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AMINES AND A PEPTIDE AS NEUROHORMONES IN LOBSTERS: ACTIONS ON NEUROMUSCULAR PREPARATIONS AND PRELIMINARY BEHAVIOURAL STUDIES

BY EDWARD A. KRAVITZ, SILVIO GLUSMAN, RONALD M. HARRIS-WARRICK, MARGARET S. LIVINGSTONE, THOMAS SCHWARZ AND MICHAEL F. GOY

Harvard Medical School, Department of Neurobiology, 25 Shattuck Street, Boston, Massachusetts 02115

SUMMARY

In this communication we report that four substances, thought to function as neurohormones in Crustacea, all produce long-term changes in the physiological properties of lobster opener muscle preparations. The substances are the amines, octopamine, serotonin and dopamine, and the peptide, proctolin. The actions of these substances are superimposed on the normal synaptic apparatus that utilizes the amino acids GABA and glutamate (probably) as the inhibitory and excitatory neurotransmitter compounds.

Serotonin acts on excitatory and inhibitory nerve endings to facilitate transmitter release and directly on muscle fibres to produce a contracture and to induce the appearance of Ca^{2+} action potentials. The latter two actions of serotonin are shared by proctolin and octopamine as well. Dopamine, on the other hand, relaxes muscle baseline tension. The mechanism of action of these substances at their target site (or sites) has been explored with electrophysiological and biochemical techniques and the results will be presented.

In addition preliminary behavioural experiments have been carried out with serotonin and octopamine. These substances produce opposite postures when injected into lobsters. The amines act on central ganglia to produce these effects where they cause a programmed readout of firing of neurones that will produce either a flexed posture (serotonin) or an extended posture (octopamine).

INTRODUCTION

In past years invertebrate nervous tissue preparations have been invaluable for unravelling the details of basic physiological processes that operate in the nervous systems of higher organisms. As examples, experiments with squid giant axons led to the ionic theory for the generation and propagation of the action potential; studies with the giant synapse in the squid stellate ganglion provided a direct demonstration of the existence of voltage sensitive Ca^{2+} currents linked to transmitter release in nerve terminals; physiological and biochemical experiments with crustacean tissues led to an understanding of the mechanism of inhibition and the evidence that GABA functioned as an inhibitory transmitter compound. In recent years, invertebrate

organisms have served as the starting point for investigations of more complex physiological phenomena often involving the integrated operation of networks of nerve cells. Some of the types of studies performed are: (1) analyses of the developmental programs that control behaviour and the differentiation and death of neurones (e.g. Truman, 1978; Sulston & Horvitz, 1977; White, Albertson & Anness, 1978); (2) attempts to understand at the cellular level the readout of motor patterns controlling complex body movements (e.g. Johnson & Stretton, 1980; Davis, 1976); (3) analyses at the cellular level of the mechanisms of habituation, sensitization and learning (e.g. Kandel, 1978).

In our laboratory we have begun an examination of the functioning of neurohormonal systems in lobsters. The hormonal systems of these animals are amenable to analysis at many levels and it should be possible to define each element in the process from the location of the hormone-containing cells and the inputs that activate them to the mode of action of the hormones at their target tissues, and ultimately to how the hormones function in the behaviour of the animals.

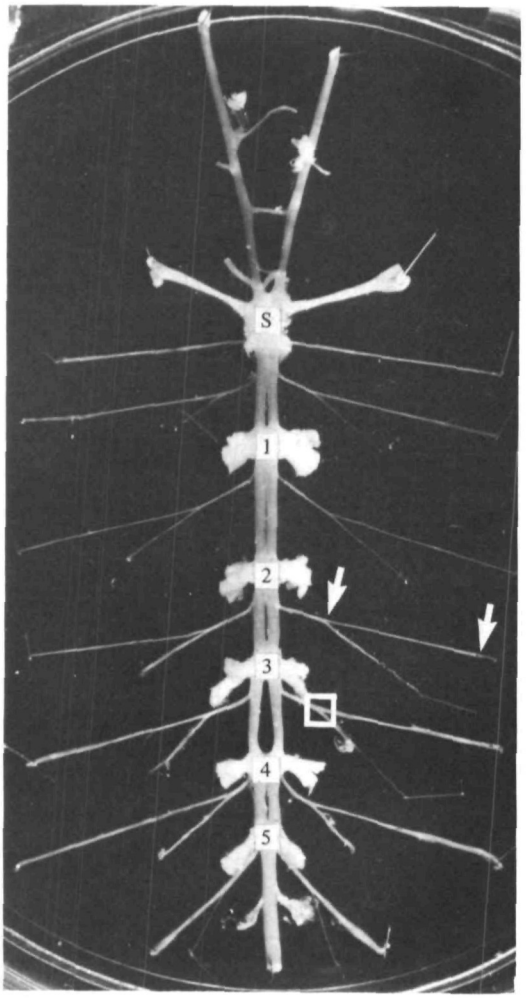
In this communication we will briefly describe the location and activation of groups of amine-associated neurosecretory neurones in lobsters. The main focus of the paper, however, will be: (1) an analysis of the effects of multiple hormones (including the amines) on a single target tissue, the lobster neuromuscular preparation; and (2) an examination of the cellular mechanisms underlying the behavioural observation that serotonin and octopamine produce opposite postures when injected into lobsters.

Octopamine and serotonin: association with neurosecretory cells in second thoracic roots

The lobster central nervous system consists of a chain of ventrally located ganglia linked together by connectives containing axons that run between the ganglia. Each ganglion is primarily concerned with the peripheral tissues that surround it and it communicates with these tissues through nerve roots containing efferent and afferent axons. The most anterior ganglion (the brain or supraesophageal ganglion) contains hundreds of thousands of nerve cells and is linked to a pair of small circumesophageal ganglia, a large subesophageal ganglion, 5 thoracic and 6 abdominal ganglia.

Amines are concentrated in the anterior half of the nervous system. They are found in highest concentration along thin nerve trunks we call the second thoracic roots, in association with clusters of neurosecretory neurones (Evans *et al.* 1976*a*, Fig. 1). About 100 of these neurosecretory neurones are found spread out along seven pairs of the 2nd thoracic roots (Fig. 1). Despite the wide dispersion of these cells, physiological studies suggest that they function as a unit (Konishi & Kravitz, 1978). At two locations along the roots amines are found in high concentrations: (1) near the somata of the neurosecretory cells where these cells are known to have elaborate arrays of endings (Evans, Kravitz & Talamo, 1976*b*); (2) near the distal ends of the nerves in the pericardial organs (well known crustacean neurohaemal organs) where the cells are believed to have second axonal aborizations (see Maynard & Welsh, 1959; Sullivan, Friend & Barker, 1977).

