SEROTONERGIC MODULATION OF THE CRAYFISH HINDGUT: EFFECTS ON HINDGUT CONTRACTILITY AND REGULATION OF SEROTONIN ON HINDGUT

by

BARBARA ELLEN MUSOLF

Under the Direction of Donald H. Edwards

ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) has long been associated with the vertebrate gut and is an important neuromodulator of crustacean foregut. This dissertation presents evidence that 5-HT initiated peristalsis in crayfish hindgut and enhanced the power of contractions in caudal regions of the hindgut. 5-HT receptor immunoreactivity studies showed that the two identified crustacean 5-HT receptors, 5-$HT_{1\alpha}$ and 5-$HT_{2\beta}$, are present on the hindgut in different and distinctive patterns.

5-HT immunoreactivity (5-HT-ir) studies revealed that the fibers from central neurons found on the hindgut showed a broad range of 5-HT-ir intensity, which led to the hypothesis that they borrowed 5-HT. This hypothesis was tested by first determining that the HGNs can take up 5-HT through a serotonin transporter and that uptake can be blocked by a serotonin reuptake inhibitor. Second, synthesis was tested by superfusing tryptophan and using 5-HT-ir to determine the presence of 5-HT. No constitutive 5-HT synthesis occurred under these conditions. Superfusion of the intermediate product of 5-

HT synthesis, 5-hydroxytryptophan (5-HTP), did lead to 5-HT-ir. The HGNs can take up 5-HT but have only one of the synthetic enzymes.

The lack of nearby sources for 5-HT led to the hypothesis that hormonally supplied 5-HT may be the source for 5-HT in the HGNs. High performance liquid chromatography measurements of 5-HT and 5-HTP levels in tissue following injection of 5-HT into the hemolymph revealed that levels of 5-HT significantly increased in the terminal ganglion and hindgut, where the HGNs cell bodies and projections are respectively located. All other areas of the central nervous system, with the exception of the brain, also showed a significant increase in 5-HT levels. Injection of tryptophan produced a significant increase in 5-HTP levels in the brain.

Quantitative 5-HT-ir indicated that feeding increased the intensity of 5-HT-ir in the HGNs. Feeding was determined to be a relevant stimulus to examine facultative synthesis of 5-HT. The enzyme that converts 5-HT to 5-HTP was blocked and 48 hrs after feeding 5-HTP-ir was used to indicate that facultative synthesis did not occur. At the same time, 5-HT-ir was used to indicate that uptake of 5-HT by the HGNs more likely occurred.

INDEX WORDS: Hindgut, Crayfish, Serotonin, 5-hydroxytryptamine, 5-hydroxytryptophan, Tryptophan, Dopamine, Borrowed transmitter, Peristalsis

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DEDICATION

This dissertation is dedicated to my parents, sisters, sons, and the many friends who have waited so patiently for me to complete it.

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LIST OF ABBREVIATIONS

5-HT serotonin

5-HTP 5-hydroxytryptophan

A1 first abdominal ganglion

A2-A5 second through fifth abdominal ganglia

A6 abdominal ganglion 6 or terminal ganglion

AAHG anterior abdominal hindgut

AINs anterior intestinal nerves

ANOVA analysis of variance

CEG circumesophageal ganglion

CM circular muscle

cm centimeter

CNS central nervous system

DA dopamine

EC enterochromaffin cells

ENS enteric nervous system

Fr statistic Friedman statistic

GPR gastropyloric receptor cells

HG hindgut

HGNs hindgut neurons

HPLC high performance liquid chromatography

hr hour

Hz hertz

ir immunoreactivity

ICC immunocytochemistry

L-AADC L-amino acid decarboxylase

LM longitudinal muscle

min minute

μM micromolar

μg micrograms

mL milliliter

mN milliNewtons

N7 intestinal nerve

nM nanomolar

NMS N-methylserotonin

n.s. not significant

NSD-1015 *m*-hydroxybenzylhydrazine

PAHG posterior abdominal hindgut

PB 0.1M phosphate buffer

PBTx PB with 0.3% Triton X-100

PCA perchloric acid

PINs posterior intestinal nerves

SERT serotonin transporter

sec second

SEG subesophageal ganglion

STG stomatogastric ganglion

T5 fifth thoracic ganglion

THG thoracic hindgut

TpOH tryptophan hydroxylase

V volt

1

General Introduction

General introduction

The hindgut of the crayfish, *Procambarus clarkii* is a deceptively simple organ; tubular in shape and limited in its diversity of functions. While the crustacean foregut has been an important model system in elucidating the behavior of central pattern generators and cataloging the large array of neuromodulators that vary the motor patterns produced by the stomatogastric nervous system, less is known of the crustacean hindgut.

This dissertation focuses on two different sets of questions. The first set of questions address how the hindgut is anatomically organized, where serotonin is located on the hindgut and what effects serotonin (5-hydroxytryptamine, 5-HT) has on the contractile properties of the crayfish hindgut. The second set of questions address the source of 5-HT and whether the serotonergic neurons located on the hindgut acquire 5-HT through synthesis or uptake. I examine how increased levels of 5-HT or the 5-HT precursor, tryptophan in the hemolymph affect levels of 5-HT in serotonergic neurons and I test whether feeding can affect the amount of 5-HT found in these neurons.

In the following general introduction, I will first provide an overview of the organization and function of the hindgut and what is known of its innervation. I will then present information for comparison on the role of 5-HT in the digestive system of other organisms. I will then briefly present what is known concerning the effects of 5-HT in crayfish foregut and will present a rationale for the novel regulation of 5-HT in crayfish. Finally I will give a more detailed overview of the dissertation aims.

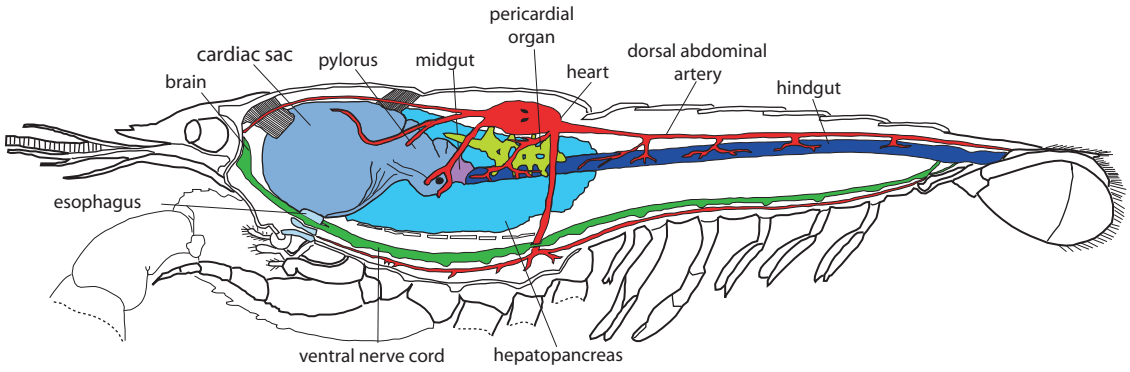
The crayfish digestive system and digestion

The digestive system of crayfish consists of a foregut, midgut, and hindgut (Fig. 1.1). The foregut includes the esophagus and the cardiac sac and pylorus. The midgut includes a short intestine and the hepatopancreas. The hindgut includes a longer intestine that extends from mid-thorax to the sixth abdominal segment, the rectum and the anus. The foregut and hindgut are ectodermal in origin while the midgut is endodermal in origin.

Crayfish are omnivores that use a number of feeding appendages to gather food that is conveyed to a ventral opening. The mouthparts break the food down into smaller chunks before it is ingested and lubricated by mucopolysaccarides secreted by esophageal glands (Brown, 1995). The lubricated food particles travel superiorly through the short esophagus to the cardiac sac where they are further broken down by a grinding apparatus that consists of teeth-like projections. Enzymes from the hepatopancreas are released into the cardiac stomach to mix with the food and facilitate digestion. Rows of setae move the food along to the cardiopyloric valve where a sorting and sieving process takes place. Large food particles that cannot pass through the valve are retained in the cardiac sac to be reground. The pylorus primarily filters particles for passage to the hepatopancreas. Only small particles pass to the hepatopancreas where they undergo further digestion and absorption. Intermediate sized particles are routed to the intestine and are mixed with waste material from the hepatopancreas. There are three chambers to the pylorus and it is in the medial chamber that the fecal pellet is formed and passes through the pylorointestinal valve to the midgut (Vogt, 2002).

Fig. 1.1 Gross anatomy of the crayfish circulatory, digestive, and nervous system. The circulatory system is in red, the digestive system is in hues of blue, and the central nervous system is in hues of green. This illustration is based on Fig. 12 from Huxley (1880).

Fig. 1.1


pericardial
organ
cardiac sac
pylorus
midgut
dorsal abdominal
artery
brain
heart
hindgut
esophagus
ventral nerve cord
hepatopancreas

The hepatopancreas has numerous functions beyond the secretion of digestive enzymes and absorption of nutrients. It has excretory functions, stores organic and inorganic reserves, engages in lipid and carbohydrate metabolism, detoxifies xenobiotics, synthesizes vitellogenins and blood proteins such as hemocyanins, and functions in the molt cycle (Factor, 1995; Vogt, 2002). The gland is well vascularized by arterial blood, which discharges into an extensive network of hemal sinuses. The midgut intestine also engages in absorption of food and storage of lipids and likely produces the peritrophic membrane that surrounds the fecal pellet formed in the pyloric medial chamber. This membrane binds the waste material together into a fecal string and serves to protect the cuticular lining of the hindgut.

The hindgut functions primarily to further process and eliminate waste material, however it is also involved in the molt cycle, engaged in reabsorption of water and ions, and it harbors bacterial symbionts that provide essential nutrients. These bacterial symbionts further digest material in the fecal pellet, engaging in both amylolytic and proteolytic activity (Mickénienė, 1999). Absorption of these nutrients probably takes place in the more anterior regions of the hindgut. The hindgut also engages in anal uptake of water, which is used to produce the hydrostatic "skeleton" following molt and may be used to move bacterial products to more anterior regions of the digestive system (Webster et al., 2000; Vogt, 2002).

Crayfish hindgut anatomy

The crayfish hindgut is a linear tube without caeca that rises from midthorax to the dorsal aspect of the abdomen. Six longitudinal ridges extend into the lumen of the hindgut. These ridges are composed of epithelium, glands, connective tissue, and

longitudinal muscles (Factor, 1995; To et al., 2004). The connective tissue is a fibrous, collagenous matrix that consists of circulating hemocytes, fibroblasts, and granulocytes. Hemal sinuses permeate the connective tissue and function much like capillaries, allowing exchange of gases and nutrients as well as collection of waste materials (Factor, 1995). The longitudinal muscle is loosely arranged in fascicles that are inserted at shallow oblique angles to the longitudinal axis of the hindgut.

The luminal surface of the hindgut is lined with a spiny cuticle (Chisaka et al., 1999) that is sloughed off during molt. The spines point towards the anus and vary in morphology along the length of the hindgut (Chisaka et al., 1999).

Surrounding the six longitudinal ridges is circular muscle, a visceral membrane and nerve fascicles that include fibers from the central nervous system (Elekes et al., 1988). The circular muscles vary in thickness and are most prominent on the rectum. The circular muscles also extend to the anus where they form an arch surrounding the anal vent. These muscles control anal movements and act as a sphincter (Winlow and Laverack, 1972c). Both longitudinal and circular muscles are striated, unlike visceral muscle in vertebrates, which is smooth.

The hindgut is well vascularized and is likely to be one of the locations of hyaline hemocytes or blood stem cells (Martin and Hose, 1995). The dorsal abdominal artery rests on the dorsal surface of the hindgut and branches at each abdominal segment. This artery is unique in that it has valves where the segmental arteries branch. GABAergic and cholinergic fibers innervate these valves and they are modulated by the amines and peptides. When the valves are open, blood flows through the segmental arteries to the

abdominal musculature. When the valves are closed, blood is shunted to the hindgut (Wilkens, 1997).

Innervation of the crayfish hindgut

Alexandrowicz (1909) was the first to investigate in detail the innervation of the hindgut. He described two nerve plexuses: a ground plexus, which is an irregular network of fibers branching off from the main fascicles, and an end plexus, which runs parallel to the muscles and innervates them. Motor neurons innervate the hindgut and form two types of neuromuscular junctions, one where the neuromuscular junction is embedded in the muscle cell cavities and another where the nerve contacts muscular protrusions (Elofsson et al., 1978). There has been some controversy on whether sensory input travels to the CNS, with Alexandrowicz (1909) and Orlov (1926) indicating respectively that bipolar sensory neurons make either peripheral or central contact onto motor neurons. Backfills of the intestinal nerve label what may be the bipolar sensory cells (Musolf and Edwards, 1999), supporting central contacts.

Central input to the hindgut arises from single neurons located in anterior abdominal ganglia and from several neurons in the terminal ganglion (A6), a trio of single cells and three clusters of neurons medially located in the caudal region of A6 that I refer to as hindgut neurons (HGNs). All of the neurons that project to the hindgut have been previously described as the hindgut efferent neurons (Elekes et al., 1988). My focus is only on the clusters of neurons that project to the hindgut. The primary role of the HGNs is innervation of the hindgut, however, they have presynaptic and postsynaptic terminals in the central ganglion, indicating that they are involved in processing signals within the distal portion of the ganglion and they have projection fibers to the neural

sheath that suggest a neurosecretory role (Elekes et al., 1988; Johansson and Schreiner, 1965). The HGN axons project through the intestinal nerve (N7) where they form collaterals that make synaptic connections with other fibers that project through N7 (Elekes et al., 1988). The HGN projections reach the hindgut at the rectum and then separate into the anterior intestinal nerves (AINs) and the posterior intestinal nerves (PINs). The AINs branch and project laterally along the hindgut giving rise to smaller branches that project to the longitudinal folds of the hindgut. An ultrastructural study of the hindgut shows that these branches become thinner as they penetrate into the hindgut tissue, with the thinnest fibers innervating the longitudinal muscle (Elofsson et al., 1978).

Peripheral 5-HT and digestion in mammals

5-HT has long been associated with the mammalian enteric nervous system (ENS). It was first discovered in the enterochromaffin (EC) cells of the guinea pig gastrointestinal mucosa (Erspamer, 1946; Erspamer and Asero, 1952) and later found to be the same substance isolated from cattle serum that was identified as a vasoconstrictor (Rapport et al., 1948a, b). Over 95% of the 5-HT found in mammals is located in the EC cells that are found in the mucosal layer of the vertebrate gut (Erspamer, 1966). A small percentage of 5-HT is found in a subpopulation of interneurons in the myenteric plexus. Early research showed that 5-HT initiates peristalsis when applied to the luminal side of the guinea pig intestine (Bulbring and Lin, 1958).

Serotonin is critical to the functioning of the mammalian ENS (Gershon, 1999) and acts as both a neurotransmitter and as a neurohormone. Mechanical and chemical stimuli lead to broadcast release of 5-HT from the EC cells following which a number of responses can occur: peristalsis, emesis, nausea, and intestinal secretion (Hansen, 2003).

5-HT also mediates nociceptive signals between the ENS and the CNS (Coates et al., 2004; Galligan, 2004).

5-HT in crustacean digestion

Serotonin is important in the functioning of the crustacean digestive system. Studies of crustacean foregut indicate that 5-HT is present and has an influence on foregut behavior. In the crabs *Cancer borealis* and *Cancer irroratus*, hormonal levels of 5-HT increase neuronally evoked muscle tension (Katz et al., 1989). In these same crabs and other decapod crustaceans, there are also pairs of proprioceptors called the gastropyloric receptor cells (GPRs) (Beltz et al., 1984; Katz et al., 1989), which provide serotonergic input to the stomatogastric ganglion (STG). The GPRs are located in the lateral ventricular nerve; their dendrites are embedded in gastric mill muscle and they project to the STG, terminating in the circumesophageal ganglia. The GPRs respond to movement of the gastric mill and modulate the pyloric motor pattern by acting on a number of neurons in the STG (Katz and Harris-Warrick, 1990). The release of 5-HT from the GPRs enhances cycle frequency through its excitatory effects on the AB neuron, which along with the PD neurons make up the pacemaker kernel in the lobster pyloric network. This role puts the serotonergic GPRs in the position of coordinating the pyloric central pattern generator with activity of the gastric mill (Katz and Harris-Warrick, 1990).

In addition to the 5-HT released from GPRs, there may also be hormonal release of 5-HT from the pericardial organ into the ophthalmic artery where the STG resides (Beltz and Kravitz, 1983). In some crustaceans, such as *Panulirus interruptus*, no 5-HT is found in the GPR cells. Instead, 5-HT released from the pericardial organs is thought to be the sole serotonergic source that leads to modulation of the STG.

Little is known of the role that 5-HT plays in later stages of digestion in crustaceans. While 5-HT is an important signaling molecule in mammalian intestine, it is unknown what role 5-HT may play in crustacean midgut, midgut glands and hindgut, the organs that are homologous to mammalian small and large intestine. The crayfish have no 5-HT containing EC cells but 5-HT is periodically present in hindgut neurons under particular experimental conditions. Either 5-HT plays a different role in crayfish hindgut than in the mammalian gut or there may be a different way to supply 5-HT to this organ to produce effects similar to what are found in the mammalian ENS.

Distribution of 5-HT

The distribution of 5-HT is a function of where it is synthesized and where it is taken up after it is released. Judging from the location of most 5-HT uptake sites, which is on axons and outside synaptic junctions, 5-HT often acts as a paracrine signal (Bunin and Wightman, 1999). In vertebrate animals, the neuronal and non-neuronal sources in the enteric nervous system (ENS) and neuronal sources in the CNS produce their own 5-HT and these supplies largely remain separate from one another as a result of the blood-brain barrier. The blood-brain barrier, an anatomical feature of organisms with a closed circulatory system, possibly led to this dichotomous arrangement of 5-HT synthesis and regulation in the vertebrate ENS.

5-HT released from EC cells supplies both the enteric and vascular systems. Following broadcast release of 5-HT from the EC cells in mammals, 5-HT is quickly taken up by enterocytes, epithelial cells of the gastrointestinal mucosa, and by 5-HT neurons (Gershon, 1999). In the vascular system, platelets take up and store 5-HT to

carry out hemostatic functions such as constriction of the blood vessels and activation of platelet aggregation (Cote et al., 2004).

Organisms such as arthropods and mollusks have an open circulatory system comprised of heart(s), arteries, and hemal sinuses. Blood is delivered to the different organs through the arteries and collects in blood cavities called hemocoels (Maynard, 1960). Muscular movement and, in some organisms, accessory hearts assist in the diffusion of blood to the gills and back to the heart. This organization provides a different rationale for the organization and regional regulation of 5-HT in arthropods, which include crustaceans, and mollusks. In these organisms, 5-HT also acts as a hormone, whereby 5-HT moves freely between the CNS and other organ systems. This leads to the question of how an open circulatory system impacts the regulation of 5-HT. Massive release of 5-HT as seen in the vertebrate gut would overwhelm the more accessible CNS and introduce problems with cross talk between the different organ systems, neuronal and non-neuronal, that respond to 5-HT.

Dissertation overview

This dissertation specifically focuses on the contractile properties of the hindgut, the effects of 5-HT on crayfish hindgut and how 5-HT is supplied to the hindgut. I propose that while 5-HT may perform the same function in the crayfish hindgut as it does in vertebrate hindgut, the lack of a blood brain barrier produces a different way to handle or regulate the movement of 5-HT. Initially I present evidence that 5-HT affects hindgut motility and then I present my investigations into the source of 5-HT in the gut; whether it is synthesized or acquired through a serotonin transporter (SERT). I then test whether increased levels of 5-HT in the hemolymph lead to increased levels of 5-HT in A6 and

hindgut, the location of the HGN cell bodies and axonal projections respectively. I also test whether increasing the 5-HT precursor tryptophan could also increase 5-HT levels in the CNS or hindgut. Finally, I examine whether a physiologically relevant stimulus, ingestion of a meal, could alter 5-HT levels and 5-HT distribution in the hindgut as compared to the crayfish brain.

The organization and focus of the different chapters of this dissertation are presented as follows.

Chapter 2 has dual foci: it presents necessary background information on the anatomy and contractile behavior of crayfish hindgut and it describes the 5-HT innervation of the hindgut and the effects that 5-HT has on the hindgut. The anatomy of the hindgut and the diversity of behavioral functions suggest that the hindgut may have variable contractile properties along its length. I identify different regions of the hindgut; describe hindgut behavior, and present evidence of the variability of contractile properties in three hindgut segments as measured by frequency, force, and power.

Anatomical studies of 5-HT-ir show that following superfusion of 5-HT, 5-HT-ir is found in a large cluster of centrally located neurons that form extensive axonal projections along the length of the hindgut. In addition, both crustacean receptors, 5-$HT_{1\alpha}$ and 5- $HT_{2\beta}$, are found on the hindgut. Not surprisingly, 5-HT does have an effect on contractile properties of the hindgut. I conclude this chapter by presenting evidence of its effects on the behavioral and contractile properties of the hindgut segments identified earlier in this chapter.

Immunocytochemical studies of 5-HT on the hindgut, presented in Chapter 2, indicate that the HGNs are the only potential neuronal source of 5-HT in the hindgut.

Furthermore, levels of 5-HT in the HGN axons on the hindgut are typically very low, but increase as 5-HT concentrations applied to them increase. Questions presented in this chapter ask whether 5-HT uptake is the sole source of 5-HT in the HGNs and whether the HGNs are able to synthesize 5-HT from superfused tryptophan. Corollary questions ask whether 5-HT is taken up through SERT and whether uptake occurs primarily along the dendrites and cell soma or along the axon and terminal endings. In this chapter, I hypothesize that the HGNs borrow 5-HT, i.e., they take up 5-HT from extracellular sources for later release. Other neurons in the terminal ganglion and on the hindgut are phenotyped as well and are shown to respond uniquely to changes in tryptophan and 5-HT levels.

The labile levels of 5-HT found in the HGNs suggest that there are inconsistent supplies of 5-HT. This leads to the question of whether *in vivo* increases of 5-HT in crayfish hemolymph can account for fluctuating supplies of 5-HT in the hindgut. To answer this question, I broadened the scope of my inquiry beyond the hindgut and terminal ganglion to include the brain and the rest of the thoracic and abdominal ganglia. Chapter 4 aims to determine whether changes in the concentration of 5-HT or tryptophan in the hemolymph affect concentrations of 5-HT in the hindgut and different areas of the crayfish CNS. Changes in the levels of 5-HT and the 5-HT precursor, 5-hydroxytryptophan (5-HTP) were used to track changes in the distribution of 5-HT in the CNS and hindgut following injection of either tryptophan or 5-HT into the ventral sinus of crayfish. This chapter also identifies the primary 5-HT synthesizing regions in the crayfish CNS and the primary uptake regions.

Results presented in Chapter 4 show that increased amounts of 5-HT in the hemolymph do increase measured levels of 5-HT in hindgut and terminal ganglion. Since 5-HT is important in initiating peristalsis, I hypothesized that feeding or stimulation of the digestive organs has an effect on hindgut 5-HT levels. Chapter 5 tests whether a physiologically relevant natural stimulus, feeding, can alter 5-HT-ir or 5-HTP-ir in the HGNs and/or measured 5-HT and 5-HTP levels in the hindgut and terminal ganglion in the 48 hr after crayfish have ingested food.

I would like to acknowledge the contributions of Brian Antonsen and Nadja Spitzer, who did the 5-HT receptor labeling presented in Chapter 2. I also received assistance from Carolin Kraft for the work done on the effects of feeding on 5-HT-ir in the HGNs and on 5-HT levels in A6 and hindgut tissue. Much of this work is published as abstracts, which are listed in the reference section.

2

The differentiated crayfish hindgut: hindgut activity and the effects of serotonin on hindgut contractions

In most organisms, the hindgut or colon functions in the reabsorbtion of water and essential ions, compaction of feces and eventual elimination of fecal matter. The serial processing of fecal material requires different contractile properties along the length of what appears to be a simple muscular tube. One type of contraction compacts fecal material and other types of contractions produce movement and elimination of the fecal pellet. In addition to these common functions, some organisms, such as crayfish have a hindgut that actively "swallows" water through the anus. The intake of water is important in osmoregulation and is essential during ecdysis, when excess water is used to shed the chitinous exoskeleton (Vogt, 2002; Muramoto, 1981).

Fecal movement results from contraction of circular and longitudinal striated muscles (Prosser, 1965). The circular muscles lie underneath a membranous sheath on the serosal (outside) surface and the longitudinal muscles are obliquely arranged in six ridges that protrude into the hindgut lumen. Two types of contractions have been identified in crayfish, peristaltic and torsional (Muramoto, 1981; Brenner and Wilkens, 2001). Feces are driven to the anus by caudally directed peristaltic contractions, whereas water is taken up from the anus and driven forward by rostrally directed peristalsis (Muramoto, 1981). The torsional contraction, which has a twisting, wringing appearance (Brenner and Wilkens, 2001), also functions to move fecal material through the hindgut. These torsional movements rely more on contractions that spontaneously arise in longitudinal muscle and are regulated by pacemakers located in each of the six longitudinal folds (Ebara, 1969).

Serotonin (5-HT) is an important neuromodulator of the mammalian gut, stimulating secretory activity and producing numerous inhibitory and excitatory effects

on visceral muscle and enteric neurons (Gershon, 1999). One prominent role of 5-HT is the initiation of peristalsis, which has more recently been described as anterior moving contractions with descending muscle relaxation (Grider, 2003). 5-HT is thus an important neuromodulator that appears primarily to function in the movement of food through the alimentary canal, eventually leading to defecation. This movement is more frequently described in an aboral direction (Bayliss and Starling, 1899); however, peristalsis is produced in the oral direction as well. Reverse peristalsis is used in emesis and segmentation and, in crayfish; it may also function in the anal uptake of water.

Numerous other neurotransmitters are involved in the peristaltic reflex, but in mammals, 5-HT is needed to initiate the release of those neurotransmitters (Grider, 1994). In crayfish, numerous neurotransmitters have been identified as affecting the contractile activity of crayfish hindgut; however, none have been described as initiating peristalsis. Dopamine (Mercier et al., 1991), proctolin (Mercier et al., 1997), FMRFamide related peptides (Mercier et al., 1997), crustacean cardioactive peptide (Stangier and Keller, 1990), orcokinins (Stangier et al., 1992; Bungart et al., 1994), orcomyotropin (Dircksen et al., 2000), and glutamate (Wrong et al., 2003) produce excitatory effects on the hindgut musculature. Allostatins inhibit the strength of hindgut contractions (Dircksen et al., 1999).

