysed by HPLC with electrochemical detection as described in the Methods. Typical chromatograms from the STG extracts of the three species are seen in Fig. 5. Table 1 shows that *C. irroratus* STG contained about 350 fmol of serotonin per ganglion, *H. americanus* STG contained about 810 fmol per ganglion, while in *P. interruptus* STG there was no detectable serotonin. The limit of detection was approximately 15 fmol. To check for the presence of serotonin below the limits of detection of the assay, three *P. interruptus* ganglia were pooled and assayed (Fig. 5C). These samples were still below the limit of detection of the assay.

To compare the amounts of serotonin in the STG from the three species, we estimated the volume of the ganglia (see Methods) and then calculated an approximate serotonin concentration in the ganglion. These values are shown in Table 1. By this method, the *C. irroratus* and *H. americanus* STG contain approximately 2–4 $\times$ 10^{-5} M serotonin. The lack of immunoreactive staining found in the *P. interruptus* STG corresponds to the lack of detectable serotonin found by HPLC.

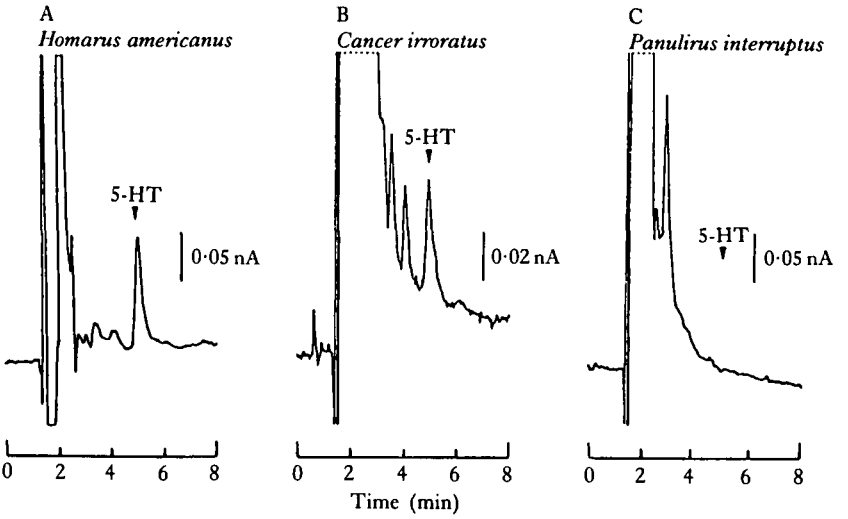

Fig. 5. HPLC determination of serotonin content in STG. (A) *Homarus americanus*, (B) *Cancer irroratus*, (C) *Panulirus interruptus*. Ganglia were processed as described in Methods. For *P. interruptus*, three ganglia were pooled and processed together. 20 μl aliquots were injected at the time indicated. The identity of the serotonin peaks (arrows in A and B) was verified as described in Methods.

Table 1. *Serotonin content in stomatogastric ganglia*

Species	Serotonin per ganglion (fmol)	Approximate neuropil volume (nl)	Approximate serotonin concentration (M)
Cancer irroratus	350 ± 230* ($N = 7$)	10	$3{\cdot}5 \times 10^{-5}$
Homarus americanus	811 ± 310 ($N = 5$)	40	2×10^{-5}
Panulirus interruptus	<5 ($N = 4$)	150	$<3 \times 10^{-8}$

* Values are means ± standard deviations.

Physiological effects of serotonin on stomatogastric motor patterns

In this section we report results of experiments that demonstrate that bath-applied serotonin can influence the pyloric motor pattern of the STG not only of *C. irroratus* and *H. americanus* but also of *P. interruptus*. Since the STG of *P. interruptus* is best characterized in the literature (Selverston *et al.* 1976; Miller & Selverston, 1982*a,b*), and the synaptic connectivity is well-known (Eisen & Marder, 1982), we present the effects of serotonin on the STG of *P. interruptus* first. To carry out these experiments, the complete stomatogastric system was dissected from the animals, and extracellular electrodes placed on the motor nerves to record the motor output of the ganglion. The pyloric rhythm is composed of repetitive sequences of activity in a number of different motor neurones. Fig. 6A shows the pyloric rhythm of the lobster, *P. interruptus*. Simultaneous intracellular recordings from four pyloric motor neurones of a *P. interruptus* STG, the pyloric dilator neurone (PD), the ventricular dilator neurone (VD),