While we have been able to demonstrate that octopamine and serotonin can be synthesized in morphologically distinguishable categories of nerve endings close to the neurosecretory neurone cell bodies, we have not yet demonstrated whether



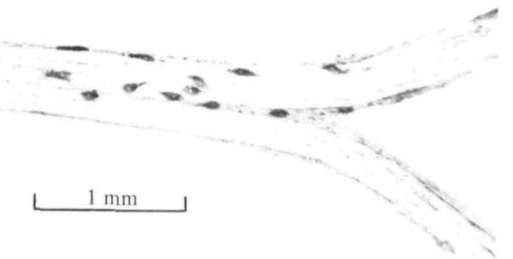


Fig. 1. Thoracic region of the ventral nerve cord – location of the amine-associated neurosecretory neurons. The ganglia are labelled S = subesophageal, 1–5 = thoracic ganglia 1–5. Each ganglion has a pair of thin second thoracic roots associated with it. The arrows indicate the regions of high concentration of amine. In the boxed-in area cell bodies are concentrated. On the right are shown a cluster of neurosecretory neurones stained with the dye neutral red from a third thoracic segment. (Both figures are reprinted from Evans *et al.* 1976, with permission.)

dings actually arise from the cell bodies in their vicinity (Livingstone, Schaeffer & Kravitz, 1980*a*). The close association of amines with the cells is revealed, however, by the proportionality seen between the number of cells in a root and the content of serotonin and octopamine (Livingstone *et al.* 1980*a*). The amines are found in a roughly 1 to 14 ratio (ser:oct) in each root. Both amines can be synthesized from radioactive precursor compounds and released from the roots in a calcium dependent manner by depolarization with K^+ (Evans *et al.* 1976*b*; see also Sullivan, Friend & Barker, 1977 for studies with spiny lobster ligamental plexus).

The nerve endings containing amines are not associated with any specialized structures and are mostly found within several microns of the surface of a root. We presume, therefore, that amines are released directly into the haemolymph at these sites (see also Sullivan, Friend & Barker, 1977). Further evidence that amines actually function as circulating neurohormones comes from recent studies demonstrating that amines are found in lobster haemolymph at concentrations within an order of magnitude of their physiologically effective concentrations in target tissues ($1–2 \times 10^{-9}$ M, Livingstone *et al.* 1980*b*). It is likely that amine-containing nerve endings will be found in lobsters in locations other than those in the peripheral roots. In fact, the highest total amounts of serotonin are found in the brain and subesophageal ganglion and all ganglia contain low levels of amines (Livingstone *et al.* 1980*a*).

Octopamine and serotonin: actions on target tissues

Our studies and those of other investigators suggest that circulating amines exert physiological actions on a wide variety of peripheral tissues (Fig. 2). Upon release, the first tissue contacted by amines is the haemolymph where octopamine produces a selective acceleration of clotting. This is associated with an increase in cyclic AMP levels in haematocytes but the details of the relationship between the cyclic nucleotides and clotting have not been explored further (Battelle & Kravitz, 1978). Both octopamine and serotonin increase the frequency and strength of the heart beat, effects that are also associated with increases in cyclic AMP (Sullivan & Barker, 1975; Battelle & Kravitz, 1978; for serotonin actions on heart see also Cooke & Hartline, 1975; Cooke, 1976). Amines also act on exoskeletal muscles and our recent studies have concentrated on these tissues. Exoskeletal muscles are responsive to octopamine, serotonin, dopamine and to proctolin, a pentapeptide originally isolated from cockroach intestine (Brown, 1975). The detailed description of the physiological actions of these substances on the muscles will be presented below. The last tissue we have examined for actions of amines are the central ganglia of the ventral nerve cord. These studies result from the observation that the injection of octopamine and serotonin into lobsters produces opposite postures, an effect that we have shown to be due to the selective activation of separate motor programs by each of the amines (Livingstone *et al.* 1980*b*). These results will be described below. Other investigators have reported additional actions of amines in crustacean peripheral tissues. For example in stomatogastric ganglia the synthesis of octopamine and dopamine have been demonstrated and selective effects of amines on the patterns of firing of neurones within the ganglia have been noted (Barker, Kushner & Hooper, 1979; Anderson & Barker, 1977). In crabs, serotonin causes changes in the beating of the scaphognathite, appendage that circulates water through the gill chamber (Berlind, 1977). Also

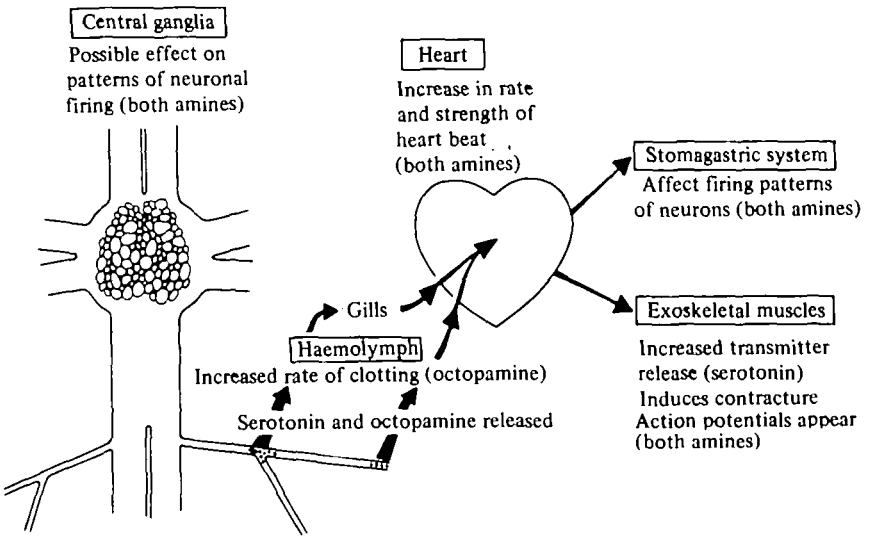


Fig. 2. Peripheral and central targets of amines.

in crabs the localization of dopamine and serotonin containing cell bodies in central ganglia and terminal varicosities in neurosecretory regions has been reported by Cooke and coworkers (Cooke & Goldstone, 1970; Goldstone & Cooke, 1971).

