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AMINERGIC AND PEPTIDERGIC NEUROMODULATION IN CRUSTACEA

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SUMMARY

Biogenic amines and peptides can act both as circulating neurohormones and as classical central and peripheral neurotransmitters. This article reviews some of the variety of roles played by amines and peptides in crustacean nervous systems. Cardiac, stomatogastric and postural systems are used to illustrate: (1) the functional versatility of amines and peptides; (2) the molecular basis of their actions; (3) the coexistence of amines and peptides with other bioactive compounds; and (4) the developmental expression of amine and peptide phenotypes. We will deal in detail with the postural neuromuscular system of the lobster, *Homarus americanus*. Physiological and pharmacological experiments have shown that the biogenic amines serotonin and octopamine are capable of regulating posture by direct neurohormonal actions on the muscles and by central actions that alter motoneuronal output. We have localized serotonin to identified neurones in the lobster ventral nerve cord and have shown further that the pentapeptide proctolin coexists with the amine in these cells. Such neurones provide a convenient system in which to study the functional interactions between peptide and amine cotransmitters. In addition, the serotonin and proctolin phenotypes of these cells are first expressed at widely different times in development. This presents the possibility of studying the regulation of these two transmitter phenotypes in a system that is readily amenable to experimental manipulation.

INTRODUCTION

Learning how behaviour is governed by sets of neurones within central nervous systems, or ultimately in terms of the gene, is one of the great challenges for the neurosciences in future decades. Higher organisms weave a rich and varied fabric of behaviour. It is difficult, however, to unravel individual behaviours at the level of circuits of neurones with vertebrates due to the large numbers of neurones and the complexity of their brains. Invertebrate organisms, with their much smaller neuronal complement, have allowed far more progress in forging the link between neurobiology and behaviour (Mackey & Carew, 1983; Kinnamon, Klaassen, Kammer & Claassen, 1984; Mayeri & Rothman, 1985; Kravitz *et al.* 1985; Gelperin, Hopfield & Tank, 1985). Much of the focus of research on these simpler systems is on amines and peptides and their ability to bias or control aspects of the behavioural repertoire

Key words: peptide, amine, serotonin, octopamine, proctolin, neurohormone, identified neurones, development.

of various invertebrate species. For example, models of learning in the sea slug, *Aplysia californica*, and in the fruit fly, *Drosophila melanogaster*, are based on particular aminergic neurones, and the actions of amines on cyclic nucleotide-linked enzyme systems (Quinn & Greenspan, 1984; Livingstone & Tempel, 1983; Abrams, 1985; Brunelli, Castellucci & Kandel, 1976; Kandel & Schwartz, 1982). Studies of swimming and feeding behaviour in the leech, *Hirudo medicinalis*, have focused on serotonin found in the haemolymph in these animals and on particular serotonergic neurones located in individual ganglia of the ventral nerve cord (Willard, 1981; Lent & Dickinson, 1984; Kristan & Nusbaum, 1983; Kristan & Weeks, 1983). The complex motor patterns demonstrated in moulting behaviour in certain insects, or in egg-laying behaviour in *Aplysia*, have in addition been related to particular peptides found in identified neurones in these animals (Reynolds & Truman, 1983; Truman & Weeks, 1985; Kupfermann, 1967; Strumwasser, 1983; Mayeri & Rothman, 1985).

Various decapod crustacean species also offer important and useful tissue preparations with which to explore the variety of roles played by amines and peptides in neuronal circuits. In this review three such systems will be described: (1) aminergic and peptidergic regulation of the heart; (2) the stomatogastric system; and (3) aminergic modulation of the neuromuscular system regulating posture.

AMINERGIC AND PEPTIDERGIC REGULATION OF THE HEART

In crustaceans, the heart beat is generated by endogenous rhythmic electrical activity in the small network of neurones comprising the cardiac ganglion. The ganglion consists of four small (30–40 μm), posterior 'pacemaker' neurones that drive the five anterior motoneurones (80–100 μm) that innervate the heart musculature. These cells are arranged along a ganglionic trunk located on the dorsal heart wall (Fig. 1). The endogenous activity of these neurones is modulated *via* excitatory and inhibitory inputs from neurones in the central ganglia of the ventral nerve cord whose axons form the dorsal nerve, and by substances released into the haemolymph from nearby neurohormonal regions, the pericardial organs (POs). Extracts of the POs that surround the heart generally cause dramatic increases in both amplitude and frequency of the heart beat (Fig. 2) (Alexandrowicz & Carlisle, 1953; Augustine, Fetterer & Watson, 1982; Miller, Benson & Berlind, 1984). For some time it was believed that serotonin, which is present in the POs, was the major cardioexcitatory factor affecting the heart (Florey & Florey, 1954). However, Maynard & Welsh (1959) found that serotonin is present in the POs at concentrations that are too low to account for the cardioexcitatory activity of the extracts and that the response of the heart to them was qualitatively unlike that of serotonin. Instead, they attributed the effects to a peptide that they partially purified. Later studies suggested that two cardioexcitatory peptides were present in crab POs (Belamarich & Terwilliger, 1966; Berlind & Cooke, 1970).

One of the cardioexcitatory peptides found in the POs is closely related or identical to proctolin, a pentapeptide that was originally isolated from cockroach hindgut (Brown & Starratt, 1975; Starratt & Brown, 1975; Sullivan, 1979). Proctolin has

direct excitatory effects on both premotor cells and motoneurones of the cardiac ganglion in *Homarus americanus* (Sullivan & Miller, 1984). The excitation of the premotor cells has a fairly rapid onset (5–10 s) and is readily reversible (3 min) while the excitatory effects on the motor neurones have a slower onset (60–90 s) and last for 10–20 min. Proctolin is effective on both premotor and motor cells at low concentrations (threshold 10^{-9}–10^{-8} mol l^{-1}), consistent with a neurohormonal role. The other peptide or peptides in the POs that excite the neurones of the cardiac ganglion have not been characterized further or isolated, although recent studies have shown the existence of high concentrations of FMRFamide-like peptides in lobster POs (Kobierski, Trimmer, Beltz & Kravitz, 1985; Trimmer, Kobierski & Kravitz, 1985). Serotonin, octopamine and dopamine have also been found to be present, synthesized and released from POs (Maynard & Welsh, 1959; Cooke, 1966;

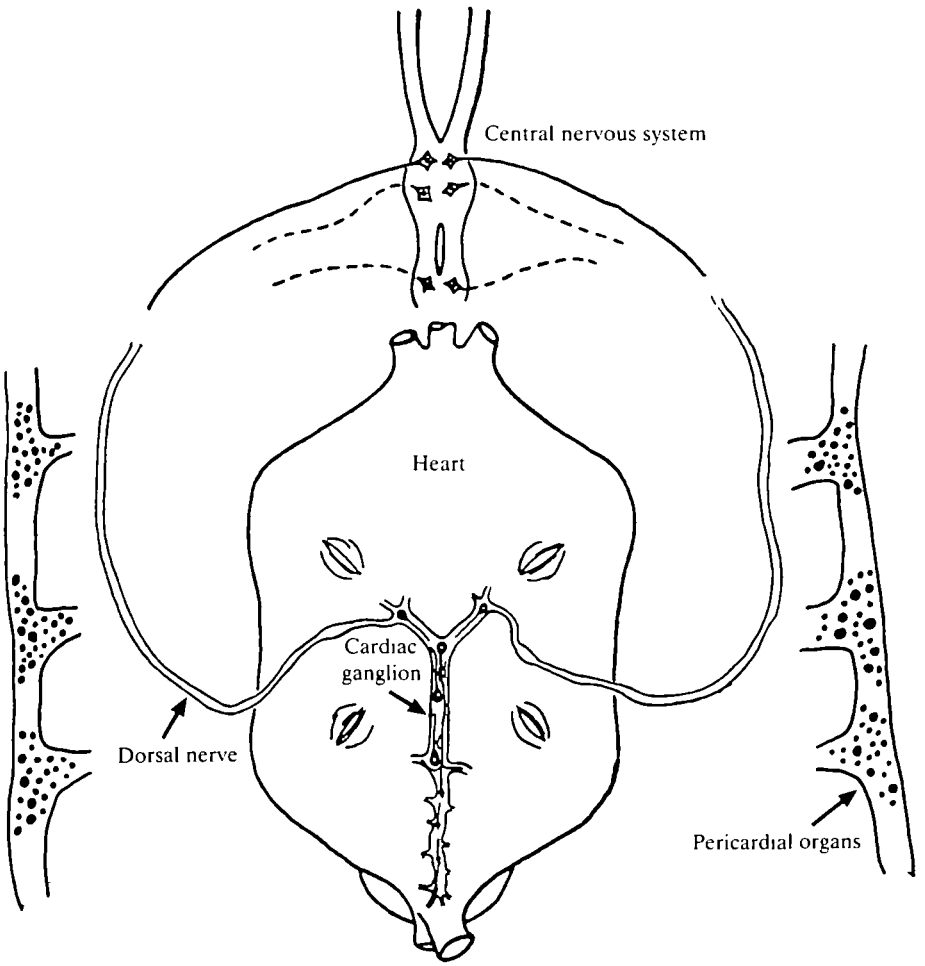


Fig. 1. Diagram of cardiac ganglion of *Homarus americanus*, located on the dorsal heart wall. The activity of the nine ganglionic neurones is modulated *via* excitatory (dashed lines) and inhibitory (solid lines) inputs from central ganglia whose axons form the dorsal nerve, and by substances released from the neurosecretory pericardial organs.

