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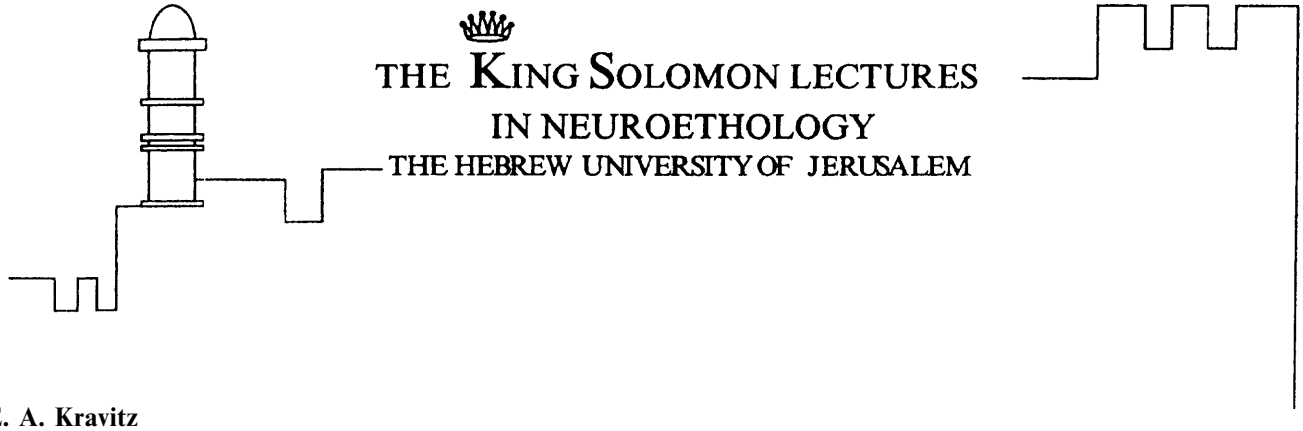
REVIEW



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Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior

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Abstract The amine serotonin has been suggested to play a key role in aggression in many species of animals, including man. Precisely how the amine functions, however, has remained a mystery. As with other important physiological questions, with their large uniquely identifiable neurons, invertebrate systems offer special advantages for the study of behavior. In this article we illustrate that principal with a description of our studies of the role of serotonin in aggression in a lobster model system. Aggression is a quantifiable behavior in crustaceans, the amine neuron systems believed to be important in that behavior have been completely mapped, and key physiological properties of an important subset of these neurons have been defined. These results are summarized here, including descriptions of the "gain-setter" role and "autoinhibition" shown by these neurons. Results of other investigations showing socially modulated changes in amine responsiveness at particular synaptic sites also are described. In addition, speculations are offered about how important developmental roles served by amines like serotonin, which have been well described by other investigators, may be related to the behaviors we are examining. These speculations draw heavily from the organizational/activational roles proposed for steroid hormones by Phoenix et al. (1959).

Key words Amine neurons · Aggression · Lobster · Neurohormone · Serotonin

Abbreviations *5,7-DHT* 5,7 dihydroxytryptamine · *5HT* serotonin · *A1* first abdominal ganglion · *CHH* crustacean hyperglycemic hormone · *CNS* central nervous system · *EPSPs* excitatory post synaptic potentials · *IPSPs* inhibitory post-synaptic potentials · *LG* lateral giant axon · *MG* medial giant axon · *OCT* octopamine · *T5* fifth thoracic ganglion

Introduction

While observing fighting behavior among behaviorally naïve juvenile lobsters, one cannot help but be struck by the elegance of the unfolding scene. When placed in a new environment, animals pause, then begin to explore the arena, generally keeping close to the walls, which they continually circle. Invariably they meet another animal, and just as invariably, display their principal

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weapons, the large claws. Mirroring moves, they remain motionless, claws up, standing high on the tips of their walking legs, or they bump, darting to and fro, fore and aft, maintaining the display. The dactyls, the movable fingers of the claws, open wide but do not close to grasp the opponent. Meetings are short, lasting about 30 s, during which, in addition to the display, animals direct streams of urine at each other from the nephropores at the base of their 2nd antennae; then they break off, only to begin exploring again. Few or many meetings may take place, during each of which the display is repeated. If a large size asymmetry exists, the fight ends usually with the smaller animal retreating then refusing to engage the larger in combat. A second component of display may be interposed with the posturing. Here is a ballet, a pas de deux on the ocean floor, in which one animal advances, antennae whipping and claws folded downward, while the other animal retreats, antennae straight up and claws up and open. Then on some unknown cue, the animals completely switch their directions of movement and the use of their appendages. If no decision is reached with either of the displays, one of the animals escalates to the next level of intensity, a move immediately paralleled by the opponent. The transition is stepwise, seamless and irreversible. Now the weapons, the claws, start to be used, but only to grasp the opponent. Like Greco-Roman wrestlers in a giant underwater arena, each combatant tries to overturn the other. If one succeeds, then here too, a decision is made by the retreat of the loser. If not, fights move to the next, highest level of intensity, a move almost always leading to a decision. Moving with great speed now, animals advance on each other with claws wide open. Their giant claws snap shut on whatever they can reach, then tail flips, contractions of the large abdominal flexor muscles, move animals back and upwards in attempts to tear their catch from the opponent. The danger of damage is high now, but we seldom see animals losing appendages. Those that do, however, usually will not survive the continued presence of the winner. Losers stop urinating, continually retreat, and tail flip to escape the advance of the winner. The ''memory'' of losing persists for many days, altering the willingness of animals to engage others in combat. In the wild, fights tend to be short in duration, with decisions made early. Mostly these are decided by the size difference that exists when two random animals meet. Under these circumstances, the making of decisions may not have significant impact on the subsequent willingness to fight, but this remains to be established (references for this paragraph: Scrivener 1971; Atema and Cobb 1980; Huber and Kravitz 1995; Karavanich and Atema 1998a, b; Breithaupt et al. 1999; Rutishauser et al. 1999).

One remarkable feature about fighting behavior in lobsters is that the entire elaborate ritual, involving stereotypical stepwise increases in intensity, and appropriate responses of animals to each other, all appears to be pre-wired in the lobster nervous system. We know this because the entire repertoire exists in socially naïve animals that have been raised in complete isolation from the 4th stage onwards. There is no indication that this has to be learned. That is not to say that it will not be modified by experience: indeed, we already know this to be the case (Scrivener 1971; Karavanich and Atema 1998a; Rutishauser et al. 1999). Another remarkable feature is the long-term nature of the behavioral consequences of winning and losing fights. Winning animals are more likely to win their next fight (Scrivener 1971), while losers are more likely to lose again. In fact, losing animals will not fight with any other animals, winners or losers of other fights, for some time after their initial fight. Thus, after an initial, approximately 10 min of actual fighting (in an average 30-min fight), lobsters appear to ''remember'' the outcome for days. Challenges for investigators attempting to understand complex behaviors like aggression, are: (1) to understand how context dependent, stereotypical, sequential pathways of events, like the intensity-linked different levels of fighting behavior, are assembled in the nervous system; and (2) to find out where and how these systems change to allow experience to alter the subsequent fighting behavior.

As with all aspects of animal behavior, hormones and neurohormones likely serve essential roles in modulating aggression. We firmly believe that amines like serotonin (5HT) are important in aggression in crustaceans. However, amines are not the only, and may not even be the most important, hormonal substances influencing aggression in these animals. In essentially all species of animals, including man, 5HT is important in aggression (cf. Coccaro 1989; Raleigh et al. 1991; Miczek et al. 1994; Olivier et al. 1995; Edwards and Kravitz 1997) but evidence implicates peptides like gonadotropin-releasing hormone and arginine vasopressin (Francis et al. 1993; Ferris et al. 1986, 1997), and steroids like testosterone (Rubinow and Schmidt 1996) in this behavior as well. Recent studies have demonstrated close interactions between amines and peptides and the neurons involved with these substances and circulating steroids (Asmus and Newman 1993; Bonson et al. 1994; Mani et al. 1994; Delville et al. 1996; Ferris et al. 1997). These probably foreshadow the ultimate unraveling of complex systems of interdigitating humoral substances, all of which contribute to optimal fighting performance. We focus only on 5HT here, because with the exception of octopamine (OCT), we have yet to positively identify other candidate hormones important in agonistic behavior in crustaceans. We anticipate, however, that several such substances exist. For example, the steroid hormone ecdysone, the lobster molting hormone, is one candidate, since lobster fighting and escape behaviors change dramatically over the molt cycle (Tamm and Cobb 1978; Cromarty et al. 1991). Levels of 20-hydroxyecdysone, the active form of ecdysone, peak just before animals show their highest levels of aggressiveness (Snyder and Chang 1991). The peptide crustacean hyperglycemic hormone (CHH), a putative lobster stress hormone, is a second (Chang et al. 1999a). CHH-like peptides have been localized very recently to a group of neurosecretory neurons whose activity is strongly influenced by both

5HT and OCT (Chang et al. 1999b). Studies presently underway in our laboratory are exploring possible linkages between the different hormonal systems, but the results are too preliminary to allow their inclusion in this article. The focus here, therefore, will be entirely on 5HT. Despite the narrow focus, we believe that useful generalizations and speculations can be made, *that should be testable*, and therefore worthy of perusal.