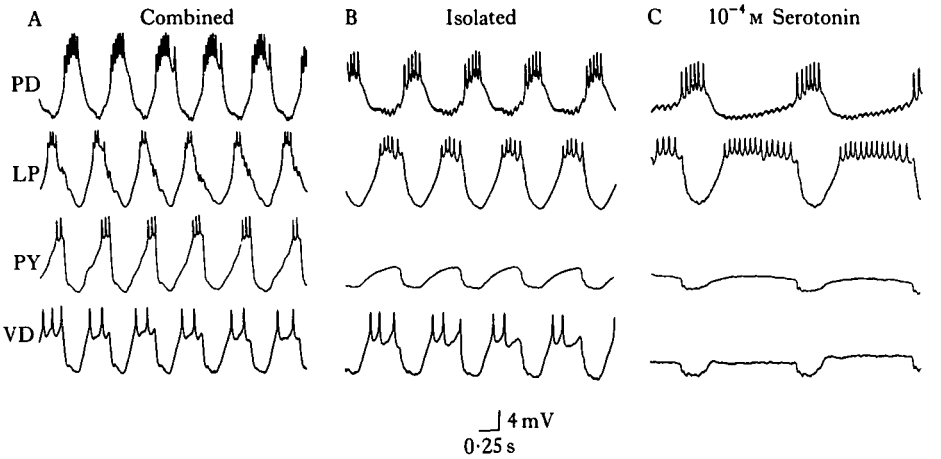

Fig. 6. Effect of 10^{-4} M serotonin on pyloric activity of *Panulirus interruptus*. Simultaneous intracellular recordings from the PD, LP, PY and VD neurones. (A) Inputs from the OG and CGs present. (B) After blocking the OG and CG input by placing isotonic sucrose in a well surrounding a portion of the stomatogastric nerve. (C) 10^{-4} M serotonin applied to the sucrose-blocked preparation. The most hyperpolarized points of the membrane potential excursions in A were: PD, −68 mV; LP, −70 mV; PY, −68 mV; VD, −62 mV. The traces in B and C are displayed to show the actual changes in membrane potential caused by sucrose block and serotonin.

the lateral pyloric neurone (LP), and a pyloric neurone (PY) show pyloric cycling at approximately 2 Hz, with each of the neurones periodically firing action potentials.

It is well-established that inputs from the commissural and oesophageal ganglia have dramatic influences on the motor output of the STG in all species studied (Russell, 1979; Robertson & Moulins, 1981*a*; Miller & Selverston, 1982*a,b*; Eisen & Marder, 1982; Russell & Hartline, 1982). Following the application of a reversible sucrose block on the STN, which functionally isolates the STG from commissural and oesophageal inputs (Russell, 1979) the pyloric activity continued to cycle, but at a lower frequency (Fig. 6B). The PY neurone although periodically inhibited during the time of PD depolarization no longer fired action potentials. The amplitude of the slow wave in the PD neurone was decreased, and the LP membrane potential trajectory was much smoother due to the removal of synaptic input from the PY neurones.

Following the application of 10^{-4} M serotonin to the isolated preparation shown in Fig. 6B, the LP neurone started to fire in long bursts of action potentials, periodically interrupted due to inhibition from the PD bursts (Fig. 6C). The PY and VD neurones were inhibited during the strong LP burst. The overall frequency was decreased, due to the pronounced inhibition of the PD neurones during the long LP bursts. In this and all experiments reported in this paper, the effects were reversible upon removing the serotonin from the bath, and were reproducible upon repeated applications of serotonin separated by 30-min washes.