Multiple hormones act on neuromuscular preparations

GABA has been well established as the inhibitory transmitter compound at lobster neuromuscular junctions and glutamate is the leading and only candidate for the excitatory transmitter (but see Shinozaki & Ishida, 1979). Three amines (serotonin, octopamine and dopamine) and a peptide (proctolin), however, modify excitatory and inhibitory synaptic effectiveness in these tissues and have direct actions on muscle fibres (the sites of action and possible mechanisms are summarized in Fig. 8). We suspect that all these substances function as circulating neurohormones in lobsters, but thus far only have direct evidence that octopamine and serotonin circulate in the haemolymph. All four modulating substances act directly on muscle fibres. Three of these, octopamine, serotonin and proctolin, lead to a sustained contracture in muscle fibres and to the generation of Ca^{2+} action potentials, while dopamine relaxes muscles. Serotonin alone directly alters incoming synaptic input: it greatly increases transmitter release from excitatory nerve endings (Dudel, 1965; Glusman & Kravitz, unpublished) and also increases the effectiveness of inhibitory nerve stimulation. In the following sections we describe the experimental observations supporting the various sites of actions of the modulating substances.

Serotonin: pre-synaptic action on excitatory nerve terminals

When serotonin at concentrations as low as 5×10^{-9} M is superfused onto lobster walking leg opener muscle preparations the size of the excitatory junctional (synaptic) potentials (e.j.p.s) are increased (Dudel, 1965; for method of preparing and superfusing tissue see Otsuka *et al.* 1966). The increase in e.j.p. size usually takes 1–2

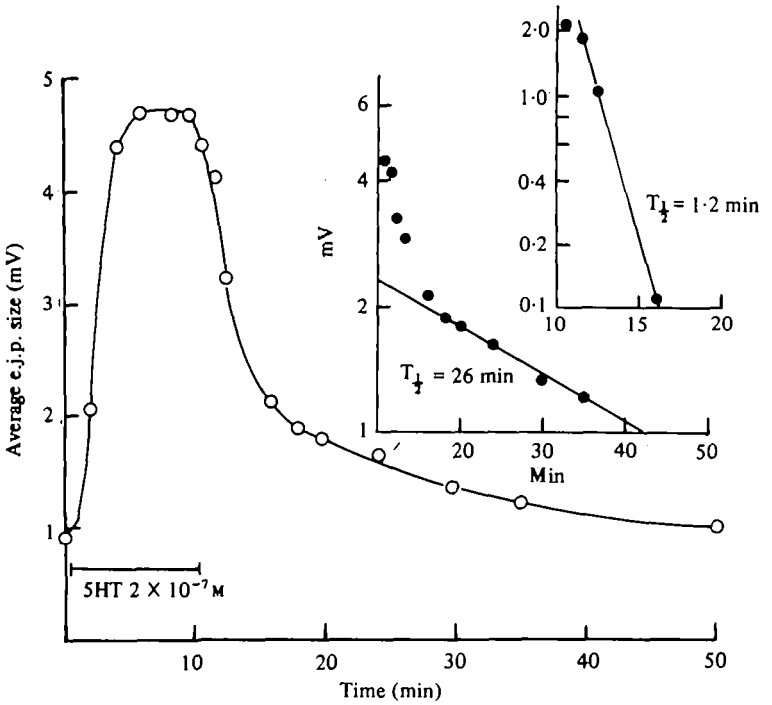


Fig. 3. Two components of the pre-synaptic action of serotonin. The opener muscle of the dactyl of the lobster walking leg was dissected and set up for superfusion as described by Otsuka *et al.* (1966). Saline was flowed through the preparation at a rate of 1–2 ml/min. At the indicated times computer averages were taken of 25 e.j.p.s at a frequency of 1 Hz. The inset graph is data from the falling phase of the curve after 5 HT washout. Points for the fast component ($T_{\frac{1}{2}}$ = 1·2 min) were obtained by extrapolating the slow component to 10 min (the washout time) and subtracting the extrapolated values from the measured points. The temperature was 17°. (Reprinted with permission from Kravitz *et al.* 1980.)

to reach its peak, remains constant during up to 10 min of superfusion with serotonin and, after washout of serotonin from the bath, decays in two steps ($T_{\frac{1}{2}}$ = 1–2 min; $T_{\frac{1}{2}}$ = 30 min) to the control size (Fig. 3). The overall increase in size induced by serotonin can be up to 4 to 5 times the control response. Almost all of this increase is due to a pre-synaptic action of serotonin. The evidence that supports this is as follows: (1) muscle input resistance shows at best a 10–15% increase, much too small an increase to account for the increase of the e.j.p.; (2) quantal analysis of the e.j.p. reveals an increase in the probability that quanta of transmitter will be released with excitatory nerve stimulation (see also Dudel, 1965); (3) the sensitivity of muscle fibres to exogenously applied glutamate is not increased by serotonin treatment; (4) the frequency of spontaneous release of quanta of transmitter is increased with no change in mean size with serotonin treatment, and the duration of the increase in frequency parallels the duration of the increased nerve-evoked release.

Preliminary studies suggest that at least a portion of the serotonin-induced increase in transmitter release may be due to an alteration of part of the calcium storage machinery within excitatory nerve terminals. This notion is supported by two cate-

gories of physiological experiments. In the first the increase in frequency of release of spontaneous miniature e.j.p.s caused by serotonin (see above) occurs both in normal saline (26 mM Ca^{2+}) and after washing preparations for over an hour in a saline solution without Ca^{2+}, with Mg^{2+} elevated to replace Ca^{2+} and with 1 mM EGTA added (Ca^{2+}-free saline). In this medium the baseline miniature frequency is decreased compared to normal saline, but the magnitude of the serotonin-induced increase above baseline is similar in both media. The duration of the increase in Ca^{2+}-free saline is shorter, however. If the frequency of spontaneous miniature e.j.p.s is related to the intra-terminal free Ca^{2+} level (see Rahamimoff *et al.* 1978*a*) the serotonin-induced increase in frequency in Ca^{2+}-free saline may reflect an alteration in the intraterminal Ca^{2+} storage machinery. The short duration of this effect could be due to a partial depletion of the affected pool after prolonged treatment in Ca^{2+}-free saline. The second category of experiment supporting the idea that Ca^{2+}-storage is changed by serotonin treatment is that in about one-third of our experiments we have been able to demonstrate a nerve-evoked release of transmitter in the Ca^{2+}-free, EGTA containing saline. Since no extracellular Ca^{2+} is available for release in these studies, and if Ca^{2+} is required for release, we would presume that Na^{+} entering during the nerve action potential may, by acting on some Ca^{2+} pool within the terminal, be providing the Ca^{2+} needed for release. This could be due to Na^{+} causing a greater release of Ca^{2+} from a storage pool after serotonin treatment than before, or that Ca^{2+} possibly released by Na^{+} entry or accumulation (see Rahamimoff *et al.* 1978*b*; Atwood, Swenarchuk & Gruenwald, 1975; Jan & Jan, 1978) brings an already elevated free Ca^{2+} pool to the threshold for release. We should note, however, that the interpretations of these results presumes that at the nerve-terminal release zones a prolonged wash with Ca^{2+}-free saline actually removes all extracellular Ca^{2+}. Since we have no direct measure of the Ca^{2+} level in synaptic clefts these results should continue to be treated with caution. We have no information as to whether nerve terminal Ca^{2+} channels might also be altered by serotonin treatment (see Kandel, 1979; Dunlap & Fischbach, 1978). An analysis of the Ca^{2+} requirement for evoked transmitter release demonstrated that at all levels of Ca^{2+} tested, serotonin caused a greater release of transmitter than in controls but there was no change in the second power relationship existing between extracellular Ca^{2+} and release (Glusman & Kravitz, unpublished).