The enigma of amines lies in their ubiquity. They are important in behavior, everyone agrees, but they are selective in the behaviors they influence, and in the sign and magnitude of their influence. 5HT, for example, has been suggested to serve important roles in memory formation (cf. Dale et al. 1987; Kandel et al. 1987), in pain perception (cf. Leung and Mason 1999), in feeding behavior (cf. Rosen et al. 1983; Schachtner and Bräunig 1993; Yeoman et al. 1994), in affective conditions like depression (cf. Stockmeier et al. 1998), in aggression (cf. Coccaro 1989; Raleigh et al. 1991; Miczek et al. 1994; Olivier et al. 1995; Edwards and Kravitz 1997), and in any number of other important aspects of animal and human behavior. Despite its obvious importance, in all species, the numbers of 5HT neurons are small compared to the total number of neurons in the nervous system (only a fraction of a percent), while their sphere of influence is large, with virtually all areas of the central nervous system receiving neuronal processes from these few cells. Often, in target area, dendrites and dendritic spines closely apposed to 5HT-containing endings show none of the specialization typical of normal synaptic contacts (for reviews see Beaudet and Descarries 1978; Descarries et al. 1990). Thus, the concept of a local hormone action of amines like 5HT has emerged (see Beaudet and Descarries 1978; Descarries et al. 1990), in which specificity is imparted by the selective distribution of amine receptors on subsets of cells in the vicinity of the amine endings (Aghajanian et al. 1990). In some species, like crustaceans (see below), amines also are released from neurosecretory neurons into the general circulation where they may function as hormones, akin to the amines, steroids and peptide hormones released from specialized vertebrate endocrine tissues or from brain neuroendocrine regions. Possibly the greatest mystery of all, is how amines and amine neurons influence the organization and/or activation of the complex patterns of behavior with which they are involved.

This communication will focus on serotonergic neurons and agonistic (fighting) behavior in lobsters, and will attempt to formulate a speculative synthesis of how these might be related.

Why a lobster model of aggression?

Amine neuron systems have been mapped and well studied in a wide variety of invertebrate species (cf. Schürmann and Klemm 1984; for reviews see Nässel 1988; Callaway and Stuart 1999; Hörner 1999). Many of those species show aggression, and in some, for example *Drosophila* (Hoffmann 1987; Dow and von Schilcher 1975), powerful molecular and genetic methods in principle allow the analysis to be brought to the level of the genes important for the behavior (Neckameyer and White 1992; Monasterioti et al. 1996). Our focus remains on crustacean systems, however, because these animals are particularly good for studies at many different levels of analysis. First, lobsters and crayfish are highly aggressive, form long-term stable dominance relationships, and the behavior has proven amenable to quantitative analysis (Huber and Kravitz 1995; see also below). Second, detailed physiological studies have been carried out in the isolated central and peripheral nervous systems of these animals. Many of the neurons found in the nervous system are large and uniquely identifiable from preparation to preparation (Otsuka et al. 1967). This allows extensive examination of the roles served by particular neurons throughout the lifetime of these animals, and in animals of differing behavioral status. Moreover, the functions of such cells have been examined at both cellular and systems levels. Finally, in recent years, genes relevant to the physiological functioning of lobster neurons have been cloned (cf. Baro et al. 1996a; McClintock et al. 1997; Xu et al. 1997) and methods have been elaborated that allow their quantitative measurement in single identified cells (Baro et al. 1994, 1996b; Schneider et al. 1999). While methods of mutational analysis are not practical in these animals because of their long generation time, the use of molecular methods to search for changes in the levels of expression of genes relating to social status and experience now are possible. A future goal is to develop methods of manipulating the levels of expression of particular genes in selected neurons at precise times during the lives of these animals.

Localization and identification of serotonergic neurons

Amines in the lobster nervous system

Serotonin is the major amine derived from tryptophan in lobsters. OCT is the main amine formed from tyrosine, but small amounts of dopamine also are found in the lobster nervous system. Thus, enzyme systems capable of hydroxylating both the phenol ring of tyrosine (to dihydroxyphenylalanine and then by decarboxylation to dopamine) and the side chain of phenylethylamines (tyramine to OCT) exist in lobster nervous systems, but not in the same neurons, as no norepinephrine has been found. Specific sets of neurons containing OCT (Schneider et al. 1993) and dopamine (Cournil et al. 1994) have been mapped in the lobster nervous system, but less is known about their function. 5HT and OCT appear to have opposite actions in postural regulation (Livingstone et al. 1980; Harris Warrick and Kravitz 1984), and thereby may serve opposite functional roles in aggression, but studies with OCT have lagged until very recently (R. Heinrich et al., unpublished observations), and for the most part, will not be considered here.

Localization

Lobster 5HT neurons originally were localized within the ventral nerve cord of the lobster central nervous system, (CNS) using immunocytochemical methods (Beltz and Kravitz 1983). The results showed that there were approximately 120 5HT neurons, widely distributed among the ganglia of the nerve cord. Recently, additional subsets of neurons have been found that show immunostaining for 5HT, but only after incubation of tissues in low concentrations of the amine. It is not certain, however, whether these neurons also contain the synthetic enzyme, tryptophan hydroxylase (Beltz et al. 1998; Musolf and Edwards 1999). Such neurons, which likely contain high levels of a 5HT transporter, are capable of release of the amine (Beltz et al. 1998; Musolf and Edwards 1999). Therefore, despite the possible absence of the key synthetic enzyme, these neurons may be utilizing 5HT as a neurotransmitter or neurohormone. It is not clear how such cells should be categorized. For the original endogenous 5HT-immunostaining cells, however, it was easy to demonstrate that they were organized into distinct subgroups on morphological grounds. The groups included short and long-distance projecting interneurons, local projection interneurons, and neurosecretory neurons. The most detailed physiological studies have been carried out with the neurosecretory neurons, but at least one key supraesophageal (brain) interneuron, the deutocerebral giant cell also has received considerable attention, at least in crayfish (Sandeman and Sandeman 1987, 1994, 1995).

Identification of amine neurons

Strong evidence that a neuron is serotonergic involves first developing a method to routinely find the same cell in different animals, and then to show by biochemical techniques that the cell actually contains the amine, and if possible, also contains the synthetic enzyme tryptophan hydroxylase. In lobsters, this has been done for the neurosecretory subset of serotonergic neurons, which are the focus of this review (Beltz and Kravitz 1987). The technique used was first to carry out immunocytochemical studies to show the approximate location within ganglia of neuronal somata staining for amines (Beltz and Kravitz 1983). With the antibodies used, the arbors of neurons also stained well, allowing tracing of axons into nerve bundles and tracts within the ventral cord. With the immunocytochemical studies as a guide, neurons were identified by stimulating nerve bundles containing the axons of the cells in question, while recording with intracellular electrodes from cell somata in the vicinity of the candidate immunostained cell. Cell bodies that showed action potentials when activated in this way were filled with electron dense or fluorescent dye markers through the intracellular electrode. Thereafter, tissues were processed to allow visualization both of the injected dye and the amine-associated immunoreactivity. Once candidate neurons were routinely identifiable in this way, their physiological properties were explored. Such studies ultimately allowed identification of the neurons using physiological criteria alone (see below). To confirm that the immunostained cells actually contained 5HT, the somata were microdissected from nerve cords to measure their amine and amine biosynthetic enzyme contents, and thereby to confirm their identity. Using this method we have shown that the average 5HT concentration in neurosecretory neuron cell bodies is 0.4–0.6 mmol l^{-1} (Siwicki et al. 1987). 5HT-neurosecretory neurons also contain the peptide proctolin at approximately 20 μmol l^{-1} (Siwicki et al. 1987), but the amine and peptide phenotypes of these cells first appear at widely different times in development (Beltz and Kravitz 1987).

Morphological and physiological properties of 5HT-containing neurosecretory neurons

Morphology

The above studies identified two pairs of large 5HT-containing neurosecretory neurons: one pair found in the 5th thoracic (T5-5HT cells) and a second in the 1st abdominal (A1-5HT cells) ganglia. The T5- and A1-5HT cells are important in postural and escape neuronal circuitries (see below), and serve as the principal and possibly the only source of 5HT reaching the circulation. Peripheral targets of amines, like muscles (Glusman and Kravitz 1982; Dixon and Atwood 1989; Goy and Kravitz 1989) and sensory neurons (Pasztor and Bush 1989), have no serotonergic innervation, and are supplied exclusively via the circulation. Once a reliable method existed for finding these neurons, intracellular injections of horseradish peroxidase, lucifer yellow, or cobalt chloride allowed mapping of their morphology (Beltz and Kravitz 1987).