A series of experiments was done to determine the approximate threshold concentration of serotonin required for physiological effects. Fig. 7 shows simultaneous intracellular recordings from the VD, PD and LP neurones in a sucrose-blocked *P. interruptus* STG in normal saline, and in the presence of 10^{-8} M serotonin. The effects of 10^{-8} M serotonin were similar to those produced by 10^{-4} M serotonin, but

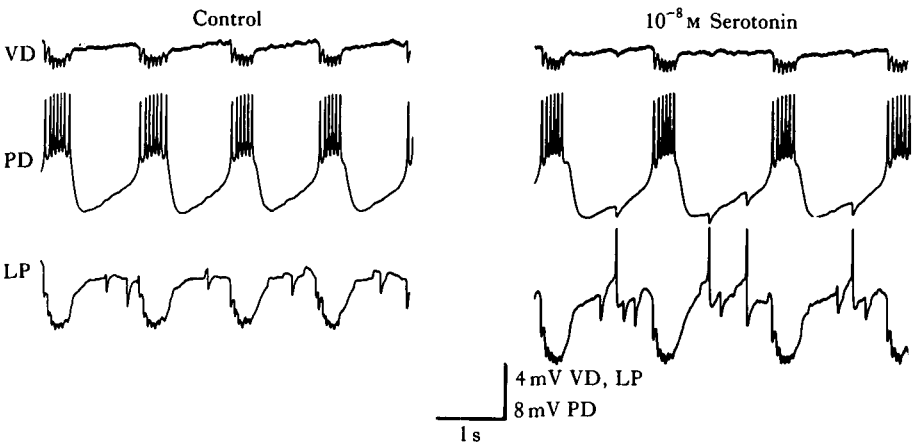

Fig. 7. Effect of 10^{-8} M serotonin on pyloric activity in *Panulirus interruptus*. Simultaneous intracellular recordings from VD, PD and LP neurones. (A) Preparation isolated from OG and CG inputs by sucrose block. (B) Activity in the presence of 10^{-8} M serotonin, still isolated by sucrose block. The most hyperpolarized points of the membrane potential excursions were: (A) VD, −42 mV; PD, −54 mV; LP, −52 mV and (B) VD, −36 mV; PD, −58 mV; LP, −52 mV.

less pronounced. Again, the frequency of the pyloric cycle decreased, and the LP neurone activity increased.

In about one-third of our experiments, serotonin (regardless of concentration applied) did not result in increased LP neurone activity. In those cases serotonin produced an increase, rather than a decrease, in the frequency of pyloric cycling. Fig. 8 shows recordings from the PD motor nerve (PDN), illustrating the timing of the PD bursts in a STG isolated from the CG and OG with a reversible sucrose block. In control saline this preparation was bursting slowly, with a frequency of about 0·5 Hz. Several minutes after the application of 10^{-9} M serotonin to the bath, the burst frequency almost doubled. In this experiment there was no LP or PY neurone activity, before, during, or after serotonin application. The acceleration of the pyloric rhythm by serotonin is likely to be explained (see Discussion) by the ability of serotonin to increase the frequency and amplitude of the slow wave pacemaker potentials in the anterior burster (AB) pacemaker neurone (Eisen & Marder, 1983). The records presented in Figs 6, 7 and 8 are typical examples of results obtained from approximately 30 experiments performed on *P. interruptus*.

It was somewhat more difficult to study the action of serotonin on the STG of *H. americanus*, because the ganglion is much less likely to continue to produce cyclic motor outputs when entirely deafferented (see also Moulins & Cournil, 1982). In the presence of inputs from the commissural and oesophageal ganglia, the STG of *H. americanus* showed cyclic pyloric activity with the usual pattern of LP, PY, PD alternation (Fig. 9A). The activity in the VD neurone is shown on the record labelled MVN. LP neurone (large unit) and PY neurone (small units) activity can be seen on the second trace. PD neurone activity is shown on the PDN trace. The intracellular recording is of the LP neurone. In this combined preparation the pyloric pattern was cycling at approximately 1 Hz.