Serotonin: action on inhibitory nerve transmission

GABA, the inhibitory neurotransmitter in these preparations acts by increasing the permeability of the membrane to Cl^{-}. Because the Cl^{-} equilibrium potential is very close to the resting level of membrane potential, small changes in membrane potential introduced by amines or in intracellular ion concentration introduced by electrodes make it difficult to measure actions of substances on inhibitory neurotransmission. Two types of experiments were performed to try to overcome these difficulties. In the first the membrane potential was shifted in a series of steps above and below the chloride equilibrium potential before, during, and after treatment of tissues with test substances, and the size of inhibitory junctional potentials was measured (see legend to Fig. 4 for experimental details). In the second type of experiment a two-electrode point voltage clamp was used to hold the membrane voltage constant at levels above and below the chloride equilibrium potential and synaptic currents were measured

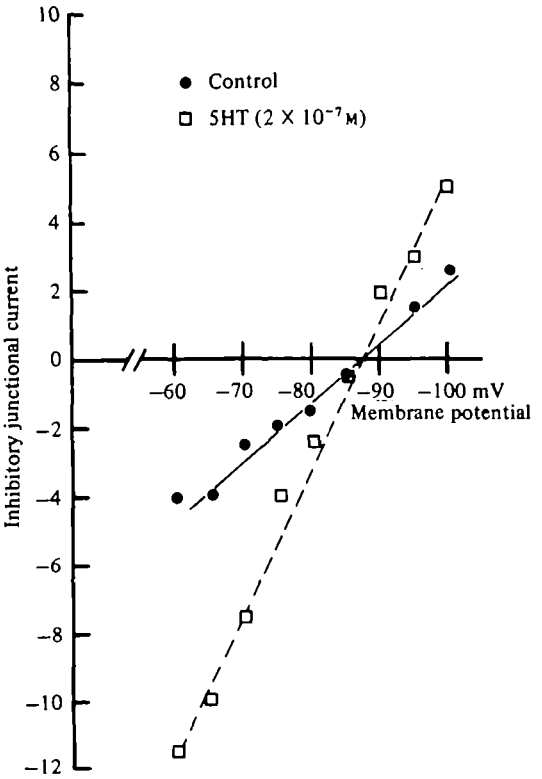


Fig. 4. Inhibitory junctional currents in opener muscle preparation with or without added serotonin. Muscle fibres (preparation set-up as in Fig. 3) were voltage clamped at different levels of membrane potential and the inhibitory currents generated in the muscle by stimulating the inhibitory nerve were monitored. The units are arbitrary measures of the size of the current; (−) = outward currents; (+) = inward currents. The chloride equilibrium potential in this experiment was at −87 mV and remained unchanged after serotonin treatment. The measurements of the currents with serotonin treatment were made 8–12 min after the start of serotonin superfusion.

Only two substances have been tested thus far: the peptide proctolin caused no change in size of the inhibitory response (Schwarz *et al.* 1980); serotonin caused a two- to threefold increase in size (Fig. 4). We have not yet determined whether this is a pre- or postsynaptic action of serotonin but it is interesting that serotonin increases the effectiveness of both excitatory and inhibitory synaptic transmission in these preparations. When the action of serotonin on muscle fibres (see below) is added to the actions on synaptic transmission, serotonin has a general activating action on the entire tissue preparation.

Actions of multiple substances on muscle fibres

The three amines, serotonin, octopamine and dopamine, and the peptide proctolin all act on muscle fibres directly. Serotonin, octopamine and proctolin all induce contractures while dopamine relaxes the muscles (Battelle & Kravitz, 1978; Schwarz *et al.* 1980). The dopamine effect has not been well studied and is not considered further here. The amine and peptide induced contractures share several features.

(1) The contractures are prolonged, far outlasting the time of application of the t substance. The most dramatic effects are seen with proctolin where contractures lasting longer than an hour are commonly seen.

(2) Low levels of test substances, as might be expected for circulating neurohormones, are required for contractures. The thresholds are proctolin-1×10^{-10} M, serotonin-5×10^{-9} M and octopamine-5×10^{-8} M.

(3) Contractures require extracellular Ca^{2+}. No contractures are seen in the absence of extracellular Ca^{2+} and removal of Ca^{2+} or the addition to the bathing medium of agents that block Ca^{2+} entry, like Mn^{2+} or Co^{2+}, reversibly inhibit contractures that have already been established (Fig. 5).

(4) Contractures are produced with little or no change in membrane potential or input resistance (R_I). With proctolin at concentrations up to 10^{-7} M no significant changes are seen in either parameter. Serotonin, on the other hand, occasionally produces 1–2 mV depolarizations and small (10–15 %) increases in R_I.

(5) The contractures are apparently voltage sensitive. Preliminary results suggest that hyperpolarizing the muscle membrane potential by 5–10 mV abolishes the contractures. Thus lowering or removing K^+ from the bathing medium or adding GABA (10^{-5} M) completely eliminates the contractures (Fig. 5). Interestingly if one stimulates the inhibitory nerve before amine or peptide treatment there is little or no effect on muscle tension, while at the peak of a contracture, inhibitory nerve stimulation causes short relaxations of the muscle analagous and opposite to excitatory nerve-evoked contractions (Fig. 5). We have no reason to suspect that the relaxations we observe are due to anything other than the hyperpolarization produced by released GABA acting on the muscles. This is an interesting result because it adds an additional significance to inhibition. In addition to decreasing excitation by causing pre- and postsynaptic conductance increases, inhibition, by producing a hyperpolarization, may reduce the size of a hormone-induced Ca^{2+} influx leading to a contracture.