The serotonin-containing neurosecretory neurons are suprasegmentally organized and both pairs show similar morphological features. The general plan is as follows. A single process emerges from the cell body, which gives off an elaborate arbor of branches and endings in the neuropil region of the hemiganglion containing the soma. One branch, the ''axon'', travels in a rostral direction in an anterior lateral nerve bundle for one segment (to the next ganglion), turns medially, and then ascends the nerve cord through all rostral thoracic ganglia to ultimately end in the subesophageal ganglion. In the neuropil of each anterior ganglion, this branch gives off a main process that divides: one branch leaves the neuropil via the 2nd nerve root to terminate in one or more extensive fields of neurosecretory endings along the root; the second branch yields an arbor of endings in the neuropil of the ganglion (Fig. 1). Amines reach peripheral targets like exoskeletal muscles and sensory neurons by release into the hemolymph from the endings along

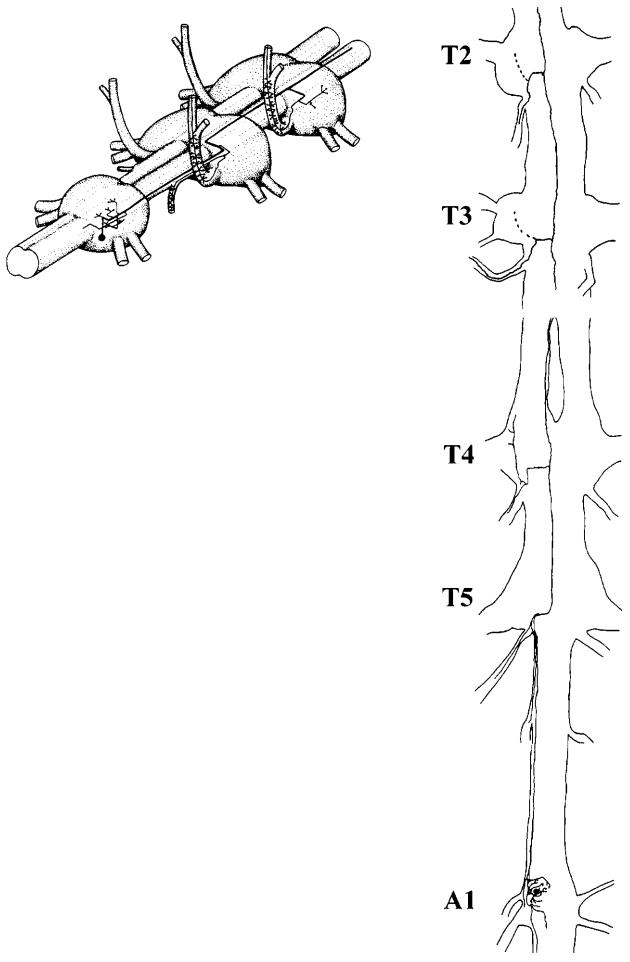

Fig. 1 Reconstruction of 1st abdominal serotonin-containing (A1-5HT) cell from intracellular injection of the enzyme horseradish peroxidase. After physiological identification of the A1-5HT cell, it was injected with the enzyme horseradish peroxidase which was allowed to diffuse for 12–72 h (details in Beltz and Kravitz 1987). Tissues then were fixed and a reaction product was generated using diaminobenzidene as substrate. The cell morphology was traced using a computer reconstruction system. The drawing is a composite of two separate injections, one of which showed better morphology in ganglia A1 through T4, the other of which showed better morphology in T2 and T3. The inset diagram (*left side of figure*) is an artist's reconstruction of a typical A1-5HT cell. The A1-5HT neurons have two sets of ending in every anterior ganglion through the subesophageal: one is in the central neuropil regions of the ganglion, the other is along the second thoracic roots in peripheral neurosecretory regions. See text for further details. This figure is slightly modified from Fig. 3A and Fig. 5 of Beltz and Kravitz (1987)

the 2nd roots, while the endings within the neuropil of the ganglion appear to influence the central circuitries concerned with motor programs (see below).

Intrinsic physiological properties

Of the two pairs of 5HT-containing neurosecretory neurons, the best studied are the A1 pair (A1-5HT cells). Although occasionally silent, these cells usually are spontaneously active in the range 0.5–3 Hz. Recordings from cell bodies show large overshooting action potentials with prominent after hyperpolarizations, both of which are typical of invertebrate neurosecretory neurons (Beltz and Kravitz 1987; Ma et al. 1992). Spontaneous firing continues in the absence of calcium or with cobalt added to the bathing medium, indicating that the action potentials are not synaptically driven, although their size and shape are altered under these conditions. In the presence of 100 nmol l^{-1} tetrodotoxin, action potentials are completely abolished leaving no residual oscillations, suggesting that a sodium current underlies the spontaneous activity (Cromarty et al. 1999). The after-hyperpolarization is reduced or eliminated by superfusion of preparations with tetraethyl ammonium chloride (0.5–2 mmol l^{-1}), 4-aminopyridine (100 nmol l^{-1}) or charybdotoxin (10 nmol l^{-1}) (Cromarty et al. 1999). These and other pharmacological studies suggest that calcium-activated BK channels are important contributors to the after-hyperpolarization. Molecular studies demonstrate that all isoforms of the *shab* form of the shaker family of potassium channels are missing in the A1-5HT cells, or are present at levels below the limit of detection of our methods, but are present in all other neuron types examined so far (Schneider et al. 1999).

Autoinhibition

A particularly interesting property of the A1-5HT neurons is that they show a pause in their firing after a period of high-frequency activation triggered by injection of current through an intracellular recording electrode (Fig. 2) (Heinrich et al. 1999). We call this pause "autoinhibition" and it resembles the "postactivation inhibition" seen in vertebrate serotonergic neurons from the midline raphe nuclei (Aghajanian and VanderMaelen 1982). The prevailing explanation of the autoinhibition in vertebrate neurons is that it is due to released 5HT acting back on $5HT_{1a}$ receptors located on the somata, axons and dendrites of the amine neurons (for review see Aghajanian et al. 1990). Although the autoinhibition seen in lobster neurons resembles that seen in the vertebrate cells, the mechanism is different in that it appears to be an intrinsic property of the cells. We believe this to be the case because: (1) we still see autoinhibition in saline with no added calcium or with cobalt added, when we see no remaining synaptic activity; and (2) we still see the inhibition in nerve cords from animals depleted of 5HT through use of the drug 5,7 dihydroxytryptamine (5,7-DHT). The duration of the autoinhibition is directly related to the magnitude and duration of the period of high frequency stimulation, but is *inversely* related to the initial firing rate of cells over their normal range of firing (0.5–3 Hz). If the tonic release of 5HT from cells is a key part of how amine neurons work (see below), such a mechanism could serve to maintain uninterrupted elevated levels of 5HT in target areas when cells are firing at the higher rates.

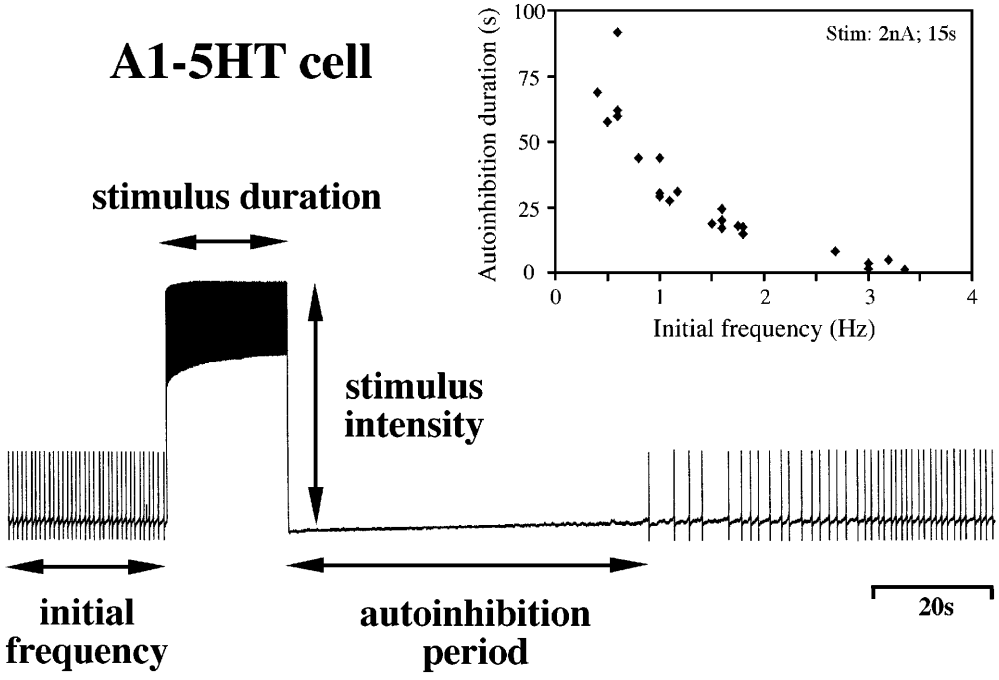

Fig. 2 Autoinhibition of A1-5HT neurons. An intracellular recording from a spontaneously active 5HT neuron is shown on the *lower part* of the figure. With high frequency firing of the cell through the intracellular electrode there is a pause in the firing of the cell (autoinhibition). The duration of the autoinhibition period is directly related to the magnitude of the stimulation, but is inversely related to the initial spontaneous firing rate (*inset diagram*). See text for details. The inset diagram is reprinted from Fig. 4 of Heinrich et al. (1999)

Synaptic inhibition and excitation

Pharmacological responsiveness

The rate of spontaneous firing of A1-5HT neurons is reduced by bath application of either octopamine or γ-aminobutyric acid (GABA), and is increased initially, followed by a prolonged reduction, after bath application of 5HT (Ma and Weiger 1993; Heinrich et al. 1999). Proctolin, which co-localizes with 5HT in these neurons, increases the firing of the cells. Thus two modulators (5HT and proctolin), which are co-localized in the same cell (Siwicki et al. 1987), have predominantly opposing physiological effects. Since the proportions of amine and peptide released at different frequencies of stimulation should vary (with more peptide released at the higher frequencies of firing), the physiological consequences of stimulating the A1 neurons also should vary, depending on the firing frequency.