The crayfish hindgut receives input from the CNS through the intestinal nerve (N7), which forms two branches, the anterior intestinal nerve (AIN) and posterior intestinal nerve (PIN). The axons in these nerves form numerous collaterals that penetrate into hindgut tissue (Elekes et al, 1988). The patterns of innervation that arise from these nerves vary in the different hindgut regions. Here I describe anatomical and functional

differences that occur along the length of the hindgut, and I tie these different regions to patterns of serotonergic innervation and receptor distribution, and to contractile responses produced by superfusion of 5-HT.

MATERIALS AND METHODS

Animals and dissection

Crayfish, *Procambarus clarkii*, were acquired from Atchafalaya Biological Supply (Raceland, LA) and maintained at 22°C in communal freshwater aquaria exposed to a 12:12 light/dark cycle. Crayfish used for physiological experiments and 5-HT immunocytochemistry (ICC) of hindgut sections were 6.2 to 10.8 cm in length from rostrum to telson. Crayfish used for receptor ICC and wholemount 5-HT ICC were 3.5 to 3.6 cm in length. They were fed shrimp pellets (Aquidine Nutritional System, Heraldsburg, CA) twice a week. Prior to experimental manipulation, crayfish were cold anesthetized and the hindgut was dissected out of the animal. For physiological studies the hindgut was irrigated with crayfish saline with the following composition: (mmol l^{-1}) NaCl, 205; KCl, 5.3; $CaCl_2$ 13.5; $MgCl_2$, 2.45; HEPES, 5; pH 7.4 (van Harreveld 1936). The hindgut was then pinned out in a Sylgard-lined (Dow Corning, Midland, MI) petri dish. Experiments were performed on irrigated sections of hindgut. Early experiments were performed on empty hindguts and subsequent experiments used a wax ovoid bead made from Surgident peripheral dental wax (Heraeus Kulzer, Hanau, Germany) inserted into the anterior end of the hindgut section. 5-HT solutions were made fresh in crayfish saline. For ICC studies, the hindgut and abdominal nerve cord with the intact seventh nerve (N7) of the A6 ganglion were removed from the crayfish.

5-HT immunoreactivity

The protocol for 5-HT ICC was derived from Beltz and Kravitz (1983). Following incubation in 1 μM 5-HT for 60 min, the hindgut and nerve cord were fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB) pH 7.4 for 18 hr at 4° C. All subsequent incubations were done on a shaker at 4° C. The tissue was rinsed twice over 24 hours in 0.1M PB with 0.3% Triton X-100 (PBTx). The tissue was then incubated in PBTx with 5% normal goat serum for 1 hr followed by incubation in rabbit anti-5-HT at a 1:200 dilution in an antiserum diluent (0.1 M PB with 0.4% Triton X-100, 0.25% bovine serum albumin, and 3% milk powder, centrifuged and filtered) for 48 hr. We used rabbit anti-5-HT antibodies from Immunostar (Stillwater, MN) at a 1:200 dilution. The tissue was then rinsed twice in PBTx over 24 hours and incubated in 1:100 goat anti-rabbit Texas Red or Alexa 488 (Molecular Probes, Eugene, OR) for 24 hr. The tissue was then rinsed in PB for 12 hr and then in 4 mM sodium carbonate hr 1 hr. Tissue was then dehydrated in an alcohol series, cleared and mounted in either Cytoseal (Fisher Scientific, Pittsburgh, PA) or methyl salicylate (Sigma-Aldrich, St. Louis, MO).

Following fixation in 4% paraformaldehyde, hindgut sections were embedded in agarose and placed in a sucrose series of 10%, 20% and 30% until they were saturated. The embedded sections were then frozen in a small beaker of isopentane that was surrounded with dry ice. The frozen chunks of agarose-encased tissue were transferred to a chuck, embedded in Tissue-Tek, and sliced at –19°C on a Leica CM 3050 cryostat. Longitudinal and cross sections 25 to 40 μm in thickness were sliced from anterior and posterior abdominal hindgut and rectum. The sections were mounted on Fisherbrand Superfrost/plus slides and dried on a slide warmer. Sections were labeled for 5-HT using

anti-5-HT 1:1000 and Alexa 488 at 1:500. Sections were mounted using 60% glycerol as a clearing agent.

Wholemount 5-HT$_{1\alpha}$ and 5-HT$_{2\beta}$ immunoreactivity

The protocol for 5-HT-ir with either 5-HT$_{1\alpha}$ or 5-HT$_{2\beta}$ immunocytochemistry (5-HT$_{1\alpha}$ir, 5-HT$_{2\beta}$ir) was derived from Spitzer et al. and Clark et al., respectively (Clark et al., 2004; Spitzer et al., 2005). 5-HT$_{2\beta}$ epitopes are identical between lobster and crayfish (Spitzer, unpublished). Preabsorption controls were done previously on the 5-HT$_{1\alpha}$ (Spitzer et al., 2005) and the 5-HT$_{2\beta}$ (Johnstone, Antonsen, and Edwards, unpublished) antibodies. The hindgut and nerve cord were dissected out of a cold anesthetized crayfish and bathed in 1 μM 5-HT for 60 min at room temperature. The tissue was then rinsed 3 times in saline at 10 min intervals and then pinned out on a Sylgard block and fixed overnight (~16 hr) at 4° C in 4% paraformaldehyde dissolved in PB. The fixed tissue was rinsed for 60 min in deionized water at room temperature and then rinsed 6 X 60 min in PBTx at room temperature. Following the rinses the tissue was placed in primary antibody: 1:50 mouse anti-5-HT (Dako, Glostrup, Denmark), 2 μg/mL rabbit anti-5-HT$_{1\alpha}$ or 5-HT$_{2\beta}$, 0.1% sodium azide, and PBTx for 40 hours on a shaker at 4° C. Following the incubation in primary antibody the tissue was rinsed 6 X 60 min in PBTX at room temperature. The tissue was then incubated on a shaker for 40 hr at 4° C in the secondary antibodies made up of 1:50 goat anti mouse CY3 (Jackson Immunoresearch, West Grove, PA), 1:50 goat anti rabbit Alexa 488, 0.1% sodium azide, and PBTx. Following incubation in secondary antibody the tissue was rinsed in deionized water for 10 min at

room temperature and then dehydrated in an ethanol series at 15 min each (10%, 30%, 50%, 70%, 80%, 90%, 100%) at room temperature. The tissue was then mounted using methyl salicylate as a clearing agent. All chemicals used were from Sigma (St. Louis, MO).

A Zeiss LSM 510 confocal microscope was used to obtain micrographs of the fluorescent immunolabeled preparations. The laser used for imaging the Alexa 488 was an Argon 488, emission: BP 505-530. The laser used for imaging Texas Red and CY3 was a HeNe 543, emission: LP560. The wholemount 5-HT-ir hindgut preparation was imaged using a 5X fluor NA 0.75 air interface objective. To image the entire hindgut we took 17 overlapping images consisting of 60-70 16.6 μm optical slices. A projected image was produced using LSM510 3.0 software and exported as a tif file into Adobe Photoshop 7.0 and assembled into a photomontage of the entire hindgut. The wholemount 5-$HT_{1\alpha}$ and 5-$HT_{2\beta}$ receptor label was imaged using a 20X Fluor NA 0.75 air interface, and 63X C-Apochromat NA 1.2 water interface objectives. Images of hindgut sections were taken using a 10X Fluor NA 0.75 air interface objective in addition to the objectives used in wholemount receptor imaging.

Physiological studies

Tension in hindgut sections was measured using a Grass PT103 transducer and recorded by Spike2 (version 4.12 by Cambridge Electronic Designs, Cambridge, England). Hindgut segments were attached to the transducer by a minutien pin attached to a larger insect pin that extended 2.5 cm in the same plane as the transducer head. The

insect pin was mounted on a screw that fit into the transducer head and then adjusted so that it was perpendicular to the axis of the transducer head. Hindgut segments were pinned at the posterior end in a 3.5 cm Sylgard-lined petri dish; the posterior segment was pinned ventral side up to expose the anus. The anterior end of each hindgut segment was attached to the transducer and adjusted so that the hindgut was taut and resting parallel to and above the Sylgard. The three segments were then superfused with saline or 5-HT using variable flow peristaltic mini-pumps for each (Fisher Scientific, Pittsburgh, PA.). Saline or 5-HT was suctioned out of the dish on the opposite side. Saline was superfused 50 min before and 50 min after a 50 min application of 5-HT. The dishes were 3.5 cm in diameter. 0.4 cm deep and held a volume of 4 to 5 mL of fluid. The rate of application was measured to be approximately 2 mL/min; with perfect mixing this rate would reach 95% of maximum concentration in 4 min.

Signals from the transducer were collected by Spike2 data acquisition software as voltage and converted to force measurements. To calibrate the force measured by each transducer, we attached a known mass to the transducer pin and measured the amplitude produced by the weight. We then converted the measurement to milliNewtons (mN). Recordings of pulsatile contractions for each preparation were performed simultaneously in parallel of all three hindgut segments.

Hindgut contractions generated brief changes in force referred to as pulses. A pulse was measured if it exceeded 0.05-0.1 V from peak to trough. Recordings of the pulsatile contractions were individually checked before automated measurements to determine the appropriate threshold for a contraction as opposed to the measurements of noise produced by the transducer prior to attaching a hindgut segment. Frequency

measurements were the inverse of the time between each pulse. Average frequency was the average of the frequency of individual pulses relative to the pulse preceding it. Changes in relative force were recorded as changes in the amplitude of the pulses.

Power measurements were collected using the Spike2 software analysis program. I used a Hanning window that had a fast Fourier transform size of 1024 (102.4 sec). The resolution was 0.0098 Hz and results ranged to 5 Hz. The data were binned into 500 sec time periods and summed for the 50 min periods. Total power measurements used the frequencies from 0.022 to 0.3 Hz because this was the frequency where the most power was observed.

Control experiments were performed on hindgut segments over the same time period as experimental measurements, three 50 min periods. The first third corresponded to baseline measurements, the second third corresponded to application of 5-HT, and the final third corresponded to the wash period.

I recorded digital movies of hindgut contractions with a Canon XL 1s digital video camcorder that was attached to a Carl Zeiss ZVS-3C75DE camera mounted on a Leica MZ6 stereomicroscope. Tension measurement records were synchronized to the videotaped contractions using a LED optical synchronizing mechanism. This mechanism produced a voltage signal on a channel of the Spike2 recording at the same time it produced a light signal on the video recording.

Statistics

All values are given as median ± 25/75 inter quartile range, except where indicated in figure legends. We used InStat3 for Macintosh (GraphPad Software, San Diego, CA) to determine significance among different groups. All analyses were done

using nonparametric statistics. When comparing baseline, experimental, and wash data we used the Friedman's test for comparisons of repeated measures of analysis of variance (ANOVA). When comparing the three segments of the hindgut we used the Kruskall Wallis ANOVA. If significance was indicated both tests were followed with Dunn's post-test. Differences were considered significant if $p < 0.05$.

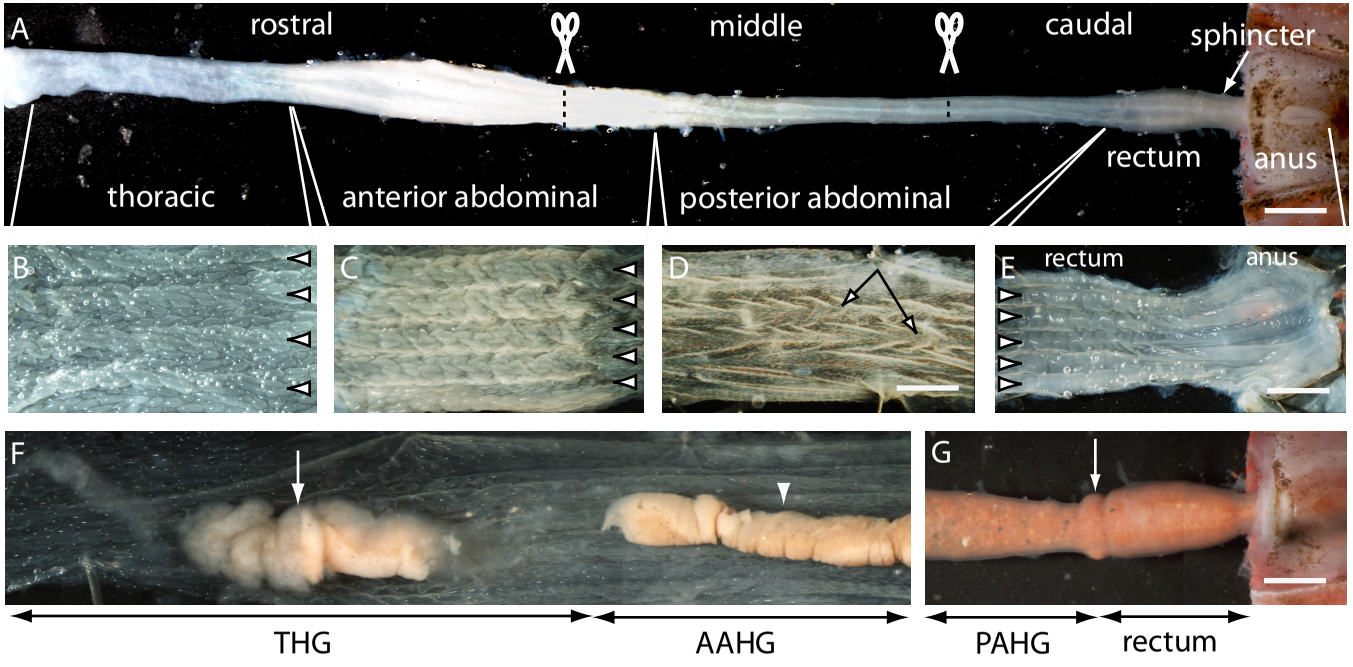
RESULTS

Hindgut regions

I distinguished six anatomically distinct regions of the crayfish hindgut. From anterior to posterior I referred to these regions as: thoracic, anterior abdominal, posterior abdominal, rectum, sphincter, and anus (Fig. 2.1A). The thoracic and abdominal regions are new distinctions; prior studies of crayfish hindgut were not explicit about the regions that were studied. The rectum and anus have been identified and discussed in previous literature (Huxley, 1880; Winlow and Laverack, 1972a, Muramoto, 1977). The thoracic hindgut (THG) is located in the thorax and superficially is distinguished by a papillate luminal surface (Fig. 2.1B). The anterior abdominal hindgut (AAHG) is located in the first two abdominal segments and has prominent longitudinal ridges (Fig. 2.1C; arrowheads) that include larger longitudinal muscle (LM) and tubular structures found within the connective tissue that extend from the ridge troughs to the ridge apex (BEM, personal observation). The posterior abdominal hindgut (PAHG) extends through abdominal segments 3-5 and lacks the glands and structures of the other anterior regions. The PAHG has the most spontaneously active LM of all hindgut regions. In Fig. 2.1D, arrows indicate two longitudinal ridges that show "V" shape indentations in the

Fig. 2.1 Gross anatomy of the crayfish hindgut. (A) The entire hindgut from its attachment at the midgut to the anus, ventral side up. Rostral, middle, and caudal experimental segments are labeled above and demarked with scissor symbols. Anatomically distinguishable regions are identified below. The correspondence of each region to detailed micrographs below (B-E) is indicated by the white lines that flank the regional label. The anatomical regions of the hindgut (HG) are the thoracic (THG), anterior abdominal (AAHG), posterior abdominal (PAHG), rectum, and anus (B-E). The luminal surface of the different hindgut regions. The longitudinal folds that show in B, C and E are indicated by arrowheads. (B) The luminal side of the THG is papillate and has the least pronounced longitudinal ridges. (C) The luminal side of the AAHG shows more pronounced longitudinal folds. (D) Two of the longitudinal ridges on the luminal side of the PAHG have V-shaped indentations that appear during a longitudinal muscle contraction (arrows). (E) The luminal side of the rectum, sphincter and anus shows more pronounced longitudinal folds. (F) Fecal material in the THG (arrow) is loose and sponge-like. Fecal material in the AAHG (arrowhead) is compacted. (G) Peristalsis is initiated adjacent to the rectum. The contraction of circular muscles produces a bulge (arrow) that travels in an anterior direction. Scale bars: A=5 mm, B-D=1 mm, E=2 mm, F,G=2 mm

Fig. 2.1


A
rostral
middle
caudal
sphincter
thoracic
anterior abdominal
posterior abdominal
rectum
anus
B
C
D
E
rectum
anus
F
G
THG
AAHG
PAHG
rectum

longitudinal fold. The apex of the “V” indicates the direction of the posteriorly moving contraction. The rectum is in the sixth abdominal segment (A6) and has large bundles of longitudinal muscle (Fig. 2.1E, arrowheads) surrounded by thick layers of circular muscle (not shown here). The sphincter and anus are in the telson and are made up of the same six longitudinal ridges. They receive central input and along with the rectum are responsible for defecation (Fig. 2.1E).

As fecal material enters the hindgut, it is wrapped in a peritrophic membrane that is thought to be secreted from midgut epithelial cells (To et al., 2004). This membrane gathers all the fecal material together into the fecal string. When the feces first enter the THG, they are loose and sponge-like (Fig. 2.1F). In the AAHG they appear compacted suggesting water reabsorption. While all regions of the hindgut initiated torsional contractions, I observed initiation of peristaltic contractions only in the PAHG (N=8) (Fig. 2.1G, arrow). Contractions of both LM and CM formed a bulge that was propagated in an anterior direction. Despite the fact that the anterior moving peristaltic wave is used for anal drinking, I observed fecal material excreted as well (data not shown, N=1). The rectum also provides support against which the sphincter contracts to regulate defecation. I observed no movement of the anal lips in hindgut preparations separated from the crayfish CNS, which is in keeping with other research describing movement of the anal lips as centrally controlled (Muramoto, 1977). In preparations where we left the CNS attached to the hindgut, dilation of the anus followed movement of the peristaltic wave (data not shown, N=3).

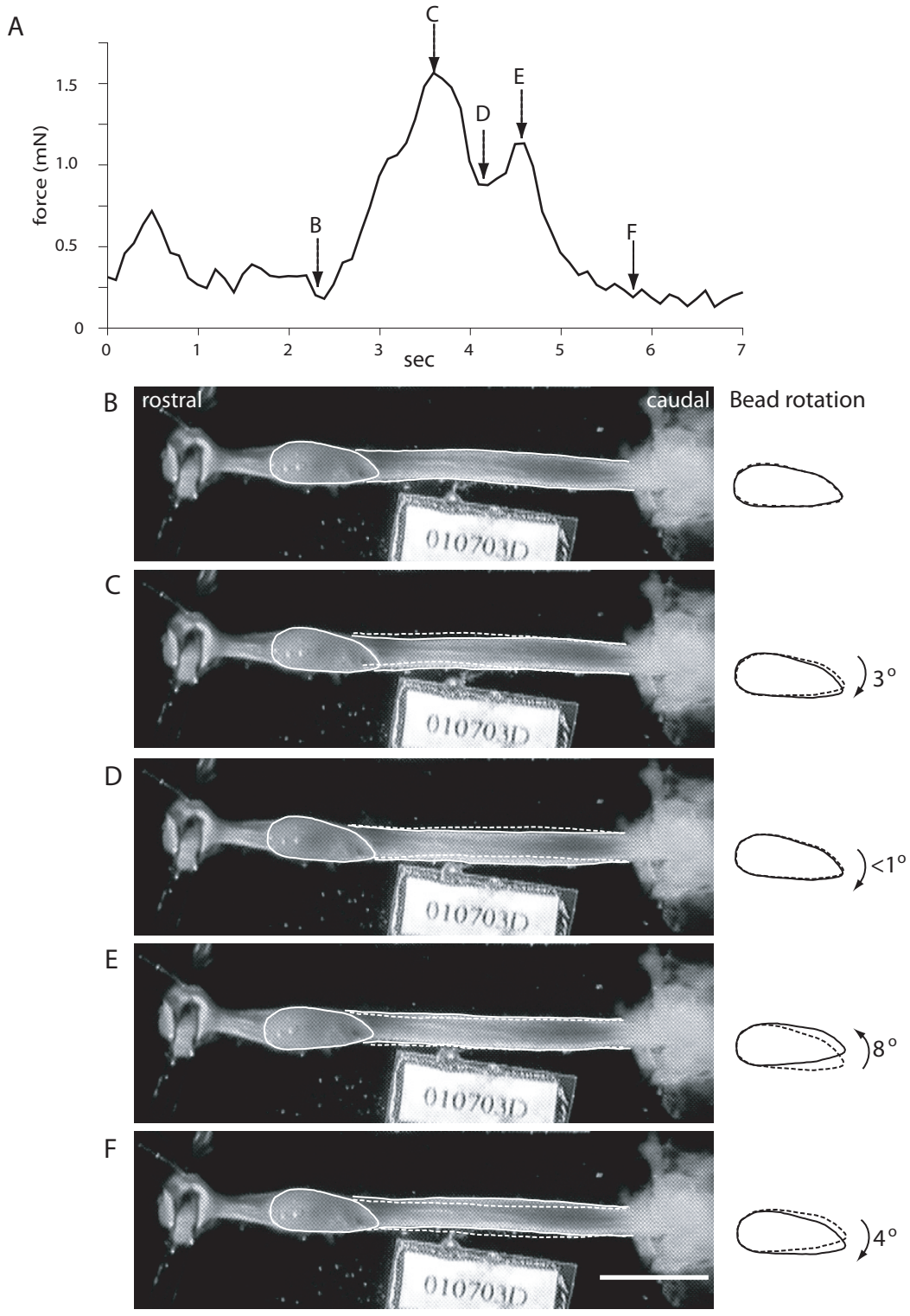
Variation in contractile properties along hindgut length

I videotaped hindgut motion in segments that included most of the PAHG, rectum, sphincter, and anus and linked images of a contraction to a measurement of tension during the contraction (Fig. 2.2A). A wax bead was inserted into the lumen of the hindgut segment to stimulate contractions. In one representative preparation, strong torsional contractions moved the hindgut from side to side and caused the wax bead to rotate through an arc of approximately 8° (Fig. 2.2B-F). The movement appeared to be linked to the contraction of one or two longitudinal muscle bundles (Fig. 2.2C). This contraction caused the posterior end of the bead to pitch to the side contralateral to the contraction. As this muscle bundle relaxed (Fig. 2.2D), other longitudinal muscle bundles contracted and pitched the bead in the opposite direction (Fig. 2.2E). The contraction of single or adjacent longitudinal muscle bundles continued rotating the bead inside the hindgut lumen (Fig. 2.2F). The torsional contractions have been described as sequential around the hindgut circumference (Brenner and Wilkens, 2001); however I was unable to determine if the recorded contractions were the result of a stereotypical sequential pattern of contractions.

To test the differences in hindgut contractile properties along the length of the hindgut, I cut the hindgut into three segments that I referred to as rostral, middle, and caudal (Fig. 2A). The rostral segment was composed of the THG and AAHG. The middle segment included the transitional area from AAHG to PAHG plus 70% by length PAHG. The caudal segment was made up of the distal 30% of PAHG plus rectum, sphincter, and anus.

Fig. 2.2 Behavior of the posterior hindgut during a contraction. (A) Tension measurement in mN (milliNewtons) of a contraction in the posterior hindgut. (B-F) Photographs of a 40 mm length of posterior hindgut taken at different times (labeled arrows) during the contraction wave shown in A. The anus is at right, the rostral end is at left, and an inserted wax bead into the anus is outlined in white. The pitching of the bead is shown at right where the current position is indicated by a solid outline and the previous position is indicated by the dashed outline. The rolling of the bead illustrates how the longitudinal contractions alternate around the circumference of the hindgut. The hindgut moved in response to contractions of discrete longitudinal muscles. This appeared in B-F as side-to-side movements that may reflect alternating contractions on opposing sides or the predominance of contractions in a particular longitudinal muscle bundle and the rebound following that contraction. Scale bar B-F=10 mm.

Fig. 2.2


A
force (mN)
1.5
1.0
0.5
0
0
1
2
3
4
5
6
7
sec
B
C
D
E
F
B
rostral
caudal
Bead rotation
C
3°
D
<1°
E
8°
F
4°

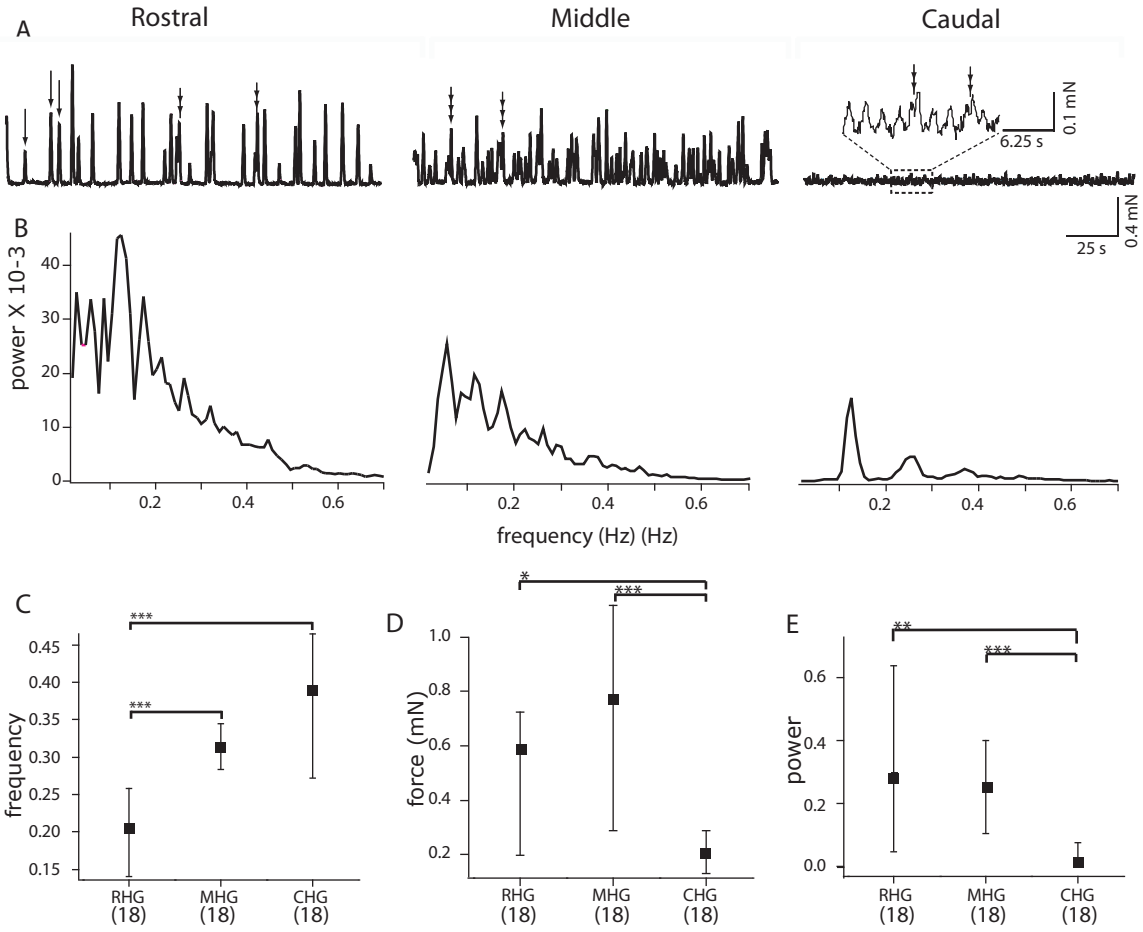
Analysis of contraction traces of all hindgut segments showed that the contractions produced by individual LM led to asynchronous contractions within a hindgut segment. Typically, each contraction pulse represented the contraction of one LM bundle, although larger pulses may represent simultaneous contractions of more than one LM bundle (Fig. 2.3A, single arrows). Contraction pulses also showed multiple peaks, suggesting that two or more LM bundles were contracting at close to the same time (Fig. 2.3A multi-headed arrows). Caudal hindgut produced more rhythmic contractions that exhibited less variation in frequency.