These experiments, although very preliminary, raise the possibility that serotonin, octopamine and proctolin all act in a similar manner to activate a voltage-sensitive Ca^{2+} entry at the resting level of membrane potential and thereby induce a contracture with little or no change in membrane potential. That serotonin actually can cause changes in voltage sensitive Ca^{2+} channels is shown below.

By far the most dramatic effect seen with intracellular recording after amine or peptide treatment is that action potentials appear in muscle fibres. This is illustrated in Fig. 6 for a preparation treated with octopamine. Action potentials are also seen in fibres treated with serotonin and they have occasionally been seen in proctolin treated preparations. In the experiment shown the muscle does not return to the baseline condition (with no action potentials visible) for at least 90 min after octopamine has been removed from the bath. These muscle fibres have no voltage sensitive Na^+ channels and when action potentials are seen in lobster muscles they are mediated by Ca^{2+} entry through voltage sensitive channels. To explore this further, voltage clamp studies have been performed. A three-electrode voltage clamp assembly (Adrian, Chandler & Hodgkin, 1970) was constructed with the counsel and cooperation of Dr John Moore and Mr Edward Harris. When such a clamp is used in lobster muscle fibres and a depolarizing voltage step is imposed, a complex current is seen that involves both inward and outward components. By pharmacological manipulatio

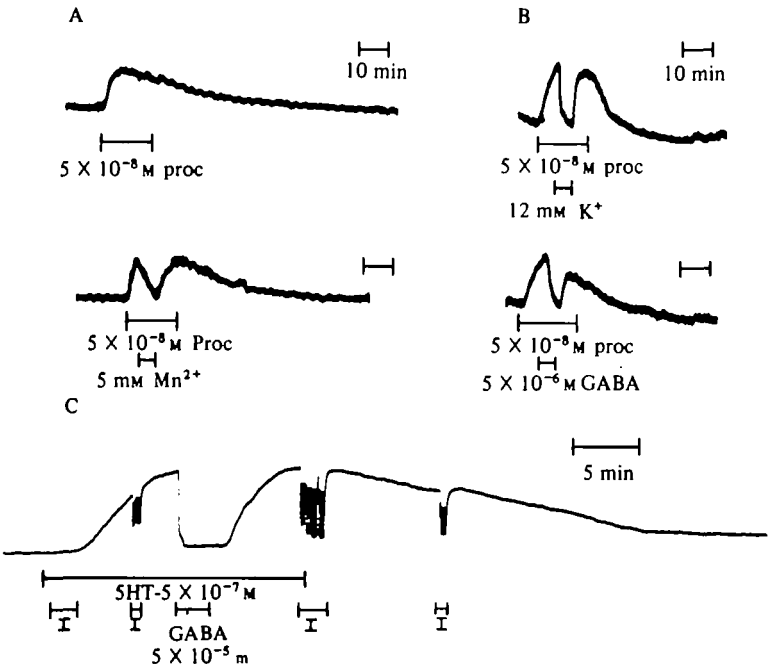


Fig. 5. The apparent voltage and Ca^{2+}-sensitivity of contractures generated by proctolin and serotonin. Preparations of the opener muscle were set-up as in Fig. 3 except that the muscle fibres and attachment of the distal end of the central tendon were cut and the tendon attached to a strain gauge for measurement of tension. The bars below the graphs indicate the duration of treatment with various substances. At I, brief trains of stimuli were delivered to the inhibitory nerve innervating the preparation (in this experiment 8 pulses at 30 Hz at the indicated times).

these can be resolved partially into the following components: (1) a voltage sensitive outward K^+ current that can be blocked by tetraethyl ammonium (TEA); (2) a voltage sensitive inward Ca^{2+} current that can be blocked by Co^{2+}. Sr^{2+} will pass through this channel and apparently not activate a slow outward current (no. 3 below) that follows the inward current. Using Sr^{2+} and TEA, it therefore seems possible to isolate this current; (3) a slow outward current dependent on the inward Ca^{2+} current. This is likely to be a Ca^{2+} activated K^+ current but it has not yet been studied separately; (4) a residual outward current that remains in the presence of 100 mM TEA and 30 mM Co^{2+}. This may only represent the portion of the K^+ current (no. 1) not blocked by TEA. In Fig. 7 current:voltage curves are graphed for the outward K^+ current (Fig. 7A) and for the inward Ca^{2+} current (Fig. 7B) in the presence and absence of serotonin. It can be seen that the Ca^{2+} current is significantly increased by serotonin treatment while no change is observed in the outward K^+ current.

Whether the results with the voltage clamp experiments are compatible with the contracture experiments will require further experimentation. One apparent discrepancy is that the major increase in Ca^{2+} conductance seen in the voltage clamp experiments does not occur until about 20 mV above the resting potential, yet we know that contractures are seen with little or no change in membrane potential. It may