Inhibition

The 5HT-containing neurosecretory neurons receive a constant barrage of spontaneous inhibitory input at a frequency of around 3–5 Hz (Ma et al. 1992; Weiger and Ma 1993). The inhibitory post-synaptic potentials (IPSPs) are synchronized among the T5 and A1 ganglion 5HT cell pairs, and fall into three distinct size categories. The most common of these are small (0.4–1.5 mV) and originate from putative GABAergic neurons in the 3rd abdominal ganglion. Inhibition arising from this source is blocked by picrotoxin and eliminated by cutting or blocking the connectives between the 2nd and 3rd abdominal ganglia. While this suggests that spontaneously active GABAergic neurons in the A3 ganglion are the source of the IPSPs, picrotoxin is not a completely selective blocker of GABAergic input in crustaceans (Marder and Paupardin-Tritsch 1978, 1980; Lingle and Marder 1981). Upon eliminating this input to the A1 cells, the spontaneous firing rates of 5HT cells are increased by about 50%, suggesting that these cells are under constant inhibitory regulation (Weiger and Ma 1993). Large, slow IPSPs can be triggered in the A1 cells with connective stimulation (Hörner et al. 1997; Heinrich et al., submitted). These seem to arise from fibers that traverse the entire length of the lobster ventral nerve cord. A particularly interesting aspect of these slow inhibitory responses is that they are simultaneous with the appearance of EPSPs in the octopamine-containing neurosecretory neurons (R. Heinrich et al., unpublished observations). Whatever the source of this input, it may be an important part of the machinery involved in governing opposing actions of the two amines. Moreover, unilateral stimulation of either anterior or posterior connectives, leads to the appearance of bilateral inhibitory synaptic responses in A1-5HT neurons. The slow IPSPs are abolished after high frequency firing of the A1-5HT cells, under conditions that produce autoinhibition in these cells, and recover back to their original size over the next several minutes. Fast inhibitory synaptic responses do not appear to be blocked by the high frequency pre-firing.

Excitation

Spontaneous excitatory post-synaptic potentials (EPSPs) also are seen in recordings from the A1-5HT neurons, but these are best seen after blocking IPSPs

through the use of picrotoxin. A complex pattern of nerve evoked excitatory synaptic responses is evoked by connective stimulation. Included in the pool of axons that trigger the excitatory responses in A1 cells are the rapidly conducting lateral (LG) and medial (MG) giant axons (Hörner et al. 1997). As in crayfish, the LG and MG axons are believed to be used in escape, and possibly in fighting behavior in lobsters. They are command interneurons, that in crayfish act through a segmental giant interneuron to trigger the readout of escape motor programs from the ventral nerve cord (Roberts et al. 1982). The LG and MG axons generate long, slow EPSPs in A1-5HT cells (durations of up to several hundred milliseconds) that usually trigger action potentials. The latter often arise from a plateau in the synaptic response.

System properties of serotonergic neurons

Gain setter role in postural regulation

The experiments that began our explorations of the roles of amines in aggression in lobsters (Livingstone et al. 1980), showed that injections of 5HT and OCT into living animals triggered the appearance of opposing static postures that resembled those seen in dominant and subordinate animals. Serotonin injections triggered the appearance of tall-standing dominant-looking animals, in which the postural flexor muscles were contracted and the extensors were relaxed, while octopamine injections resulted in low to the substrate subordinate-looking animals showing the opposite profile of muscular responses. Studies with the isolated nervous system and bath applied amines yielded similar results: the two amines activated opposing motor programs. These studies were the origin of our suggestions that dominance status might be associated with enhanced serotonergic neuron function in lobsters, while subordinate status might result from or might lead to enhanced octopaminergic neuron function.

To test these suggestions we felt it first necessary to find candidate amine neurons in the lobster CNS, then define their physiological properties, and finally ask whether the properties of these cells or their targets changed with changes in social status. The studies ultimately focused on the T5- and A1-5HT neurosecretory neurons for two reasons: (1) the cells appeared to be the major source of 5HT circulating in the hemolymph, and the only route to peripheral tissues responsive to amines like the exoskeletal muscles and sensory neurons (Beltz and Kravitz 1987; Glusman and Kravitz 1982; Goy and Kravitz 1989; Pasztor and Bush 1989); and (2) since the cells were the source of 5HT acting on muscles, but also had sets of central endings, we felt that they might be important in postural regulation as well, which was the initial observed effect of amine injection. In particular, we were concerned with whether the firing of these serotonergic neurosecretory cells produced the same effect as injected or bath applied 5HT. In other words, when activated, did these cells trigger the appearance of a dominant-looking stance in lobsters? We attempted to address these questions in isolated tissue preparations by firing the A1- and T5-5HT neurosecretory neurons using intracellular electrodes while recording from the nerve trunks innervating the postural flexor and extensor muscles in order to monitor the patterns of firing of the excitatory and inhibitory motoneurons innervating the muscles.

The result we obtained, however, was initially disappointing. We found that increasing or decreasing the rates of firing of single A1- or T5-5HT neurons neither increased nor decreased the rates of firing of motoneurons. With further study, however, the roles of these neurons in postural control pathways turned out to be much more interesting than simply serving to turn on or off particular motoneurons. This was shown in experiments in which flexor and extensor command neurons were activated. Such neurons are identified by teasing fibers out of connectives of the ventral nerve cord, stimulating them at high frequency and triggering the readout of motor programs from the CNS. Flexor commands increase the rates of firing of excitatory neurons to flexors and inhibitory neurons to extensors, while simultaneously decreasing the firing of excitatory neurons to extensors and inhibitory neurons to flexors. The net result of firing a flexor command, therefore, is to cause animals to go into a flexed posture, which makes them stand tall (in intact animals), just as dominant animals do in approaching a subordinate. Extensor commands do just the opposite, triggering postures resembling those seen in subordinate animals.

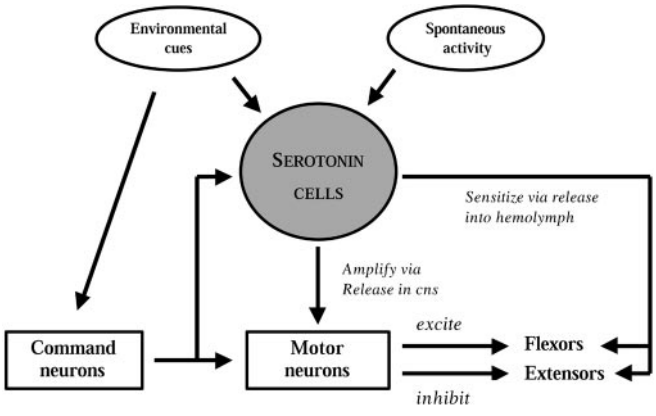

Fig. 3 The gain-setter role of A1-5HT neurons. Flexor command neurons excite tonic flexor muscles and inhibit tonic extensors through activation of central motor programs. The same command neurons increase the rate of firing of A1-5HT cells, which enhances the output of the command through release of 5HT within the central nervous system (CNS) and increases the strength of contraction of muscle fibers through release of 5HT into the general circulation (see text). Thus these spontaneously active neurons act as "feed forward" amplifiers. Further details are presented in the text and see Ma et al. (1992)

By simultaneously isolating flexor command neurons for stimulation from abdominal connectives, and recording from A1- and T5-5HT cells with intracellular electrodes, and nerve roots with extracellular electrodes, it was possible to define the role of 5HT neurosecretory neurons in postural control circuitries (Beltz and Kravitz 1987; Ma et al. 1992). Flexor commands tended to excite the 5HT neurons, which in turn enhanced the output of the command. This was demonstrated by either allowing the 5HT cell to fire upon command activation, or preventing the cell from firing by passing current through the intracellular electrode. Since the 5HT neurosecretory neurons have peripheral and central sets of ending, these neurons not only enhance the command output via central actions, they also enhance motor effectiveness through their pre- and post-synaptic actions on neuromuscular preparations (Glusman and Kravitz 1982; Dixon and Atwood 1989; Goy and Kravitz 1989). This is diagrammed in Fig. 3, which illustrates what we have termed the "gain-setter" role of these neurons. They in essence act as "feed-forward" amplifiers. If instead of exciting a flexor command neuron, we fire an extensor command (these elicit opposite postures to the flexor commands), then the 5HT cells are inhibited. However, if we force the 5HT cells to fire by depolarizing the cell through the intracellular electrode while activating an extensor command, then the 5HT cells *enhanced* the output of the extensor circuitry as well. Thus, the cells are "general" gain-setters, capable of enhancing *flexor or extensor* motor output, but the circuitry determines that they are used only to enhance the appropriate behavioral responses.