The power spectrum of the contractions showed that in all segments tested most of the power was in the frequency range of 0.022 to 0.3 Hz (Fig. 2.3B). The rostral and middle segments showed power in a greater range of frequencies than the caudal segment. The frequencies in which the caudal segment showed power were more discrete, occurring at the central frequency and harmonics of the oscillation recorded there.

Measurements of average frequency, force and power show that the different hindgut segments significantly differed in their contractile properties. Median frequency (see Methods) was significantly higher in the middle and caudal segments than in the rostral segment (Fig. 2.3C). The amount of force was greatest in the rostral and middle segments; the caudal segment produced the least amount of force (Fig. 2.3D). I also compared total power within the range of 0.02 to 0.3 Hz among the different hindgut segments (Fig. 2.3E). I chose this range because total measured power of the contractions in the three segments showed the greatest differences in this range. The analysis showed

Fig. 2.3 Tension measurements of contractile activity in hindgut segments. Average measurements of force, amplitude and power were based on contractions produced over a 50 min period. The entire hindgut was divided into 3 segments, labeled above each column as illustrated in Fig 2 and described in Methods. (A) Contraction patterns of individual LM bundles differed among the hindgut segments. Contraction of an individual LM bundle produced an impulse of force (single arrows). It was not uncommon to see contractions with multiple peaks produced by the contractions of several individual LM bundles at close to the same time (multiple headed arrows). (B) Power spectrum analysis (see Methods) of the contractions showed that the greatest power was in very low frequencies. In the rostral and middle segments, power varied continuously across the frequency spectrum while the caudal segment showed power in more discrete frequency bands. (C-E) The frequency, amplitude, and power of muscle pulsatile contractions (see Methods) in the three segments. (C) The rostral hindgut had significantly fewer pulsatile contractions than middle and caudal hindgut. (D) The force of pulsatile contractions was significantly less in caudal hindgut than in rostral and middle segments. (E) Contractions in caudal hindgut had significantly less total power than in the rostral and middle hindgut. (C-E) Means and standard deviations. Significance is indicated by asterisks with * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$. The number of preparations for each experiment is indicated in parentheses.

Fig. 2.3


A
Rostral
Middle
Caudal
0.1 mN
6.25 s
0.4 mN
25 s
B
power X 10-3
frequency (Hz) (Hz)
C
frequency
D
force (mN)
E
power
RHG (18)
MHG (18)
CHG (18)

that rostral and middle segments had significantly greater power in the range of 0.02 to 0.3 Hz than the caudal segment.

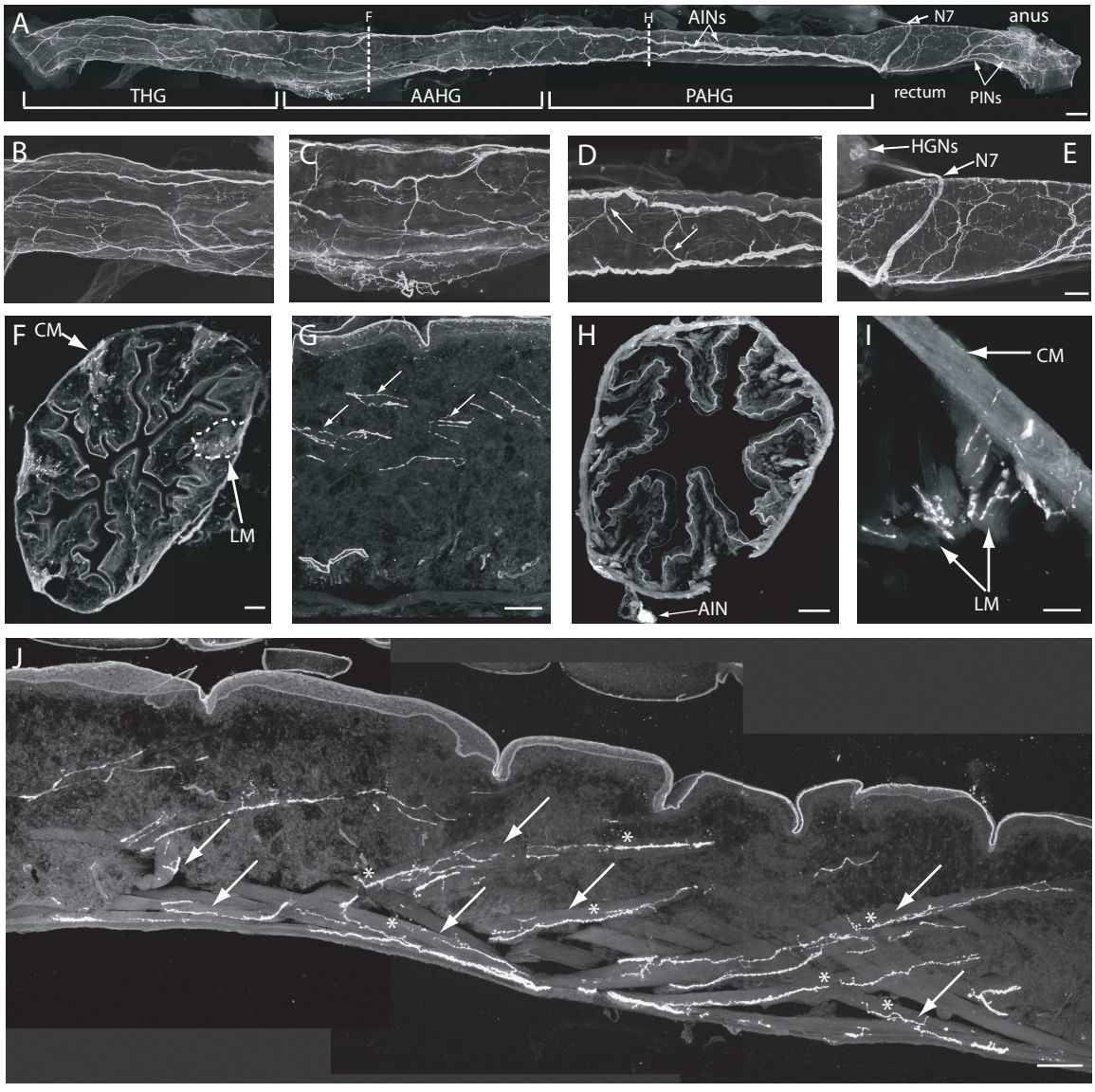
5-HT-ir on the hindgut

5-HT immunocytochemical studies of the hindgut reveal that following superfusion of 5-HT, 5-HT-ir is found in hindgut neuron somata and the AIN and PIN projections on the hindgut. On the THG, the AINs formed four branches that run along the anterior-posterior axis (Fig. 2.4B). Fine varicose 5-HT-ir fibers in the THG are in the region of the longitudinal muscles, possibly lying along them (Fig. 2.4B). On the AAHG, the AIN branches exhibited numerous projections that formed an irregular network of fibers; branches from the AINs extended anteriorly along the hindgut serosal surface and split to form branches that penetrated the longitudinal ridges (Fig. 2.4C). In the PAHG region, the AINs were organized in two large fascicles on the serosal surface of the hindgut. Short collaterals projected perpendicular to a fascicle and split into either a T-shaped or Y-shaped trajectory, before innervating the longitudinal muscles in the PAHG (Fig. 2.4D). On the rectum, the PINs split into numerous branches that eventually formed varicose connections on the circular muscles of the rectum (Fig 2.4E). On the anus, the PINs branched more profusely (Fig. 2.4A).

Fine varicose nerve fibers labeled for 5-HT were on both the LM and CM of the hindgut (Fig. 2.4F-I). These 5-HT-ir fibers projected along the LM bundles that were embedded in the six longitudinal folds of the hindgut and along the CM fibers situated below the hindgut sheath (Fig. 2.4F). The 5-HT-ir fibers in most instances clearly innervated muscle, but some varicose fibers in AAHG are not associated with muscle

Fig. 2.4 5-HT-ir in the hindgut. (A) The entire hindgut as in Fig. 2, with experimental and anatomical sections indicated. 5-HT-ir was found on the entire hindgut located primarily in the HGNs that project through N7 of A6 to the rectum. At the rectum the HGNs formed two major collaterals, the AINs and the PINs. The AINs formed projections on the anterior half of the rectum, the PAHG, AAHG, and THG. The PINs formed projections on the posterior half of the rectum, sphincter, and anus (scale=500 μm). (B-E) Magnified views positioned below the appropriate anatomical segment identified in (A). (B) 5-HT-ir of THG shows further branching of AINs along the longitudinal axis of the hindgut. (C) 5-HT-ir of AAHG. (D) 5-HT-ir of PAHG shows large AIN fascicles and small branches that extend to adjacent longitudinal folds. (E) 5-HT-ir of A6 and rectum shows labeling of the HGN somata and their projections through N7 to the rectum. 5-HT-ir on the rectum is found on circular muscles (scale for B-E= 250 μm). (F) A cross section of the AAHG (location of section indicated by dashed line in A) showed 5-HT-ir on longitudinal (LM, outlined) and circular muscles (CM) (scale=100 μm). (G) In a longitudinal section of AAHG, varicose 5-HT-ir fibers (arrows) were sometimes found unassociated with muscles (scale=100 μm). (H) In a cross section of PAHG the AINs are found external to the hindgut sheath (location of section indicated by dashed line in A; scale=100 μm). (I) A higher magnification of the transition from CM to LM showed that the same varicose 5-HT-ir fibers projected to both muscles (scale=20 μm). (J) A longitudinal section of the AAHG shows 5-HT-ir varicose fibers (arrows) following the interwoven longitudinal muscle bundles (*) (scale=100 μm).

Fig. 2.4


A
F
H
AINs
N7
anus
THG
AAHG
PAHG
rectum
PINs
B
C
D
HGNs
N7
E
F
CM
LM
G
H
AIN
I
CM
LM
J

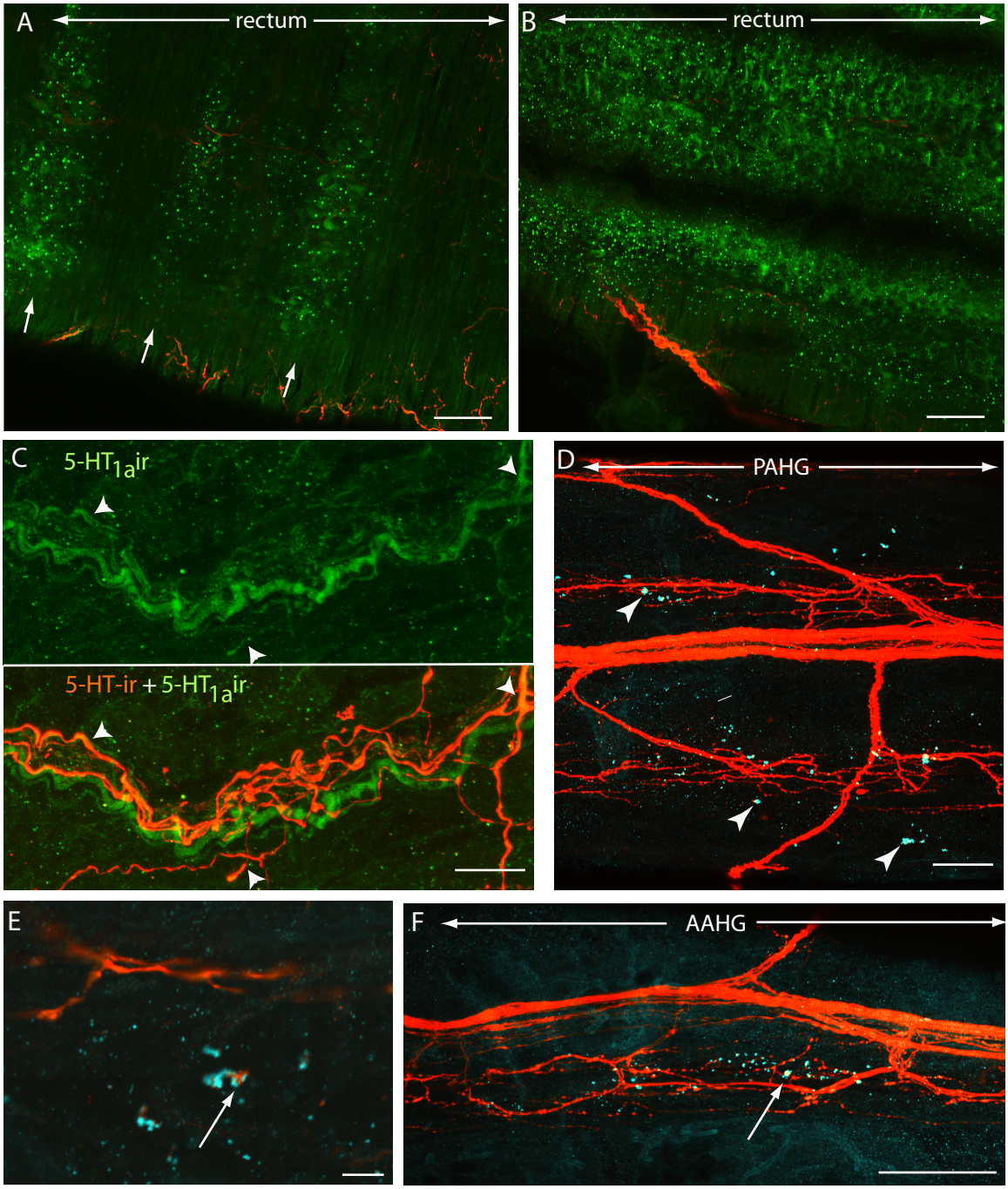
(Fig. 2.4G, arrows). They either innervated a different tissue or are involved in paracrine signaling. In a cross section of PAHG (Fig. 2.4H, I), individual varicose 5-HT-ir fibers were seen that innervated both CM and LM. I observed that most of the 5-HT-ir in THG, AAHG and PAHG regions was found on LM. The arrangement of LM and 5-HT-ir of AAHG illustrated in Fig. 2.4J showed an interwoven pattern of LM fibers that ran oblique to the anterior-posterior axis.

5-HT$_{1\alpha}$ and 5-HT$_{2\beta}$ receptor distribution on the hindgut

Two 5-HT receptors, 5-HT$_{1\alpha}$ and 5-HT$_{2\beta}$, have been identified in decapod crustaceans (Clark et al., 2004; Spitzer et al., 2005). ICC labeling for both receptors was found on the hindgut, but the pattern of labeling differed. 5-HT$_{1\alpha}$ immunoreactivity (5-HT$_{1\alpha}$ir) was primarily associated with CM (Fig. 2.5A) and with LM (Fig. 2.5B) of the rectum, and in axons along the length of the hindgut (Fig. 2.5C). Labeling of the CM appeared as distinct bands of diffuse 5-HT$_{1\alpha}$ir plaques. The fine receptor plaques on the CM and the LM were not directly adjacent to 5-HT varicosities. The anatomical regions anterior to the rectum exhibited a low density of fine 5-HT$_{1\alpha}$ir plaques. AIN axons and their collaterals co-labeled for 5-HT-ir and 5-HT$_{1\alpha}$ir, however most of the 5- HT$_{1\alpha}$ir appeared in AIN fibers that were not labeled for 5-HT. The receptor labeling in the AINs appeared diffuse. In THG, AAHG, and PAHG, 5-HT$_{1\alpha}$ir appeared in the larger AIN fascicles, with intense label in all regions of the hindgut except the distal region of THG, which showed very little axonal 5-HT$_{1\alpha}$ir.

Fig. 2.5 Differential expression of 5-$HT_{1\alpha}$ (green) and 5-$HT_{2\beta}$ (blue) receptors on the hindgut. (A) 5-$HT_{1\alpha}$ir is expressed in bands on the CM of the rectum but not with 5-HT-ir. (B) 5-$HT_{1\alpha}$ir is also found in the six longitudinal folds of the rectum, where the LM is located. (C) Co-labeling of 5$HT_{1\alpha}$ir and 5-HT-ir in the area of the hindgut where PAHG adjoins the rectum. In images of the same section, 5-$HT_{1\alpha}$ir is shown alone at top and together at bottom. Arrowheads indicate areas where 5-$HT_{1\alpha}$ir and 5-HT-ir are co-localized in AIN axons. 5-$HT_{1\alpha}$ir is diffuse in the axons, which suggests that trafficking of receptor protein occurs there. (D) 5-$HT_{2\beta}$ir is expressed as large plaques in PAHG (arrowheads). (E) A large 5-$HT_{2\beta}$ir plaque (arrow) is associated with a 5-HT-ir varicosity in the PAHG. (F) Small 5-$HT_{2\beta}$ir plaques in anterior regions of AAHG (shown) and THG appear to be on fine fibers that innervate the longitudinal muscles. Some plaques are associated with 5-HT-ir varicose fibers (arrow). Scale bar for A, B, D, F=100 μm; C=20 μm; E=10 μm.

Fig. 2.5



In contrast to 5-$HT_{1\alpha}$ir, very few 5-$HT_{2\beta}$ir plaques were found on the rectum and no 5-$HT_{2\beta}$ir was in projection fibers (not shown). The PAHG had larger and more prominent plaques associated with the longitudinal folds (Fig 2.5D). Similar plaques were found in the proximal THG. Occasionally, a large plaque of 5-$HT_{2\beta}$ receptors was found associated with a 5-HT varicosity, suggesting a synaptic relationship (Fig. 2.5E). Larger 5- $HT_{2\beta}$ir plaques were also found in AAHG associated with the longitudinal folds. In THG, the 5-$HT_{2\beta}$ir plaques were smaller and appeared to be associated with both 5-HT-ir fibers and unlabeled fibers more often than in other hindgut regions (Fig. 2.5F).

5-HT initiates and modulates contractions of the hindgut

Initial experiments that examined hindgut contractions using irrigated, empty caudal segments (the distal 30% of PGHG plus rectum, sphincter, and anus) showed spontaneous contractions in approximately 30% of these segments. 10 nM and 30 nM applications of 5-HT failed to initiate contractions in such preparations, whereas application of 100 nM to 1 μM 5-HT reliably initiated contractions and in caudal hindgut would initiate peristalsis (Fig. 2.6A, Table 1). A wax bead inserted into the lumen of an unmodulated hindgut superfused with saline reliably produced contractions.

The caudal hindgut was videotaped and contractions were simultaneously recorded to describe the peristaltic behavior that was initiated by 1 μM 5-HT. The contraction trace captured a weak rostrally moving peristaltic wave (Fig. 2.6B_1), which is the contraction of one segment with a relaxation of a contiguous segment. Following application of 5-HT, peristaltic contractions were initiated by a contraction around the circumference of the hindgut that was anterior to the rectum (Fig. 2.6B*i*, *ii*).

Table 2.1 Initiation of contractions by 5-HT in quiescent, empty posterior hindgut. Concentrations of 5-HT above 100 nM reliably initiated hindgut contractions whereas below 100 nM did not induce contractions in empty hindgut.

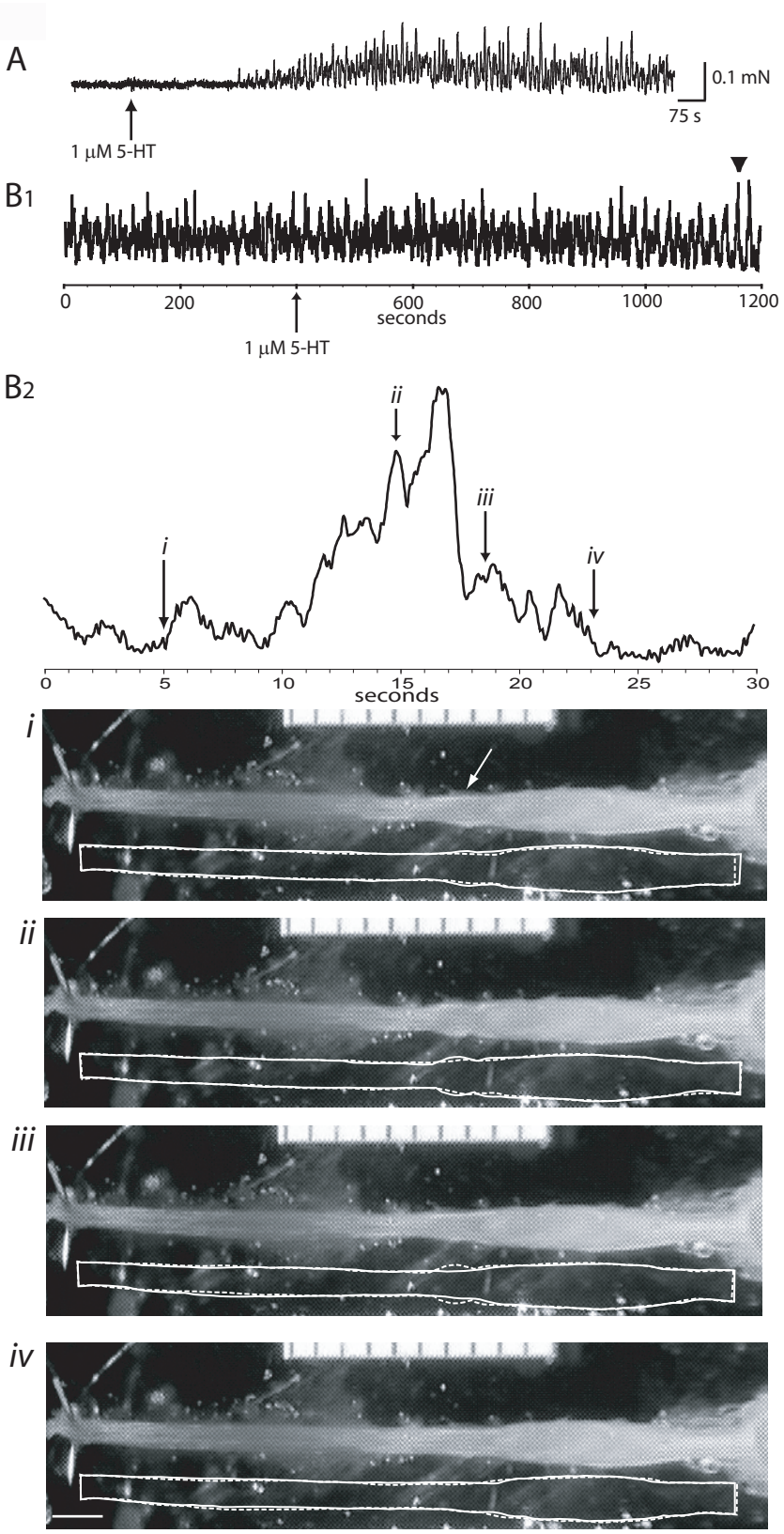
Table 2.1

Initiation of contractions by 5-HT

[5-HT]	# quiescent	# initiated
30 nM	2	0
100 nM	2	2
300 nM	1	1
1 μM	4	4

Fig. 2.6 Response of hindgut to application of 1 μM 5-HT. (A) Tension recording of a quiescent caudal hindgut where 1 μM 5-HT initiated contractions. (B) Tension recordings of hindgut in which 1 μM stimulated peristalsis. (B_1) Application of 1 μM 5-HT initiated weak peristaltic waves at approximately 975 sec. The arrowhead shows the location of the peristaltic wave shown in B_2. (B_2) Single peristaltic contraction wave. Points (*i-iv*) are marked on the tension trace of a peristaltic contraction and videotaped images are shown below that are synchronized to those times. (*i)* The hindgut created tension on the transducer by coordinated contractions of longitudinal and circular muscles that form a slight bulge anterior to the rectum (arrow). The solid white line is the shape of the contraction; the dotted white line indicates the shape of the contraction in *iv*. (*ii)* The bulge became more prominent as the contraction became stronger. The dotted white line is the outline of the contraction in *i*. (*iii)* In strong peristaltic waves, the bulge is reproduced in more anterior sections. Here it appears that circular muscles narrow the hindgut circumference and moving tension measurements towards the baseline. The dotted white line is the outline of the contraction in *ii*. (*iv)* The hindgut prepares for another peristaltic motion through circular muscle contractions that produce a sphincter-like narrowing of the area anterior to the rectum. The dotted white line is the outline of the contraction in *iii*. In *iii* and *iv* it is possible to observe in the more proximal region a slight side-to-side motion that is seen in torsional contractions of the hindgut under control conditions (Fig. 3).