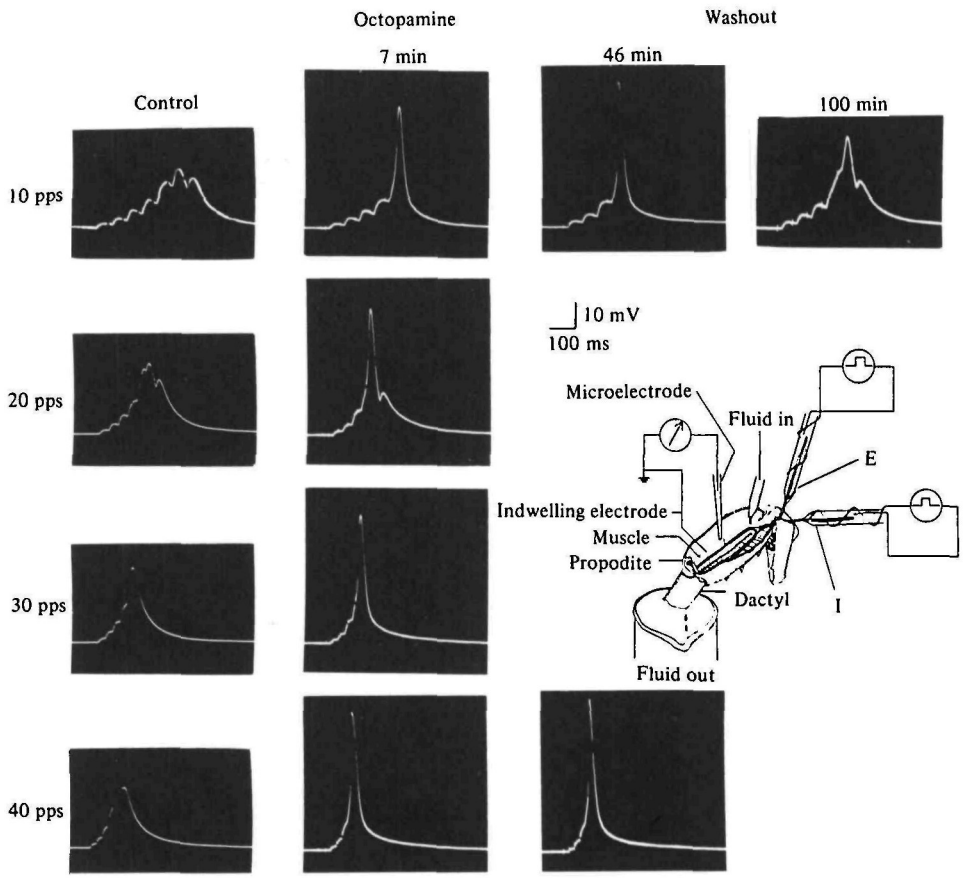


Fig. 6. Action potentials generated in muscle fibres after treatment with octopamine. Stimuli at different frequencies were delivered to the excitatory nerve innervating an opener muscle preparation (*left side of figure*). Several minutes after octopamine treatment (5×10^{-6}M) large action potentials are seen. After 100 min of washout in control saline, action potentials begin to disappear in this experiment. (Reprinted with permission from Kravitz *et al.* 1980.)

be that the contracture we observe can be accounted for by a very small entry of Ca^{2+} through a hormone induced change in a population of voltage sensitive channels that are ordinarily closed at rest. The failure to measure this change in our voltage clamp records may only represent the low sensitivity of the assay we are using.

In several reports in the literature actions of multiple hormones on Ca^{2+} channels have been noted. For example, in sensory neurones in culture the calcium component of the sensory neurone action potential is reduced by GABA, norepinephrine, serotonin and the pentapeptide enkephalin (Dunlap & Fischbach, 1978; Mudge, Leeman & Fischbach, 1979; Konishi, Tsumoo & Otsuka, 1979). In parotid gland slices and in isolated secretory cells from these glands, muscarinic, α-adrenergic and substance P receptors all seem to activate a single population of Ca^{2+} channels (Putney, 1977). The lobster muscles that we are studying may represent another tissue where multiple hormones funnel their actions through a common locus of action. The long duration of action of the amines and peptide may suggest that the hormo

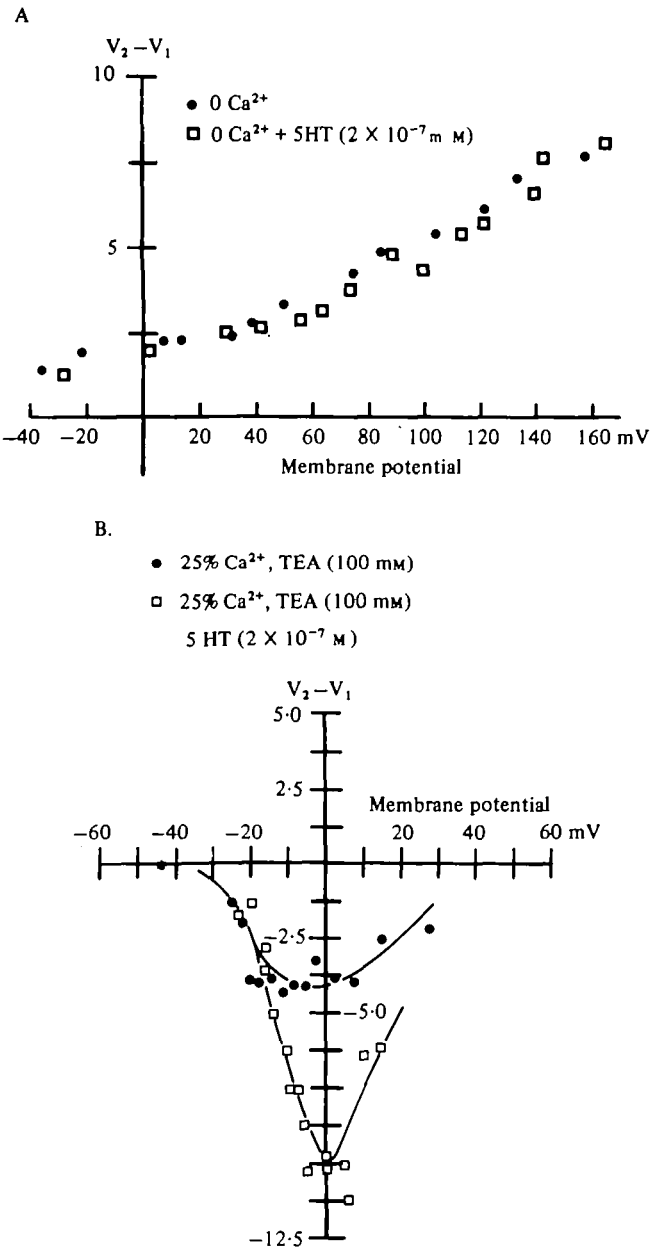


Fig. 7. Current-voltage relationships for the outward K^+ current (A) and inward Ca^{2+} current (B) in the presence and absence of serotonin. A three-electrode voltage clamp assembly was used to measure currents (Adrian *et al.* 1970). The outward K^+ current was isolated by leaving Ca^{2+} out of the bath while the inward Ca^{2+} current was measured in 100 mM TEA and in lowered (25 %) Ca^{2+}. Measurements with serotonin (5 HT) were made 8–12 min after serotonin was added to the bath. The voltage difference between two of the electrodes in the three electrode assembly ($V_2 - V_1$) is proportional to the current. The current can be calculated by equation (2) of Adrian *et al.* (1970)