Cooke & Goldstone, 1970; Evans, Kravitz & Talamo, 1976*a*; Evans, Kravitz, Talamo & Wallace, 1976*b*; Sullivan, Friend & Barker, 1977).

The primary sites of action of serotonin, and of the PO extracts, on the heart are the nine neurones of the cardiac ganglion, rather than the heart muscle itself, or the sensory endings in the heart, or the cardiac neuromuscular junctions (Cooke, 1962, 1966; Cooke & Hartline, 1975; Miller & Sullivan, 1981; Sullivan & Miller, 1984). Moreover, Berlind, Cooke & Goldstone (1970) showed that serotonin directly affected the heart and did not act by releasing the cardioexcitatory peptide substances.

In addition to serotonin, Florey & Rathmayer (1978) have demonstrated that octopamine, adrenaline, noradrenaline and dopamine also accelerate the heart beat in the crayfish, *Astacus*, and in the crab, *Eriphia*. The effect of octopamine on the heart is preceded by a brief inhibitory phase. This inhibition is unlikely to be caused by octopaminergic excitation of the presumed GABA-containing inhibitory nerve terminals in the heart, since picrotoxin does not antagonize this effect even though it blocks the effects of stimulating the cardioinhibitory nerves (Florey & Rathmayer, 1978). Octopamine is effective on the heart at low concentrations (10^{-9}–10^{-8} mol l^{-1}), and its excitatory actions are long-lasting. Octopamine, like serotonin, is therefore likely to act as a neurohormone in the heart. Dopamine is known to increase the frequency and/or duration of bursts of action potentials in cardiac ganglia of the crabs *Portunus* and *Podophthalmus* (Miller *et al.* 1984). Within each burst, the number of motoneurone action potentials increases; this would cause an increase in the force and amplitude of contraction in an intact heart. The threshold for these effects is 10^{-8} mol l^{-1} or lower. The primary actions of dopamine are on a group of pacemaker interneurones in which slow tetrodotoxin-resistant driver potentials are stimulated. These actions are believed to be responsible for the excitatory action of dopamine in cardiac ganglia (Miller *et al.* 1984).

Serotonin, octopamine and proctolin also have effects on the cardioarterial valves of the heart (Kuramoto & Ebara, 1984). By enhancing the heart beat and relaxing the valves, serotonin increases the amount of cardiac outflow into the arteries. In contrast, octopamine increases the tension of the posterior valves and relaxes the anterior ones, so as to bias blood flow towards the head. Proctolin, by contracting all the valves and enhancing the heart beat, could prevent a backward blood flow during strong or sudden movements. All three substances act on the valves at low concentrations (10^{-10}–10^{-7} mol l^{-1}), which is consistent with a neurohormonal role in the heart.

THE STOMATOGASTRIC SYSTEM

The stomatogastric ganglion (STG) of decapod crustaceans contains approximately 30 neurones that control the movements of the animal's stomach (Fig. 3). About half of the STG neurones are responsible for the rhythmic dilations and constrictions of the posterior, pyloric end of the stomach, while the others are used to operate the teeth of the gastric mill. Food that has been macerated by the gastric mill

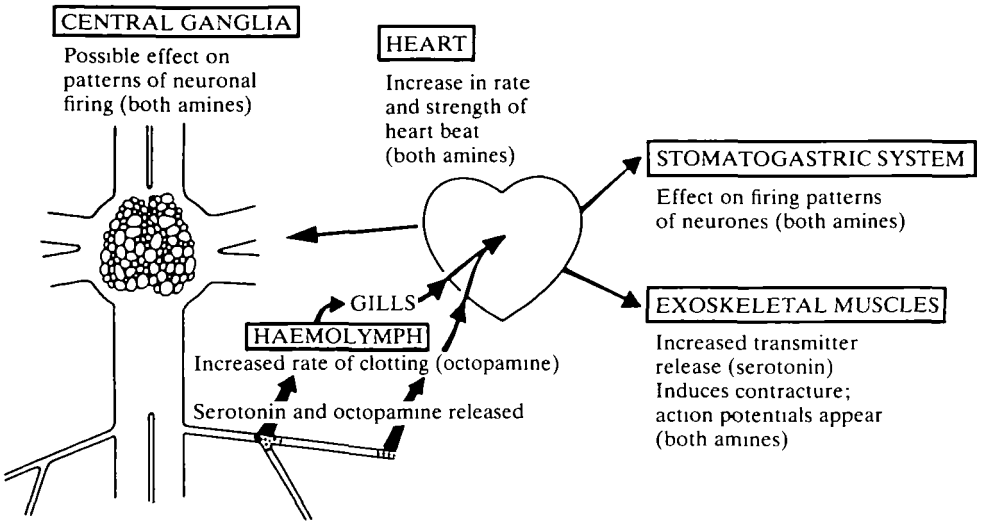


Fig. 2. Peripheral and central targets of serotonin and octopamine released from thoracic neurosecretory regions. Peptides are also released from these areas and follow the same route of delivery to their targets.

passes into the pyloric region where it is filtered, kneaded further, and then passed into the gut (Fig. 3).

The neurones and muscles that control the rhythmic activity of the stomach are sensitive to a number of peptides and amines. Stomatogastric neurones are responsive to dopamine, octopamine, serotonin, histamine and the peptides proctolin, and FMRF amide (Beltz *et al.* 1984; Flamm & Harris-Warrick, 1984; R. E. Flamm & R. M. Harris-Warrick, in preparation; Harris-Warrick & Flamm, 1984; Heinzel & Selverston, 1985; Hooper & Marder, 1984; Trimmer *et al.* 1985). Dopamine, serotonin and octopamine also enhance nerve-evoked contractions of the foregut muscles (Lingle, 1981).

Using immunocytochemical and biochemical procedures, serotonin has been found in the stomatogastric ganglia of *Cancer irroratus* and *Homarus americanus*, but has not been found in this ganglion in the spiny lobster *Panulirus interruptus* (Beltz *et al.* 1984). Physiological studies have shown that the pyloric motor output of the stomatogastric ganglion of all three species is modified by serotonin superfusion. The sensitivities to the amine, however, are quite different in the three species. In *Homarus* and *Cancer*, serotonin concentrations of 10^{-6} mol l^{-1} or higher are required to influence the pyloric rhythm, while in *Panulirus* the threshold for serotonin is about 10^{-9} mol l^{-1}. Immunocytochemical studies showed a dense serotonin immunoreactive neuropile region in stomatogastric ganglia in *Homarus* and *Cancer*, but not in *Panulirus*. In *Homarus* and *Cancer*, therefore, the stomatogastric neurones are probably regulated by local release of serotonin from ganglionic neuropile. In *Panulirus*, on the other hand, it is probably serotonin circulating in the haemolymph as a neurohormone that regulates the ganglionic neurones. In this species neurones are sensitive to nanomolar concentrations of the

amine and the circulating levels in the haemolymph are predicted to be in the nanomolar range (Sullivan, 1978). The low levels of serotonin measured in haemolymph in *Homarus americanus* (Livingstone, Schaeffer & Kravitz, 1981) probably do not influence the activity of the neurones in the stomatogastric ganglion in this species. The interesting observation here is that different strategies are used for the delivery of serotonin to target neurones in this series of closely related species. The threshold responsiveness of the target neurones, in turn, is regulated to adapt to the different routes of delivery.

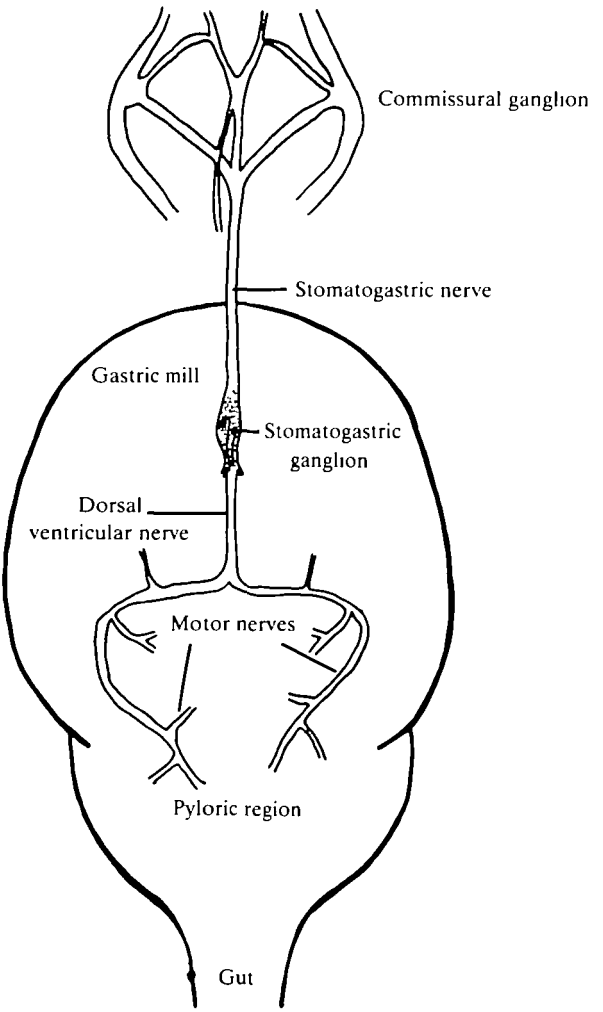


Fig. 3. Schematic diagram of a crustacean stomatogastric system. Represented here are the paired commissural ganglia which are part of the ventral nerve cord. The stomatogastric ganglion (STG) is located on the dorsal stomach wall. Motoneurones innervating the gastric mill and pyloric muscles, part of the striated musculature of the crustacean foregut, exit principally from the dorsal ventricular nerve. Input to the STG from the ventral nerve cord comes *via* the stomatogastric nerve.