Fig. 2.6


A
0.1 mN
75 s
1 μM 5-HT
B1
0
200
600
800
1000
1200
seconds
1 μM 5-HT
B2
ii
iii
i
iv
0
5
10
15
20
25
30
seconds
i
ii
iii
iv

This contraction appeared as a bulge in the hindgut (Fig. 2.6B*i*, arrow). Following, the hindgut narrowed around the circumference in that same area (Fig. 2.6B*iii*). The peristaltic contractions displayed less of the twisting and side-to-side motion seen during the torsional contractions that appeared produced by longitudinal muscles (Figs. 2.3; 2.6B*i-iv*)

5-HT modulates frequency, force, and power of contractions in hindgut segments

5-HT modulated contractions in all three areas of the hindgut. I measured frequency, force and power during a 50 min baseline period, a 50 min period during which different concentrations of 5-HT were superfused, and a final 50 min washout period. All segments of the hindgut appeared responsive to superfusion of 1 μM 5-HT (Fig. 2.7A). In the rostral segment, 1 μM 5-HT gave rise to an increase in small amplitude contractions, which were similar to those observed when 300 nM 5-HT was applied (Fig 2.7B_1).

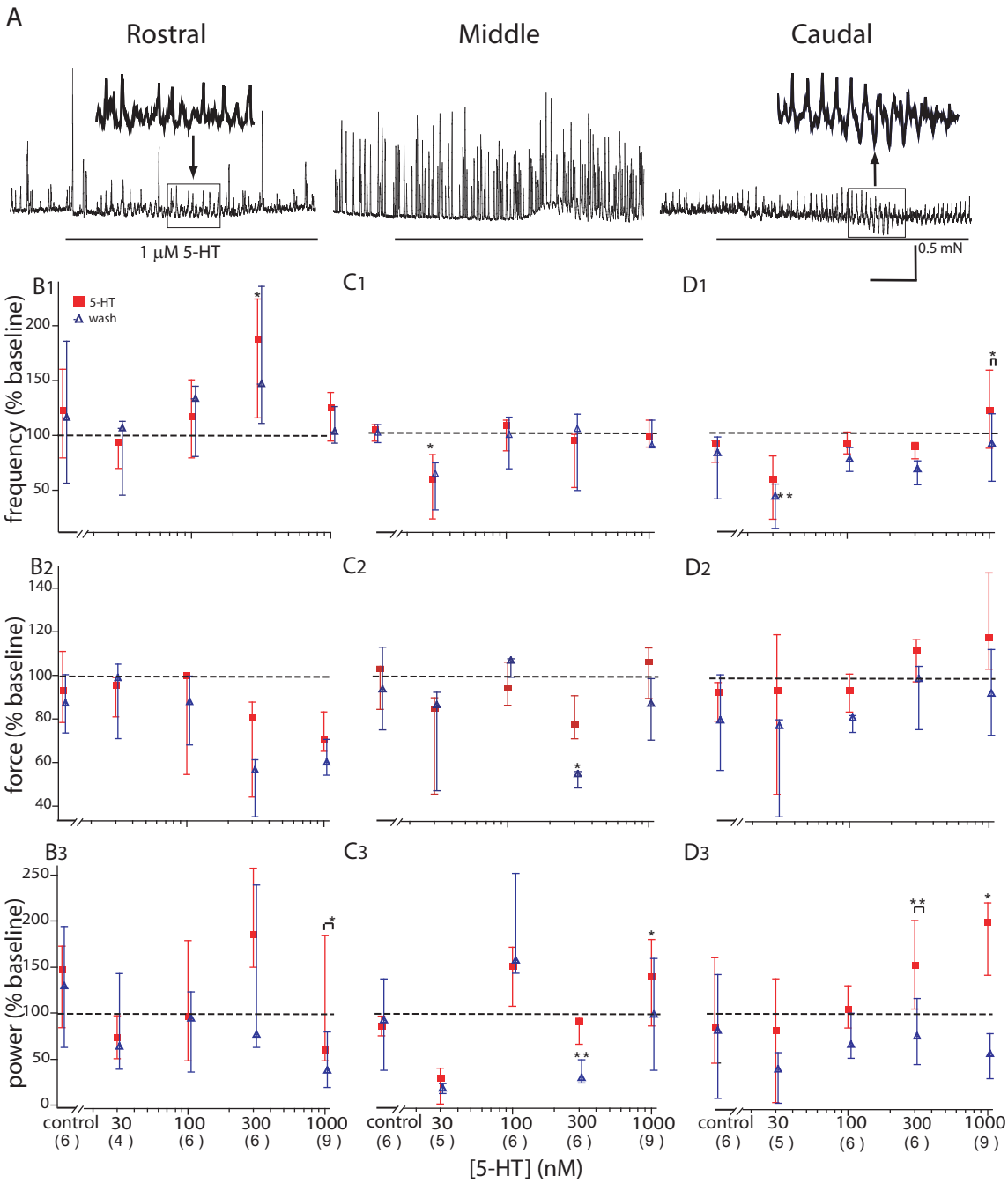
In middle hindgut there was a brief increase in tonus after approximately 5 min. In caudal hindgut, there was a period where the contractile pulses reversed sign and became negative. These occurred as peristaltic waves transiently unloaded the transducer, producing brief changes in force below the basal tonus. Application of 1 μM 5-HT reliably produced peristalsis in caudal hindgut (7 of 8).

Application of 30 nM, 100 nM, 300 nM and 1 μM 5-HT did not produce dose response effects in frequency, force or power (Fig. 2.7B-D), (control results at left in all graphs). In rostral hindgut, application of 300 nM 5-HT led to significantly increased contraction

Fig. 2.7 Effects of 5-HT on frequency, force, and power of hindgut contractions. (A) Tension traces from separate segments of the same hindgut showing response to application of 1 μM 5-HT (bar). The rostral hindgut trace exhibited a brief increase in small amplitude contractions. The middle hindgut tension trace exhibited a brief increase in tone. The caudal hindgut tension trace produced a downward deflection, which is characteristic of anterior moving peristaltic waves. (B-D) Dose-response plots for each segment where pulsatile contraction frequency (B_1-D_1), amplitude (force) (B_2-D_2) and power (B_3-D_3) are presented for responses to four different 5-HT concentrations and a control. Control measurements are on the left of each graph. Median measurements are shown normalized to baseline, represented by the broken line. Error bars represent 25/75 inter quartile range, and Friedman statistics were performed on raw means of the 50 min bins. Red boxes represent responses to 5-HT and dark blue open triangles represent responses to washout of 5-HT. (B_1) In rostral hindgut, 300 nM 5-HT produced a significant increase in contraction frequency compared to baseline (Friedman (Fr) statistic = 7.000, Dunn's post-test). (B_2) Rostral hindgut showed no significant change in force (Fr statistic = 2.333). (B_3) In rostral hindgut, washout of 1 μM 5-HT significantly decreased power compared to when 1 μM 5-HT was applied (Fr statistic = 8.222, Dunn's post-test). (C_1) In middle hindgut, 30 nM 5-HT produced a significant decrease in contraction frequency from baseline (Fr statistic = 7.600, Dunn's post-test). (C_2) In middle hindgut, washout of 300 nM 5-HT significantly reduced force from baseline (Fr statistic = 9.333, Dunn's post-test). (C_3) In middle hindgut, washout of 300 nM 5-HT significantly power from baseline (Fr statistic = 10.333, Dunn's post-test). Application of 1 μM 5-HT significantly increased force from baseline (Fr statistic = 8.222, Dunn's

post-test). (D_1) In caudal hindgut, washout of 300 nM 5-HT decreased contraction frequency from baseline (Fr statistic = 10.333, Dunn's post-test) and significantly decreased during wash following application of 1 μM 5-HT (Fr statistic = 7.750, Dunn's post-test). (D_2) Caudal hindgut showed a trend for force to increase as the concentration of 5-HT increased. (D_3) In caudal hindgut, 1 μM 5-HT significantly increased power from baseline levels and decreased power during washout of 5-HT (Fr statistic = 13.000, Dunn's post-test). The number of preparations used for quantification is in parentheses in the power row. Significance is indicated by asterisks with * = $p<0.05$, ** = $p<0.01$.

Fig. 2.7


A
Rostral
Middle
Caudal
1 μM 5-HT
0.5 mN
B1
C1
D1
5-HT
wash
frequency (% baseline)
B2
C2
D2
force (% baseline)
B3
C3
D3
power (% baseline)
control
30
100
300
1000
(6)
(4)
(5)
(9)
[5-HT] (nM)

frequency over baseline measurements (Fig. 2.7B_1). This increase in small amplitude contractions negatively impacted the force of contractions. Rostral hindgut also showed a reduction in force during the wash following superfusion of 1 μM 5-HT (Fig. 2.7B_3). In middle hindgut, application of 30 nM 5-HT significantly reduced frequency from baseline measurements (Fig. 2.7C_1). 300 nM 5-HT significantly reduced force and power from baseline during the wash (Fig. 2.7C_2, C_3). Application of 1 μM 5-HT significantly increased power over baseline levels (Fig. 2.7C_3). In caudal hindgut, application of 30 nM 5-HT reduced frequency from baseline during the wash and 1 μM 5-HT reduced frequency during wash compared to 5-HT application (Fig. 2.7D_1). Force and power measurements appear to show dose response effects (Fig. 2.7D_2) with power significantly increased during application of 1 μM 5-HT (Fig. 2.7D_3).

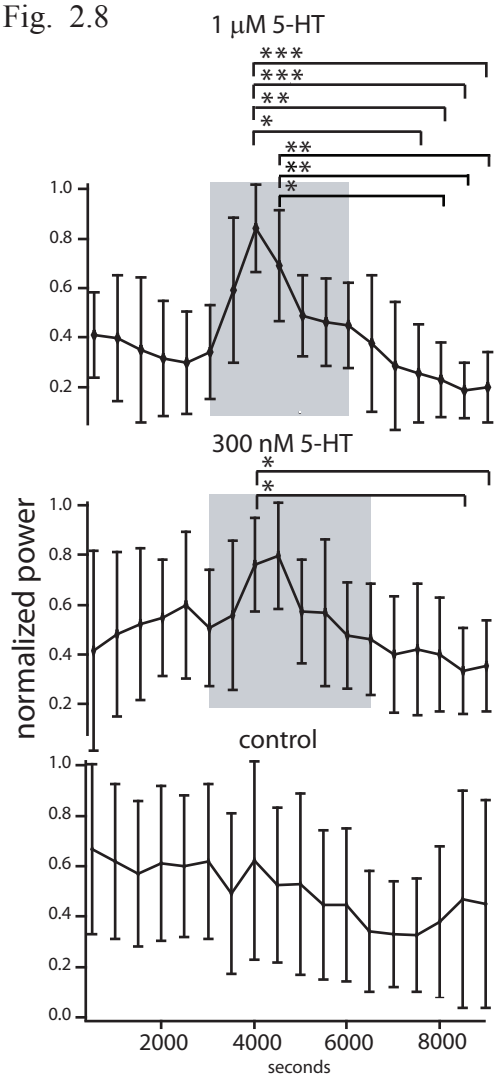
Changes in power over time in caudal hindgut showed that 5-HT effects were phasic (Fig. 2.8). During application of 300 nM or 1 μM 5-HT, it took approximately 15 min for power to peak before declining. Peak power measured following application of 1 μM 5-HT was significantly greater during wash out of 1 μM 5-HT. The peak power measured during application of 300 nM 5-HT was significantly higher than at time points measured during the wash; peak power measured during application of 5-HT did not differ significantly from baseline measurements of power. Control measurements of total power over time indicated a steady decline from initial measurements.

Effects of 5-HT on the power spectra of contractions

The power spectra of contractions indicate the power of contractions at particular frequencies. Greatest power occurred in the frequency range of 0.022 to 0.7 Hz; only

Fig. 2.8 Total power of contractions over time in the caudal hindgut. Total power peaked close to 15 min following application of 1 μM and 300 nM 5-HT. Following application of 1 μM 5-HT total power significantly varied ($p<0.0001$, Fr statistic = 56.159, Dunn's post-test) and was significantly different at 4000 s and 4500 s then at the indicated time periods during wash. Following application of 300 nM 5-HT total power also significantly varied ($p<0.001$, Fr statistic = 35.385, Dunn's post-test) and was significantly different at 4000 s than at the indicated time periods during wash. Control measurements over the same time period showed no such peak in total power and no significant differences ($p>0.15$, Fr statistic 22.878). Error bars in the graph show SEM. Shaded area of the graph indicates period of time when 5-HT was applied (see Methods for details). Total power measurements were normalized to highest measured total power within each preparation and then the normalized measurements were averaged. Significance is indicated by asterisks with * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Fig. 2.8


1 μM 5-HT
***
***
**
*
**
**
*
300 nM 5-HT
*
*
control
normalized power
1.0
0.8
0.6
0.4
0.2
0.0
2000
4000
6000
8000
seconds

power in this frequency range was analyzed further. Non-parametric ANOVA statistics were used to determine differences in power during baseline, 5-HT application, and wash conditions, using the mean normalized value at each frequency. To control for natural variations in hindgut contractions over time, I compared the power spectra recorded from untreated hindgut segments to the power spectra of the experimental hindgut segments (see Methods). I focused on the experimental results from application of 300 nM and 1 μM 5-HT because these concentrations produced significant changes in hindgut contractile properties (Fig. 2.7-8).

The rostral and middle hindgut showed the most pronounced effects on the power spectra of contractions when 300 nM 5-HT was superfused. In rostral hindgut, power of contractions in the frequency range of 0.022 Hz to 0.55 Hz significantly increased and then significantly decreased during washout of 300 nM 5-HT (Fig. 2.9A, B). In control rostral hindgut, there was a modest increase in mean power during the second 50 min time period (when 5-HT was applied to experimental hindgut segments), which persisted during the last 50 min time period (Fig. 2.9C). In middle hindgut segments, application of 300 nM 5-HT decreased mean power from baseline in the frequency range of 0.022Hz to 0.55 Hz (Fig. 2.9D,E). During washout of 300 nM 5-HT, mean power decreased further. In control middle hindgut segments, mean power increased from the first 50 min time period to the second 50 min and this persisted during the final 50 min (Fig. 2.9F).

Application of 1 μM 5-HT had the strongest effects on the middle and caudal hindgut power spectra (Fig. 2.10). In middle hindgut segments, application of 1 μM 5-HT increased mean power in the frequency range of 0.022 to 0.7 Hz (Fig. 2.10A,B). Removal of the 5-HT by wash eliminated that increase in mean power.

Fig. 2.9 Power spectrum analysis of contractions in rostral and middle hindgut before, during, and after application of 300 nM 5-HT. (A,B) In rostral hindgut, the power of contractions increased significantly over pre-exposure levels (baseline) during application of 300 nM 5-HT. Power during washout of 5-HT was less than baseline and less than power during application of 300 nM 5-HT (ANOVA $p<0.0001$, Fr statistic 121.04, Dunn's post-test $p<0.001$). (C) In control rostral hindgut segments, mean power in the low frequencies increased in the second and final 50 min time period from the first 50 min (ANOVA $p<0.0001$, Fr statistic 69.831, Dunn's post-test, $p<0.001$). There was a significant difference between the second and final 50 min time period in the power spectrum ($p<0.05$). (D,E) In the middle hindgut, power in the low frequencies significantly declined during application and during washout of 300 nM 5-HT (ANOVA $p=0.0001$ Fr statistic = 142.00, Dunn's post-test, $p<0.001$). (F) In control middle hindgut segments, mean power increased significantly in the second and final 50 min over the first 50 min (ANOVA $P<0.0001$, Fr statistic = 84.615, Dunn's post-test $p<0.001$). Mean power measurements in A and D show standard deviation.

Fig. 2.9

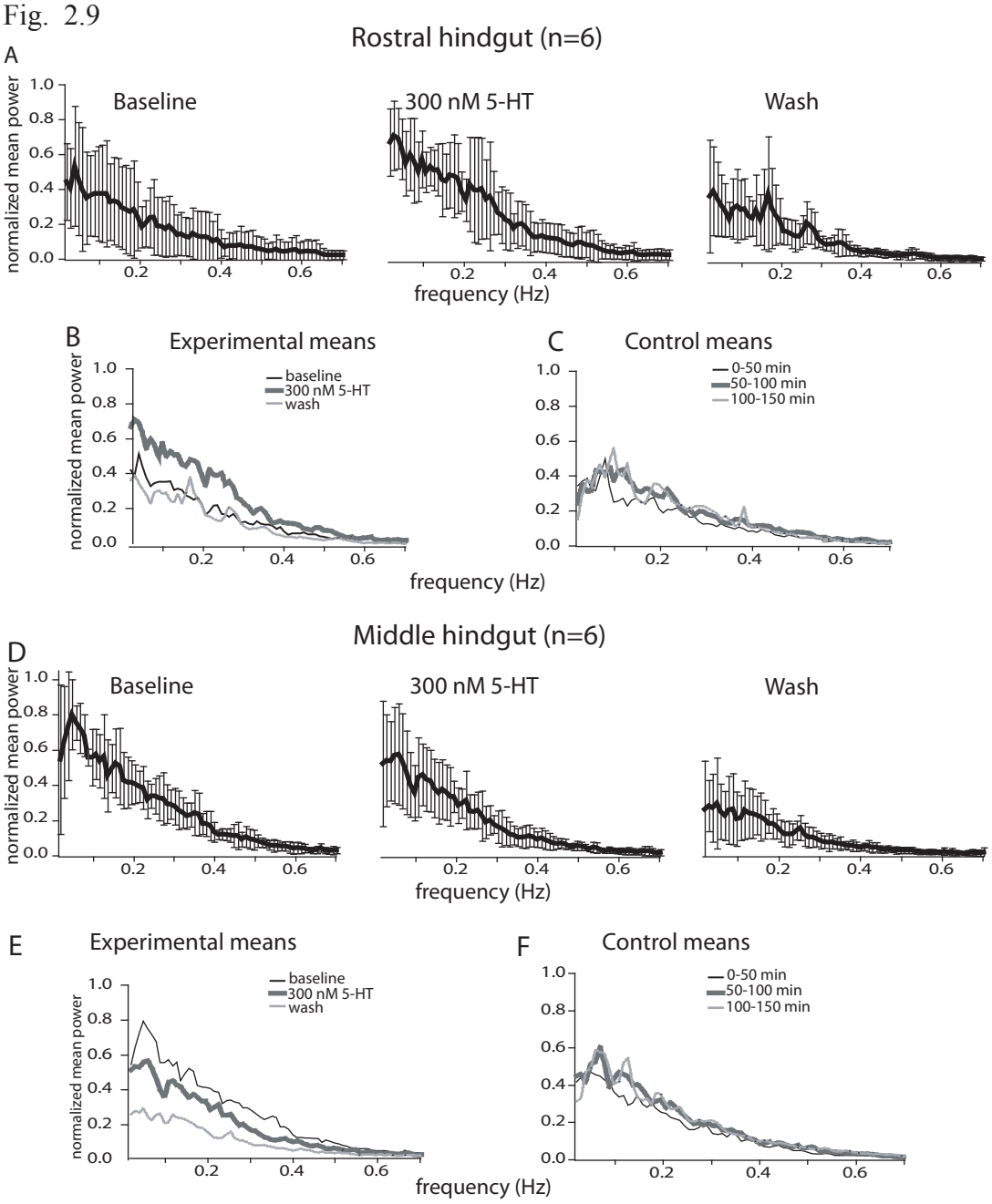
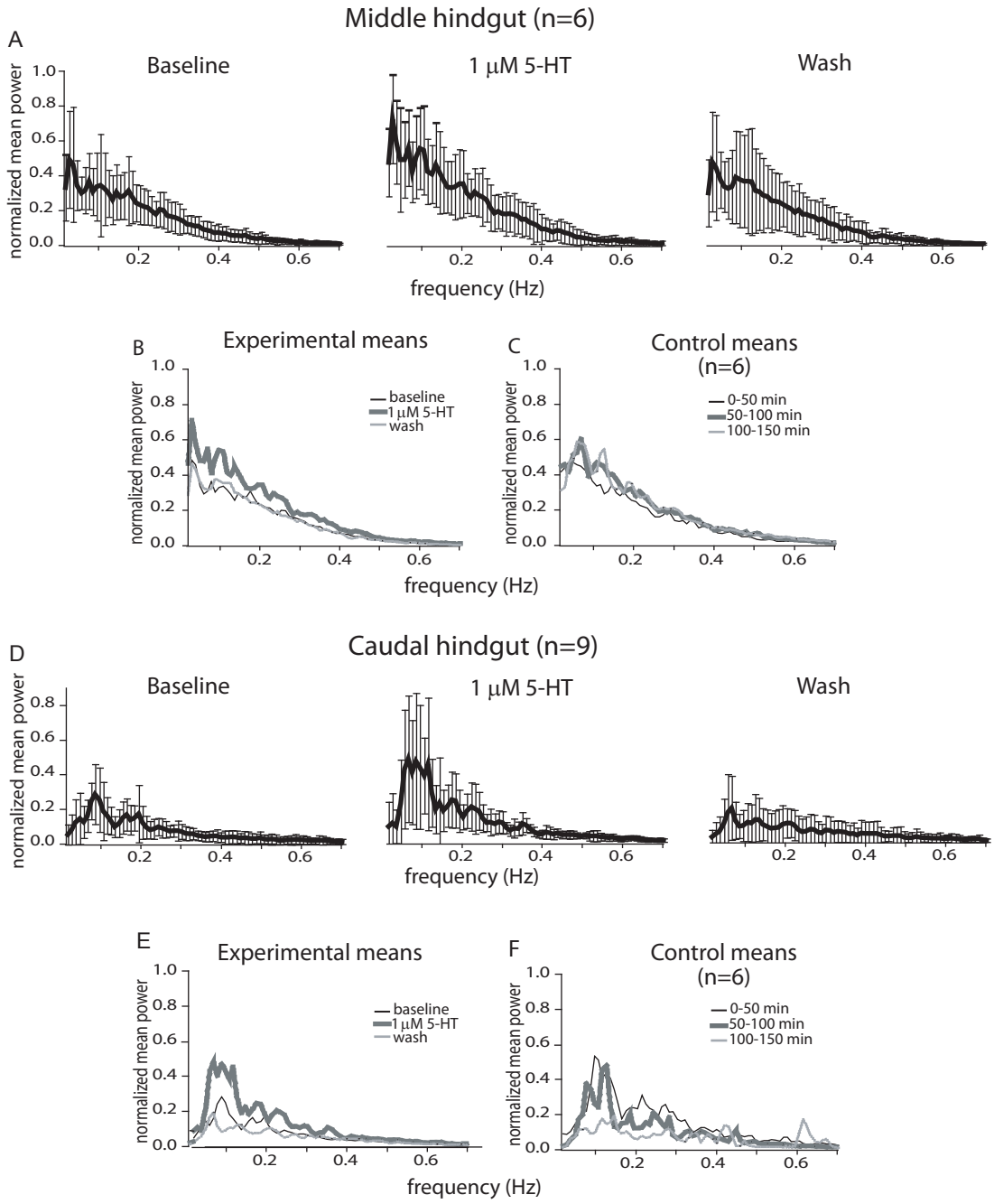

Rostral hindgut (n=6)
A
Baseline
300 nM 5-HT
Wash
normalized mean power
frequency (Hz)
B
Experimental means
baseline
300 nM 5-HT
wash
C
Control means
0-50 min
50-100 min
100-150 min
Middle hindgut (n=6)
D
E
F

Fig. 2.10 Power spectrum of contractions of the middle and caudal hindgut before, during, and after application of 1 μM 5-HT. (A,B) In middle hindgut, power significantly increased over baseline during application of 1 μM 5-HT (ANOVA $p<0.0001$, Friedman's test). During the wash, power significantly decreased from power during application of 1 μM 5-HT (ANOVA $p<0.0001$, Fr statistic = 115.94, Dunn's post-test baseline vs. 1 μM 5-HT or wash $p<0.001$, 1 μM 5-HT vs. wash $p<0.01$) (C) Control middle hindgut was discussed in the legend of Fig. 2.9 F.. (D,E) In caudal hindgut, 1 μM 5-HT significantly increased power in the frequencies range of 0.022 to 0.125 Hz (ANOVA $P<0.0001$, Fr statistic = 103.69, Dunn's post-test $p<0.001$). The power in those frequencies significantly decreased during the wash from baseline and from levels reached during application of 1 μM 5-HT ($p<0.001$). There was no significant difference between baseline and washout of 1 μM 5-HT. (F) In control caudal hindgut, mean power significantly decreased between the first 50 min time period and both the middle and final 50 min time period (ANOVA $p< 0.0001$, Fr statistic = 67.127, Dunn's post-test $p<0.001$ for both). There was no significant decrease in mean power between the middle 50 min time period and the final 50 min time period. Error bars in A and D show standard deviation.

Fig. 2.10


Middle hindgut (n=6)
A
Baseline
1 μM 5-HT
Wash
normalized mean power
frequency (Hz)
B
Experimental means
baseline
1 μM 5-HT
wash
C
Control means
(n=6)
0-50 min
50-100 min
100-150 min
Caudal hindgut (n=9)
D
E
F

The power spectra of control middle hindgut, as mentioned above, showed a less robust increase in mean power than that measured during the experimental application of 1 μM 5-HT (Fig. 2.10C). During the final 50 min in control middle hindgut there was no decline in mean power as was measured during washout of 1 μM 5-HT (Fig. 2.10B).

In caudal hindgut segments, application of 1 μM 5-HT significantly increased mean power from baseline levels in the frequency range of 0.022 to 0.125 Hz (Fig. 2.10E). The power in those same frequencies decreased during washout of 1μM 5-HT. In control caudal hindgut, the power spectrum showed a decrease in mean power over the three time segments examined (Fig. 2.10F). The decrease in mean power from the first 50 min time period and the middle 50 min time period contrasts with the increase measured in caudal hindgut during application of 1 μM 5-HT.

DISCUSSION

The crustacean hindgut differs in its anatomy, contractile properties, and serotonergic profile along its length. Newly identified anatomical regions include the thoracic and anterior and posterior abdominal regions. Contraction frequencies increased on the anterior-posterior axis and the force and power of contractions decreased along the same axis (Fig. 2.3). All segments of the hindgut produced wringing torsional contractions sometimes spontaneously but always in response to luminal stimulation by a wax bead. Movement of the bead occurred in a posterior direction through the middle and caudal segments (Fig. 2.2). Contractions in the rostral segment did not propel the wax

bead. Torsional contractions were produced by alternating contractions of one or a few longitudinal muscle bundles.