$$i_m(l) = \frac{p^2(V_2 - V_1)}{3l^2 r_t}$$

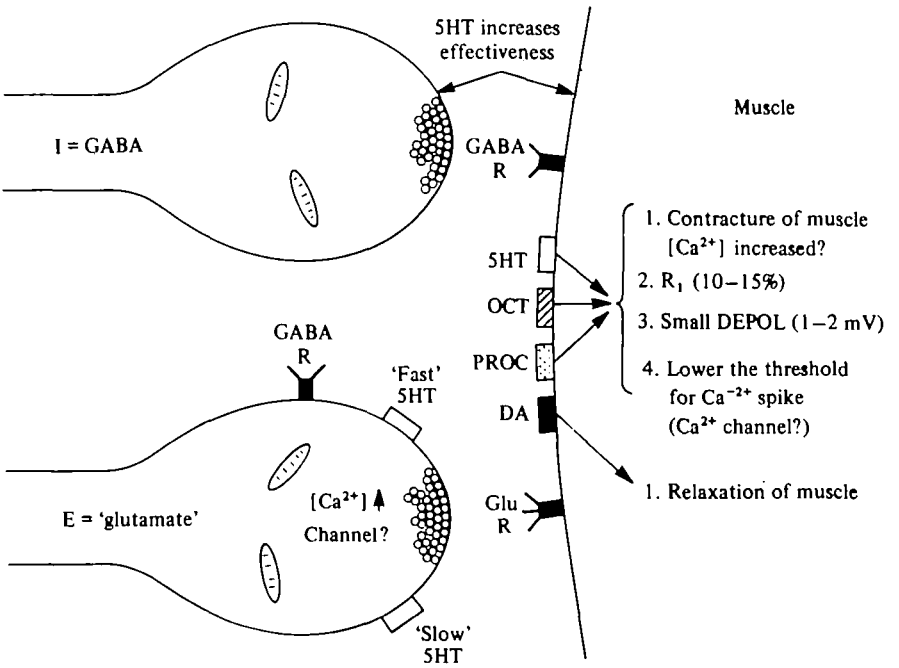


Fig. 8. Summary diagram of sites of action and effects of serotonin (5 HT), octopamine (OCT), dopamine (DA) and proctolin (PROC) on the lobster opener muscle neuromuscular preparation.

receptors are not directly linked to an ionic conductance channel. The finding that amines induce increases in the levels of cyclic AMP in these tissues (Battelle & Kravitz, 1978) may provide further support for this notion but additional experiments will be needed to convincingly demonstrate any relationship between cyclic nucleotides and the physiological changes we observe.

A summary diagram of the effects we have observed thus far with serotonin, octopamine, dopamine and proctolin on the lobster opener muscle preparation is shown in Fig. 8. It is clear that a complex set of hormonal controls exists superimposed on the normal synaptic machinery that functions in this preparation. How this hormonal control system relates to the behaviours of lobsters in which the neurohormones play a role remains a challenge for future exploration.

Behavioural experiments – serotonin and octopamine induce opposite postures in lobsters

We have begun a series of studies examining the role of amines in lobster behaviour. In these studies, amines are directly injected into lobster haemolymph and the effects on behaviour are noted. The injection of high levels (several mg) of octopamine or serotonin into lobsters induces a characteristic posture and the postures are opposite to each other (Livingstone *et al.* 1980*b*). Octopamine injection causes lobsters to assume a sustained, hyper-extended posture. The animals lie close to the substrate with their claws and walking legs pointed forward and elevated off the substrate and their abdomens arched upward. If animals are disturbed by tapping the side of th

Table 1. *Summary of the actions of serotonin and octopamine on the excitatory and inhibitory neurones innervating lobster abdominal slow flexor and extensor muscles*

	Octopamine	Serotonin
Extensor activity		
Excitatory	↑↑↑ (13/16)	+ or ↓↓↓ (3/6) (2/6)
Inhibitory	↓↓ or → (3/6) (2/6)	↑↑↑ (4/4)
Flexor activity		
Small excitatory (tonically active)	↓↓ (15/15)	↓ or → (9/14 (5/14)
Large excitatory (phasically active)	→ or ↑, no bursts (1/8) (7/8)	↑↑, bursts (9/11)
Inhibitory	↑↑↑ (13/13)	↓↓↓ (6/7)

Preparations were dissected that included central ganglia still attached to peripheral flexor or extensor muscles. Intracellular recordings were performed from individual muscle fibres to monitor excitatory and inhibitory synaptic activity. The nerve trunk innervating the muscles was drawn into a suction electrode for recording to allow correlation of the firing of specific units with the appearance of synaptic responses in the muscles. Preparations were superfused with saline carrying the amines at high ($10^{-6}-3 \times 10^{-5}$M) concentrations. ↑ = increase in firing; ↓ = decrease; → = no change. Individual muscle fibres did not receive synaptic input from all of the units identified in the roots.

tank they relax from the posture, then quickly resume it. Serotonin injection yields the opposite posture. Animals stand high on the tips of their walking legs with their claws open and slightly drawn back and their abdomens loosely tucked under them. The postures induced by both amines last for long periods of time (ranging from minutes to hours depending on dose).

We have begun to explore the mechanism of these postural changes at the cellular level with isolated tissue preparations. Examination of amine effects on peripheral flexor and extensor muscles did not suggest any possible explanation for the opposite postures induced by the two amines. We therefore turned our attention to the central ganglia and here we found clear differences that could account for the amine-induced postural differences. Using preparations that consisted of abdominal ganglia attached by nerve roots to flexor or extensor muscles we found that: (1) only slow (postural) flexor and extensor muscles were activated by amine treatment; (2) each amine induced an opposite pattern of activation of the excitatory and inhibitory neurones that innervated the slow flexor and extensor muscles (see Table 1). Thus octopamine caused excitatory neurones to extensors and the inhibitory neurone to flexors to dramatically increase their firing rates and the inhibitory neurone to extensor muscles and excitatory neurones to flexors to decrease or stop in firing (Table 1). The result of these changes in patterns of firing in response to octopamine is that postural extensor muscles contract and postural flexor muscles relax, thereby inducing bodily extension. Serotonin produced just the opposite pattern of alterations in the firing of neurones (Table 1, Livingstone *et al.* 1980*b*).