After the STG had been isolated from the CG and OG inputs with a reversible sucrose block on the STN, the pyloric rhythm slowed down dramatically, and only the PD neurones continued to fire (Fig. 9B). Superfusion of 10^{-4} M serotonin activated or increased activity in all pyloric neurones (Fig. 9C). The PD neurones showed increased burst duration and increased spike frequency. Furthermore, the LP, VD and PY neurones were activated. As was the case in *P. interruptus*, in certain preparations the LP neurone was not activated by serotonin applications. In those preparations, as in *P. interruptus* (Fig. 8), serotonin applications increased the frequency of the pyloric cycle. In contrast to *P. interruptus*, the threshold concentration required for physiological effects in *H. americanus* was about 10^{-6} M, but activation of the LP neurone and VD neurone was only seen at concentrations above 10^{-5} M.

The STG of the crab, *C. irroratus*, retains a high level of activity when isolated from the CGs and OG. Fig. 10A shows recordings of pyloric cycling in a sucrose-blocked *C. irroratus* STG. The top four traces are extracellular recordings from the pyloric motor nerves. The VD neurone is seen on the MVN recording. PY neurone activity is seen on the PYN trace. PD neurone activity is shown intracellularly, and can be seen on the PDN trace. LP neurone activity is also shown intracellularly, and can also be seen as the largest unit on the LVN trace. Note the sequential firing of the LP, PY and PD neurones.

In the presence of 10^{-4} M serotonin (Fig. 10B) the normal pyloric pattern of activity is replaced with a qualitatively different one. This novel pattern is typified by long bursts of high frequency firing of the LP neurone which inhibits PD neurone

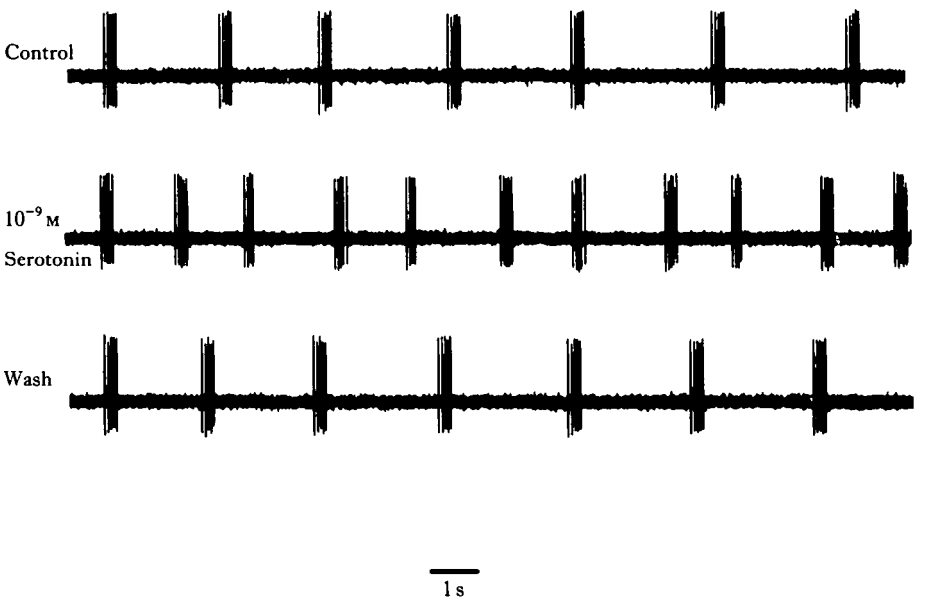

Fig. 8. Effect of 10^{-9} M serotonin on pyloric cycle frequency in *Panulirus interruptus* when the LP neurone is silent. Extracellular recordings from the PDN showing the activity of the PD neurones. Preparation was isolated from OG and CG inputs by sucrose block. 10^{-9} M serotonin reversibly increased PD burst frequency.

bursting. In the LP neurone interburst interval, the PD neurones continue to burst, often (as in Fig. 10B) firing three or four bursts between each LP neurone burst. The intracellular recordings show that the amplitude of the slow membrane potential oscillations in the PD neurone is decreased, the PD neurone is depolarized, and that the LP neurone shows plateau-like behaviour (Russell & Hartline, 1978, 1982). This pattern was stable and continued in this fashion for many minutes. Washing the preparation in serotonin-free saline produced a rapid return (within several minutes) to the normal pattern.