Changes in amine neuron function with changes in social status

Sensory input to LG neurons

The first clear demonstration of an important effect of social status on synaptic responsiveness to amines comes from elegant experiments from the Edwards laboratory (Yeh et al. 1996, 1997) in which they examined the actions of 5HT on the modulation of synaptic transmission between mechanosensory afferents of the tailfan and the LG neuron in crayfish. In these animals, activation of mechanosensory afferents leads to a complex pattern of synaptic activity in LG neurons. The activity can be broken into a series of components (α, β, γ) depending on whether the activation is monosynaptic and direct, or is through interneurons. In all cases, the input to the LG neuron is mainly through rectifying electrical synaptic contacts. Amines were found to modulate this synaptic input some time ago (Glanzman and Krasne 1983), but the modulation was only recently found to be dependent on the social status of the animals (Yeh et al. 1997). Crayfish were divided into three groups for these studies: isolates, dominants, and subordinates. Isolates were animals housed alone for 1 month or longer, while dominant/subordinate pairs were generated by housing animals together for 12 days or longer. In isolates and in dominant animals, 5HT had a facilitating effect on synaptic transmission between the sensory afferents and the LG, while in subordinate animals, 5HT reduced the magnitude of the synaptic response. Moreover the facilitation by 5HT of the response in isolates and dominants was thought to be through different kinds of receptors, as suggested by differences in the duration of the response to 5HT. The changes from the kinds of modulation seen in isolates to that seen in dominants and subordinates occurred linearly over a 12-day period, suggesting a gradual change in receptor subtype distribution with the pairing of the animals. Reversals of the changes caused by the pairings of animals could be brought about by new pairings of animals (losers with losers, winners with winners), but the time required for change depended on the direction of the change. It took longer for winners to revert to the loser pattern than for losers to change to the winner pattern.

While it is not yet clear how such changes contribute to the behavioral differences seen between dominant and subordinate animals, recent experiments on synaptic activation of the A1-5HT cells in lobsters (described above, Hörner et al. 1997), may give some insights into how these changes fit into the underlying circuitry (see Fig. 4). Mechanosensory afferents excite LGs, which in turn trigger the activation of motor programs for upwards and backwards movements used by animals in escape, but possibly also used in fighting behavior. At least such movements are a prominent part of the high-level aggression seen in lobster fights. In lobsters, LG neurons also excite the A1-5HT cells (see above), usually causing the cells to fire. This should release 5HT both within central neuropil regions where it may enhance motor output, and into the general circulation from peripheral release sites where it acts on both tonic and phasic muscles to enhance their effectiveness (Glusman and Kravitz 1982; Harris-Warrick and Kravitz 1984). The 5HT also may reach ganglionic sites by release into the general circulation, but this has not yet been demonstrated. Thus, the circuitry from mechanosensory neurons to LG to A1-5HT cells to release of 5HT should be more efficient in dominant and less efficient in subordinate animals in the presence of 5HT. How this serves in the behavior remains unknown.

In another series of studies in crayfish, an altered excitability is seen in the firing of the LG neuron in animals of different social status (Krasne et al. 1997). The excitability of the LG falls substantially in subordinate animals, but only slightly in dominants. How and if this interesting effect relates to serotonergic neuron function, however, remains unknown.

Serotonin in fighting behavior in lobsters

In studies using paired socially naïve juvenile lobsters, Huber and Kravitz (1995) devised a quantitative method

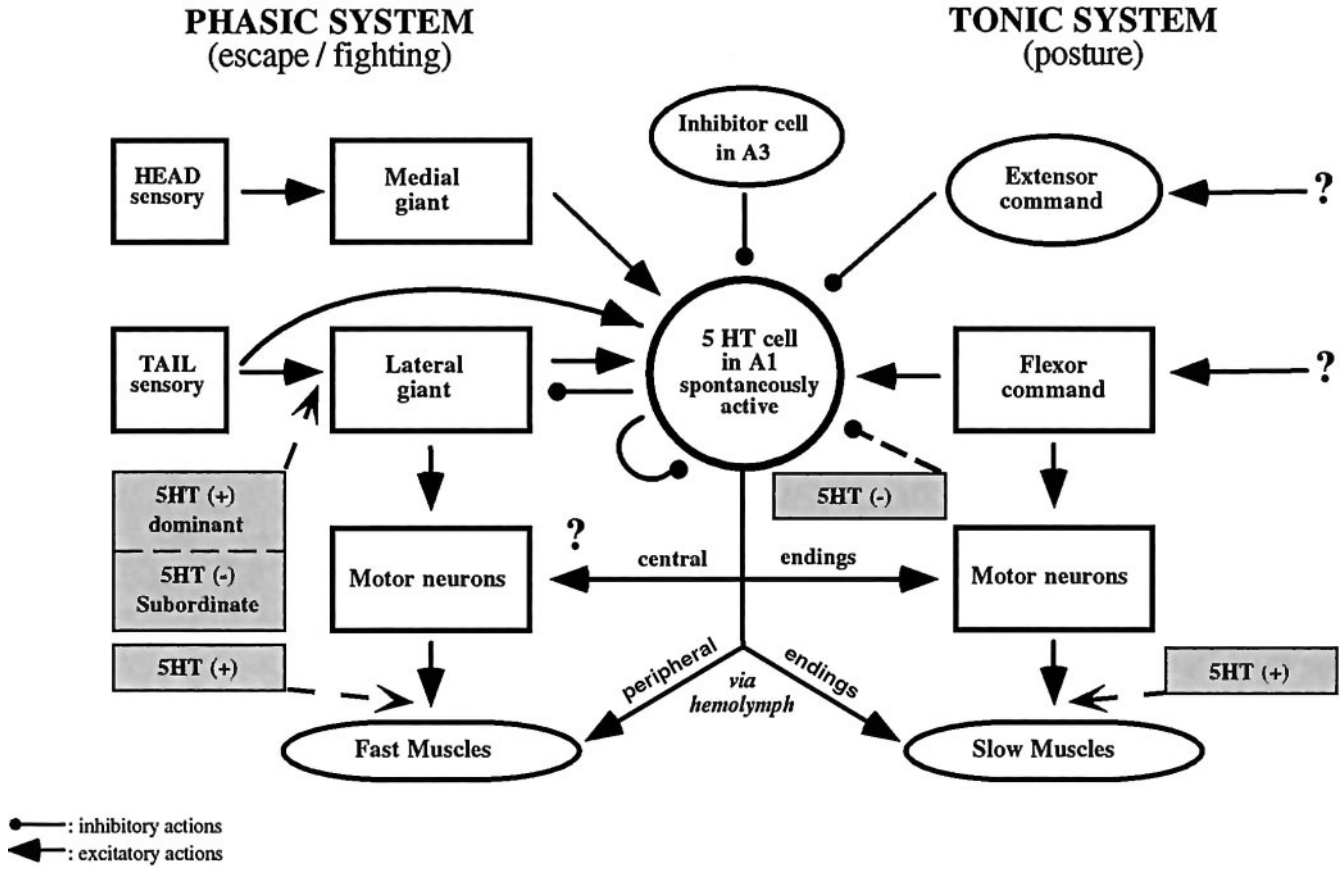

Fig. 4 Schematic of the known pathways involved in activation and inhibition of A1-5HT neurons. The right side of the figure shows linkages between the A1 cell and the tonic (postural) muscle system. In addition to the information already shown in Fig. 3, extensor commands inhibit the firing of A1 cells (Ma et al. 1992) and a major source of inhibitory input to these cells comes from an unidentified spontaneously active putative GABAergic neuron in the A3 ganglion (Weiger and Ma 1993). On the *left side* of the figure, known interactions with the phasic muscle system involved in escape and fighting behavior are shown. The LG and MG axons excite A1-5HT neurons, while prefiring the A1 cells reduces the magnitude of the excitatory input from these sources (Hörner et al. 1997). The phasic (fast) muscles activated by the lateral giant (LG) and medial giant (MG) axons also show enhanced contractility when treated with 5HT. The synaptic contacts between sensory receptors in the telson (tail) of the crayfish and the LG neuron are modulated by 5HT in opposite directions in dominant and subordinate animals (Yeh et al. 1997). See text for further details. This figure is slightly modified from Fig. 10 of Hörner et al. (1997)