The hindgut is innervated by the HGNs and other neurons in A6 and more anterior ganglia. The HGNs are a prominent cluster of cells that project from the distal medial portion of A6 to the rectum and that exhibit 5-HT-ir. The 5-HT-ir showed here indicates different innervation patterns of the HGNs in the identified hindgut regions (Figs. 2.1 and 2.4). Serotonin produces measurable effects that are in part mediated by the5-$HT_{1\alpha}$ and 5- $HT_{2\beta}$ receptors located on the hindgut. It is likely that other 5-HT receptors exist as well. In leech pharynx, application of different 5-HT agonists produced distinct patterns of contractile activity leading to the conclusion that several receptors were likely responsible for the complex contractile responses (O'Gara et al., 1999). In crayfish, 5-$HT_{1\alpha}$ is found on the CM and LM of the rectum and 5- $HT_{2\beta}$ is on the LM of PAHG and AAHG (Fig. 2.5). The 5-$HT_{1\alpha}$ir was more diffuse and less often associated with 5-HT-ir, although it was co-localized in some 5-HT-ir fibers (Fig. 2.5C). The co-localization with some 5-HT-ir fibers (Fig. 2.5C) is similar to that observed throughout the crayfish nerve cord (Spitzer, 2005). 5-$HT_{2\beta}$ir was also diffusely distributed, but exhibited larger receptor plaques that were occasionally co-localized with 5-HT varicosities (Fig. 2.5D-F).

Serotonin both enhanced and reduced contractile properties of the hindgut segments. Superfusion of 300 nM 5-HT and greater initiated contractions in empty and quiescent caudal hindgut segments (Table 2.1, Fig. 2.6) and 1 μM 5-HT reliably initiated peristalsis in caudal hindgut (Figs. 2.6, 2.7). Hindgut segments did not show dose responses to increasing concentrations of 5-HT. Superfusion of 30 nM 5-HT led to a

decrease in frequency of pulsatile contractions in middle hindgut. Superfusion of 100 nM 5-HT had no effect on frequency force or power in any hindgut segments. Superfusion of 300 nM 5-HT produced antagonistic responses between rostral and middle hindgut. The frequency of pulsatile contractions increased in rostral hindgut and increased power in all frequencies of the power spectrum (Figs. 2.7, 2.9), however 300 nM 5-HT decreased contraction force of middle hindgut segments and decreased power in all frequencies of the power spectrum (Fig. 2.7, 2.9). 1 μM 5-HT increased total power and power in all frequencies of the power spectrum in middle and caudal hindgut segments (Figs. 2.7, 2.8, 2.10). The contrasting effects of 300nM and 1 μM 5-HT in middle hindgut suggest that this region may operate as a switch in regulating movement of the fecal string with lower levels inhibiting movement and higher levels stimulating fecal movement.

Washout of 5-HT led to reduced contractile responses. Power decreased compared to baseline following washout of 30 nM 5-HT in caudal hindgut. Washout of 300 nM 5-HT reduced force and power in middle hindgut and during washout of 1 μM 5-HT, the total power of contractions in rostral and caudal segments was reduced compared to the power of contractions produced during 5-HT superfusion (Figs. 2.7). Similar washout effects have been recorded in leech pharynx (O'Gara et al., 1999).

Hindgut anatomy and contractile properties

The muscular motions of the hindgut must support ion and water reabsorption, compaction of feces, and propulsion and defecation of the fecal pellet. The unmodulated hindgut produced primarily torsional contractions, which relied more on asynchronous contractions of the LMs. In their study of the lobster *Homarus gammarus*, Winlow and

Lavarack (1972b, 1972c) posited that the uncoordinated contractions were produced by separate oscillators in LM and CM. These oscillators functioned independently of central input, as our results show occurs in crayfish and effectively moved matter posteriorly in the middle and caudal segments of the hindgut (Fig. 2.3). Measurements of the contractile properties of LM strips suggest that these muscles are responsible for the variation in force and higher frequency contractions (Mercier and Lee, 2002). Measurement of CM rings suggests that the measured lower frequency contractions would alter muscle tone of the hindgut (Mercier and Lee, 2002).

The crayfish hindgut produces peristalsis and reverse peristalsis, which may function in several physiological processes. Reverse peristalsis is speculated to produce water uptake during the molt, generate fluid pressure to assist defecation, and to permit rumination, propelling the contents in the anterior portion of the gut back to the cardiac sac to be further digested (Muramoto, 1977; Fox, 1952; Vogt, 2002). Cuticular spines that project towards the anus function to prevent anterior movement of the compacted fecal pellet during reverse peristalsis and to provide channels for water to flow anteriorly (Chisaka et al., 1999).

Most studies of crustacean gut indicate that peristalsis is centrally initiated (Miller, 1910; Winlow and Laverack, 1972a, 1972b, 1972c). Central control of defecation in the lobster arises in the brain and local input is from the A6. Peristalsis was not reversed and led to defecation (Winlow and Laverack, 1972b). Winlow and Laverack (1972b, 1972c) speculated that sensory input from ventral soft cuticle receptors, proctodaeal cuticle receptors, and pyloric sensory cells fed back to the brain and activated two types of interneurons, one which produced phasic input and regulated the

contractions in longitudinal muscle and another which produced tonic input and produced the peristaltic wave. In crayfish reverse peristalsis has been more frequently described, probably because the preparations examined local input from A6 and not central input from the brain (Alexandrowicz, 1909). Our results indicated that local input from A6 was not necessary to produce forward moving or reverse peristalsis, and that the motor program that produced peristalsis could be initiated by 5-HT (Fig. 2.7).

Serotonin and serotonin receptors on the hindgut

Serotonin is an important neurohumoral transmitter (Hansen, 2003) that has clear effects on mammalian visceral muscle. The responses to 5-HT we describe in crayfish hindgut are not unlike what has been described in other invertebrate and vertebrate visceral muscle. A study of *C. elegans* showed that 5-HT increased the contraction-relaxation cycle of pharyngeal muscle (Niacaris and Avery, 2003). Another study on leech pharynx attributed four separate contractile responses to 5-HT; it increased basal tonus, increased large, slow, phasic contractions and smaller phasic contractions with a). frequency of 1 Hz, and it led to a relaxation of pharyngeal muscle following washout of 5-HT (O'Gara et al., 1999). In mammals, depending on what section of the digestive tract was studied, 5-HT increased amplitude of contractions; it stimulated circular muscle and longitudinal muscle; it stimulated motility by inducing phasic contractions; it activated microcircuits that initiate peristalsis; and it evoked hypermotility (Hansen, 2003).

As in mammalian gut, hormonal levels of 5-HT may also be responsible for some of the measured 5-HT effects. The 30 nM application reduced activity in middle hindgut. The diffuse punctuate labeling seen for the 5-$HT_{1\alpha}$ receptor suggests that the hindgut

would be sensitive to hormonal levels of 5-HT. 5-HT already functions as a hormonal signal in decapod crustacean foregut activity (Beltz et al., 1984; Katz et al., 1989). 5-HT is released directly into the hemolymph by the pericardial organs and by plexuses associated with ganglionic nerve roots (Beltz, 1999). The 5-HT released is pumped by the heart through the dorsal abdominal artery, which supplies the hindgut with blood. This humorally supplied 5-HT would then act on receptors associated with the hindgut muscles. In mammalian gut, broadcast release of 5-HT from the enterochromaffin cells leads to excitation of sensory neurons that release calcitonin gene receptor protein (CGRP) and it is the CGRP that actually initiates peristalsis (Grider, 1994).

The increase in motility and initiation of peristalsis, however, required higher concentrations of 5-HT and it is not obvious where concentrations close to synaptic levels are found. In addition, the effectiveness of higher concentrations of 5-HT but the scarce co-localization of 5-HT-ir with receptor plaques suggests that 5-HT more likely operates as a paracrine signal rather than synaptically. The effectiveness of concentrations equal to or greater than 1 μM 5-HT was observed in studies of peristalsis in leech pharynx where concentrations between 1 μM and 1 mM produced more robust effects (O'Gara et al., 1999).

Levels of 5-HT in crayfish hindgut are normally low as measured by HPLC and typically 5-HT-ir is faint in the HGNs (Musolf and Edwards, 2003). The 5-HT-ir presented here required superfusion of 5-HT to produce a signal that would allow for visualization of the HGNs and their projections (Fig. 2.4). It is not known whether these neurons can release the 5-HT they take up. The HGNs, a subset of the previously identified efferent neurons, contain vesicles in the synaptic connections on the hindgut

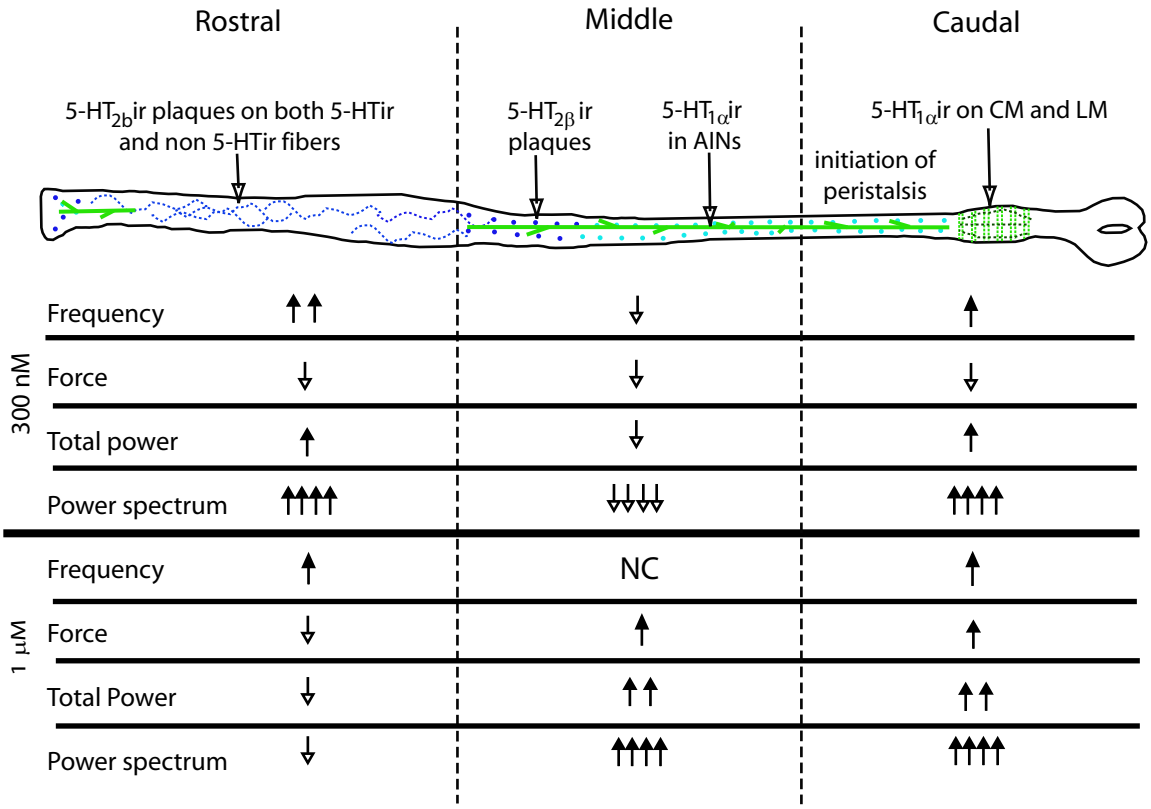
(Elekes et al., 1988), however, the 5-HT that is taken up needs to be repackaged into vesicles for release. Similar neurons exist in *C. elegans* that are cholinergic and take up 5-HT and repackage it using the vesicular monoamine transporter (Duerr et al., 2001). The HGNs could be taking up 5-HT from the hemolymph, concentrating it and then re-releasing the 5-HT to produce the different contractile effects that I observed with higher concentrations of 5-HT. However, further studies are needed to determine that the 5-HT that is taken up by the HGNs is released. The anatomical variations of 5-HT-ir in the different hindgut regions, the different branching patterns of the 5-HT-ir fibers and the differential distribution of the two 5-HT receptors and their association and lack of association with 5-HT varicosities and fibers may underlie some of the regional variations found in 5-HT effects (Fig. 2.11).

Serotonin effects on the hindgut

In response to superfusion of 5-HT, the changes in frequency, force, and power in the three hindgut segments suggest that 5-HT may play a role in coordinating the processing and movement of fecal material through the hindgut. The functions of the crustacean hindgut are to compress undigested particles, regulate water uptake through either reabsorption from the digesta or through anal swallowing, reabsorb ions from digesta, lubricate fecal material to ease passage of the fecal string, and propel and eliminate the fecal string through torsional contractions and peristaltic waves. Serotonin is likely to be involved in at least two of these processes: 5-HT effects on middle hindgut may regulate movement of the fecal string, and 5-HT effects on posterior hindgut may regulate defecation by means of stimulating strong peristaltic waves. In middle hindgut,

Fig. 2.11 Summary of regional differences in 5-$HT_{1\alpha}$ir and 5-$HT_{2\beta}$ir expression and serotonergic modulation of contractile activity in hindgut sections. Regional differences in 5-HT receptors may underlie the different responses of hindgut segments to 5-HT. 5-$HT_{1\alpha}$ir (green) plaques are found on both LM and CM of the rectum and in the axons of HGNs located in middle and caudal hindgut. Large 5-$HT_{2\beta}$ir (blue) plaques are found in middle and caudal hindgut and smaller plaques are found opposite 5-HT-ir varicosities and appear to be associated with other non-5-HT-ir fibers in rostral hindgut. Applications of 300 nM and 1 μM 5-HT produced different hindgut behaviors. At 300 nM 5-HT, frequency significantly increased in rostral hindgut without attendant increases in force or total power. There was a significant increase in very low frequency contractions as seen in the power spectrum. At this same dose in middle hindgut, force and low frequencies in the power spectrum were significantly reduced. The middle hindgut likely plays a major role in moving the fecal string through the hindgut. The reduction in force and power would slow the movement of the fecal string at 300 nM 5-HT. At 1 μM 5-HT, there is little change from baseline in rostral hindgut. In middle hindgut, the force of contractions is no longer significantly reduced, total power and mean power in the low frequencies is significantly increased. In caudal hindgut, both force and total power are significantly increased and peristaltic waves are often induced. This increase in contractile activity would assist the middle hindgut in moving the fecal string through the hindgut. One arrow indicates a variation from the median (frequency, force, power) or mean (power spectrum), two arrows = $p<0.05$, and four arrows = $p<0.001$.

Fig. 2.11


Rostral
Middle
Caudal
5-HT2bir plaques on both 5-HTir and non 5-HTir fibers
5-HT2β ir plaques
5-HT1αir in AINs
initiation of peristalsis
5-HT1αir on CM and LM
300 nM
Frequency
Force
Total power
Power spectrum
1 μM
Frequency
NC
Force
Total Power
Power spectrum

application of 300 nM 5-HT led to a reduction in force and power, which likely shows movement of the fecal string. Application of 1 μM 5-HT to middle hindgut led to little change in force but led to a significant increase in power in low frequency contractions, which could enhance movement of the fecal string. These two opposing effects suggest that the middle hindgut can function as a switch to regulate movement of the fecal string. Application of 1 μM 5-HT to caudal hindgut initiated peristalsis and increased force and power of contractions, which could reflect the strong peristaltic waves that accelerate movement of the fecal string and lead to defecation (See Fig. 2.11 for summary).

Serotonin effects on rostral hindgut are more difficult to interpret; the increase in small amplitude contractions when 300 nM 5-HT is applied may be used to compact or mix fecal material. Unlike the other two segments rostral hindgut did not propel the inserted wax bead and is likely not involved in propelling the fecal string through the hindgut. It is of note, though, that 300 nM 5-HT reduces force in the middle hindgut, which may reduce movement of the fecal string. I suggest that when digesta first enters into the hindgut it could be necessary to slow down fecal movement and give the anterior hindgut time to reabsorb water and salts from the digestive juices and waste. The application of 300 nM 5-HT appeared to induce this behavior. In contrast, superfusion of 1 μM 5-HT restored the force and increased power of contractions in middle hindgut. At 1 μM 5-HT the fecal string would move more rapidly through this hindgut segment, aided by the initiation of peristalsis and the significant increase in power and force produced in posterior hindgut.

This study of crayfish hindgut shows that, just as in vertebrate gut, 5-HT initiates the peristaltic response and modulates hindgut musculature. Interestingly, there is no

crayfish equivalent of the vertebrate enterochromaffin cells, endocrine cells that synthesize 95% of the 5-HT in the mammalian enteric system and which release 5-HT in response to mechanical or chemical stimulation (Erspamer, 1966). My studies indicate that one possible source for 5-HT could be the HGNs that have projections on the hindgut that exhibit 5-HT-ir. Another source for 5-HT is the hormonal release of 5-HT from the pericardial organs and neurosecretory sites associated with the CNS. While the anatomical details and the source of 5-HT appear to be very different between crayfish and vertebrate gut, the same neurotransmitter plays similar roles in these systems.

3

Borrowed serotonin in crayfish hindgut neurons

The most salient phenotypical features of a serotonergic neuron consist of the enzymes that synthesize and metabolize serotonin (5-HT), a vesicular transporter to transport 5-HT into vesicles for release, and a plasma membrane transporter to curtail the activity of 5-HT. Neurons that do not have this full complement of serotonergic elements have been described as orphan neurons (Weihe and Eiden, 2000) or as accumulating neurons (Schutte and Witkovsky, 1990). These neurons most often lack the enzymes for synthesizing 5-HT and instead express the vesicular monoamine transporter (VMAT) and/or the 5-HT plasma membrane transporter (SERT). The orphan neurons or 5-HT accumulating neurons acquire 5-HT through gap junctions formed with 5-HT synthesizing neurons or through uptake (Vaney et al., 1998; Bunin and Wightman, 1999).

Typically, 5-HT is taken up by a serotonin transporter (SERT) found either on the presynaptic neuronal terminals or along the axon (Zhou et al., 1998). In most organisms, the 5-HT taken up can be either metabolized or transported into vesicles by a VMAT for subsequent release. In the latter case, this relocation of 5-HT into neuronal circuits downstream from the original 5-HT neurons adds further complexity and range to 5-HT effects.

Two kinds of 5-HT accumulation are described in the literature: transient 5-HT accumulation, which is thought to play a role in neuronal outgrowth and morphogenesis (Lebrand et al., 1996; Hansson et al., 1998; Beltz et al., 2001; Richards et al., 2003); and persistent 5-HT accumulation, which may play a role in fine tuning neurotransmission and regulating hormonal levels of 5-HT (Jackowski et al., 1988; Schutte and Witkovsky, 1990). This latter type of accumulation may be particularly important in an organism that has an open circulatory system, such as the crayfish, where the nervous system is not

buffered by a blood brain barrier (Fig. 3.1A). Hormonal release of 5-HT provides opportunities for non-serotonergic neurons that have SERT to accumulate 5-HT.

The variability of 5-HT-ir and the ability of an accumulating neuron to acquire 5-HT led to the speculation that 5-HT is possibly borrowed and reused (Ehinger and Floren, 1976; Beaudet and Descarries, 1979; Schutte, 1994; Vanhatalo and Soinila, 1994). I found that the hindgut neurons (HGNs) in the terminal ganglion (A6) of crayfish do not normally display 5-HT-ir, but can be induced to do so when exposed to 5-HT. Because serotonin promotes motility of the hindgut and the two crustacean 5-HT receptors, 5-$HT_{1\alpha}$ and 5-$HT_{2\beta}$ are located on the hindgut, (Chapter 2; Musolf and Edwards, 2004; Musolf et al., 2005), I asked whether the HGNs are 5-HT accumulating neurons that cannot make 5-HT, but can take it up to use in modulating hindgut activity.

I found that the HGNs persistently take up 5-HT through a transporter that is blocked by a selective serotonin reuptake inhibitor (SSRI) and that 5-HT uptake can occur both centrally and peripherally, allowing 5-HT to undergo both anterograde and retrograde transport. The HGNs have the enzyme to convert the 5-HT precursor, 5-hydroxytryptophan (5-HTP) to 5-HT, but they are unable to convert tryptophan to 5-HTP under *in vitro* conditions. The HGNs either do not have the rate-limiting enzyme, tryptophan hydroxylase (TpOH), or it is infrequently active.

MATERIALS AND METHODS

Animals and Dissection

Adult crayfish, *Procambarus clarkii*, ranging in size from 4.6 to 6.5 cm from rostrum to telson, were acquired from Atchafalaya Biological Supply (Raceland, LA). Juvenile crayfish, 2.5-3.8 cm from rostrum to telson, were hatched from adults acquired from Atchafalaya Biological Supply and raised in the lab. Crayfish were maintained at 22°C in freshwater aquaria while exposed to a 12:12 light:dark cycle. Prior to experimental manipulation, crayfish were anesthetized in a 0-4°C icy bath for 15-30 min and the ventral nerve cord and hindgut were dissected out of the animal in crayfish saline of the following composition (in mM): 202 NaCl, 5.37 KCl, 13.53 $CaCl_2$, 2.6 $MgCl_2$, and 2.4 HEPES, pH 7.5 (van Harreveld, 1936). The hindgut was irrigated with crayfish saline and both the hindgut and nerve cord were pinned out in a Sylgard-lined petri dish or processed for quantification of 5-HT levels. Pharmacological reagents applied to the hindgut and nerve cord were dissolved in crayfish saline.

Pharmacology

Uptake was examined in 3 different groups of crayfish (2.5 to 4.5 cm from rostrum to telson). Group 1 was incubated in saline 80-150 min. Group 2 was incubated in 10-50 μM of 5-HT (serotonin creatine sulfate, # H7752, Sigma, St. Louis, MO) 260 min for 10 μM 5-HT and 70-150 min for 50 μM 5-HT. Group 3 was incubated in 10 μM of 5-HT plus 10 μM paroxetine 145-265 min (GlaxoSmithKline, London, UK).

Retrograde and anterograde transport was investigated in crayfish (5.5 to 6.5 cm from rostrum to telson) by placing the hindgut and nerve cord in separate chambers linked by the intestinal nerve (N7). The nerve cord was pinned out in a Sylgard-lined petri dish and N7, which projects from the distal end of A6 to the hindgut, was threaded through a groove in a smaller incubation chamber. The hindgut was placed in the smaller incubation chamber and the groove was filled with petroleum jelly (Vaseline) to separate the two chambers. To investigate anterograde transport, the nerve cord was incubated in 1 μM 5-HT for 120 min and the hindgut was in saline, rinse for the hindgut in 5-HT was 15 min. To investigate retrograde transport, the hindgut was incubated in 1 μM 5-HT and the nerve cord was in saline over the same time period.

Synthesis of 5-HT was examined by incubating the hindgut and nerve cord in either tryptophan or 5-HTP and labeling for 5-HT or 5-HTP. Application of tryptophan tested for activity of both enzymes in the 5-HT synthetic pathway: TpOH, which converts tryptophan to 5-HTP, and L-amino acid decarboxylase (L-AADC), which converts 5-HTP to 5-HT. The hindgut and nerve cord were incubated in 100-200 μM of L-tryptophan for 120 to 150 min with and without 1 mM *m*-hydroxybenzylhydrazine (NSD-1015), an L-AADC inhibitor, and rinsed for 15 min. Conversion of 5-HTP to 5-HT was examined through application of 1 mM 5-HTP for 135 min and rinsed for 20 to 45 min. All of the above reagents used were from Sigma-Aldrich (St. Louis, MO).

Whole-mount Immunocytochemistry

Experimental sets of animals were processed together. The immunocytochemical protocol was identically and simultaneously applied to each tissue in a set. The 5-HT

immunocytochemical protocol was one modified from Beltz and Kravitz (1983). Both hindgut and nerve cord were fixed in 4% paraformaldehyde for 18 hr. They were rinsed twice over 24 hr in 0.1 M phosphate buffer with 0.3% Triton X-100 (PBTx). The tissue was then incubated in PBTx with 5% normal goat serum for 1 hr followed by incubation in rabbit anti-5-HT at a 1:200 dilution in an antiserum diluent (0.1 M PB with 0.4% Triton X-100, 0.25% bovine serum albumin, and 3% milk powder, centrifuged and filtered) for 48 hr. Several rabbit anti-5-HT antibodies were used: Eugene Tech, Incstar, and DiaSorin (Stillwater, MN). The rabbit anti-5-HTP antibody was purchased from DiaSorin and Immunostar (Stillwater, MN). The tissue was then rinsed twice in PBTx over 24 hr and incubated in 1:100 goat anti-rabbit Texas Red or Alexa 488 (Molecular Probes, Eugene, OR) for 24 hr. The tissue was then rinsed in 0.1 M phosphate buffer for 12 hours and then in 4 mM sodium carbonate for 1 hr. Tissue was then dehydrated in an alcohol series, cleared, and mounted in either Cytoseal (Fisher, NJ) or methyl salicylate (Sigma-Aldrich, MO).

Confocal imaging

I used a Zeiss LSM 510 confocal microscope with 10x and 20x fluor air interface lenses to produce micrographs of the immunolabeled preparations. In order to produce images for comparison purposes, I took the following steps to reduce variability in capturing images of the immunolabeled preparations. Prior to imaging 5-HT-ir in the HGNs, the laser was turned on and allowed to equilibrate for 1 hr. I then chose an area of the preparation that best matched the intensity of the most brightly labeled HGNs within the set to be imaged. The microscope detector settings were adjusted so that the image

was not saturated. These settings were maintained while imaging the entire set of preparations. Each set of preparations was imaged in one sitting. The z-axis for imaging the HGNs and the A6 5-HT synthesizing cell was set to include all visible fluorescent labeling of the cells being imaged. I used projections of the z-axis images for a qualitative comparison within a set of experiments. In most instances, the resulting differences among the experimental groups were visibly evident compared to control groups that often showed no 5-HT-ir. The images were imported into Adobe Photoshop where they were enhanced using identical settings within an experimental group.

5-HT injection and tissue measurement with high performance liquid chromatography

5-HT levels in A6 and hindgut were measured using high performance liquid chromatography (HPLC) coupled to an electrochemical detection system using a protocol derived from Fickbohm et al. (2001). A model 528 pump and Coulochem II detector with a flow through model 5011 Analytical Cell (ESA, Inc. MA) made up the HPLC system. The guard cell was set at 350 mV, the screen electrode set at +50 mV, and the analytical electrode set at +325 mV. The column was 150 X 3.2 mm, 3 μm RP-C18 (MD-150; ESA, Inc.). The mobile phase composed for this system was made up of 75 mM sodium dihydrogen phosphate monohydrate (Sigma-Aldrich), 1.7 mM sodium octyl sulfate (Sigma-Aldrich), 0.01% (v/v) triethylamine (Fisher), 25 μM ethylenediaminetetraacetic acid (Sigma-Aldrich), 15% acetylnitrile, pH 3.2.