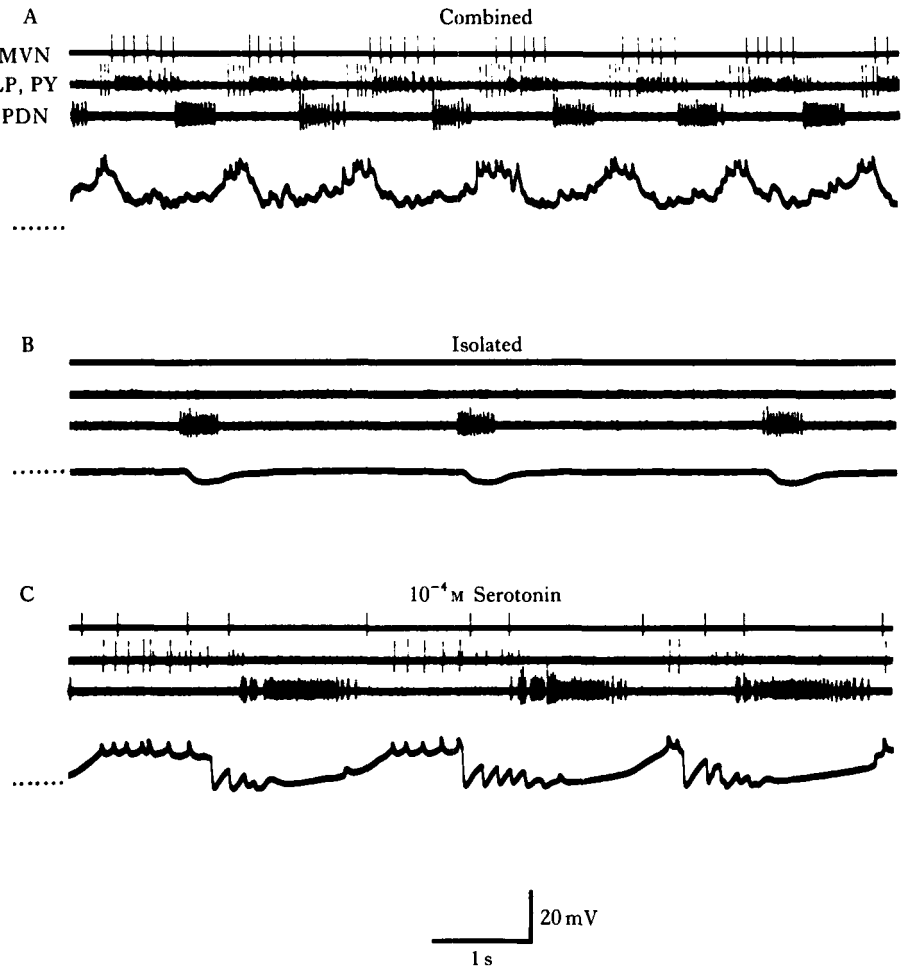

Fig. 9. Effect of 10^{-4} M serotonin on pyloric motor output in *Homarus americanus*. VD neurone activity is shown on the MVN trace. Activity of LP neurone (large unit) and the PY neurones (small units) are shown in the second trace. PD neurone activity is shown on the PDN (third trace). The bottom trace is an intracellular recording from the LP neurone. The dotted line is the same level of membrane potential in all traces. (A) Pyloric activity in the presence of inputs from OG and CGs. (B) Pyloric activity after the application of a sucrose block to the STN. All pyloric units stopped firing with the exception of the PD neurones. (C) Activity after the application of 10^{-4} M serotonin to the sucrose-blocked preparation shown in B.