The effects of dopamine, octopamine and serotonin on the pyloric neurones have been studied in detail at both the cellular and circuit levels in *Panulirus interruptus* (R. E. Flamm & R. M. Harris-Warrick, in preparation; Harris-Warrick, 1986). Under conditions in which external inputs were removed from the stomatogastric ganglion, the neurones of the pyloric circuit generated an endogenous and weakly rhythmical pattern of firing. However, this pattern turned out to be only a framework upon which exogenously added amines could impose a series of different and highly characteristic rhythms. The amine-induced patterns of firing depended upon the amine and its concentration. Considerable malleability was seen in the resulting firing pattern. In addition, changes in the patterns of activation or inhibition of neurones were not necessarily predictable from observing amine effects on isolated neurones. In some cases, amine actions could be explained only when a knowledge of the interconnections among the pyloric neurones was added to information about amine effects on isolated neurones. Even more flexibility in the circuit was demonstrated when it was shown that amine-induced changes in the properties of certain superficially similar neurones could result from apparently very diverse basic mechanisms.

For example, in the Anterior Burster (AB) neurone, which is a very important cell in the circuit for timing the pyloric cycle, all three amines induce slow oscillations in membrane potential (R. E. Flamm & R. M. Harris-Warrick, in preparation; Harris-Warrick, 1986). Dopamine and serotonin cause the appearance of large amplitude (20 mV), biphasic, slow membrane potential oscillations (bursting pacemaker potentials) and very attenuated (2 mV) action potentials. While octopamine also enhanced AB burst activity, the membrane potential oscillations were smaller (about 10 mV) and were not biphasic. Pharmacological and physiological studies suggest that the ionic mechanisms underlying the oscillations in membrane potential may be different for each amine. For instance, calcium entry appears to be essential for bursting activity in dopamine solutions but not in serotonin or octopamine (R. M. Harris-Warrick & R. E. Flamm, in preparation). Serotonin and octopamine require sodium entry through tetrodotoxin-sensitive channels to sustain their burst mechanism, while the dopamine bursting continues (without spikes) even in the complete absence of sodium. These studies were done with physiologically isolated neurones. It appears, therefore, that more than one burst mechanism exists in this single neurone. Such differences could lead to greatly increased plasticity of the AB cells and allow them to change the timing of the pyloric rhythm in many different contexts (Harris-Warrick, 1986).

The peptides proctolin and FMRFamide also modify the pattern of neuronal output that is generated by the pyloric neurones (Hooper & Marder, 1984, 1985). Bath application of 10^{-5} mol l^{-1} FMRFamide or 10^{-6} mol l^{-1} proctolin causes an increase in the frequency of the pyloric cycle, while 10^{-6} mol l^{-1} proctolin can also induce robust pyloric cycling in previously quiescent preparations (Hooper & Marder, 1984). Proctolin-like and FMRFamide-like immunoreactivities have been seen in the neuropile of stomatogastric ganglia. The proctolin-like material appears to be authentic proctolin; the FMRFamide-like peptide is related but not identical

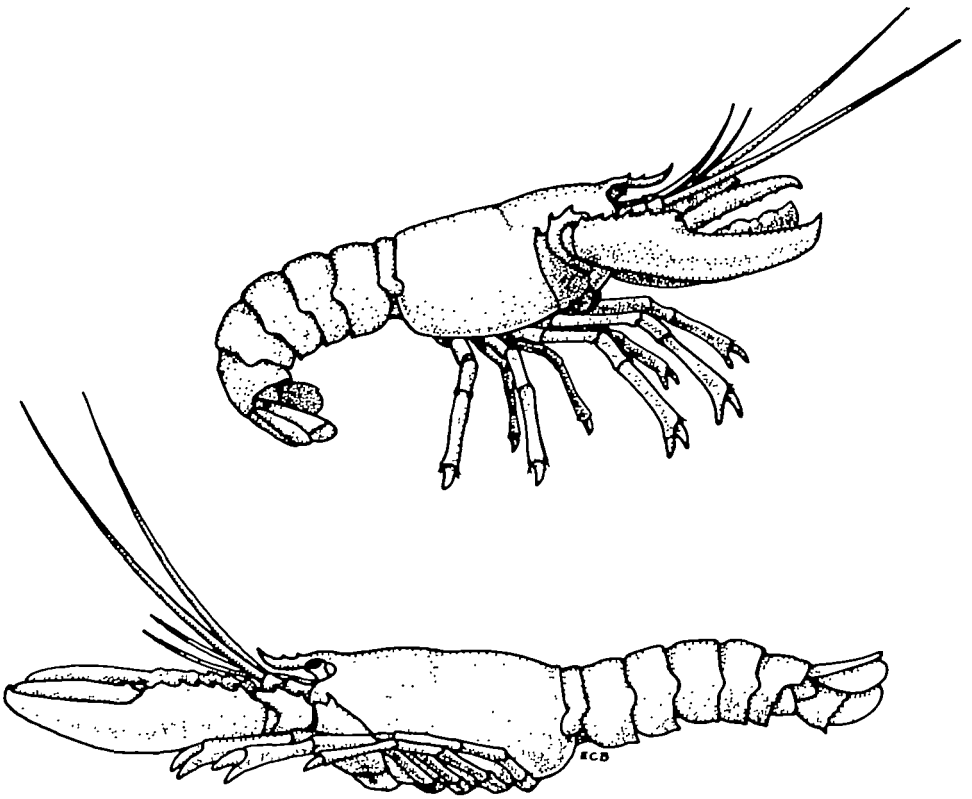


Fig. 4. Postures generated when serotonin (top) or octopamine (bottom) is injected into freely moving lobsters. These flexed and extended poses can last for several hours following injection (see text for details). (Reprinted from Beltz, 1986, with permission.)

to FMRFamide (Marder, Hooper & Siwicki, 1986; E. Marder, R. Calabrese, M. Nusbaum & B. Trimmer, in preparation). While many studies have emphasized the role of these peptides as modulators of neuromuscular preparations or muscle contractility (Painter, 1982; Schwarz, Harris-Warrick, Glusman & Kravitz, 1980; Watson, Augustine, Benson & Sullivan, 1983), the work of Hooper & Marder (1984, 1985) demonstrates these substances are likely to have important effects on ganglionic neurones as well.

AMINERGIC MODULATION OF THE NEUROMUSCULAR SYSTEM REGULATING POSTURE

Serotonin and octopamine affect postural control systems in lobsters when these amines are injected into the circulation of freely moving animals (Livingstone, Harris-Warrick & Kravitz, 1980). Serotonin injection produces sustained flexion of the limbs and abdomen (Fig. 4, top), while octopamine injection produces sustained extension (Fig. 4, bottom) (Livingstone *et al.* 1980). To discover how serotonin and octopamine might trigger these opposing postures we have examined the actions of

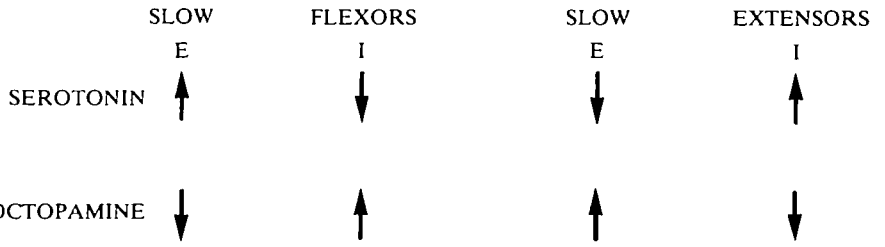


Fig. 5. Actions of serotonin and octopamine on the firing of excitatory and inhibitory motoneurones innervating postural flexor and extensor muscles. Arrows indicate increase (↑) and decrease (↓) in activity of postural excitor (E) and inhibitor (I) motoneurones. (Reprinted from Kravitz *et al.* 1985, with permission.)

the amines: (1) on peripheral flexor and extensor muscle pairs, and (2) on central ganglia of the ventral nerve cord where the motoneurones that innervate these muscles are found.

Amine actions on flexor and extensor muscles

When postural flexor and extensor muscles are perfused with saline containing octopamine or serotonin at low concentrations (10^{-9}–10^{-8} mol l^{-1}) we observe: (1) a sustained increase in the resting tension of the muscles; and (2) increases in the size of nerve-evoked contractions (Livingstone *et al.* 1980). For both effects, the action of serotonin is more dramatic than that of octopamine. Since the two amines cause similar changes in the properties of flexor and extensor muscles, the site of selectivity, the choice between flexion *vs* extension, cannot reside in the peripheral effects of these substances.