Crayfish (5 to 6.5 cm, rostrum to telson) were injected with either 50 μL of either saline or 0.05 mg/mL 5-HT from a 26 gauge (BD precision glide) Hamilton syringe

needle. The saline or 5-HT was given 30 min to diffuse in the crayfish before they were cold anesthetized for another 30 min. The hindgut, A6 trimmed of its nerves, and half of the A5 /A6 connective were then dissected from the crayfish, blotted for excess saline, and placed in previously weighed tubes with 100 μL of 0.1N perchloric acid. The tubes with perchloric acid were weighed again to determine the weight of the tissue sample. At this time, 5 pg of N-methylserotonin was pipetted into the tubes to function as an internal standard, adjusting for loss of sample during processing. The tissue was homogenized with a Teflon pestle (Kontes, NJ) attached to a drill. Grinding consisted of 30 pulses with the drill at top speed. The homogenized tissue was centrifuged at 14000 rpm for 15 min at 4°C and the supernatant was filtered and centrifuged through a 0.22 mm centrifuge filtration device (Ultrafree-MC; Millipore, MA) at 14000 rpm for 15 min at 4°C. The filtrate was then diluted to 100 μL with mobile phase and stored at -4°C until analysis.

On the day samples were analyzed, standard curves were run of 5-HT, 5-HTP, and N-methylserotonin. The amounts of these compounds were determined using software provided by the ESA 5011 HPLC-EC system.

RESULTS

HGN serotonin immunoreactivity

Published maps of 5-HT-ir in the crayfish and lobster CNS display sets of neurons in each abdominal ganglion (Beltz and Kravitz, 1983; Real and Czternasty, 1990). In A6 there is only one neuronal soma and a set of descending projections that terminate in different sections of neuropil. No neuron somata in the caudal portion of the ganglion are shown, nor any projections out of N7.

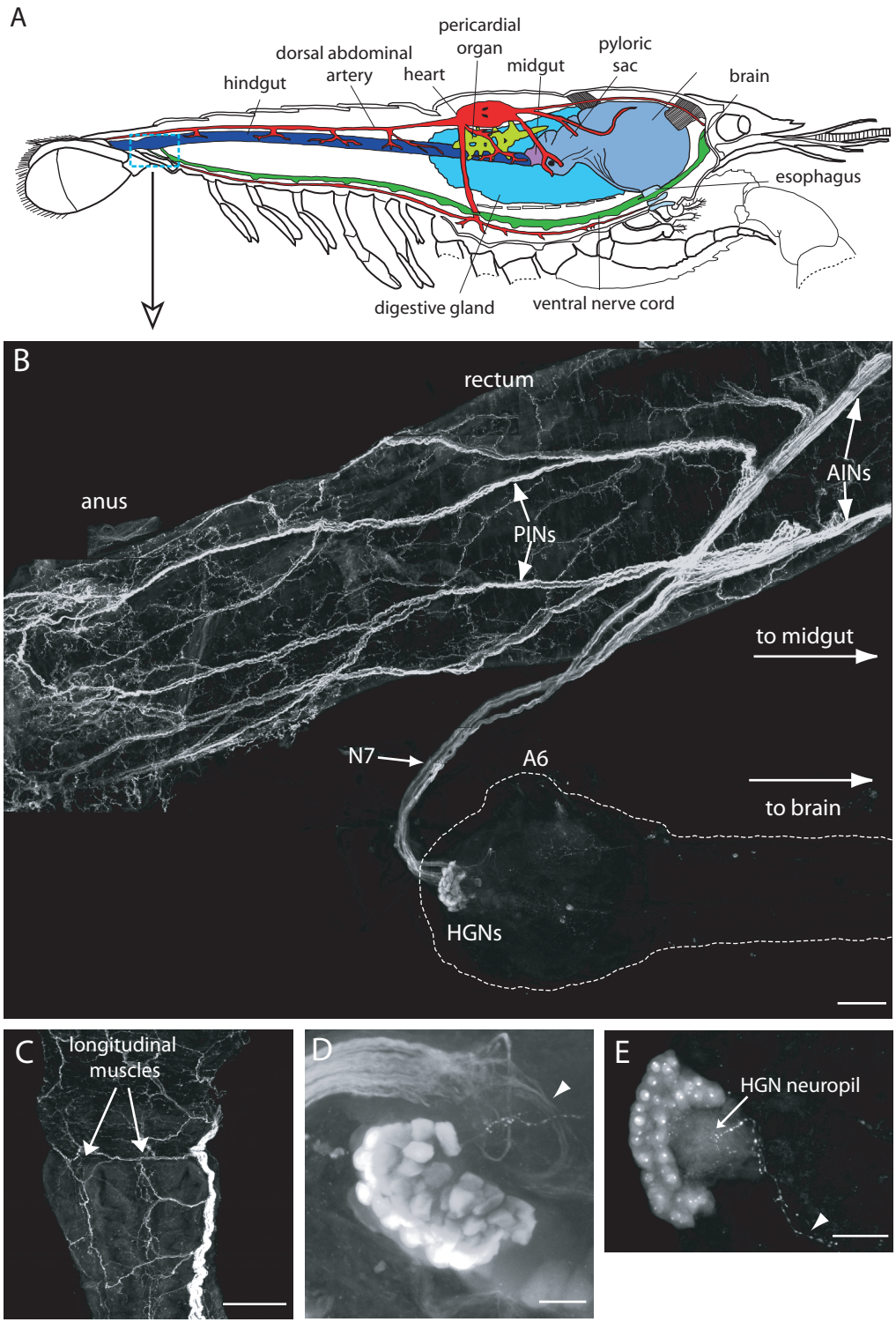
The central nervous system of the crayfish is a string of 14 ganglia, some of which are fused, that is located on the ventral surface of the animal (green in Fig. 3.1A). The nerve cord extends from the rostrum to the last abdominal segment, where A6 is found. A6 has six pairs of nerves that innervate the last abdominal segment and the tailfan, and it has a fused N7, which innervates the hindgut. The crayfish hindgut extends from the midthorax to the last abdominal segment (dark blue in Fig. 3.1A). It starts behind the midgut and below the pericardial organ and then ascends to the dorsal side of the animal when it reaches the abdomen. The dorsal abdominal artery lies on the dorsal surface of the hindgut and provides a rich supply of blood to the hindgut and the abdominal musculature.

Upon incubating the abdominal nervous system in 1 μM 5-HT, 5-HT-ir in the descending projections is intensified relative to unincubated preparations, whereas labeling of the single medial soma is usually reduced. The greatest change, however, is the appearance of a large cluster of immunoreactive somata, the HGNs at the caudal margin of the ganglion, together with their ganglionic projections out of N7 to the hindgut (Fig. 3.1B).

The HGNs are a subset of efferent neurons in A6 that project out of N7 to innervate the hindgut (Elekes et al., 1988) and label for 5-HT-ir. Their axons form two distinct fascicles that project through N7 to the rectum of the hindgut (Fig. 3.1B). The HGN collaterals on the hindgut are organized into two major projections: the posterior intestinal nerves (PINs) that project towards the anus and the anterior intestinal nerves (AINs) that project towards the midgut (Fig. 3.1B) (Elekes et al., 1988). The AINs send out perpendicular projections that split into a Y-shape fibers that follow the longitudinal

Fig. 3.1 Orientation of the HGNs in crayfish CNS and on the hindgut (A) A sagittal view of the crayfish nervous system, digestive system, and partial circulatory system adapted from Huxley (1880). The nervous and endocrine systems are colored in shades of green. The digestive system is in shades of blue, and the circulatory system is in red. The area appearing in 1B is at the caudal end of the animal and outlined in cyan. (B) Photomontage of the serotonergic HGNs and their projection to the rectum of the hindgut as shown by 5-HT-ir following incubation in 1 μM 5-HT. A6 and the hindgut are displayed as they are oriented in the; they are parallel to each other with the hindgut on the dorsal surface and the nerve cord on the ventral surface. The roughly 80 HGN cell bodies are in the medial posterior section of A6. The HGN axons appear as two fascicles and project through N7, a fused nerve that projects to the rectum and then splits into the AINs and the PINs. The AINs form two large fascicles laterally located along the hindgut and the PINs split into 3 to 5 fascicles that project to the anus. Both the AINs and the PINs undergo further divisions. A dorsal view of the A6 ganglion and its connective to the A5 ganglion is outlined. (C) Innervation of the abdominal hindgut longitudinal muscles by AIN collaterals. Axons project perpendicular to the main fascicle at regular intervals along the hindgut and then split into anterior and posterior projections that innervate the longitudinal muscle bundles. (D) Lateral view of the HGNs and N7 in A6 showing the cluster of cell somata, the projections of axons into N7 (arrowhead) and a descending varicose axonal fiber. (E) Dorsal view of the HGN soma cluster and neuropil, including a prominent varicose 5-HT fiber that traverses the perimeter of the A6 and terminates inside this neuropil (arrowhead). Scale bar B,C = 200 μm. Scale bar D,E = 50 μm.

Fig. 3.1


A
pericardial organ
dorsal abdominal artery
heart
midgut
pyloric sac
hindgut
brain
esophagus
digestive gland
ventral nerve cord
B
rectum
anus
AINs
PINs
to midgut
N7
A6
to brain
HGNs
C
longitudinal muscles
D
E
HGN neuropil

muscles of the hindgut, where they form specialized neuromuscular contacts (Elofsson et al., 1978) (Fig. 3.1C). Centrally, these axonal projections emerge from A6 to join N7 close to the cluster of their cell somata. A lateral view of A6 shows that some of the HGN axons project anteriorly from the soma cluster and loop up to the dorsal segment of the ganglion before exiting to N7 with other HGN axons (Fig 3.1D). Dendrites of the HGNs project into a small neuropil that makes up the core of the larger and more distal cell body cluster (Fig. 3.1E). The HGNs make up a major portion of the third, or last neuromere of A6 (Kondoh and Hisada, 1986), where a cluster of somata lies ventrally, and the neuropil and axonal projections out N7 are found dorsally (Fig. 3.1B-E). Descending serotonergic fibers project into the HGN neuropil from the ventral medial tract and from pathways around the perimeter of the ganglion (arrowhead, Fig. 3.1D).

The 5-HT-ir displayed in the HGN cell bodies of Fig. 3.1E showed a concentration of 5-HT in what appeared to be the nucleus, with less labeling in the cytoplasm. Labeling of the nucleus and the more intense labeling of a pair of nuclear elements were frequently seen in images of the HGN cell bodies.

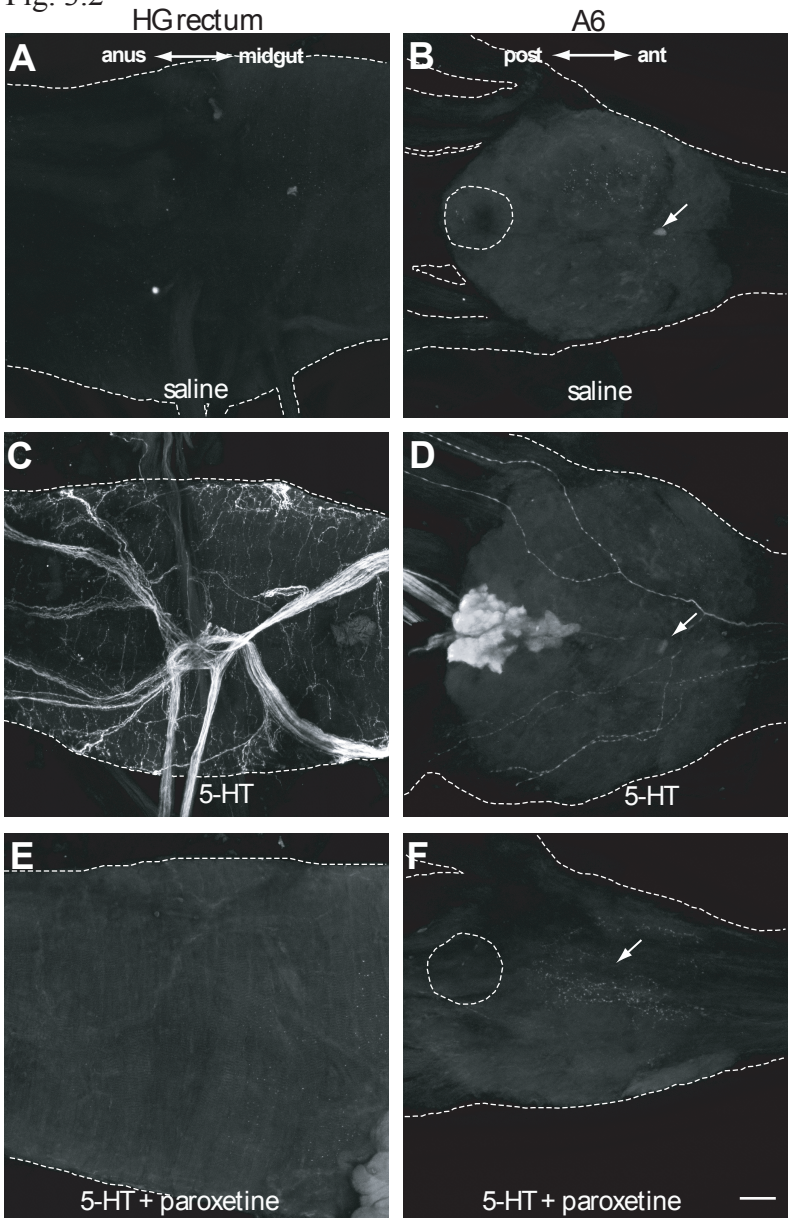
Uptake of 5-HT in the HGNs

Exposure of the HGNs to 5-HT was necessary for them to display distinct 5-HT-ir. Superfusion of the tissue with saline alone failed to produce labeling of the HGN projections on the hindgut or their cell bodies in A6 (Fig. 3.2A, B). The A6 medial neuron, which is marked on the published maps of 5-HT-ir in crustaceans (Beltz and Kravitz, 1983; Real and Czternasty, 1990), showed 5-HT-ir of the cell soma in 6 of 10 preparations superfused with saline. Robust 5-HT-ir of the HGNs occurred after I

Fig. 3.2 Uptake of 5-HT by the HGNs through a paroxetine-sensitive transporter. (A) The rectum and hindgut of crayfish, incubated in saline, showed no 5-HT-ir. (B) A6, incubated in saline, showed no 5-HT-ir in the HGN cell bodies (dotted line circle at left), but did show 5-HT-ir in the medial 5-HT neuron (arrow). (C) The rectum of a crayfish, incubated in10 μM 5-HT, showed intense 5-HT-ir in the AIN and PIN collaterals of the HGNs and in fine varicose fibers. (D) A6, incubated in 10 μM 5-HT, showed intense 5-HT-ir in two clusters of HGNs. 5-HT-ir of the medial 5-HT neuron was faint (arrow). Fibers that appeared in some of the paired nerves also showed 5-HT-ir. (E) The rectum and hindgut, incubated in 10 μM 5-HT and 10 μM paroxetine, showed no 5-HT-ir. (F) A6, incubated in 10 μM 5-HT and 10 μM paroxetine, showed no 5-HT-ir in the region of the HGNs (dashed circle) or in the medial 5-HT neuron (location indicated by arrow). There was faint 5-HT-ir of the dorsal descending varicose 5-HT fibers. Scale for all figures = 50 μm. (G) Changes in the levels of 5-HT, measured by HPLC (see Methods), in A6 and the hindgut after injection of saline (n=7) or 50 μL of 0.5 mg/mL 5-HT (n=8) into the hemolymph of crayfish. Kruskal-Wallis Test KW statistic for A6 = 17.748, $p<0.001$; HG = 15.023, $p<0.001$; Dunn's post-test used for pairwise comparisons (*** = $p<0.001$,

** = $p<0.01$).

Fig. 3.2


HG rectum
A6
A
anus
midgut
saline
B
post
ant
saline
C
5-HT
D
5-HT
E
5-HT + paroxetine
F
5-HT + paroxetine
G
***
**
Saline
5-HT
pmol/mg
4
3
2
1
A6
Hindgut

incubated the ventral nerve cord in 10 μM (2 preparations) or 50 μM (9 preparations) of 5-HT (Fig. 3.2C, D). The AINs and projections on the hindgut longitudinal muscles were labeled in every preparation, with labeling of the PINs and N7 visible in 4 of 5 preparations. The HGN cell bodies showed 5-HT-ir in every preparation (9 of 9 at 50 μM 5-HT and 2 of 2 at 10 μM 5-HT); however the A6 medial neuron was less reliably labeled following application of 50 μM 5-HT (1 of 9) and not labeled at all following application of 10 μM 5-HT (0 of 2).

To test whether 5-HT was taken up by the HGNs via a serotonin transporter (SERT), we exposed the nerve cord and hindgut to 10 μM paroxetine plus 10 μM 5-HT. Paroxetine is a SERT antagonist that has been described as very effective at blocking 5-HT uptake in *Drosophila melanogaster*, another arthropod (Blakely et al., 1994). I compared 5-HT-ir labeling of the HGNs with and without exposure to paroxetine to determine whether blocking uptake would affect the 5-HT content of the HGNs. When 10 μM paroxetine and 10 μM 5-HT were applied to the nerve cord and hindgut, 5-HT-ir was very faint or absent in N7, AINs, and PINs (positive label, 1 of 8), and absent on longitudinal muscles (Fig 3.2E). The HGN cell bodies also were not labeled (positive label, 0 of 8), whereas labeling of the A6 medial neuron was comparably labeled to that seen in the saline incubated preparations (Fig. 3.2F).

The increase in 5-HT-ir in the HGNs led to the question of how much 5-HT is taken up by the HGNs. I used HPLC to measure 5-HT levels in the hindgut and A6. I injected 50 μL saline into the ventral sinus of members of one group of adult crayfish and 50 μL of 0.5 mg/mL 5-HT into members of a similar group of animals 30 min prior to

dissection and harvesting of tissue. Large increases in 5-HT levels were measured in A6 and the hindgut (Fig. 3.2G). While the increase per mg of tissue was greatest in A6, the total amount of 5-HT measured in the hindgut was almost 25 times greater than that measured in A6, reflecting the much larger size (and presumably, the number of uptake sites) of the hindgut. Taken together, these results indicate that much of the 5-HT increase in A6 and the hindgut is the result of 5-HT uptake into the HGNs.

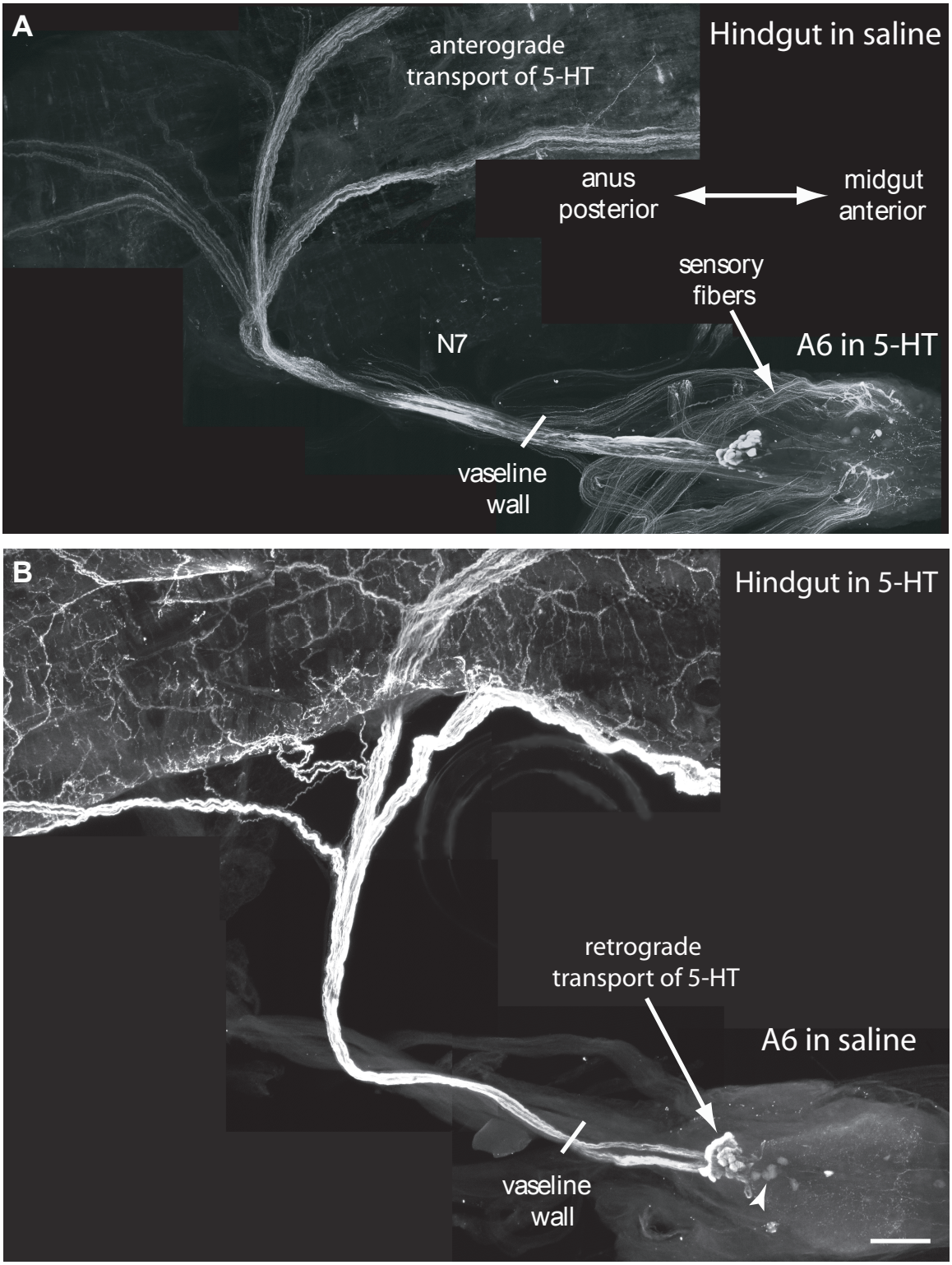
Central and peripheral uptake of 5-HT and anterograde and retrograde transport

In using 5-HT-ir to examine uptake, I observed that 5-HT underwent both anterograde (n=2) and retrograde transport (n=10). When the abdominal nerve cord was selectively incubated in 1 μM 5-HT, 5-HT-ir was detected in the HGN axonal projections on the hindgut (Fig. 3.3A). Both the AINs and PINs showed labeling and the perpendicular branches of the AINs were visible. There was no labeling of the fibers on the hindgut longitudinal muscles. In general, the axonal projections on the hindgut exhibited diminished labeling as they projected further out on the hindgut. The cell bodies were intensely labeled as expected following incubation in 5-HT as were the proximal projections. Furthermore, as is typical of long incubations in 5-HT, sensory fibers in paired nerves 1 through 6 also showed 5-HT-ir (arrow).

In preparations used to examine retrograde transport, the hindgut was incubated in 1 to10 μM 5-HT, which led to 5-HT-ir in the HGN cell bodies (Fig. 3.3B). Hindgut incubation led to intense 5-HT-ir of the AINs and their perpendicular branches as well as the PINs. The number and extent of 5-HT-ir on the HGN projections to the hindgut was greater

Fig. 3.3 Anterograde and retrograde transport of 5-HT in the HGNs. The nerve cord and hindgut were placed in adjacent containers and linked by N7 which passed through an opening filled with petroleum jelly, so that either the nerve cord or the hindgut and axonal projections could be exposed to 5-HT while the other was in saline. (A) Anterograde transport: incubation of the abdominal nerve cord in 1 μM 5-HT led to 5-HT-ir in the axonal projections on the hindgut. (B) Retrograde transport: incubation of the hindgut in 1 μM 5-HT led to intense 5-HT-ir in the HGN cell bodies in A6. The arrowhead indicates cell bodies that do not typically show 5-HT-ir. Scale bar for A,B = 200 μm.

Fig. 3.3


A
anterograde
transport of 5-HT
Hindgut in saline
anus
posterior
midgut
anterior
sensory
fibers
N7
A6 in 5-HT
vaseline
wall
B
Hindgut in 5-HT
retrograde
transport of 5-HT
A6 in saline
vaseline
wall

than that seen in the preparations that examined anterograde transport. Projections on the circular muscles of the rectum showed 5-HT-ir and so did fine varicose fibers innervating the longitudinal muscles of the hindgut. The HGN axons in N7 showed intense 5-HT-ir as did the cell bodies in A6. In addition, unidentified cells along the midline showed light 5-HT-ir (Fig 3.3B, arrowhead). Unlike in the preparations that examined anterograde transport, the sensory nerves showed no 5-HT-ir, indicating that there was no leaking of 5-HT into the saline incubation chamber.

Testing for 5-HT synthesis in the HGNs

Two enzymes act to convert tryptophan to 5-HT: TpOH hydroxylates tryptophan to produce 5-HTP, and L-AADC decarboxylates 5-HTP to produce 5-HT. I tested for conversion of tryptophan to 5-HT by incubating the crayfish hindgut and nerve cord in 100 μM tryptophan and dibutyryl cAMP, which enhances enzymatic activity (Johansen et al., 1996; Mockus and Vrana, 1998), and assayed for conversion of tryptophan to 5-HT. Tryptophan application led to no 5-HT-ir of the HGN projections found on the hindgut preparations (positive label, 0 of 5) (Fig. 3.4A). In A6, there was no 5-HT-ir in the HGNs in 3 of 5 preparations and very faint 5-HT-ir in the remaining two (Fig 3.4B), whereas there was consistently strong labeling of the A6 medial neuron (open *, Fig. 3.4B) and labeling of an anterior lateral set of neurons in 3 of 5 preparations (open arrowhead, Fig. 3.4B). This latter result confirmed that the application of tryptophan did lead to synthesis of 5-HT. The A6 medial neuron was labeled in all the preparations, an increase in the percentage labeled from saline incubated preparations (Table 3.1). The anterior lateral

Fig. 3.4 Lack of conversion of tryptophan to 5-HT in the HGN cell bodies and projections. (A) 5-HT-ir of the rectum after exposure to tryptophan. There was no 5-HT-ir of HGN axonal projections. (B) 5-HT-ir in A6 after exposure to 100 μM tryptophan. The A6 5-HT medial neuron (open *) showed intense 5-HT-ir, as did a pair of lateral anterior neurons (open arrowheads), whereas the HGN somata (dotted outline) were not labeled. Scale bar for A and B = 100 μm. (C) Blocking L-AADC with NSD-1015 and labeling for 5-HTP also showed that there was no conversion of tryptophan to 5-HTP on the hindgut. (D) In the A6 ganglion both the medial 5-HT cell and the lateral anterior neurons converted tryptophan to 5-HTP (open *, open arrowheads). The neuropil also showed 5-HTP-ir as did some unidentified structures, which showed punctate labeling (arrows). There was no build up of 5-HTP in the HGNs (dotted outline). Scale bar for C and D = 50 μm.