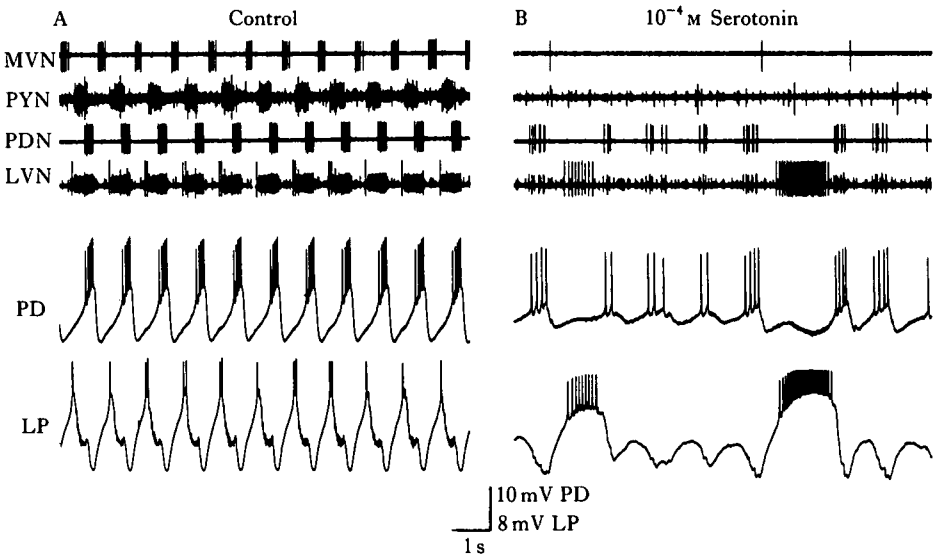

Fig. 10. The effect of 10^{-4} M serotonin on the motor output of the *Cancer irroratus* STG. In both panels the top trace is a recording of the MVN, showing VD neurone activity. The second trace is a PYN, showing PY neurone activity. The third trace is the PDN, showing PD neurone activity, and the fourth trace is a LVN recording, showing the LP, PY and PD neurones. (A) Motor output of the STG after the STN was blocked with sucrose. The LP and PD neurones alternate. (B) Motor output in the presence of 10^{-4} M serotonin. Note the disruption in the normal cycle pattern, and that the LP neurone (large unit on the LVN and bottom trace) is active in long bursts. PD neurone bursts no longer alternate with LP neurone bursts. After washing (not shown) the pattern returned to that shown in A. The most hyperpolarized points of the membrane potential excursions shown in these traces were: (A) PD, −78 mV; LP, −68 mV and (B) PD, −74 mV; LP, −69 mV.

Similar results were obtained in 11 experiments on *C. irroratus*. In four other experiments in which the pyloric activity was slow before serotonin application, serotonin applications terminated pyloric cycling entirely. Serotonin concentrations of less than 10^{-6} M were without effects on either cycling frequency or LP neurone firing in all experiments on *C. irroratus*. The lowest concentration required to produce physiological effects varied from animal to animal, but was between 5×10^{-6} M and 5×10^{-5} M.

DISCUSSION

As a first step in describing the cellular mechanisms of serotonergic modulation of a rhythmic motor pattern, we have begun to study the effects of serotonin in a well-defined neuronal circuit, the pyloric system of the crustacean stomatogastric ganglion. Using immunocytochemical and biochemical criteria, we have shown that serotonin is present in the stomatogastric nervous systems of the crab, *C. irroratus*, and the Maine lobster, *H. americanus*, but not in the spiny lobster, *P. interruptus*. Despite this difference in distribution of the amine, serotonin superfusion modifies the pyloric motor output of the stomatogastric ganglion in all three species. The differences in the threshold concentrations required to produce physiological effects suggest that serotonin may play a neurotransmitter role in *C. irroratus* and *H. americanus*, bu

may have hormonal actions in *P. interruptus*, as has previously been suggested (Sullivan *et al.* 1977; Sullivan, 1978).

The general pattern of serotonin-like immunoreactivity was quite similar in *C. irroratus* and *H. americanus*. The neuropil of the STG was covered by fine immunoreactive processes, which appeared somewhat more dense in *C. irroratus* than in *H. americanus*. Our biochemical measurements of serotonin by HPLC reinforce this result; both *C. irroratus* and *H. americanus* have significant amounts of serotonin, with a somewhat higher concentration present in *C. irroratus*. Fibres were seen in both the STN and the DVN, the main nerves entering and leaving the ganglion. Immunoreactive cell bodies were seen in the CGs of both species; however, we have not been able to determine whether these contribute processes which enter the STG itself. Fibres were observed in motor nerves of both species; although we have not yet followed these processes to muscles, it is possible that they interact with stomach muscles as well as with motor neurones in the STG.