Amine actions on motoneurones of the ventral nerve cord

Perfusion of the abdominal ganglia with saline containing one or the other of the amines produces opposite changes in the firing pattern of motoneurones that innervate the postural flexor and extensor muscles. These changes in firing pattern require much higher concentrations of the amines (10^{-5} mol l^{-1}) than were necessary to elicit the peripheral effects (Harris-Warrick & Kravitz, 1984; Livingstone *et al.* 1980). With octopamine treatment there is a dramatic reduction in the frequency of firing of excitatory motoneurones that innervate flexor muscles and an increase in activity of the inhibitor to the flexors, with accompanying increases in the frequency of firing of excitatory motoneurones to extensors and decreases in activity of the inhibitor to the extensor muscles. On exposure to serotonin the opposite result occurs, although the effect is somewhat weaker. These general patterns of changes in motoneurone activity are illustrated in Fig. 5. Serotonin, therefore, appears to activate a central motor programme for flexion, and octopamine a central motor pattern for extension. These programmes are elicited by the amines regardless of whether individual ganglia or chains of ganglia are used (Harris-Warrick & Kravitz, 1984; Livingstone *et al.* 1980). Similar patterns of activation and inhibition of flexor and extensor motoneurones can be generated by stimulation of 'command fibres'

found in the connectives of the ventral nerve cord (Evoy & Kennedy, 1967; Kennedy, Evoy, Dane & Hanawalt, 1967). Therefore, the effects of the amines could be a result of direct actions on the command fibres themselves or on various interneurones associated with their circuitry, or on excitatory and inhibitory motoneurones. Preliminary studies designed to distinguish between these alternatives have shown that octopamine enhances the motor pattern evoked by firing an extension command fibre while serotonin diminishes the evoked motor output resulting from firing the same command fibre (Fig. 6) (Harris-Warrick, 1985). Intracellular recordings from motoneurones demonstrated that octopamine enhanced and serotonin reduced the amplitude of the command fibre-evoked EPSPs in flexor inhibitor (F5) and extensor excitor (M15) neurones. The effects of octopamine were, however, much more dramatic than those of serotonin. It remains uncertain where the primary site of amine action is in this system. The amines could be the transmitters of the command neurones or of a driver interneurone in the network that coordinates the motor output for extension, or the amines could modulate the activity of one or more interneurones between the command fibres and the motoneurones (Harris-Warrick, 1985).

The monoamines serotonin and octopamine can thus modulate motor activity in lobsters at two independent levels. First, they act as peripheral neurohormones released from secretory sites into the haemolymph where, at very low concentrations, they activate peripheral neuromuscular systems. Second, in the lobster central nervous system, octopamine and serotonin activate motor programmes for extension and flexion, respectively. These central actions require much higher concentrations of amines. It is probable that the amines reach their targets in the CNS through synaptic contacts or through nerve terminals in the vicinity of their targets rather than as hormones circulating in the haemolymph.

Identified serotonergic neurones

To understand how the amines might affect lobster posture *in vivo*, it is important to identify 'behaviourally relevant' neurones that contain and utilize serotonin and octopamine. To this end, immunocytochemical procedures have been used to locate cells, processes and endings in the lobster nervous system that are immunoreactive for serotonin and octopamine (Beltz & Kravitz, 1983; B. Trimmer, unpublished results). The results are more complete for serotonin where over 100 immunoreactive neurones have been found, primarily located in central ganglia (Fig. 7). Serotonin immunoreactivity was found in cell bodies, in central neuropile regions and in peripheral neurohormonal organs (Figs 7, 8). Earlier studies showed that serotonin was found, synthesized and released at two locations along the second roots emerging from lobster thoracic ganglia: in the proximal regions of the thoracic second roots and the peripheral ends of these roots that form the pericardial organs (Fig. 8A). Previous ultrastructural studies identified four morphologically distinguishable types of nerve endings in the proximal region of the second roots, one of which was shown by electron microscopic autoradiography to be associated with serotonin, and another to be associated with octopamine (Livingstone *et al.* 1981).

In several cases it was possible to trace the serotonin immunoreactive plexuses of endings to their origins in pairs of large neurones located in the fifth thoracic (T5) and first abdominal (A1) ganglia (Fig. 8B). The immunocytochemical studies showed that each of these cells had a large, anteriorly directed process that projected

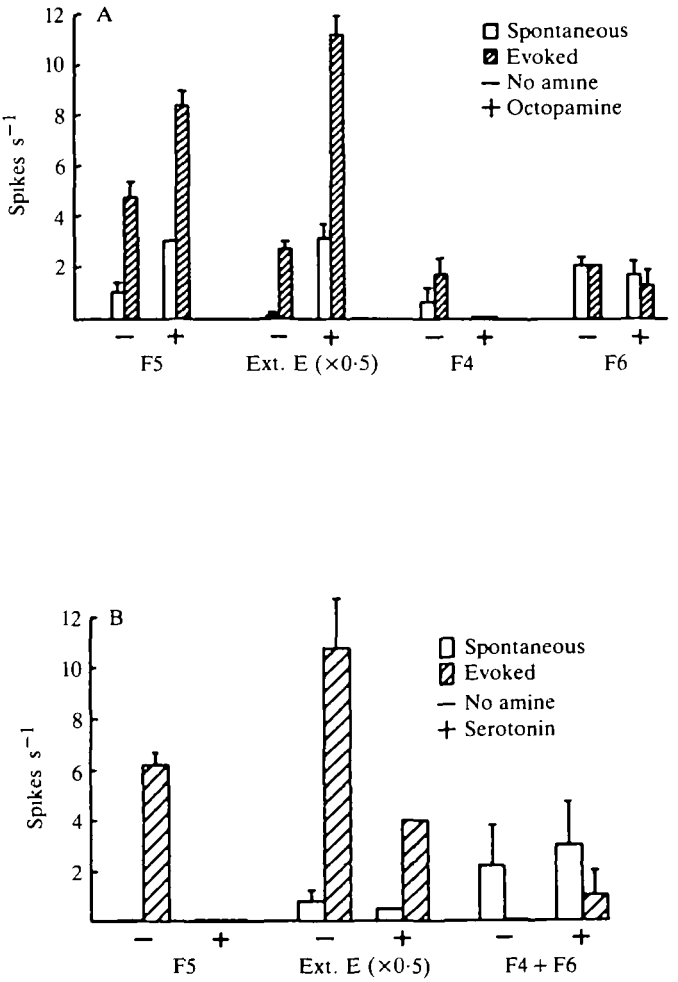


Fig. 6. Effect of octopamine and serotonin on spontaneous and extension command fibre-evoked motor activity. (A) Histogram of octopamine's effect on spontaneous (open bars) and CF-evoked (hatched bars) spike frequency in postural motoneurones before (−) and during (+) bath superfusion with 3×10^{-5} mol l^{-1} octopamine. Excitatory extensor motoneurones (Ext. E) are grouped together. Extensor activity was twice as large as shown, and has been reduced to fit into the histogram. CF-evoked spike frequency was counted during a 1-s 30-Hz train of CF pulses. F5, peripheral inhibitor to postural flexor muscles; F4, F6, excitors to postural flexor muscles. (B) Histogram of serotonin's effect on spontaneous (open bars) and CF-evoked (hatched bars) spike frequency before (−) and during (+) bath superfusion with 3×10^{-5} mol l^{-1} serotonin. Excitatory extensor motoneurones (Ext. E) are grouped together; their activity was twice as large as shown. F4 and F6 activity have also been grouped together. (Reprinted from Harris-Warrick, 1985, with permission.)