Fig. 3.4

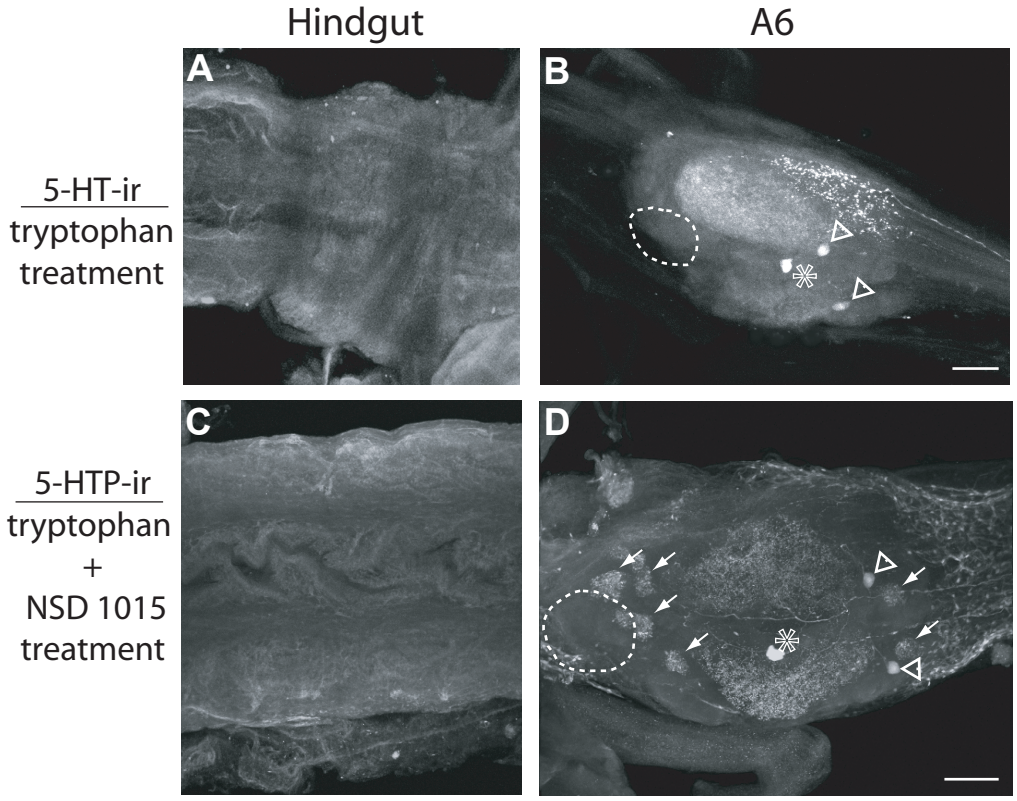

Hindgut
A6
A
B
5-HT-ir
tryptophan
treatment
C
D
5-HTP-ir
tryptophan
+
NSD 1015
treatment

Table 3.1 Survey of the HGNs, its projections, and other cells exhibiting 5-HT-ir in A6. (A) 5-HT-ir in A6 shows that a few cells show 5-HT-ir when superfused with 5-HT, whereas a number of cells following superfusion of tryptophan and/or 5-HTP show 5-HT-ir. (B) 5-HT-ir on hindgut shows that the HGN projections take up 5-HT. HGN projections and small cells on the hindgut show 5-HT-ir following superfusion of 5-HTP. Each dot represents 20% of the labeled cells in an experimental group and a half dot represents 10%. The dark dots indicate more intense labeling and the light dots represent labeling that is just slightly above background and sufficient to identify the cell. LM=longitudinal muscle fibers

Table 3.1

A

5-HT-ir in A6

Uptake	HGN cell bodies	A6 medial cell	Lateral medial cells	Anterior lateral cells	Anterior medial cell	Posterior lateral cells	Midline cells
saline		●●●					
50μM 5-HT	●●●●●	●					
10μM 5-HT+10μM Paroxetine		●●●					
Synthesis							
100μM tryptophan	●●	●●●●●		●●●			
1mM 5-HTP	●●●●●	●●●	●	●●●●●	●●●●	●●◖	●●◖
100μM 5-HTP + 100μM parox	●●●●●	●●●	●	●●◖	●●◖		●
100μM 5-HTP	●●●●●	●●◖	●	●	●●◖		

B

5-HT-ir on hindgut

Uptake	N7	AINs	PINs	LMs	HG cell bodies
saline					
50 μM 5-HT	●●●●	●●●●●	●●●●	●●●●●	
10 μM 5-HT+10μM Paroxetine	●	●	●		
Synthesis					
100 μM tryptophan					
1mM 5-HTP	●●●●	●●●●	●●◖	●●●●●	●●●●
100 μM 5-HTP+ 100 μM parox					
100 μM 5- HTP					

neurons were a new group of 5-HT-ir neurons that did not show 5-HT-ir under control conditions or when incubated in 5-HT (Table 3.1).

To further test conversion of tryptophan to 5-HT, I blocked activity of L-AADC with NSD-1015 and immunolabeled for the intermediate product, 5-HTP. Abdominal nerve cords with attached hindguts were incubated in 200 μM tryptophan plus NSD-1015. The 5-HTP-ir in A6 and the hindgut was very similar to the distribution of 5-HT-ir seen in the tryptophan incubated A6 and hindgut preparations (Fig. 3.4C, D). In the tryptophan plus NSD-1015 incubated nerve cords with hindgut, there was no accumulation of 5-HTP found in the HGN cell bodies, nor in the HGN axonal projections on the hindgut (Fig. 3.4C). Overall the hindgut showed no 5-HTP-ir. In A6, 5-HTP-ir was found in the A6 medial cell (7 of 7) (open *, Fig. 3.4D) and in the anterior lateral neurons (5 of 7) (open arrowheads, Fig. 3.4D). There was also labeling of fine varicose fibers in the A6 neuropils and a pair of larger descending fibers. In addition to 5-HTP-ir found in the three cell bodies and neuropil, there was punctate labeling of structures located in the cell body layer of the ganglion (Fig. 3.4D, arrows). The appearance and number of theses structures varied greatly between individual A6 ganglia, with some ganglia showing no labeling of such structures and others, as in Fig 3.4D, showing labeling of nine. Groups of these structures appear in the caudal third of A6 and a pair was found in the anterior region of A6. These structures were approximately 20-30 μm in diameter. 5-HTP-ir was also detected in epithelial cells attached to the nerve cord sheath where the nerve cord connective merged into the ganglion. This result indicated that tryptophan application consistently led to 5-HTP synthesis in the A6 medial neuron and the anterior lateral neurons. I concluded that TpOH was either not present or inactivated in the HGNs

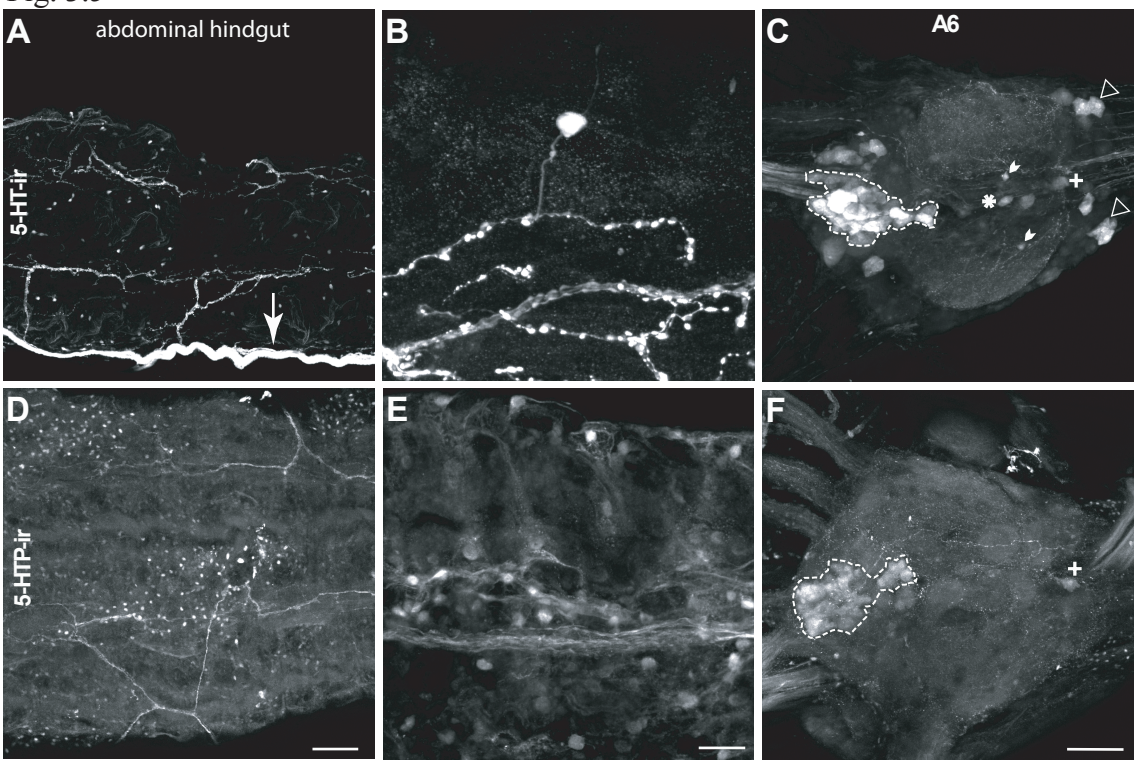
because incubation in tryptophan did not result in 5-HT-ir and incubation in tryptophan and blocking conversion of 5-HTP to 5-HT did not result in 5-HTP-ir.

I then tested for the conversion of 5-HTP to 5-HT by incubating the hindgut and nerve cord in either 100 μM or 1 mM 5-HTP and labeling for 5-HT. 5-HT-ir was prominent in HGN projections along the hindgut and in HGN somata in A6 after incubation in 5-HTP (Fig. 3.5A-C). The projection pattern of the AINs was similar to that seen in hindguts incubated in 5-HT, with the fascicles, their perpendicular branches, and fine fibers on the longitudinal muscles brightly labeled (Fig. 3.5A). In addition, there were numerous, small, bipolar 5-HT-ir neurons located in the longitudinal folds of the hindgut, some of which were observed to join the same fascicles populated by the HGN projections (Fig. 3.5B). These neurons, in reduced numbers, were occasionally observed following a prolonged incubation of the hindgut in 5-HT (B.E. M., unpublished observation). The distribution of 5-HT-ir differed between a hindgut incubated in 5-HT and one incubated in 5-HTP. The hindgut incubated in 5-HTP showed a more prominent labeling of varicosities than the one incubated in 5-HT, suggesting that this synthetic step may take place in the varicose regions of the neuron.

In most preparations incubated in 1 mM 5-HTP, the HGNs showed moderate to intense 5-HT-ir (11 of 12) (Fig. 3.5C). The lateral and medial anterior cells were the next most consistently labeled neurons, with all 12 preparations showing some 5-HT-ir of the anterior lateral cells (open arrow) and 10 showing 5-HT-ir of an anterior medial cell (open +). There were additional clusters of neurons in A6 that exhibited 5-HT-ir following incubation in 1 mM 5-HTP: the posterior lateral cells that flanked the HGNs (6 of 12 preparations), cells along the midline (6 of 12 preparations), and the medial lateral

Fig. 3.5 Conversion of 5-HTP to 5-HT in HGNs and in numerous other cells in A6 and hindgut. (A-C) Enhanced 5-HT-ir in the hindgut and A6 followed exposure of both tissues to 1 mM 5-HTP. (A) HGN projections on the hindgut and in numerous small cells embedded in the longitudinal folds of the hindgut showed 5-HT-ir. The AINs show intense 5-HT-ir (arrow) (B) A bipolar neuron showing 5-HT-ir was embedded in the longitudinal folds of the hindgut and projected into the bundle of HGN processes that innervate the longitudinal muscles. (C) In A6, multiple sets of neurons were labeled, including both clusters that make up the HGNs (outlined with dotted line), two other clusters of neurons that flank the HGNs, the medial 5-HT neuron (star), 3 other medial neurons, a pair of cells on either side of the medial 5-HT cell (arrow feathers), and the lateral anterior cells that had labeled following application of tryptophan (open arrowheads). (D-F) 5-HTP-ir in the hindgut and A6 followed exposure to 1 mM 5-HTP. (D) Fine neuronal fibers that innervate the longitudinal muscles displayed 5-HTP-ir. (E) 5-HTP-ir was found in numerous small hindgut cells, which are possibly epithelial cells embedded in the longitudinal folds of the hindgut. (F) 5-HTP-ir in A6 was confined to both HGN clusters and one anterior medial cell (+). Scale bars for A,C,D,F = 100 μm, scale bars for B,E = 20 μm

Fig. 3.5



paired cells (2 of 12 preparations, arrowhead). Interestingly, the A6 medial neuron was labeled in only 7 of 12 preparations (open *) (Table 3.1). The anterior lateral pairs of neurons that are consistently labeled under these conditions likely include the one pair of neurons that labels for 5-HT and 5-HTP following incubation in tryptophan.

Use of an antibody against 5-HTP showed that N7 and the hindgut accumulated 5-HTP as did a subset of the same neuronal cell bodies that exhibited 5-HT-ir in A6 (Fig. 3.5D-F). On the hindgut, the fine fibers on the longitudinal muscles were consistently labeled while the AINs and PINs labeled only half of the time (Fig. 3.5D). In addition, there were many small, unidentified neuronal and non-neuronal cells that showed 5-HTP-ir (Fig. 3.5E). There appeared to be more hindgut cells that showed 5-HTP-ir than showed 5-HT-ir (see Fig 3.5B), indicating that other hindgut cells take up 5-HTP and are not able to convert 5-HTP to 5-HT. In A6, the HGN cell bodies and an anterior medial cell were part of the subset that exhibited 5-HTP-ir (Fig. 3.5F). The A6 medial neuron and the anterior lateral cell pairs, which consistently labeled for 5-HT-ir and 5-HTP-ir following incubation in tryptophan, did not show 5-HTP-ir. This may indicate that they converted 5-HTP to 5-HT rapidly, although as we have shown, incubation in 5-HTP did not always lead to 5-HT-ir in the A6 medial neuron as it did in the anterior lateral cells. Comparisons of 5-HT-ir and 5-HTP-ir in both A6 and the hindgut indicated that, with the exception of the non-neuronal cells on the hindgut, all the cells that took up 5-HTP did convert it to 5-HT. Because there were many cells that displayed 5-HT-ir but not 5-HTP-ir after incubation in 5-HTP, it was not clear whether neurons that showed 5-HTP-ir were sequestering 5-HTP or whether conversion 5-HTP to 5-HT was regulated differently in each neuron (Young et al., 1994; Zhu et al., 1994).

Incubation of preparations in 100 μM 5-HTP with 100 μM of the SERT antagonist paroxetine did not diminish accumulation of 5-HTP in comparison to those incubated only in 100 μM 5-HTP (n=4, not shown). Thus, 5-HTP is not being taken up by SERT but instead may be taken up by some other transporter, perhaps an amino acid transporter. As a result of these experiments, I concluded that the HGNs do have an active form of the L-AADC enzyme, particularly located in the varicosities of the HGN axonal projections. However, because of the buildup of 5-HTP within the HGNs, the conversion of 5-HTP to 5-HT is either slow or incomplete.

DISCUSSION

The HGNs showed strikingly different intensities of 5-HT-ir, depending on the experimental conditions. In unmanipulated preparations, the HGNs typically showed no 5-HT-ir, which initially led to them being overlooked as serotonergic. However, they displayed intense 5-HT-ir following incubation with 1 to 50 μM 5-HT. The 5-HT-ir resulted from uptake that occurred centrally or peripherally and the occurrence of 5-HT-ir was eliminated or reduced by the superfusion of 10 μM paroxetine with 10 μM 5-HT. Furthermore, injection of 5-HT greatly increased measured levels of 5-HT in A6 and the hindgut (Fig. 3.3G). These results indicated that the HGNs accumulate 5-HT and may perhaps ‘borrow’ 5-HT.

The HGNs as borrowing neurons

A borrowing neuron does not synthesize its transmitter but instead acquires it through uptake from the extracellular medium by a plasma membrane transporter or from

another cell through gap junctions (Schutte, 1994; Lebrand et al., 1996; Richards et al., 2003). The transmitter is then repackaged into vesicles for re-release.

I found that, unlike other serotonergic neurons in the crayfish CNS, the HGNs are not able to synthesize 5-HT from tryptophan. They appear to lack an effective TpOH enzyme, as seen by their inability to convert applied tryptophan to 5-HTP. The HGNs did convert applied 5-HTP to 5-HT, consistent with an effective L-AADC. I concluded that the HGNs could not synthesize 5-HT, but that they could take up 5-HT from the surrounding space, both centrally and peripherally, and then transport it in either direction.

Central uptake and anterograde transport of 5-HT in the HGNs appear less effective than peripheral uptake and retrograde transport (Fig. 3.2). 5-HT-ir of axons on the hindgut following incubation of the A6 ganglion alone was less intense than after incubation of the hindgut alone. Vertebrate studies of SERT protein indicate it has a polarized expression; it is associated with dendrites and axons and is not found at all on cell bodies (Schroeter et al., 1997; Tao-Cheng and Zhou, 1999). HGNs may exhibit this same polarized expression whereby uptake does not occur on the HGN cell bodies but instead occurs on the dendrites and axons. Because the dendrites represent a much smaller percentage of the neuronal membrane (Elekes et al., 1988), less 5-HT may be taken up centrally than peripherally.

Although I have not tested whether the borrowed 5-HT is subsequently released, a couple factors suggest this is possible. As presented in Chapter 2, 5-HT receptors are located on the hindgut in patterns that suggest that 5-HT has hormonal and paracrine effects (Musolf and Edwards, 2005) and 5-HT affects contractile properties and promotes

peristalsis of the hindgut when applied at low (30 nM to 1 μM) concentrations (Chapter 2; Musolf and Edwards, 2004). It appears likely, therefore, that 5-HT is reduced or absent in the HGNs and that some sensory stimulus such as ingestion or digestion of a meal leads to release of 5-HT from neurosecretory organs and cells. Release of 5-HT in the blood would make it available for uptake by the HGNs. Whether the accumulated 5-HT is packaged into vesicles and released to promote peristaltic movements of the hindgut or 5-HT undergoes retrograde transport and is released centrally to modulate intraganglionic connections remains to be investigated, however 5-HT-ir in A6 reveals very few projections. The HGNs are purported to have central and peripheral synapses, however the majority of the synapses are peripheral (Elekes et al., 1988).

Cells with an incomplete 5-HT phenotype

Weihe and Eiden (2000), in their review of vesicular amine transporters, coined the term 'orphan neuron' to describe a neuron that is missing either one of the enzymes or the vesicular transporter to package the neurotransmitter for release. This definition includes the HGNs and the lateral medial cells, one pair of anterior lateral cells, the posterior lateral cells, and some midline cells, all of which lack the TpOH enzyme and have the L-AADC enzyme (Table 3.1). The L-AADC enzyme functions in both the indoleamine and catecholamine pathways, converting 5-HT to 5-HTP, L-DOPA to dopamine, and tryptophan to tryptamine, and so it is not surprising that a number of cells are capable of converting 5-HTP to 5-HT (Fig. 3.5). The HGNs, however, are distinct in that they are able to take up 5-HT (Table 3.2).

Table 3.2 Phenotypic differences in the cells that exhibited 5-HT-ir. The HGN cell bodies and axonal projections were the only neurons to take up 5-HT. The A6 medial cell and the pair of anterior lateral neurons are the only neurons that appear to synthesize 5-HT, but they do not appear to take up 5-HT (trp = tryptophan).

Table 3.2

Cell phenotypes

A6 cells	Control	5-HT uptake	trp→5-HT	5-HTP→5-HT
HGNs		X		X
A6 medial	X		X	X
Lateral medial				X
Anterior lateral			X	X
Anterior medial				X
Posterior lateral				X
Midline				X
HG				
N7		X		X
AINs		X		X
PINs		X		X
LMs		X		X
HG cells				X

Another phenotypically distinct group of neurons are those that have both synthesizing enzymes, but do not appear to take up 5-HT. The A6 medial cell, which is the only cell body that shows 5-HT-ir under control conditions, did not show an increase in 5-HT-ir following incubation in 5-HT but instead showed a reduction in the number of times the cell was labeled (Fig. 3.2). The lateral anterior cells showed 5-HT-ir when incubated in tryptophan or 5-HTP (Fig. 3.5), but not when incubated in saline or 5-HT (Fig. 3.2). This lack of label in both the medial and lateral anterior cells following incubation in 5-HT suggest a scarcity or lack of SERTs in addition to feedback inhibition of 5-HT synthesis when exposed to elevated concentrations of 5-HT.

Why borrow 5-HT?

The HGNs accumulate 5-HT and possibly release it. Release could occur in A6, at synaptic sites in N7, or on the hindgut (Elekes et al., 1988). If release could be established, the question is why HGNs 'borrow' 5-HT rather than synthesize it. A parsimonious explanation is that the 5-HT synthesized in anterior parts of the nervous and endocrine systems and released to modulate earlier digestive processes is available through the blood for use by the HGNs.

Uptake of extracellular 5-HT is an efficient way to acquire and redistribute a neurotransmitter. Evidence from HPLC indicates that decreasing amounts of 5-HT are measured in crayfish CNS from the brain to the abdominal ganglia (Kulkarni and Fingerman, 1992) with most of the 5-HT synthesizing neurons found in the brain, subesophageal, thoracic, and first abdominal ganglia (Chapter 4; Musolf and Edwards,

2003). Serotonin is also synthesized in the pericardial organ situated below the dorsally located heart and in contact with the anterior region of the hindgut (Beltz and Kravitz,1983) (Fig. 3.1A). Our studies show that very little 5-HT is found in the hindgut, unlike in mammals where the enteric system produces 95% of the 5-HT (Gershon, 1999). Yet 5-HT is important in the initiation of the peristaltic reflex in both mammalian gut and crayfish hindgut (Chen et al., 2001; Musolf and Edwards, 2004). Unlike in mammals, the lack of a blood brain barrier in crayfish makes it possible for 5-HT, produced in the pericardial organ and other thoracic neurosecretory sites, to be hormonally released and distributed to the hindgut via the arterial system. 5-HT is released during the early stages of digestion and is involved in modulating foregut activity (Katz et al., 1989). Gastric mill activity excites the gastropyloric receptor (GPR) neurons, which in addition to the pericardial organ are the primary sources of 5-HT in the stomatogastric ganglion of such decapod crustaceans as *Cancer borealis, Cancer irroratus,* and *Homarus americanus* (Beltz et al., 1984; Katz et al., 1989; Tierney et al., 1999).

The impressive ability of the HGNs in A6 and the hindgut to take up large quantities of 5-HT (Fig. 3.2) suggests that the HGNs may act as a sink for excess 5-HT in the hemolymph, and as a secondary source of hormonal 5-HT. Uptake occurs both centrally and peripherally in the HGNs. This extensive spread of SERT-bearing axons on the hindgut would bring excess 5-HT levels in the hemolymph produced during digestion or stress down rapidly (Lee et al., 2001). This process of regulating hormonal 5-HT signaling is assisted by 5-HT's actions on the arterial valves of the dorsal abdominal

artery, which redirect hemolymph to the hindgut rather than the abdominal musculature (Wilkens, 1997).

Uptake that occurs centrally may help supply the HGNs with 5-HT needed for modulating hindgut contractility, and it may act to clear A6 of 5-HT released by any of several sources there. These sources include descending serotonergic fibers that project into distinct regions of A6 (Figs. 3.1D, E; 3.4B) and along the integrative segment of the lateral giant (LG) command neuron for escape (Yeh et al., 1997), and the bilateral projections of the A6 medial neuron into both A6 neuropils. In A6, 5-HT modulates local and ascending mechanosensory interneurons, telson motor circuitry (Nagayama, 2002) and the LG command neuron (Edwards et al., 2002). Because 5-HT is involved in many processes, inactivation after release is essential to prevent crosstalk among different behaviorally important circuits that are affected by 5-HT, particularly when it is being used as a paracrine or hormonal signal.

Having acquired 5-HT through uptake, the HGNs may metabolize it through the N-acetylation pathway (Dubbels and Elofsson, 1989) or release it. The sulfonation pathway is thought to be active in the cellular walls lining the blood vessels (Kennedy, 1978). Nothing is known of 5-HT metabolism by the HGNs.

The possibility exists that 5-HT released by the HGNs onto the hindgut will rapidly move into the circulation, providing an opportunity for it to act as an endocrine signal. Hormonal 5-HT promotes the release of crustacean hyperglycemic hormone (CHH) from the eyestalk (Lee et al., 2001; Escamilla-Chimal et al., 2002), which may be appropriate in the period between meals. Curiously, it also inhibits the release of CHH from neurons in the second thoracic roots (Basu and Kravitz, 2003). CHH is also a stress

hormone, so that the release of 5-HT from the HGNs may be part of a stress response (Chang et al., 1999b). It is often observed that the severe stress of crayfish dissection leads to hindgut contractions. Release of 5-HT at this time may account for the very low levels of 5-HT found in the HGNs in dissected preparations where the levels were not augmented by 5-HT exposure (Fig. 3.2). Finally, serotonin release may also promote CHH release in the hindgut leading to ecdysis (Chang et al., 1999a; Chung et al., 1999). 5-HT itself could support ecdysis through the anal uptake of water, which is necessary to produce the hydrostatic forces needed to split the exoskeleton and provide the temporary hydroskeleton.