Cell bodies with serotonin-like immunoreactivity were observed in motor nerves in *C. irroratus*, but not in *H. americanus* or *P. interruptus*. The physiological properties and functions of these neurones are still unknown, although neurones in a similar location have been described previously which are thought to have sensory functions (Maynard & Dando, 1974; Dando & Maynard, 1974). As described above, these immunoreactive cells could interact both with muscles of the stomach and the STG neurones. In this context, it is relevant that low concentrations of serotonin appear to have modulatory effects on some of the nerve-muscle junctions of the stomatogastric system (Lingle, 1979, 1981; S. Lin & M. O'Neil, unpublished results). The discovery that these cells stain for serotonin raises the question of which direction the other immunoreactive processes in both the STN and motor nerves are going: do they descend from the CGs and other higher ganglia, do they provide peripheral inputs to these ganglia, or do they have both ascending and descending functions? While no peripheral immunoreactive cell bodies have been observed in *H. americanus*, they could still be present along branches of the motor roots distal to our dissection.

In contrast, no serotonin-like immunoreactivity was seen in the STG of the spiny lobster, *P. interruptus*, although staining was seen in the CG. In an attempt to detect weakly staining fibres, we looked for immunofluorescence in 20-μm sections of the stomatogastric ganglion, but again no fluorescence was observed. This failure to detect serotonin-like immunoreactivity in *P. interruptus* STG was reinforced by our HPLC measurements of pooled ganglia, which also failed to detect any serotonin. Clearly there is a large quantitative difference in the amounts of serotonin in *C. irroratus* and *H. americanus* compared to the amount in *P. interruptus*.

Despite these differences in the levels of serotonin, bath-applied serotonin modulated the motor output from the STG in all three species. The threshold for detectable effects of serotonin in *P. interruptus* was about 10^{-9} M. Sullivan (1978) evoked release of serotonin from pericardial nerve plexuses in *P. interruptus* by nerve stimulation, and calculated that haemolymph concentrations of serotonin in the nanomolar range could result from pericardial plexuses-derived release. Thus, it is possible that haemolymph concentrations of serotonin high enough to influence pyloric activity may be present in *P. interruptus*. The circulating serotonin concentration in *H. americanus* haemolymph is about 10^{-9} M (Livingstone *et al.* 1981), but in

H. americanus and *C. irroratus* at least 10^{-6} M serotonin is required to influence the pyloric rhythm. Therefore, in these animals the STG presumably are not influenced by circulating serotonin levels, but would respond to serotonin released from the neuropilar processes we observed immunocytochemically.

For a number of reasons, it is unlikely that the difference in sensitivity to serotonin displayed by the STGs in the different species is due to differences in the rates of penetration of serotonin into these ganglia, or in the rate at which serotonin is inactivated. First, all these experiments were performed with desheathed preparations, and the STG of all three species, including that of *P. interruptus*, responds very rapidly to changes in extracellular ion concentration or bath-applied pharmacological agents (Eisen & Marder, 1982). Second, the STG of *P. interruptus* is the largest of the three ganglia, has the most connective tissue, but is sensitive to the lowest serotonin concentration. Third, the difference in threshold sensitivity in the three species we found for serotonin is reversed in *C. irroratus* and *P. interruptus* for dopamine, an amine similar in size (S. L. Hooper, unpublished results). Fourth, the superfusion rate employed was rapid with respect to the bath and ganglion volume, and the serotonin actions were observed within several minutes of the start of application in all three species. Since the serotonin-containing saline was continuously applied at a rapid rate inactivation sufficient to produce a three orders of magnitude drop in concentration seems unlikely.

It should be stressed that these physiological experiments show only that there are receptors that respond to serotonin on some neurones of the STG. These experiments were not designed to be, and certainly do not constitute, a complete description of the serotonin receptors on these neurones. However, we do believe it likely that we have activated physiological serotonin receptors. A number of other amines are present in the stomatogastric nervous system including octopamine, dopamine and histamine (Barker, Kushner & Hooper, 1979; Claiborne & Selverston, 1983). Bath-application of each of these amines produces a characteristic and different effect on the pyloric motor pattern (unpublished observations), and each of these effects is different from the serotonin actions described in this paper. Furthermore, each of these amines acts differently on pyloric neurones isolated with the Lucifer yellow photoinactivation technique (Eisen & Marder, 1983, and unpublished results). Therefore, although it is conceivable that bath-application of serotonin may partially activate octopamine or other amine receptors, the actions of serotonin cannot be due only to activation of other amine receptors. Certainly, much further research will be necessary to characterize pharmacologically the various amine responses on the neurones of the STG.