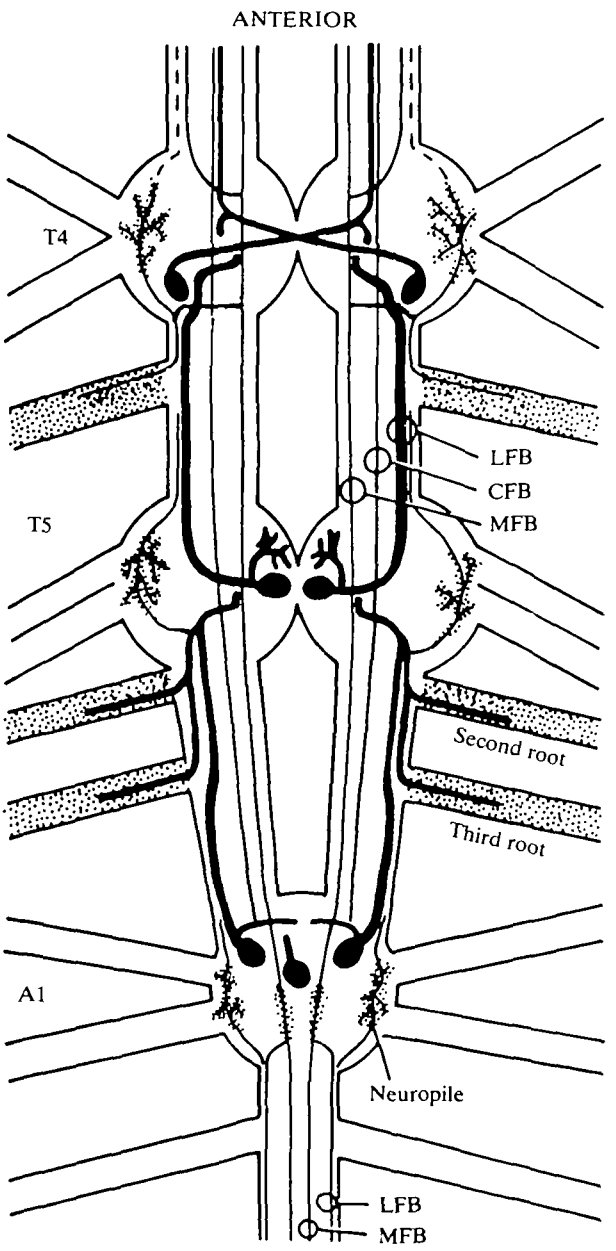


Fig. 7. Schematic diagram of the serotonin immunoreactive cell bodies, fibres, and neuropile of a part of the ventral nerve cord (T4, fourth thoracic ganglion; T5, fifth thoracic ganglion; A1, first abdominal ganglion). This is a composite drawing of wholemount preparations of 10 ventral nerve cords. Cell bodies are drawn as large, filled, round or elongate circles. Heavy black lines represent immunoreactive fibres that have been traced to their cell bodies of origin. Fine lines indicate immunoreactive fibres that have not been connected with cell bodies. Each of the fine lines of the lateral fibre bundles (LFBs), central fibre bundles (CFBs) and midline fibre bundles (MFBs) represents several fibres. Dashed lines indicate fibres that have not been directly visualized in these immunocytochemical preparations, but which we believe exist because the patterns of staining are similar from ganglion to ganglion. Stippled regions represent fine processes and varicosities of neuropile and neurohormonal release regions. (Further details are available in Beltz & Kravitz, 1983, from which this figure has been reprinted in a slightly modified form with permission.)

at least into the next anterior ganglion, where it branched and formed central neuropile (Fig. 8C). The fact that the T5 and A1 immunoreactive cells had central and peripheral release sites alerted us to the possibility that these cells might be involved both in the interganglionic coordination of neuronal activity and in the neurohormonal modulation of peripheral tissues. Since our earlier studies of the postural system showed that amines exerted both central and peripheral effects, these neurones seemed possible candidates for a role in the regulation of posture. We have therefore undertaken extensive morphological and physiological studies of these neurones.

To identify these two pairs of neurones in living preparations, a method was devised to locate the cells by size, position and physiological criteria (B. S. Beltz & E. A. Kravitz, in preparation). After the cells had first been tentatively identified as candidate neurones, we injected Lucifer Yellow into the neurones and then processed the ganglia for serotonin immunocytochemistry using a horseradish peroxidase (HRP) label. Cells that were correctly identified were therefore double labelled with Lucifer Yellow and HRP. We next pressure injected HRP into T5 and A1 identified cells to obtain detailed pictures of their neuronal geometry. These studies showed that each T5 and A1 paired neurone projects anteriorly through at least four segmental ganglia. In each ganglion a distinctive, repeating pattern of branching is seen (Fig. 9A,B). From these studies, several other morphological features of these neurones were defined: (1) processes of the T5 and A1 cells accounted for all the serotonergic axons emerging *via* thoracic second roots to form the peripheral nerve root plexuses (see Fig. 8A, and Livingstone *et al.* 1981); (2) immunocytochemical studies showed that a single right-angle branch on either side of the midline in each thoracic ganglion that gave rise to a central neuropile region and a peripheral nerve root plexus came from the A1 cells (compare Fig. 8C with Fig. 9A,B). These four large neurones, therefore, have widespread fields of innervation throughout the entire thoracic region of the ventral nerve cord and supply the serotonin released from some neurosecretory regions.

The possibility that these cells play a role in the regulation of posture is currently being investigated by testing whether the T5 and/or A1 cells, by themselves or in combination with postural command fibre stimulation, are capable of altering the motor programmes for extension or flexion. Our preliminary results show that the T5 and A1 neurones apparently are not command cells (i.e. stimulation of any of these neurones does not evoke postural motor output). They are, however, wired into the postural circuitry in a fashion compatible with a role in modulating posture: when flexion command fibres are stimulated, the T5 and A1 cells are excited; when extension command fibres are stimulated, the T5 and A1 cells are inhibited (B. S. Beltz & E. A. Kravitz, in preparation). Further experiments will test whether stimulation of T5 or A1 cells alters the efficacy of command fibre input to postural motoneurones.

The immunocytochemical studies show further that one pair of neurones in each of the posterior abdominal ganglia (A2,3,4,5,6) share certain central projections with the T5 and A1 paired cells. These cells are smaller than their suspected homologues

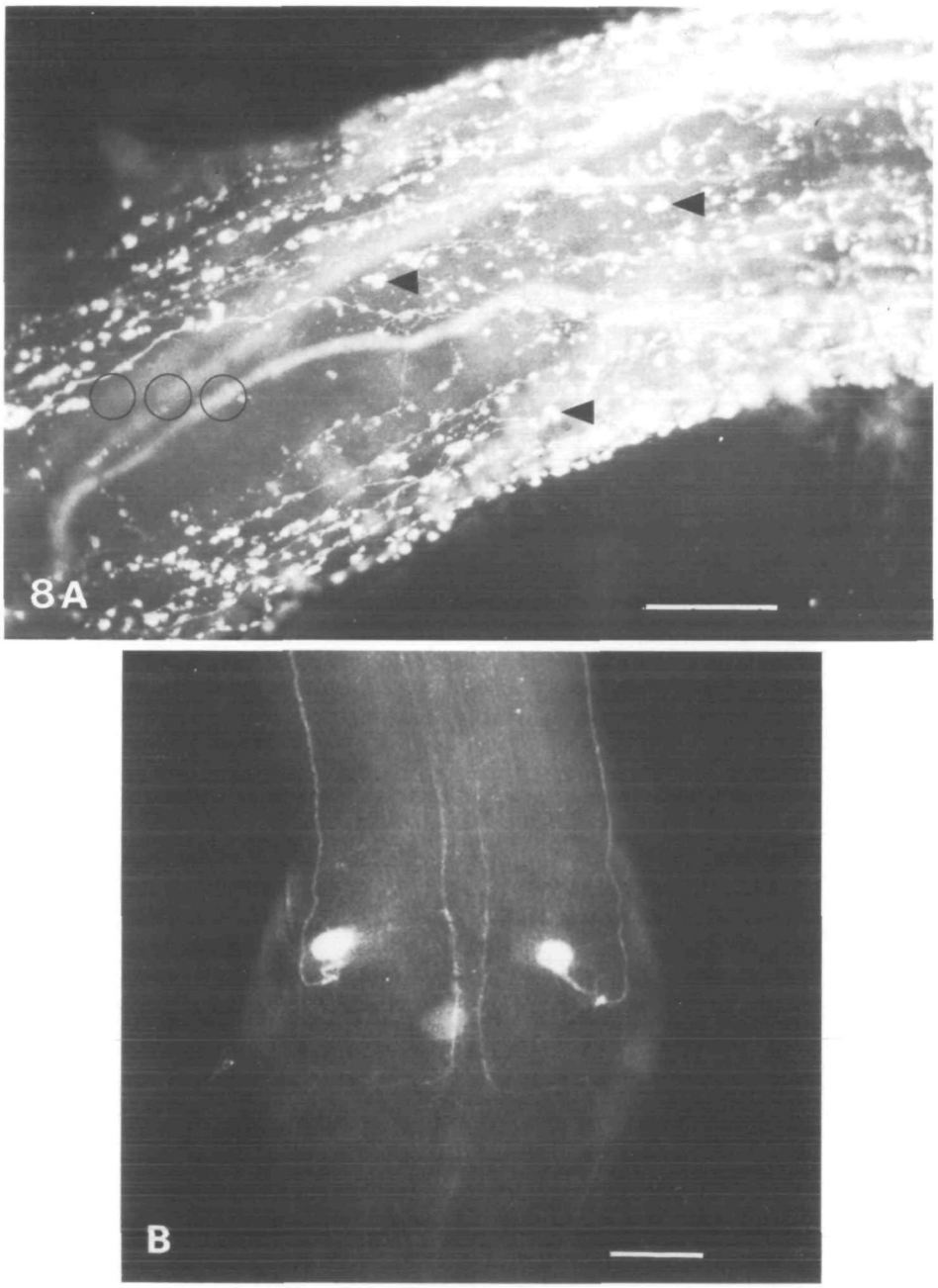
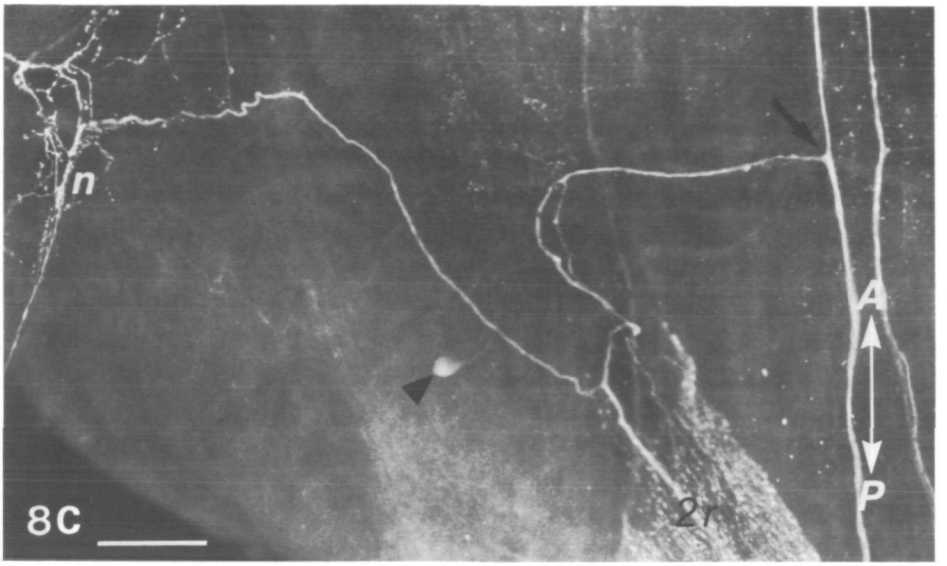