for analyzing lobster agonistic encounters (fights). Animals that had been visually and physically isolated from other lobsters since the 4th stage (when they begin their benthic existence), were used in an attempt to eliminate the influence of experience on fighting behavior. Our analyses demonstrated that lobster fights include three highly stereotypical components (described above, in the Introduction): (1) *display*, during which animals stand tall and prominently show their claws, which are their major weapons; (2) *limited aggression*, in which the claws are used to grasp and attempt to overturn an opponent; and (3) *high level aggression*, in which animals grab whatever they can of an opponent, and with short upward tail flips attempt to tear off the appendage. At present we analyze fights by making a videotape of the entire fight (usually 30 min), and then scoring the times animals approach and are within one body length of each other (called an encounter). Usually we have animals fight three times: once to establish a hierarchy; a second time 1 h later to confirm that a hierarchy is established; and a third time either after a variable time period (to test the "memory" of the initial result), or after some kind of pharmacological manipulation has been performed. For each encounter we score: who initiates, who retreats, the duration, and the maximum intensity on a 0–3 scale (0 = one animal continually retreating, usually late in a fight; 1 = display; 2 = limited aggression; 3 = high level aggression). A statistical analysis then identifies the components changed by repeated fights, or by pharmacological intervention.

Using this method we demonstrated that the most significant variable changed during fights in crustaceans was the duration of individual encounters (Huber et al. 1997a). After a hierarchy was established the average durations of encounters in lobsters were reduced from about 30 s in first fights to about 5–10 s after a hierarchy was established. Since our studies showed a close linkage between duration and maximum intensity (Huber and Kravitz 1995; Huber et al. 1997a), we usually found a statistically significant decrease in intensity as well. We next asked what the consequences

would be of acute injections of 5HT on fighting behavior. Studies were carried out using both crayfish and lobster pairs, subordinate crayfish receiving continuous infusions of test substances, subordinate lobsters being removed from the tank and injected with the test substances. The results with both lobsters and crayfish were qualitatively the same: 5HT infusion into subordinate animals, after a variable but lengthy (ca. 45 min) delay, increased the duration and the maximum intensity reached during subsequent encounters. Subordinate animals, who hardly ever initiate encounters, could be seen to advance on the former dominants. The effect was transient, and in all but a very few cases, was completely reversible, with the former dominant reestablishing its dominant position once more. Thus, 5HT injection appeared to increase the willingness of animals that had just lost fights to engage in combat again.

In asking why it took so long for the effect to appear, we considered two options: (1) 5HT, via surface receptors, activated slowly-acting second messenger pathways that altered the willingness to fight; and (2) 5HT was taken back into serotonergic neurons (Livingstone et al. 1981; Huber et al. 1997b), thereby increasing the pool of amine available for release, and the subsequent release of "extra" 5HT contributed to the effect. We believe that the latter is an important part of the explanation, because Prozac (fluoxetine), as in vertebrates, blocks the uptake of 5HT in lobsters (Huber et al. 1997b), and when co-injected with 5HT, prevents the behavioral reversal. Injections of Prozac alone into subordinate animals have no effect on fighting behavior. It is interesting that acute treatment with Prozac in patients also is not effective in the treatment of depression (for review see Stokes 1993). It takes several weeks for a fully therapeutic effect to be seen, suggesting that the ability of Prozac to block 5HT uptake is only part of the explanation for its effectiveness. We carried out chronic treatments of crayfish and lobsters with Prozac using osmotic mini-pumps glued to the backs of animals that continuously infused the drug into the cardiac sinus. Prozac was injected over a 2-week period in this way and then animals were paired against larger and smaller opponents to search for drug effects. The effects seen were not dramatic, but animals that had received chronic Prozac treatment fought for longer periods of time than saline-infused control groups (A. Delago, unpublished observations).

We then lowered 5HT levels in animals by injections of the neurotoxin 5,7-DHT. In general, this treatment does not destroy serotonergic neurons in invertebrates. Instead it seems mostly to deplete neurons of 5HT by blocking uptake and synthesis and by enhancing release, while allowing the uptake of 5,7 DHT into the neurons (reviewed in Cook and Orchard 1993). Injections were given over a period of several weeks (either six or eight injections), and animals were then paired against larger and smaller opponents. Once again three fights were carried out: a first to establish a hierarchy, a second 1 h later, and a third 1 day later. The goal was to test not only whether 5HT-depleted animals would fight, but also how effectively they would fight, and whether they "remembered" the outcome of an initial encounter. After the final fight, animals were sacrificed to measure their amine contents by high performance liquid chromatography or for immunocytochemistry to examine the arbors of processes of the known 5HT neurons in lobsters. In all cases the 5,7-DHT treatments significantly lowered the amine content in animals. Animals with reduced levels of 5HT still would fight, and still could win fights. In fact, the main change we observed in preliminary results, was mainly in the duration of encounters, which were significantly increased.

While many more experiments of these types are required, the main conclusion that we can offer is that 5HT is not important in determining whether animals will fight, or even if they will win fights, but only for how long they are willing to fight. Moreover, as in the developmental studies, where elevated or lowered levels of 5HT caused developmental abnormalities, too much or too little 5HT both seem to cause an increased willingness of animals to fight. It remains to be established whether this translates as 5HT serving mainly a "motivational" role in fighting behavior in lobsters.

A speculative synthesis: amine neurons and fighting behavior

As in the past, when invertebrate models provided essential information to explain fundamental properties of neurons like how action potentials are generated (for review see Hodgkin 1964), how synaptic processes like inhibition work (Fatt and Katz 1953; Boistel and Fatt 1958; Kuffler and Edwards 1958; Otsuka et al. 1966), and how modulators function (cf. Adams and Levitan 1982; Glusman and Kravitz 1982; Dixon and Atwood 1989; Harris-Warrick and Marder 1991), many of these same models now provide key insights into how complex sensory systems function (for reviews see Hildebrand 1996; Ache et al. 1998; Passaglia et al. 1998) and to how behaviors are constructed and organized by nervous systems (for reviews see Altman and Kien 1987; Kravitz 1988; Bicker and Menzel 1989; Harris-Warrick and Marder 1991; Morton and Chiel 1994; Katz 1995). Invariably it is the ability to repeatedly find and identify single large neurons that is an important part of why these systems have proven so valuable. At the behavioral level, elegant studies are leading to important advances in our understanding of behavior. The systems used for study range from relatively simple ones, in which the potential exists for all the neurons involved to be identified and defined, like food processing mediated by the stomatogastric systems of decapod crustaceans (for review see Harris Warrick and Marder 1991), through more complex models, in which relatively smaller numbers of the neurons involved have been identified, like swimming (cf. Kristan and Weeks

1983; Friesen 1989), feeding (cf. Lent 1985; Schachtner and Bräunig 1993; Yeoman et al. 1994), and fighting (Adamo et al. 1995; Edwards and Kravitz 1997) behaviors. At the start we must acknowledge that it is a formidable challenge to construct a model of how amine neurons function in aggression. This is true despite the growing numbers of experimental results we and others are obtaining: in behavioral studies with living lobsters and crayfish; in physiological experiments defining the intrinsic properties of amine neurons; in systems studies exploring the function of these neurons in circuits; and in combined behavioral/physiological studies finding changes in the properties of amine neurons or their targets that relate specifically to changes in social status.

Watching amine neurons at work during fighting behavior would be an important way to learn more about their role in aggression. Unfortunately, so far no recordings have been made from amine neurons in awake behaving lobsters, as have been done in vertebrate (Veasey et al. 1995, 1997; Leung and Mason 1999) and in a few invertebrate (Kupfermann and Weiss 1982; Schachtner and Bräunig 1993; Yeoman et al. 1994) systems. In the absence of such recordings, the speculations we offer rest on what we now know from in vitro studies about amine neurons in crustaceans, and on information and suggestions gathered from studies of other invertebrate and vertebrate neuron systems.

The model proposed here borrows conceptually from the hypothesis originally put forth by Phoenix et al. (1959) that steroid hormones serve two distinct roles in modulating behavior: one *organizational* and the other *activational*. Important modifications of the original hypothesis were made by Arnold and Breedlove (1985) who pointed out that the divisions between these two roles were not sharp. The hypothesis was based on studies of testosterone action on mating behavior in newborn and adult guinea pigs. Phoenix et al. (1959) confirmed and extended studies showing that gonadal steroids had actions at around the time of birth, that were essential for the much later in development, behavioral responses evoked by release of the same steroids. It was suggested that the initial exposure had an *organizational* effect, a carving out of future gonadal steroid responsive territories within the brain and other body tissues. Much later in development, upon release of the gonadal steroids again, this time in sexually mature animals, the appropriate male and female behavioral patterns are triggered. The later responses represent the *activational* component of steroid hormone action.