In more recent studies, we have attempted to carry this analysis one step farther by recording from identified neurones within central ganglia. In the experiment shown in Fig. 9 intracellular recordings were made from the cells I_1 – the slow flexor inhibi-

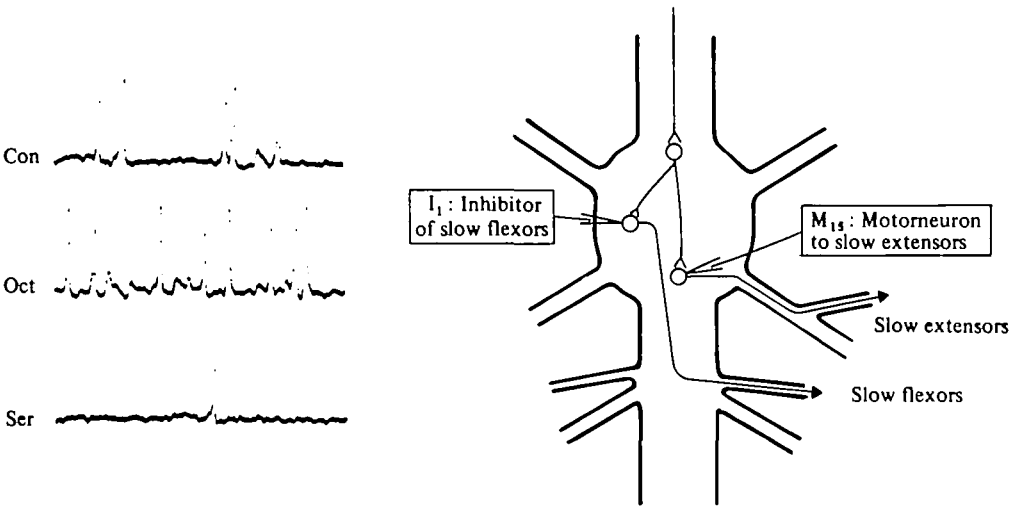


Fig. 9. Intracellular recordings from I_1, the common inhibitor to the slow flexors – effects of octopamine (oct) and serotonin (ser). An abdominal ganglion was isolated and the connective tissue surrounding the cell bodies was removed. Intracellular recordings were performed from the identified neurones M_{15} and I_1. Octopamine (3×10^{-6} M) is seen to dramatically increase action potentials and synaptic activity in I_1 (inset record) while serotonin (3×10^{-5} M) has just the opposite effect.

tory neurone and M_{15} – an excitatory neurone to slow extensor muscles. As might be expected both of these neurones show a similar pattern of excitation to octopamine and inhibition to serotonin. There is a highly significant increase in the frequency of spontaneously generated excitatory synaptic potentials recorded in these cells when preparations are superfused with octopamine and a decrease in this activity with serotonin treatment (inset, Fig. 9). Several lines of evidence indicate that this increase in frequency is not just an artifact of bringing more synaptic input out of the 'noise' level of the recordings by, for example, increasing the input resistance of the cell: (1) the input resistance and resting potential recorded in cell bodies are not changed by amine treatment; (2) a known synaptic input to the cell M_{15} (from abdominal stretch receptors) is not altered in size by amine treatment; (3) action potentials, which passively spread to cell bodies, are not altered in amplitude by amines. These results raise the possibility that at least a part of the action of amines is on cells that provide excitatory synaptic input to the motor neurones. In addition, however, there may be a direct action on the motor neurones. In lobster central ganglia, the motor neurones are generally not capable of generating an action potential in the soma, but an action potential can be triggered in the neuropil by passing current in the cell body. The threshold for generating this action potential remains relatively constant during an experiment. Amine treatment, however, changes this threshold such that motor neurones are activated more or less easily in parallel with an amine treatment that would increase or decrease their firing rate. This result could have several different explanations; recordings from the neuropil, closer to the action potential intiation site, will be necessary to explore the alternatives.

In intact animals and in the ganglia-muscle preparations high concentrations

amine ($10^{-6}-10^{-5}$ M) were required to observe changes in the pattern of neuronal firing. The concentrations are several orders of magnitude above the effective concentrations of amines on peripheral targets ($10^{-9}-10^{-8}$ M, see above) and it is not reasonable that such high concentrations of amine could ever be achieved in lobster haemolymph. One possible explanation for the high concentrations required for the central actions is that perfusion barriers exist in central ganglia limiting the access of amines to their sites of action in the neuropil. Another possibility is that it is not amine circulating in the haemolymph that is important in the central actions of amines, but amine released at specific synaptic contacts in central ganglia between amine-containing nerve terminals and central target neurones. At such contact points the released amine level could be high locally and could account for the observation that high levels of bath-applied amine are required for the physiological effects we observe.

The effects of octopamine and serotonin on central ganglia are similar to the effects observed by stimulating command neurones that modify posture in Crustacea (see Kennedy, 1976; Evoy & Kennedy, 1967). Command neurones are single fibres teased out of central nerve trunks whose activation leads to the generation of complex behavioural acts. The command neurones that modify posture are not thought to directly contact the effector motor neurones; rather an unspecified number of interneurones coordinate the motor output triggered by stimulation of the command fibre. Octopamine or serotonin could serve as neurotransmitters at some points in this circuitry. Another possibility is that the amines alter the release of transmitter at some parts of the circuitry into which the command neurones fit. The exploration of this circuitry has begun in this laboratory (see above) and these studies will hopefully lead to the discovery of the amine-sensitive elements.

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Note added in proof

This text, originally a symposium talk, was not intended as a comprehensive review of the role of amines as crustacean neurohormones. Accordingly many important contributions to this field were omitted. In this addendum we list some articles by other investigators that are clearly relevant to the work we have presented. Florey & Florey (*Z. Naturforsch.* (1954), **9b**, 58–69) first reported that serotonin enhanced contractions in crustacean muscles and Grundfest & Reuben (In *Nervous Inhibition*, Ed. E. Florey. Oxford. Pergamon Press, 1961, pp. 92–104) first published that serotonin increased the size of e.j.p.'s and caused spiking in muscle fibres. Maynard & Welsh (*J. Physiol.* (1959), **149**, 215–227) showed that serotonin was found in crab pericardial organs and Cooke & Goldstone (*J. Exp. Biol.* (1970) **53**, 651–668) and Goldstone & Cooke (*Z. Zellforsch.* (1971), **116**, 7–19), using fluorescent histochemical procedures, demonstrated the location in crab nervous systems of dopamine and serotonin containing cell bodies in central ganglia and of the terminal arborizations of the cells in

pericardial organs. Sullivan, Friend & Barker (*J. Neurobiol.* (1977), **8**, 581–605), showed the synthesis and release of octopamine and serotonin from the spiny lobster ligamental nerve plexuses (a morphological analogue of pericardial organs). These same authors noted the appearance of several morphologically distinguishable categories of nerve endings in these structures. Sullivan (*J. Exp. Biol.* (1979), **210**, 543–552) also reported on the presence of a proctolin-like peptide in crab pericardial organs. Florey & Rathmayer (*Com. Biochem. Physiol.* (1978). **61C**, 229–237), have reported actions of octopamine on crustacean heart and exoskeletal muscle preparations. In the latter tissues they report that octopamine has both pre- and postsynaptic actions. Finally a large literature (not covered here) exists on the role of octopamine as a neuromodulatory substance in insects and on the role of amines as neurohormones in other invertebrate species.

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