Fig. 8. Serotonin-like immunoreactivity in lobster central and peripheral tissues. (A) A photograph of a thoracic second root neurosecretory region. Axons are circled and arrowheads point to varicosities. (B) Three cell bodies stain for serotonin in the A1 abdominal ganglion: a pair of densely stained anterior cells and a large unpaired medial weakly staining cell. (C) A view of a second thoracic ganglion showing a single fluorescent process (at arrow) that gives rise to arbors of central and peripheral varicosities. This process originates in one of the paired cells in the A1 ganglion (see also Fig. 9). *n*, neuropile; *2r*, second root; *A*, anterior; *P*, posterior. Experimental details are outlined in Beltz & Kravitz, 1983, from which this has been reprinted with permission. Scale bars, 50 μm.



in T5 and A1, and there is no evidence that they project to peripheral neurohormonal areas. However, their central projections are sufficiently similar to the T5 and A1 cells that we are investigating the possibility that a large system of neurones (perhaps a total of 14 cells) is involved in coordinating the same central function(s). If further studies implicate the T5 and A1 cells in the modulation of posture, it is possible that the entire system of neurones must be activated to evoke a coordinated behaviour.

It is not clear how peptides and other amines interact with this postural system (Kravitz *et al.* 1985; Lingle, 1981). Dopamine, proctolin and an FMRFamide-like peptide are found along with serotonin and octopamine in the pericardial organs of decapod crustaceans, and a variety of neuromuscular systems are sensitive to these compounds at concentrations compatible with neurohormonal roles (Beltz *et al.* 1984; Flamm & Harris-Warrick, 1984; R. E. Flamm & R. M. Harris-Warrick, in preparation; Glusman & Kravitz, 1982; Heinzel & Selverston, 1985; Hooper & Marder, 1984, 1985; Kuramoto & Ebara, 1984; Lingle, 1981; Schwarz *et al.* 1980, 1984; Sullivan & Miller, 1984). Of particular interest in the present context, however, is the observation that the pairs of large serotonin immunoreactive neurones in the T5 and A1 ganglia contain proctolin (Fig. 10) (Siwicki, Beltz, Schwarz & Kravitz, 1986; K. K. Siwicki, B. S. Beltz & E. A. Kravitz, in preparation). This has been confirmed by immunocytochemistry and by dissecting single T5 and A1 cell bodies and measuring their proctolin and serotonin contents by high performance liquid chromatography followed by radioimmunoassay for proctolin (K. K. Siwicki, B. S. Beltz & E. A. Kravitz, in preparation; Siwicki *et al.* 1986). It is highly likely, therefore, that the T5 and A1 neurones release both proctolin and serotonin when stimulated. The consequences of release of both the amine and the peptide are not known at the present time.

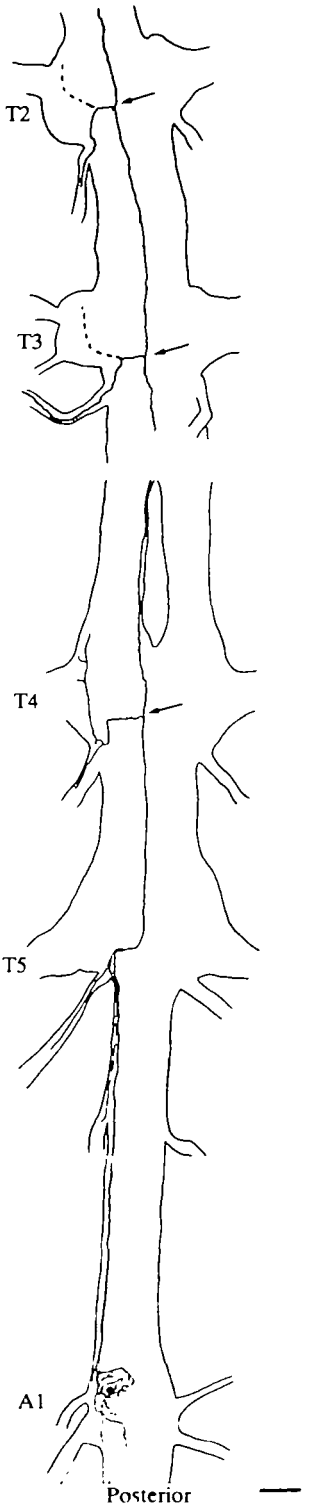


Fig. 9A

EMBRYONIC AND LARVAL STUDIES: DEVELOPMENT OF TRANSMITTER PHENOTYPES IN IDENTIFIED NEURONES

The large size, characteristic location and identified axonal arbors of the T5 and A1 neurones provide an opportunity to study the developmental expression of amine and peptide phenotypes in particular identified neurones. Furthermore, behavioural studies of larval lobsters suggest that posturally relevant circuitries likely to involve serotonin and octopamine may be 'turned on' during development. For example, at the transition from the third to the fourth larval stage, animals spend much of their time swimming with claws and tails fully extended in an octopamine-like posture. Only at the end of the fourth larval stage do they settle to the bottom and begin to behave like adult lobsters: they burrow in the sand, move away from light, and when

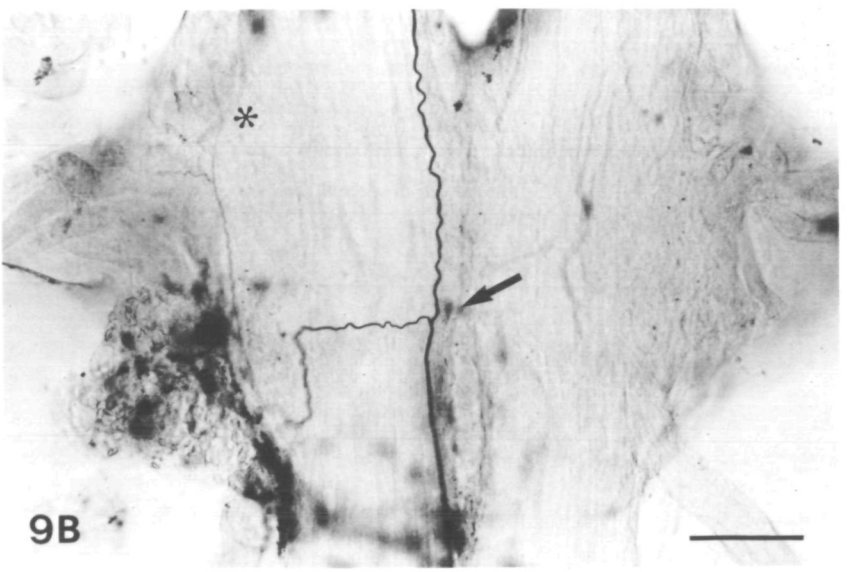


Fig. 9. Horseradish peroxidase (HRP) injection of the left A1 serotonin/proctolin neurone, and comparison of its branching pattern with fibres stained by serotonin immunocytochemistry. (A) *Camera lucida* drawings of HRP injections of portions of two different left A1 serotonin-immunoreactive cells. The top part of the drawing shows the projection of one A1 cell into the second (T2) and third (T3) thoracic ganglia. The bottom portion shows a different left A1 cell body and its projection in the fourth (T4) and fifth (T5) thoracic ganglia. Note that the A1 cell forms a right-angle branch in T2, T3 and T4 (arrows). This cell is also known to form a lateral neuropile in each of these ganglia, as seen in T4 (and represented by a dashed line in T2 and T3). A branch of this cell also projects out the ipsilateral thoracic second root of T2, T3, T4 and T5. Scale bar, 500 μm. (Reprinted from Kravitz *et al.* 1985, with permission.) (B) Photograph of the fourth thoracic ganglion from the preparation shown in the lower part of Fig. 9A showing the HRP-filled projection of the left A1 serotonin/proctolin neurone. Note the similarity between the branching pattern of the HRP-injected cell (see the right-angle branch, at arrow) and the photograph of a serotonin immunoreactive branch in a T2 ganglion shown (Fig. 8C). The HRP-filled fibre that projects out of the ipsilateral thoracic second root of this ganglion is out of the focal plane of the photograph. The region of lateral ganglionic neuropile is labelled with an asterisk in this figure. Scale bar, 200 μm. (Reprinted from B. S. Beltz & E. A. Kravitz, in preparation, with permission.)