The organizational role of amines

Amines like 5HT also are found early in development of the nervous system in many species of animals (for review see Lauder 1990; also see Wallace and Lauder 1983; Wallace 1985; Glover et al. 1987; Aitkin and Tork 1988; Konig et al. 1988; Goldberg and Kater 1989; Beltz et al. 1990, 1992). In lobsters, for example, the first visible 5HT immunostaining is seen at about 10% of embryonic life, and the complete set of 5HT neurons is found by 50% of development (Beltz et al. 1992). These times are well before most of the targets of the 5HT neurons have formed. Why are amines seen so early in development? A confusing, but compelling, literature suggests that amines and other classical neurotransmitters have important roles in development that far precede their later roles as neurotransmitters and neurohormones (for reviews see Lauder 1990; Buznikov et al. 1996). These earlier roles involve the earliest cleavage divisions of the embryo (or even gamete formation), and include morphogenetic roles in cell movement and cell shape changes during early embryogenesis and later roles when the nervous system begins to form and differentiate (for review see Buznikov et al. 1996). Various non-neuronal sites, such as yolk granules and notochord, appear capable of synthesis of 5HT in the early embryo, at least in some species (see Buznikov et al. 1996). Highly specific serotonin transporters and particular receptor subtypes show transient patterns of expression in the early embryo and early nervous system as well (Bennett-Clarke et al. 1993, 1996; Lebrand et al. 1998). The first neurons expressing amine transmitters appear well before their targets are formed (Lauder et al. 1982; Wallace and Lauder 1983; Wallace 1985). Thus, serotonergic neurons, expressing the transmitter phenotype, are seen growing through primitive epithelial layers that have not yet differentiated to form neurons. Indeed, 5HT, possibly released from growth cones, has been suggested to serve critical roles in the growth and differentiation of certain of its target neurons and in the activation of glial cells that then secrete growth and differentiation factors of their own (Haydon et al. 1987; Goldberg and Kater 1989; Lauder 1990). Too little, or too much 5HT, both lead to morphogenetic abnormalities (Goldberg and Kater 1989; Cases et al. 1996; Upton et al. 1999). For example, in mice missing the monoamine oxidase A gene, which results in elevated levels of 5HT and norepinephrine in the brain, various abnormalities are seen. The cortical barrel fields fail to develop (Cases et al. 1996) and retinal ganglion cell fibers that ordinarily segregate into layers in the lateral geniculate body, fail to do so in the mutant animals (Upton et al. 1999). Inhibiting the synthesis of 5HT at early developmental stages prevents the formation of these abnormalities (Upton et al. 1999). In monoamine oxidase A knock-out mutants, 5HT immunostaining is seen in a subpopulation of ganglion cell neurons that contain a serotonin transporter, while in control animals immunostaining is not seen (although the transporter is expressed in the same transient manner as in the mutant). The machinery for loading the amine into vesicles also is present in some of these neurons, as is a protein thought to be involved in vesicular binding of 5HT (Upton et al. 1999). Thus, the cells that take up the amine may be capable of releasing it as a borrowed transmitter in inappropriate places or amounts in the mutant animals (Cases et al.

1998). In *Helisoma* neurons, 5HT inhibits growth-cone elongation in a specific subpopulation of cells in vitro, and influences the growth and morphological appearance of these same neurons in vivo (Haydon et al. 1987; Goldberg and Kater 1989). These and related findings have led several investigators to propose that 5HT serves an *organizational* role in the nervous system (see Goldberg and Kater 1989; Lauder 1990), but precisely what that role is has not been spelled out. The suggestion offered here is that the role may be similar to that proposed for steroids by Phoenix et al. (1959). Perhaps the early arrival of amine neurons in relatively undifferentiated areas of the brain helps to carve out later amine responsive territories. In fact, possibly the *organizational* role of steroids only represents a specific case of more general developmental roles served by modulators in defining the areas of the brain they later will influence in behaviorally important ways.

The activational role of amines

This role, by contrast, deals with how amine neurons function within already formed responsive territories. The sequence of highly stereotypical motor acts that make up fighting behavior have been described above. How this context-dependent sequence is assembled in the nervous system, and how to determine the parts of the nervous system that are concerned with the behavior, are issues of paramount importance. Many models have been put forward to attempt to explain how complex behaviors are assembled in nervous systems (for reviews see Altman and Kien 1987; Kravitz 1988; Bicker and Menzel 1989; Harris-Warrick and Marder 1991; Morton and Chiel 1994; Katz 1995). Invariably the models deal with how sensory cues assemble the appropriate neuronal circuits to respond to the cues, and with the roles served by hormonal substances in consolidating those choices. One particularly elegant illustration of the complexity of the issues involved comes from studies from the Cohen laboratory on voluntary and evoked contractions of the gill mantle in the sea slug, *Aplysia* (Wu et al. 1994). These authors used optical recording methods in the abdominal ganglion to monitor the population of neurons active during reflex withdrawal of the gill, during respiratory pumping and during small spontaneous gill contractions. They observed a very great overlap of the populations of neurons involved in each of these types of contractions. Even more surprising, they found that over 20% of 900 neurons in the ganglion were involved in this "simple" reflex. The authors discuss two alternative models for how the "behavior" is organized: one is a dedicated circuit model in which sensory elements activate small sets of neurons each subserving a fragment of motor behavior; the other is a distributed organization in which sensory elements arrange patterns of behavior de novo from interneuronal pools of cells, depending on the context, to perform various motor acts. As is usually the case when two extremes are offered, probably both models are correct in how behaviors are assembled in nervous systems.

In fighting behavior, the survival of the animal is at stake. Much of the nervous system probably is involved in this important and essential behavior, including the regions controlling the changing motor patterns we observe, but also including regions concerned with the regulation of cardiac function (heart rate changes during fights, Hernández-Falcón and Kravitz 1999), respiration, excretion (see above, Breithaupt et al. 1999), and possibly other physiological processes as well. In unpublished preliminary observations we have found that losing animals not only refuse to engage other animals in fights, but they also show dramatic reductions in their feeding behavior. The challenge is to sort out the circuitries involved, and to figure out how and where amines and amine neurons fit into these circuits. *The suggestions are that amines (and almost surely other modulatory substances) serve important roles: in assembling the arrays of neurons that pattern the behavior; in the smooth transitions between one behavioral pattern and the next (e.g., limited aggression to high level aggression); and in the temporal domain concerned with how long animals are willing to perform the sequences that make up the behavior.*

These suggestions are not original and derive heavily from explorations on the roles served by amines and other modulatory substances on the motor output of systems of neurons like the stomatogastric ganglion of crustaceans (for review see Harris-Warrick and Marder 1991). This approximately 30 neuron network governs the processing of food on its way through the stomach, and much detailed information is available on how it works. Many modulators influence the activity of the two circuits that govern the behavior (Christie et al. 1997), but some of the best studied are the amines 5HT, OCT, and dopamine (Flamm and Harris-Warrick 1986a, b). Each amine causes a unique change in the way that a circuit functions. The changes are amine specific and concentration dependent, i.e., the same amine can cause different effects depending on its concentration (Flamm and Harris-Warrick 1986a). Almost all neurons in the circuit respond to all three amines, but through an elegant cell by cell analysis, it was demonstrated that each amine has selective actions on the conductance properties of the cell under study (Flamm and Harris-Warrick 1986b). The cellular studies allowed a simple model to be made that explained the circuit effects of applied amines. In our understanding of the circuitry dealing with aggression, we have not yet done any cell-by-cell analysis of the actions of 5HT on its target neurons (although much work has been done on amine actions on neuromuscular junctions – Glusman and Kravitz 1982; Dixon and Atwood 1989; Goy and Kravitz 1989). We believe, however, that the same general principals seen in the neurohormonal modulation of behavioral output in the stomatogastric system, also will apply in the more complex behavior as well. Thus,

amines, once released and through various receptors in target territories will selectively influence the properties of many central neurons, and probably also peripheral targets like exoskeletal muscles, the heart, and sensory neurons (Battelle and Kravitz 1978; Florey and Rathmayer 1978; Dixon and Atwood 1989; Pasztor and Bush 1989). These actions will alter the ways in which the target neurons function in circuits, generating new outputs from existing circuits or perhaps even molding new circuits. These, in turn, will help shape the behavioral patterns that have been initiated by sensory signals announcing the arrival of an adversary.

Are the serotonergic neurosecretory neurons important in fighting behavior?

One serious concern is that the A1- and T5-5HT neurosecretory cells, which have been the focus of our studies and those of other investigators, appear to be mainly involved with the postural components of fighting behavior. While posturing is an important part of fighting behavior in essentially all species of animals, its control probably lies with decision-making neurons in higher centers of the CNS. In lobsters, these neurons most likely will be found in the supraesophageal ganglion, the brain of decapod crustaceans. While our studies also suggest that 5HT plays an important role in decision making, since 5HT injections reverse the unwillingness of losing animals to fight (Huber et al. 1997a), much less is known about how brain serotonergic neurons function (see Sandeman and Sandeman 1987, 1994; Sandeman et al. 1995). In addition, there are short- and long-term consequences of winning and losing fights, and these too must be a part of any discussion of the role of 5HT in aggression. Defeated animals will not fight with winners for many days after an initial encounter and show reduced levels of fighting for up to a week, when animals are separated after their first fights (Rutishauser et al. 1999; for studies with adults see Karavanich and Atema 1998a). If animals are housed together after the first fight, then no changes are seen in the status of the paired animals, unless some traumatic event occurs, like the winning animal undergoing a molt (shedding of its old cuticle).