While the experiments presented in this paper are consistent with a role for serotonin as a neurotransmitter or modulator of the STG, we do not claim from these experiments using bath-application of serotonin that we have necessarily mimicked the actual motor patterns which would result from physiologically released serotonin. However, now that we have succeeded in identifying serotonin-staining neurones and pathways, we can hope in the future to be able to record from and stimulate these serotonergic neurones, and therefore to study directly the physiological roles of serotonin. With the above qualifications, what can we say about the action of serotonin on the motor outputs of the STG from the three species?

Serotonin applications to the STG of *P. interruptus* and *H. americanus* appeared

to increase cycling frequency under some conditions, and decrease cycling frequency under other conditions. The critical factor in determining which of these effects was seen was the state of activity in the LP neurone. In ganglia in which the LP neurone was active or became active with serotonin, the cycle frequency was decreased by serotonin applications. Preliminary experiments suggest that the increased firing of the LP neurone is due to actions of serotonin directly on the LP neurone (R. Flamm & R. M. Harris-Warrick, unpublished experiments). Since the LP neurone inhibits the PD neurones (Fig. 6; Eisen & Marder, 1982) increased LP neurone activity can result in decreased pyloric cycle frequency. In experiments in which the LP neurone did not fire in the presence of serotonin, the pyloric cycle frequency was increased by serotonin. Eisen & Marder (1983) showed that serotonin increased the frequency and amplitude of membrane potential oscillations in isolated AB neurones (the actual pacemaker for the pyloric rhythm) but had no effect on isolated PD neurones, to which the AB neurone is electrically coupled. Therefore, the increase in frequency seen in ganglia in which the LP neurones were inactive, can be explained by direct actions of serotonin on the AB pacemaker neurones. It is likely that the activity of the LP neurone is influenced by a number of the other inputs to the STG. Thus, it is possible that another STN input may modulate the activity of the LP neurone, and, in so doing, modify the physiological effects of serotonin on the pyloric motor output.

The pyloric motor pattern elicited by serotonin in *C. irroratus*, was qualitatively different from that seen in *H. americanus* and *P. interruptus*, although patterns similar to those found in *C. irroratus* were sometimes seen transiently in the other animals. In *C. irroratus* the basic pattern of pyloric motor output was significantly altered by serotonin; the PD neurones gave several bursts followed by a prolonged inhibition due to LP firing, rather than a regular alternation of LP and PD bursts. At present all we know is that bath superfusion of serotonin can dramatically change the pyloric motor pattern in this species, but not that this finding is physiologically or behaviourally relevant. The density of serotonergic innervation of the *C. irroratus* ganglion suggests that activation of serotonergic inputs might release serotonin over a large region of the ganglionic neuropil, and therefore could be mimicked by bath applications of serotonin, but this remains speculative. However, we hope in the future, it will be possible to stimulate selectively the fibres and neurones which stain for serotonin to determine if they also evoke this dramatic modification of the pyloric motor pattern. Should this be the case, this would constitute a clear example of a neuronally-induced modification of a motor pattern.

In conclusion, we have begun to study the mechanisms whereby neuromodulators and neurotransmitters modify the action of neuronal central pattern generator circuits to change the motor pattern underlying simple behaviour patterns. Serotonin is present and appropriately localized to have such a role in the normal modulation of the pyloric rhythm of the stomatogastric ganglion of *C. irroratus* and *H. americanus*, and it may be a neurohormonal modulator in *P. interruptus*. Since the neuronal circuitry underlying the pyloric rhythm is well understood, it will now be possible to analyse cellular targets of serotonin action in this circuit.

The work presented in this paper represents collaboration among three laboratories and the names of the authors are consequently listed in alphabetical order. We thank

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