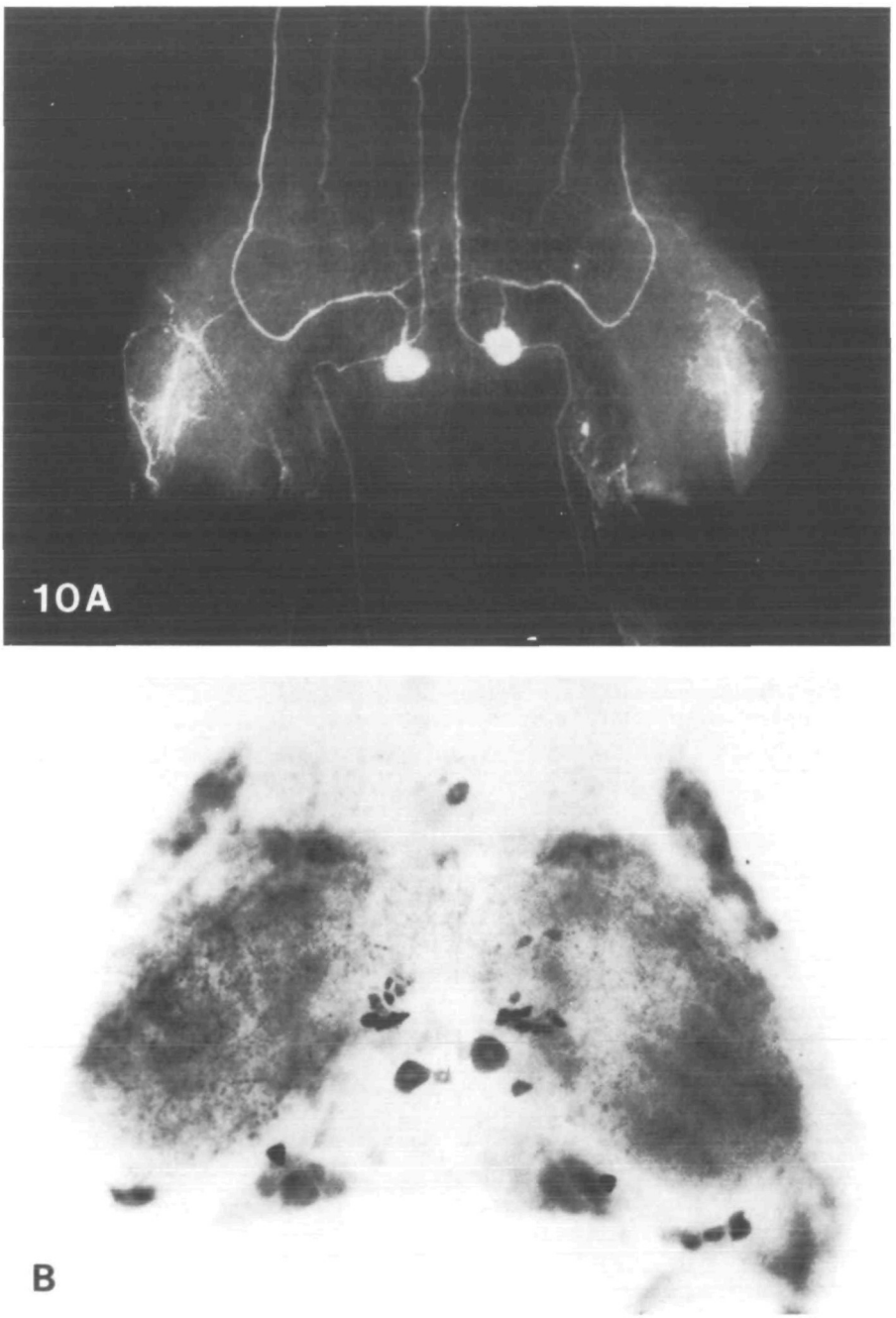


Fig. 10. Immunocytochemical staining of the fifth thoracic ganglion (T5) for serotonin (A, immunofluorescence) and proctolin (B, immunoperoxidase technique). Note the large paired cells staining in the posterior–medial quadrant of the ganglion in both preparations. These cells have been dissected out and shown to contain authentic serotonin and proctolin. (Top plate reprinted from Beltz & Kravitz, 1983, with permission; bottom plate reprinted from Kravitz *et al.* 1985, with permission.) Magnification, ×70.

disturbed open their claws and raise themselves into a 'threatening attitude' (Herrick, 1896). The 'threatening attitude' is one in which animals assume a posture comparable to what we recognize as a 'serotonin-like' stance. The following studies represent our initial efforts to explore the possible involvement of neuromodulators in the dramatic behavioural transitions experienced by developing lobsters.

Immunocytochemical studies have been carried out with ventral nerve cords from animals ranging in age from embryos (about halfway through development) to the fourth larval stage. These studies showed that even mid-stage embryos have a system of neurones staining for serotonin that is similar in its main features to the adult system. All of the approximately 100 presumptive serotonergic neurones can be accounted for; their positions and relative sizes are typical of the same neurones in the adult lobster. In comparison with neighbouring neurones, the embryonic serotonin neurones are quite large and their arbors are well elaborated at these early stages. However, in contrast to the early appearance of serotonin immunoreactivity, proctolin staining (seen in about 1500 neurones in adult lobsters, see Siwicki & Bishop, 1986) is observed in only a few hundred anteriorly located cell bodies in embryos (with one exception, see Fig. 11). Staining appears in the remaining neurones during late embryonic and early larval development. In certain neurones proctolin staining is seen only in embryos, suggesting either a lower concentration of the peptide in adult cell bodies, or death of those neurones (as with A2 stained neurone, Fig. 11B).

In the T5 and A1 serotonin/proctolin neurones the cell bodies show serotonin immunoreactivity in mid-stage embryos (Fig. 11A). The characteristic branch points of these cells also appear well developed at this time (see Figs 8C, 9), as are the plexuses of varicosities in the proximal regions of the thoracic second roots (see Figs 8A, 9). Therefore, we must examine even earlier embryonic stages to find the first immunocytochemically detectable appearance of serotonin in these neurones. In other systems, it is thought that serotonergic neurones synthesize transmitter soon after they have completed their terminal division (Lauder *et al.* 1982; Taghert & Goodman, 1984). We do not know if a similar situation exists in lobsters. Our preliminary immunocytochemical studies of proctolin, however, demonstrate no proctolin-like staining of the T5 and A1 neurones in mid-term embryos and only very light or no staining in stage I larvae (Fig. 11B). This is therefore an interesting system for examining the differential development of amine and peptide synthetic systems in the same identified neurones, and for investigating the physiological consequences of this differential development.

MOLECULAR BASIS OF AMINE AND PEPTIDE ACTIONS

Many potential targets of amines and peptides have been identified in Crustacea, including exoskeletal muscles, chromatophores, haemolymph, heart, stomatogastric, cardiac ganglia and ganglia of the ventral nerve cord. What is the molecular basis of hormone action on these effectors? Are there unifying principles underlying their actions? Cyclic nucleotides mediate many neurohormonally regulated physiological

responses (Gade & Beenakkers, 1977; Hanaoka & Takahashi, 1977; Dunwiddie & Hoffer, 1982; Greengard, 1979), and have been implicated as second messengers of amine and peptide actions on crustacean targets. Cyclic AMP mimics the action of chromatophorotropic neuropeptides by causing pigment dispersion in melanophores and erythrophores of crabs and shrimp (Fingerman, 1969; Hallahan & Orsi, 1972; Lambert & Crowe, 1976); cyclic AMP and cyclic GMP are thought to be involved in the action of crustacean hyperglycaemic hormones (Sedlmeier & Keller, 1981;