Why do fights end?

Fights appear to be decided when one animal "gives up"; it refuses to fight any longer. While this is decided on rare occasions by damage to the losing animal, generally we see no precipitous event that causes one animal to "give up". If fairly closely matched in size, animals readily engage each other in combat and easily move to higher levels of intensity throughout the fight. Suddenly, at some point, the loser backs off and avoids further encounters. We have no clear idea of why this happens. Perhaps some balance of humoral factors within the brain is involved in decision-making of this sort, like for example, changing 5HT/OCT ratios in key brain areas. Perhaps animals recognize chemical cues released in the urine of their opponents during the fights (Breithaupt et al. 1999). Animals unable to release urine, or unable to "smell" the released urine of opponents, fight for longer periods of time than control animals (Karavanich and Atema 1998b). In this regard, key metabolites of amines, like the expensive-to-synthesize sulfate conjugates (Kennedy 1978; Huber et al. 1997b), are released in the urine of animals. If lobsters can detect these metabolites, and can distinguish between the 5HT and OCT forms, then each animal would be capable of evaluating the patterns of usage of the two amines in the nervous system of the other. Whatever the reason, it is unlikely that changes in gene expression account for "giving-up". The event happens too suddenly and after too short a time for much in the way of changes in levels of expression of genes to reach synaptic regions of neurons. The decision once made is not reversed. It is very rare to see a "loser" advance on a "winner" after a fight has been decided, *unless* 5HT is injected into the "loser". Then we see a transient "willingness" of the loser to fight again, an event invariably reversed after some tens of minutes. From preliminary studies, it appears that uptake of 5HT, and presumably subsequent release, plays an important role in this reversal (see above). If animals are housed together after a hierarchy is established, however, there are longer-term consequences of being winners and losers, which may involve changes in gene expression (see below). We do not yet know if 5HT injection will cause a behavioral reversal in animals sharing a tank for extended periods of time.

Postulated roles of the A1- and T5-5HT neurosecretory neurons during fights

How might the 5HT-neurosecretory neurons function during fights? In dissected preparations, A1-5HT neurons usually are spontaneously active, firing at 0.5–3 Hz. If the cells fire at similar rates in intact animals, 5HT should be released tonically into the general circulation and into neuropil regions in the central nervous system via the two sets of endings of the cells. The different rates of spontaneous firing of cells presumably were set at some earlier time, possibly relating to earlier experiences of the animals. Although small, these differences may contribute in important ways to the functioning of the neurons. For example, neurons firing more rapidly should release more 5HT and may also release higher levels of the proctolin that co-localizes with 5HT in the cells. Moreover the duration of the autoinhibition seen after high frequency firing of the cells (see above) is inversely related to the initial firing rate, suggesting that small differences in firing rates could play large roles in how cells are used (Heinrich et al. 1999). We anticipate that there will be a phasic component to the actions of these neurons as well: that is that the rate of firing will

change during fights, under the influence of synaptic inputs of the types we have identified. Changes in the rates of firing of serotonergic neurons in behaving animals have been recorded in other species (Kupfermann and Weiss 1982; Schachtner and Bräunig 1993; Yeoman et al. 1994). Invariably these changes relate specifically to the behaviors the neurons originally were postulated to be involved with. In some cases, the changes in firing anticipate the behavior. For example, in locusts, serotonergic neurons of the satellite nervous system begin firing tens of seconds before the initiation of feeding behavior in the animals (Schachtner and Bräunig 1993).

With the information gathered so far from in vitro studies, we can propose different scenarios for how fighting behavior might alter amine neuron function, and in turn, how changes in amine neuron function might influence the behavior. Early in fights, when animals stand tall displaying their claws, increases in the rates of firing of the 5HT cells in both combatants could: (1) through central actions of amines, maintain animals in an elevated posture and facilitate the maintenance of the postural displays; and (2) through 5HT released into the circulation, increase the strength of muscular contractions (through pre- and post-synaptic facilitatory actions on exoskeletal junctions). There are several ways to effect such changes. The firing rates of A1-5HT cells would be immediately increased by blocking the spontaneous inhibitory input that holds the firing of the cells in check (see above). Rates also would be increased by firing flexor commands, which make animals stand in the elevated posture seen when animals approach each other, and the increased firing, in turn, should enhance the output of the command through central and peripheral actions of 5HT (the ''gain-setter'' role; Ma et al. 1992 and see above). Later in fights, after a hierarchy has been established, dominants alone stand tall as they approach subordinates. At this point, perhaps only winners show increased firing of their serotonergic neurosecretory neurons. Extensor commands trigger an opposite, submissive-looking stance, and inhibit the firing of serotonergic neurosecretory cells. Such commands may become more important in the repertoire of losing animals, since these animals maintain a lowered posture as they continually retreat from dominants. All of these speculations, derive from the pool of knowledge gained from our in vitro studies (see Figs. 3, 4). They would be greatly strengthened by recording from the A1- or T5-5HT cells in fighting animals, which is a high priority for future studies.

In the temporal domain, changes in the firing rates of amine neurons might influence how long animals are willing to fight (the ''motivational'' role). As noted above, the main effect we see of altering amine levels in behaving lobsters is in how long animals are willing to fight, not in whether or not they will fight. Amine actions on most subtypes of target receptors are slower in on- and offset than the actions of classical transmitters. Amines trigger these slower effects through activation or inhibition of a wide variety of 2nd-messenger pathways, including cyclic nucleotides, phosphatidyl inositides, calcium and other substances (for review see Hen 1992). Activation of the cascades triggered by these substances can produce cellular changes whose durations range from seconds to minutes if covalent modifications of proteins are involved, or to days if transcriptional regulation is altered. The durations of amine actions, therefore, are not determined by the continued presence of the amine, but by how long it takes target cells to reverse the changes initiated by activation of the 2nd-messenger systems. Advance firing of amine neurons in anticipation of behavior, through such pathways, could prime amine responsive circuitries in order to optimize their subsequent effectiveness in behavior (see above).

Long-term consequences of changes in status

Long-term changes in the behavior of both animals follow the establishment of a hierarchy. Thus, winners are more likely, and losers less likely to win their next fights (Scrivener 1971). Moreover, losing animals will not fight with winners for even a week after their initial meeting, if animals have been separated from each other (Karavanich and Atema 1998a; Rutishauser et al. 1999). If not separated after the first fight, the social positions (winner/loser) and accompanying behavioral patterns are maintained indefinitely. The maintenance of the behavioral status quo, with dominant animals regularly chasing the retreating subordinates, seems to consolidate whatever changes were initiated in both animals by their first fights. Ultimately, some of these changes are likely to be in the form of changes in gene expression. Studies supporting this idea were described above (Yeh at al. 1996, 1997), when it was seen that 5HT-receptor subtype distribution changes markedly at particular synaptic sites after changes in social status. These changes are slow in onset, however, taking some 2 weeks to reach a maximum effect. While it has not yet been determined that they are due to changes in amine receptor gene expression in lateral giant neurons, that seems a likely possibility with the very slow time-course of the changes. It is very unlikely, however, that these represent the entire spectrum of behaviorally modulated changes taking place.

An attractive feature of the ''gain-setter'' model for how amine neurosecretory cells function, is that in addition to the short-term influences on postural circuitries described above, longer-term changes in the properties of the amine cells, or in the sensitivity of their targets to amines, could alter the output of the circuits in longer-term ways, without any changes required in the hard-wiring of the system. Any of a variety of changes in the serotonergic neurons or their targets could effect such changes. These include: (1) modifying the membrane ion channel composition of amine neurons, or reducing or enhancing the levels of spontaneous synaptic input to the cells from inhibitory or excitatory sources, all of which would change the spontaneous

firing rates of the cells; (2) changing the levels of expression of tryptophan hydroxylase, the 5HT packaging machinery, or the 5HT membrane transporter, all of which would alter the size of the pool of 5HT available for release; and (3) changing the distribution of amine receptors on target neurons, which already has been seen and documented as described above. Future studies will be directed at the search for behaviorally linked changes in neuronal function relating specifically to the amine neurons.

Conclusions

Crustacean systems offer an exciting model with which to explore the role of amines in a complex behavior like aggression. Studies at many levels of analysis are possible beginning at the behavioral, and including physiological, morphological, biochemical and molecular approaches. Much progress has been made in defining the important components of the system of amine neurons in crustaceans, and in learning how they function. We are poised at the start of the investigation of how the behavior we are studying influences the function of these neurons, and of how in turn, these neurons influence the behavior.

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