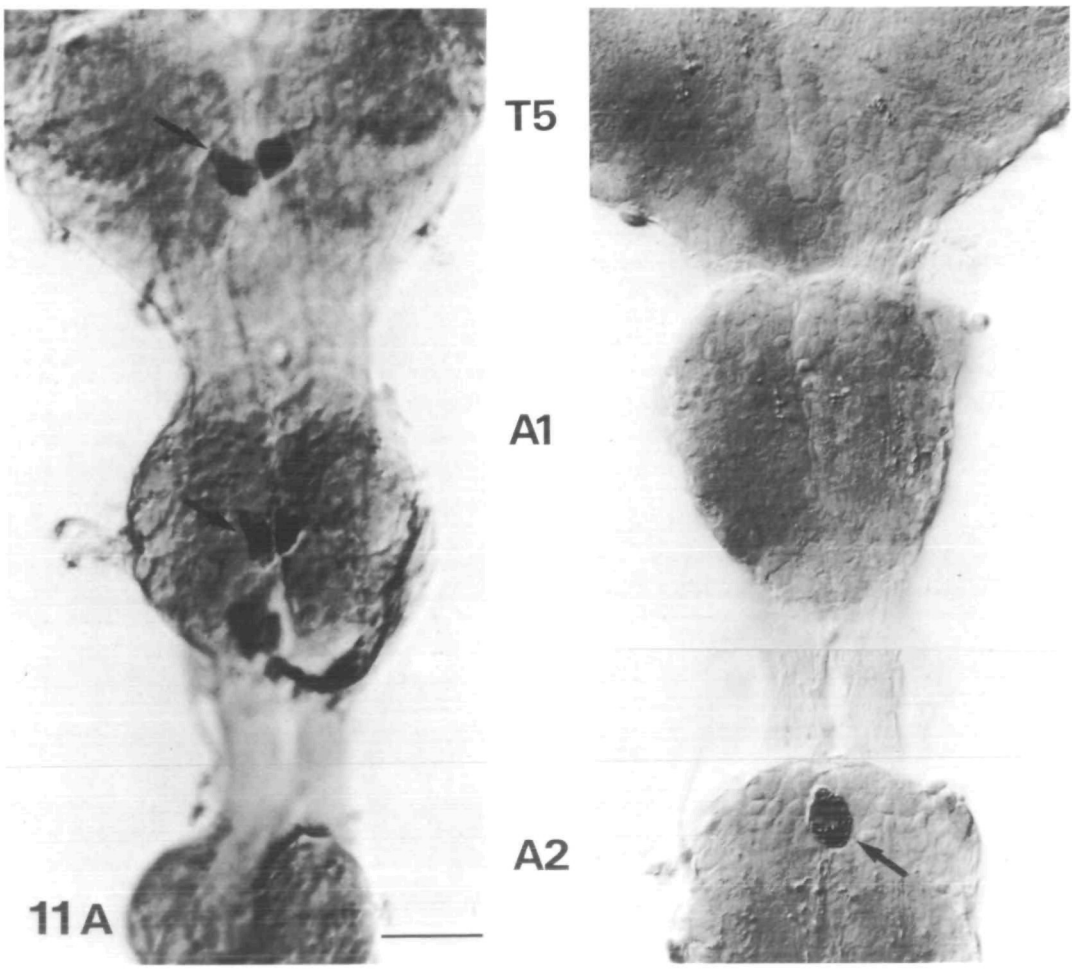


Fig. 11. Wholemounts of the T5, A1 and A2 ganglia of lobsters approximately halfway through embryonic development stained for (A) serotonin- and (B) proctolin-like immunoreactivities. At this stage, the identified T5 and A1 cell pairs stain for serotonin (arrows in A), but do not stain with antibody to proctolin (B) even though other proctolin-positive cells are present (see unpaired stained cell at arrow in A2, which is not immunoreactive in adult A2 ganglia). Proctolin-like staining appears in the T5 and A1 cells only in late embryonic and larval stages. Scale bar, 50 μm. (Reprinted from Siwicki, Beltz, Schwarz & Kravitz, 1986, with permission.)

Sedlmeier, 1982, 1985). Serotonin causes increases in cyclic AMP in lobster heart and exoskeletal muscles (Battelle & Kravitz, 1978) and in the cardiac ganglion (Lemos & Berlind, 1980), while octopamine stimulates cyclic AMP production in lobster haematocytes and cardiac muscle (Battelle & Kravitz, 1978). In addition, one of the cardioexcitatory peptides released from crustacean POs increases cyclic AMP levels in intact cardiac ganglia (Lemos & Berlind, 1980); cyclic AMP and phosphodiesterase inhibitors mimic the physiological effects of PO extracts on the cardiac ganglion.

Cyclic nucleotides and lobster exoskeletal muscle preparations

Three substances, serotonin, octopamine and proctolin, influence the responsiveness of lobster exoskeletal muscle preparations. These muscles have a dual innervation. They are innervated by excitatory axons using glutamate and inhibitory axons using GABA as transmitter compounds (Otsuka, Iversen, Hall & Kravitz, 1966; Otsuka, Kravitz & Potter, 1967). Studies with the dactyl opener muscle of the walking leg of the lobster, *Homarus americanus*, have shown that the three neurohormonal substances have similar actions on the fibres of the opener muscle: they induce long-lasting contractures that are accompanied by little or no change in membrane potential or input resistance of the muscle fibres; they produce action potentials in previously quiescent muscle fibres (Glusman & Kravitz, 1982; Kravitz *et al.* 1980; Schwarz *et al.* 1980). The effect of serotonin on the muscle fibres has been studied using voltage-clamp techniques; no change was seen in an outward voltage-sensitive potassium conductance, but a large increase was seen in an inward current, probably carried by calcium ions (Glusman & Kravitz, 1982; Kravitz *et al.* 1985). It is not clear whether this increase in inward current results from a direct action of serotonin on calcium channels, or from an indirect action, for example, on calcium-activated potassium channels. Nor has it yet been shown whether this increased inward current can account for the appearance of the serotonin-induced action potentials. Serotonin also acts directly on excitatory and inhibitory nerve endings in this preparation to facilitate transmitter release. When serotonin is washed out of the bathing medium, there is a biphasic decay of the enhanced synaptic response: one component decays with a time constant of several minutes, while the other requires more than an hour to return to control levels (Goy & Kravitz, 1984; Kravitz *et al.* 1980). Pharmacological experiments suggest that different serotonin receptors mediate these two components (Goy & Kravitz, 1984; Kravitz *et al.* 1980). Octopamine has much smaller presynaptic actions on excitatory nerve terminals in this preparation (but see Florey & Rathmayer, 1978; Breen & Atwood, 1983) and proctolin has no measureable presynaptic effect.

Incubation of intact opener muscle preparations with serotonin leads to large and with octopamine to small increases in cyclic AMP levels. Proctolin causes no detectable increase in cyclic AMP levels (Goy, Schwarz & Kravitz, 1984). Serotonin also causes phosphorylation of a 29 kDa protein; octopamine, in the presence of

isobutylmethylxanthine (IBMX, a phosphodiesterase inhibitor) can cause phosphorylation of the same protein, while proctolin never causes phosphorylation of the 29 kDa protein. The ability of these three compounds to increase cyclic AMP levels closely parallels their ability to enhance phosphorylation of the 29 kDa protein. Other agents that increase intracellular cyclic AMP levels lead to phosphorylation of this protein and incubation of tissue homogenates with cyclic AMP or cyclic GMP in the presence of ^{32}ATP causes phosphorylation of an identical protein. These studies show that increases in cyclic AMP levels and phosphorylation of this protein are linked (Goy *et al.* 1984). To explore whether cyclic AMP is involved in the serotonin-induced physiological changes, agents that elevate cyclic AMP levels, act like cyclic AMP or potentiate or inhibit the ability of serotonin to alter intracellular cyclic AMP levels have been applied to the dactyl opener nerve–muscle preparation (Goy & Kravitz, 1984). Phosphodiesterase inhibitors and adenylate cyclase activators do not cause significant contractions of opener muscle, nor do they potentiate serotonin-induced contractures. These results suggest that cyclic AMP is not causally involved in the sequence of events by which serotonin increases muscle tension. This is further substantiated by the fact that exposure to 8-bromo-cyclic AMP also does not increase muscle tension. Cyclic AMP may be involved, however, in part of the presynaptic action of serotonin. Agents that increase intracellular cyclic AMP levels all enhance transmitter release in much the same way as serotonin. Furthermore, the slower-decaying phase of serotonin's presynaptic effect is selectively potentiated by IBMX while the rapidly decaying phase appears to be unaffected by the drug. This suggests that serotonin enhances transmitter release by two mechanisms, only one of which is dependent on cyclic AMP (Goy & Kravitz, 1984).

Cyclic GMP also may be involved in the action of some crustacean neurohormones. Arthropod tissues, in contrast to most vertebrate tissues, have high levels of cyclic GMP-dependent protein kinase (Takahashi & Hanaoka, 1977). Sedlmeier & Keller (1981) have studied in detail the mechanism of action of hyperglycaemic hormone of the crayfish, *Orconnectes*. In all tissues studied, both cyclic AMP and cyclic GMP were elevated after hormone administration *in vivo*. The cyclic GMP increases, however, were larger. In hepatopancreas tissue, studied *in vivo*, cyclic GMP levels increased in response to hyperglycaemic hormone while cyclic AMP levels were not altered; the cyclic GMP rise was followed by glucose release into the medium. Spindler, Willig & Keller (1976) have shown *in vivo* that cyclic GMP is somewhat more effective in eliciting hyperglycaemia than is cyclic AMP. Recently, an eyestalk factor from the lobster, *Homarus americanus*, has been isolated that causes large selective increases in cyclic GMP levels (Goy, York & Mandelbrot, 1985). It is not yet known whether this factor also causes hyperglycaemia. The high levels of enzymes of cyclic GMP metabolism and the availability of hormones that selectively increase cyclic GMP levels in tissues suggest that crustacean preparations may be excellent tissues for exploring the second messenger function of this nucleotide.

SUMMARY AND CONCLUSIONS

The experimental observations described in this article reveal that crustacean tissues offer a rich source of material with which to explore the multitude of actions of amines and peptides in nervous systems. The experiments with the cardiac ganglion of the heart illustrate some actions of these substances as neurohormones released into the general circulation. The studies with the stomatogastric ganglion demonstrate that at the level of circuits of neurones: (1) the output of the circuit is flexible, with individual amines and peptides being capable of programming unique, concentration-dependent changes in output; (2) it is difficult to predict the consequences of amine actions on sets of neurones solely from a knowledge of amine actions on isolated neurones; and (3) even in closely related species, the route of delivery to a target of a particular modulating substance (e.g. *via* the circulation or *via* synaptic contacts) can differ dramatically. Finally, our studies with postural control systems in lobsters demonstrate that one can begin to link amine-induced alterations in neuronal output to aspects of lobster behaviour. Moreover the analysis of the alterations can be applied to individual amine neurones. The developmental studies with this system open possible avenues for exploring the relationships between the phenotypic expression of transmitter subtypes in identified neurones and the emergence of behaviour in these animals.

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