5-HT as a borrowed transmitter

The crayfish HGNs may provide one clear example of how 5-HT functions as a borrowed transmitter. Numerous other instances appear to exist: 5-HT appears to be borrowed or accumulated by other neurons across phyla, in crustaceans, amphibians, numerous mammals and humans (Schutte, 1994; Hansson et al., 1998; Beltz et al., 2001; Verney et al., 2002). The most efficient way that borrowing neurons can acquire 5-HT is through uptake mediated by a SERT; some of the most widespread drugs used today, the SSRIs, block uptake of 5-HT through SERT. It may be that part of the medical effectiveness of the SSRIs, particularly in mammalian gut, is through their effect on the redistribution of 5-HT.

4

Effects of increased 5-HT and tryptophan levels in the crayfish hemolymph on 5-HT, 5-HTP, and DA distribution in CNS and hindgut

The synthesis of serotonin (5-hydroxytryptamine, 5-HT) relies first on the availability of tryptophan, which is an essential amino acid that cannot be synthesized by crayfish but must be acquired by ingesting tryptophan-containing food Following digestion of a tryptophan-containing meal, levels of this amino acid increase in the blood and can give rise to newly synthesized 5-HT (Young and Gauthier, 1981). In crustaceans, sites of 5-HT synthesis include rostral regions of the central nervous system, the perineural sheath, and the pericardial organ (Beltz and Kravitz, 1983). While 5-HT acts on sites close to where it is synthesized and released, there are also sites at a distance from where 5-HT is synthesized that rely on hormonally distributed 5-HT (Chapter 3). In crayfish, the diffuse distribution of 5-HT receptors on muscle, nerve plexus (Spitzer et al., 2005), and on the hindgut (Chapter 2) suggests that 5-HT has numerous hormonal effects. Evidence presented in Chapter 3 suggests that much of the 5-HT is subsequently taken up and concentrated in the HGNs and used in a more targeted manner, at synapses on the hindgut and perhaps as a paracrine signal.

In Chapter 3, I showed that populations of 5-HT-ir neurons, in response to either tryptophan or 5-HT superfusion, appeared to exhibit different regulatory mechanisms in regards to their supplies of 5-HT. In the crayfish terminal ganglion (A6), three neurons that project to the local neuropil showed increased 5-HT immunoreactivity (5-HT-ir) following application of tryptophan, while other 5-HT-ir neurons showed no change in 5-HT-ir. Superfusion of 5-HT increased 5-HT-ir in the cluster of hindgut neurons in A6 that project to the hindgut (HGNs) and levels of 5-HT increased in hindgut tissue as measured by high performance liquid chromatography (HPLC). Superfusion of 5-HT led to reduced

5-HT-ir in the primary 5-HT synthesizing neuron in A6 and had no effect on other 5-HT-ir neurons that appeared following tryptophan superfusion.

This chapter further examines the effects of tryptophan and 5-HT on levels of 5-HT in crayfish CNS and hindgut. To simulate changes in blood levels of tryptophan and 5-HT, I injected either one into the crayfish hemolymph and measured the changes in levels of 5-HT and 5-hydroxytryptophan (5-HTP), the intermediate product in 5-HT synthesis. In effect, this simulates the release of tryptophan after ingestion of a meal that contains tryptophan and it simulates the hormonal release of 5-HT from neurosecretory sites. While HPLC does not provide the spatial resolution given by immunocytochemistry, it does provide a quantitative measurement of changes that I used to examine most of CNS and the hindgut. The measurement of 5-HTP following injection of tryptophan should show how the availability of tryptophan affects the first step in 5-HT synthesis in areas that putatively produce high levels of 5-HT (Real and Czernasty, 1990; Kulkarni and Fingerman, 1992). The measurement of 5-HT will show whether levels of 5-HT increase as suggested by research that indicates that the production of 5-HTP is the rate-limiting step (Boadle-Biber, 1993) or whether there are regulatory mechanisms that may exist beyond the conversion of tryptophan to 5-HTP (Young and Gauthier, 1981). Measurements of 5-HTP and 5-HT in CNS and hindgut following injection of 5-HT should indicate areas of uptake and the effects of possible feedback of extracellular 5-HT on 5-HTP and 5-HT levels.

In addition to measuring changes in 5-HT and 5-HTP, I also examined how increases in circulating tryptophan and 5-HT affect levels of dopamine (DA) in the CNS. In crustaceans, both DA and 5-HT, along with numerous peptides, operate to enhance

contractions in the hindgut (Mercier et al., 1991) and to modulate the central pattern generator that regulates foregut activity (Johnson and Harris-Warrick, 1990; Katz and Harris-Warrick, 1990). The two amines share at least one enzyme in their synthetic pathway, L-amino acid decarboxylase (L-AADC), which converts tryptophan to 5-HTP and converts L-DOPA to DA. There is also evidence in *Drosophila* that they share an hydroxylating enzyme, which converts tryptophan to 5-HTP and phenylalanine to tyrosine, the precursor to L-DOPA (Coleman and Neckameyer, 2004). This leads to the question of whether competition for access to these enzymes affects levels of either amine. Finally, there is evidence that the DA transporter is capable of transporting 5-HT into dopaminergic neurons (Chen et al., 2001; Vanhatalo and Soinila, 1994).

METHODS

Animals, dissection, and injection

Crayfish, *Procambarus clarkii*, were acquired from Atchafalaya Biological Supply in Raceland, LA and maintained at 22° C in freshwater aquaria exposed to a 12:12 light:dark cycle. They were fed twice a week. Crayfish ranged in size from 4.1 to 6.9 cm from rostrum to telson. Experiments were performed on four groups of animals, those that did not receive an injection and those injected from a 26 gauge (BD precision glide) Hamilton syringe needle into the thoracic ventral sinus with 50 μL of either saline, 500 μg/mL of 5-HT, or 1 mg/mL tryptophan. I used the amount injected and the concentration to determine the computed average amount of tryptophan in the blood. The amount of blood was determined by taking 30% of the average crayfish weight and converting this weight into mL (1 g of blood = 1.043 mL). The average amount of blood

in tryptophan-injected crayfish was 1.51 mL. The computed average amount of tryptophan injected was 5 μg, which would produce a concentration of 16.3 μM. The computed average amount of 5-HT injected was 2.5 μg, which would produce a concentration of 3.71 μM in the blood. The average amount of blood in the 5-HT injected crayfish was computed to be 1.75 mL.

Tryptophan and 5-HT were injected 30 min prior to anesthetization to allow diffusion of chemicals in the hemolymph and to allow synthesis and uptake to occur. The time between injection and tissue harvest was chosen based on preliminary experiments that varied the time between injection and anesthetization. The times tested for the tryptophan and 5-HT injections were 0, 10, 20, 30, and 45 min. Results from these preliminary test injections indicated that 30 min was the optimum time to wait between injection and anesthetization. To examine synthesis, I injected 50 μL of 1 mg/mL tryptophan and measured 5-HT and 5-HTP concentration 30 min after injection of tryptophan. To examine uptake, I injected 50 μL of 500 μg/mL 5-HT and measured 5-HT and 5-HTP levels 30 min after injection of 5-HT.

Crayfish were cold anesthetized in an icy bath at 0° C for 30 min. The hindgut was removed first, irrigated with crayfish saline (van Harreveld, 1936) to remove fecal matter, blotted for excess saline, and placed in a previously weighed tube with 100 μL of 0.1N perchloric acid (PCA). The ventral nerve cord was then removed and trimmed of its nerves, and separated into brain (without eyestalks), the circumesophageal ganglion (CEG) and subesophageal ganglion (SEG) with anterior connectives, the thoracic nerve cord with anterior connectives and half of the fifth thoracic and first abdominal ganglia connective (T5/A1), the A1 ganglion with half of T5/A1 and the first abdominal and

second abdominal (A1/A2) connectives, the A2-A5 ganglia with half of A1/A2 and A5/A6 connectives (A2-A5), and the terminal ganglion with half of the A5 /A6 connective (A6). These sections were also blotted of excess saline and placed in previously weighed tubes with 100 μL of 0.1 N PCA. The tubes were then weighed again so that the weight of the tissue could be determined. I then pipetted 5 μL of N-methyl serotonin (NMS) into the tubes to function as an internal standard.

5-HT quantification

The protocol for measuring 5-HT was derived from Fickbohm et al. (2001). A model 528 pump and Coulochem II detector with a flow-through model 5011 Analytical Cell (ESA, Inc. MA) made up the HPLC system. A more detailed description of this protocol is in Chapter 2.

On the day samples were analyzed, standard curves were run of 5-HT, DA, 5-HTP, and N-methylserotonin (NMS), the internal standard. The amounts of these compounds were determined using software provided by the ESA HPLC system.

Statistics

All values are given as mean ± SD, except where indicated in figure legends. Category plots were produced with the help of Igor Pro (WaveMetrics, Portland, OR) and error bars on the different categories represent standard error of measures. Significance among different groups was determined using InStat3 for Macintosh (GraphPad Software, San Diego, CA). All analyses were done using nonparametric statistics. We used the Kruskal-Wallis test for analysis of variance (ANOVA) to compare data derived

from saline injected, 5-HT injected, and tryptophan injected crayfish and to compare different sections of the CNS and hindgut to one another. Dunn's post-test followed the Kruskal-Wallis for pair wise comparisons. Differences were considered significant if $p < 0.05$.

RESULTS

5-HTP and 5-HT in CNS and hindgut

While studies have measured levels of 5-HT along the anterior-posterior axis (Kulkarni and Fingerman, 1992; Panksepp and Huber, 2002), no similar studies of 5-HTP have been conducted. A comparison of concentrations of 5-HTP among the different CNS regions and the hindgut showed that there were comparable levels of 5-HTP throughout most of the CNS with the exception of significantly higher levels in the brain than in the A2-A5 ganglia ($p<0.01$) and the hindgut ($p<0.001$). The A6 ganglion had the greatest variability of measured 5-HTP (Fig. 4.1A).

The profile of 5-HT concentrations along the CNS axis differed considerably from that of 5-HTP, indicating that no fixed relationship between the precursor and product exists in the CNS. The concentration of 5-HT decreased dramatically along the anterior-posterior axis of the ventral nerve cord (Fig. 4.1B). The greatest concentration of 5-HT was found in the brain (Fig. 4.1B, Table 4.1), where a pair of large serotonergic neurons, the metacerebral giants, have major arborizations in the accessory and olfactory lobes (Sandeman and Sandeman, 1987). Concentrations similar to the brain were found in the thoracic ganglia (TG), which control chelipeds and walking legs, and the circumesophageal and subesophageal ganglia (CEG/SEG), which control the mouthparts

Fig. 4.1 Levels of 5-HTP and 5-HT in CNS and hindgut without injection, with saline injection and following injection of 50 μL of 1 mg/mL tryptophan into the ventral sinus. (A) The distribution of 5-HTP (pmol/mg tissue) differed between brain and A2-A5 and the brain and hindgut (Kruskal-Wallis (KW) test $p<0.0016$; KW 21.296; Dunn's post test, brain vs. A2-A5, $p<0.01$ and brain vs. hindgut, $p<0.001$). Saline injection (black) did not significantly alter measured amounts of 5-HTP over non-injected preparations (white). Injection of tryptophan (gray) led to a significant increase in 5-HTP in the brain ($p=0.004$), Kruskal-Wallis Test (KW statistic=10.929, saline injected n=7, tryptophan injected, n=9, Dunn's post-test $p<0.01$). (B) The distribution of 5-HT showed a decrease along the anterior-posterior axis. Distribution of 5-HT was tested using the KW test (KW statistic=42.835, n=7-9, $p<0.0001$, Table 1). Saline injection (black) did not significantly alter measured amounts of 5-HT over non-injected preparations (white). Injection of tryptophan (gray) led to no significant changes in 5-HT levels in any CNS tissue or hindgut (KW Tests followed by Dunn's post-tests). The diagram of crayfish CNS illustrates the tissue regions that were analyzed.

Fig. 4.1

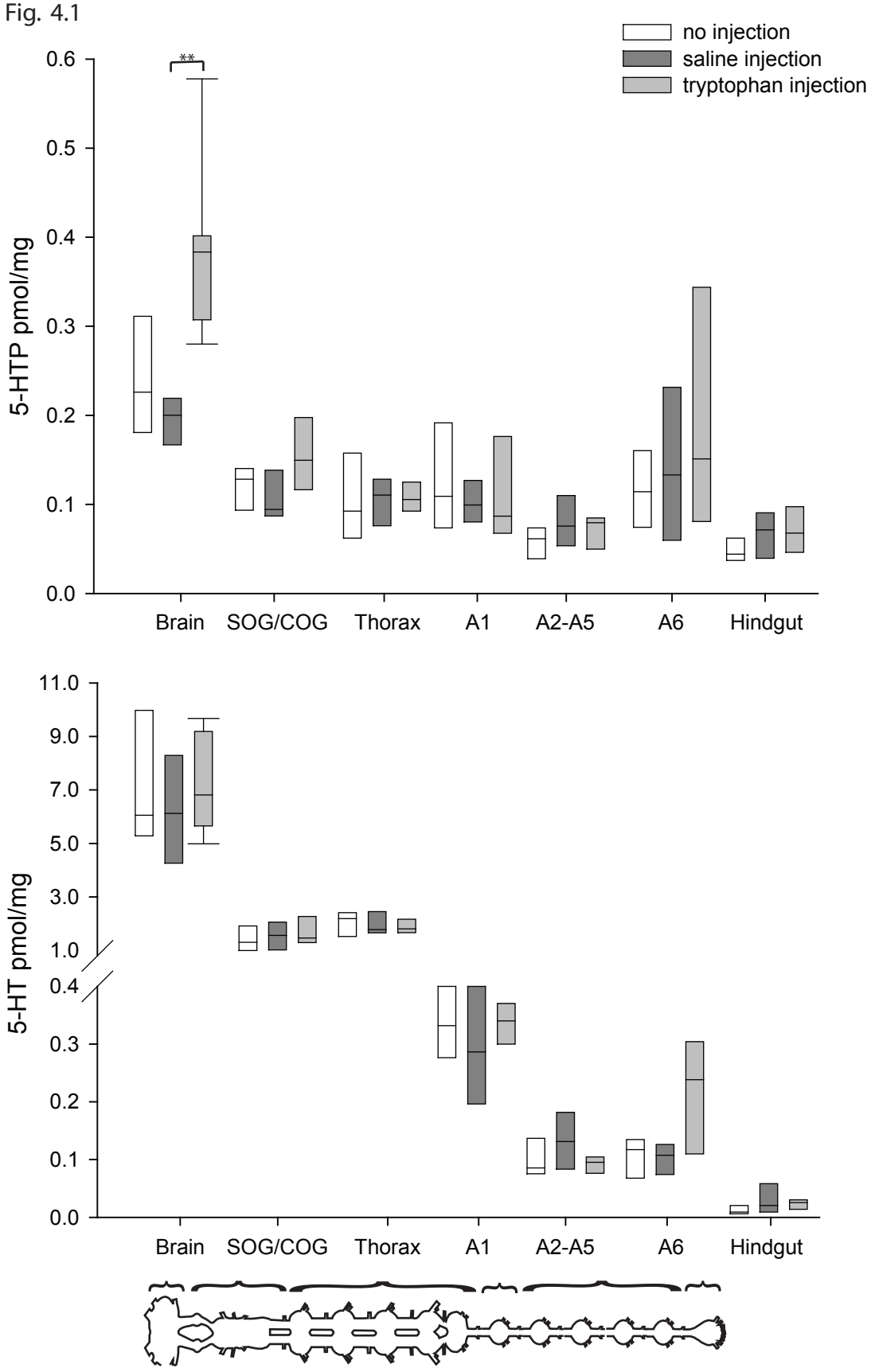

no injection
saline injection
tryptophan injection
**
5-HTP pmol/mg
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut
5-HT pmol/mg
11.0
9.0
7.0
5.0
3.0
1.0
0.4
0.3
0.2
0.1
0.0
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut

Table 4.1 Statistics comparing the amounts of 5-HT in different areas of the CNS. Dunn's post test was used to compare differences between two different CNS tissues. The CEG/SEG and thoracic ganglia had similar densities of 5-HT per mg of tissue as did the A2-A5 and the A6 ganglia. Light gray, $p<0.05$; dark gray, $p<0.01$, n.s., not significant.

Table 4.1

Differences in 5-HT levels between specific tissues (saline injected animals)

	Dunn's post test					
	CEG/SEG	Thorax	A1	A2-A5	A6	Hindgut
Brain	n.s.	n.s.	n.s.	p<0.01	p<0.001	p<0.001
CEG/SEG		n.s.	n.s.	n.s.	n.s.	p<0.01
Thorax			n.s.	n.s.	p<0.05	p<0.001
A1				n.s.	n.s.	n.s.
A2-A5					n.s.	n.s.
A6						n.s.

and feeding Levels of 5-HT in the abdominal ganglia were lower than those found in the cephalothorax. Overall, the concentration of 5-HT in the abdominal ganglia did not vary significantly among the ganglia, which was somewhat surprising given the number of 5-HT-ir neurons located in the first abdominal ganglion (A1). Here is located a pair of major 5-HT synthesizing neurons along with five other identified 5-HT-ir neurons (Real and Czternasty, 1990). The second through fifth abdominal ganglia have small, paired 5-HT neurons and A5 has an additional large unpaired 5-HT neuron (Real and Czternasty, 1990). The concentration of 5-HT in the terminal ganglion (A6) was not significantly different from the other abdominal ganglia, but it did significantly differ from both the brain and thorax. The hindgut had levels of 5-HT that were similar to the abdominal ganglia but that differed significantly from the brain, SEG/CEG and thorax (Table 4.1).

The injection alone did not have a significant effect on measured concentrations of 5-HTP or 5-HT. A comparison of 5-HTP and 5-HT in saline-injected animals and those that were not injected showed that there were no statistical differences in the amount of 5-HTP measured in any region of the nerve cord and hindgut (Fig. 4.1A, B).

Injection of tryptophan increases CNS levels of 5-HTP but not of 5-HT

5-HTP levels were measured in the brain, A6, and hindgut at several intervals following injection of 50 μL of 1 mg/mL tryptophan into the thoracic ventral sinus. Increased 5-HTP levels occurred in the brain 30 min after injection. The increases were significant when compared with the changes produced by injection of the same amount of saline ($p<0.01$) (Fig. 4.1A). The rest of the CNS and the hindgut showed no significant change in 5-HTP levels.

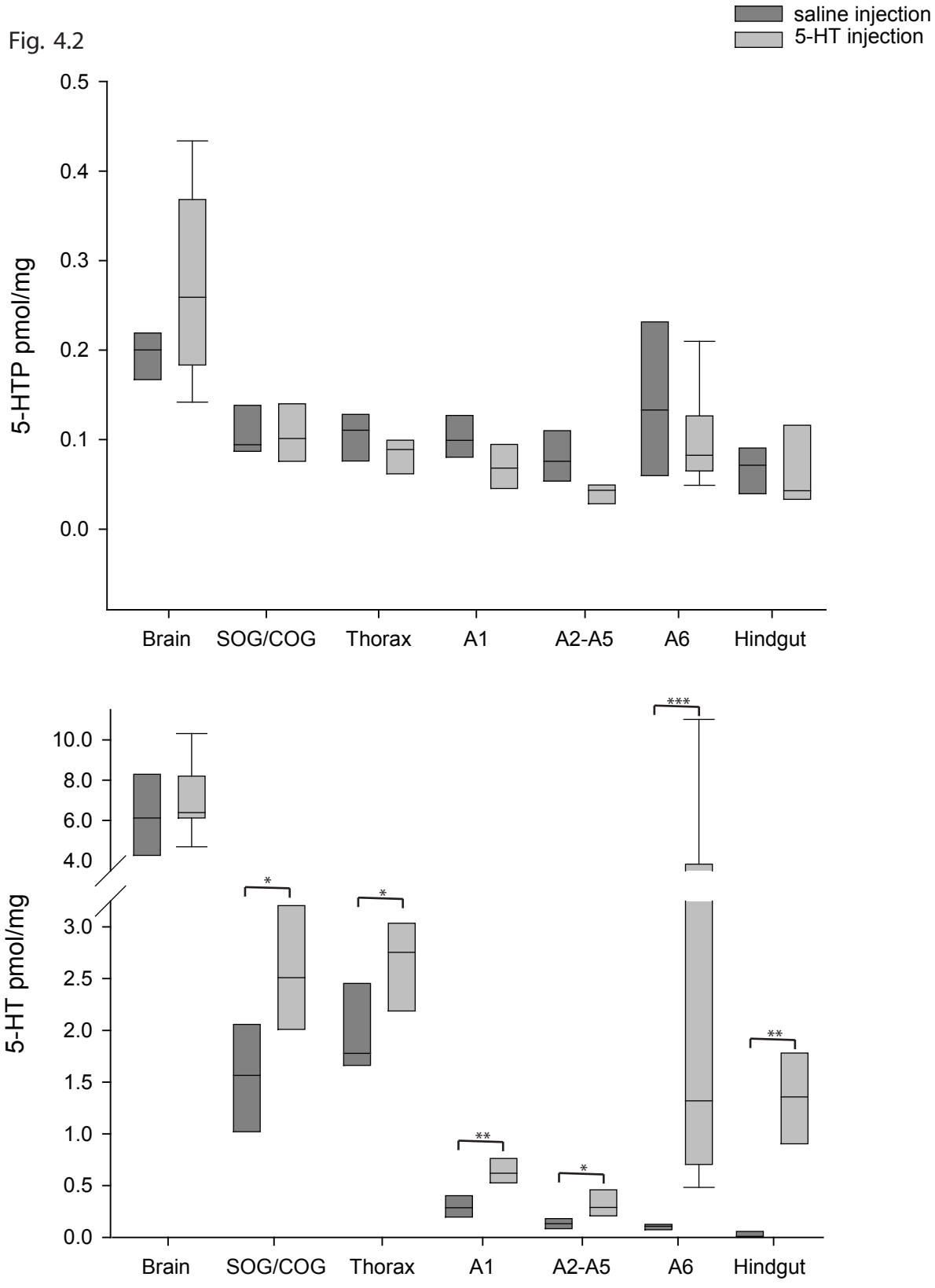
5-HT levels throughout the CNS also were unchanged 30 min after injection of tryptophan in comparison to 5-HT levels measured at the same time in saline injected controls (Fig. 4.1B).

Injection of 5-HT alters the distribution of 5-HT but not 5-HTP in crayfish nerve cord and hindgut

Injection of 50 μL of 500 μg/mL 5-HT into the thoracic ventral sinus of crayfish led to a statistically insignificant increase in 5-HTP in the brain, no change in the SEG, TG, A1, or the hindgut, and an insignificant reduction of 5-HTP in A2-A5 (Kruskal-Wallis, $p<0.03$, Dunn's post-test n.s.) (Fig. 4.2A). In A6, the reduction in 5-HTP levels also did not reach statistical significance. Injection of 5-HT significantly increased measured amounts of 5-HT in the hindgut and all sections of CNS but the brain, when compared to saline-injected controls (Fig. 4.2B). In the brain, while there was an average accumulation of an additional 1 pmol/mg 5-HT, concentrations were variable and thus did not significantly differ from the 5-HT levels in saline-injected crayfish. The most robust changes in the CNS occurred in the abdominal ganglia. Levels in A1 and A2-A5 increased 2 to 2.5 times that of saline injected controls. A6 showed the greatest increase; 5-HT levels increased 25 times over that measured in saline injected controls. Levels of 5-HT in A6 per mg of tissue were similar to what was measured in CEG/SEG and thorax. The largest change in 5-HT concentrations occurred

Fig. 4.2 Levels of 5-HTP and 5-HT in CNS and hindgut following injection of saline and 5-HT into the ventral sinus. (A) following injection of 50 μL of 500 μg/mL 5-HT into the ventral sinus, 5-HTP levels showed a downward trend in the A2-A5 ganglia following injection of 5-HT (KW Test, KW statistic=7.052; $p<0.05$; Dunn's post-test, not significant). (B) Injection of 5-HT increased 5-HT levels over saline injected crayfish in all tissues but the brain. The A6 and hindgut took up the largest amount of 5-HT (KW Test, A6 KW statistic=17.748, $p<0.0001$; hindgut KW statistic=15.023, $p<0.001$). Dunn's post-test was used for pairwise comparisons between saline injected and 5-HT injected tissue (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$)

Fig. 4.2


saline injection
5-HT injection
5-HTP pmol/mg
0.5
0.4
0.3
0.2
0.1
0.0
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut
5-HT pmol/mg
10.0
8.0
6.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
*
***
**
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut

in the hindgut where 5-HT levels increased to over 40 times more than in saline-injected controls.

Changes in tryptophan or 5-HT levels do not affect the distribution of DA

Like 5-HT, the distribution of dopamine (DA) also decreased along the anterior-posterior axis of the crayfish CNS, and like 5-HT there was much less DA per mg of tissue in the hindgut than measured in the CNS (Fig. 4.3A, Table 4.2). DA concentrations were highest and equivalent in the brain and CEG/SEG. Levels of DA in the thorax were a third of that measured in the CEG/SEG and were similar to levels in all the abdominal ganglia. A comparison of DA measurements in saline injected animals and those that were not injected showed that no statistical difference in the amount of DA measured in any region of the nerve cord and hindgut (Fig. 4.3A). Thus, injection itself did not have an effect on measured DA levels. Injection of tryptophan or 5-HT also did not have a significant effect on DA levels in comparison to those animals injected with saline (Fig. 4.3B).

Data from the non-injected groups of crayfish were used to compare the distribution and concentration of 5-HT and DA (Fig. 4.3B). Levels of 5-HT were comparable to DA in the CNS minus eyestalks (5-HT median 10.03 pmol/mg, DA median 9.96 pmol/mg). Levels of both amines were highest in the brain and diminished in the posterior regions of the CNS; however the anterior/posterior gradient of DA concentration was not as steep as the gradient of 5-HT concentration. Concentrations of 5-HT in the brain and thorax were almost twice that of DA, while DA concentrations in the CEG/SEG were over twice that of 5-HT. The A1 ganglion had twice as much DA as

Fig. 4.3 Levels of DA in CNS and hindgut. (A) DA levels did not change in CNS or hindgut in response to 5-HT or tryptophan injection. Saline injection did not significantly alter measured amounts of DA over non-injected preparations. (B) The distribution of DA differs from that of 5-HT in crayfish CNS. There was significantly more 5-HT in the brain and thorax and more DA in the CEG/SEG, the abdominal ganglia and hindgut (See Table 4.2 for statistics; *=$p<0.05$, **=$p<0.01$).

Fig. 4.3

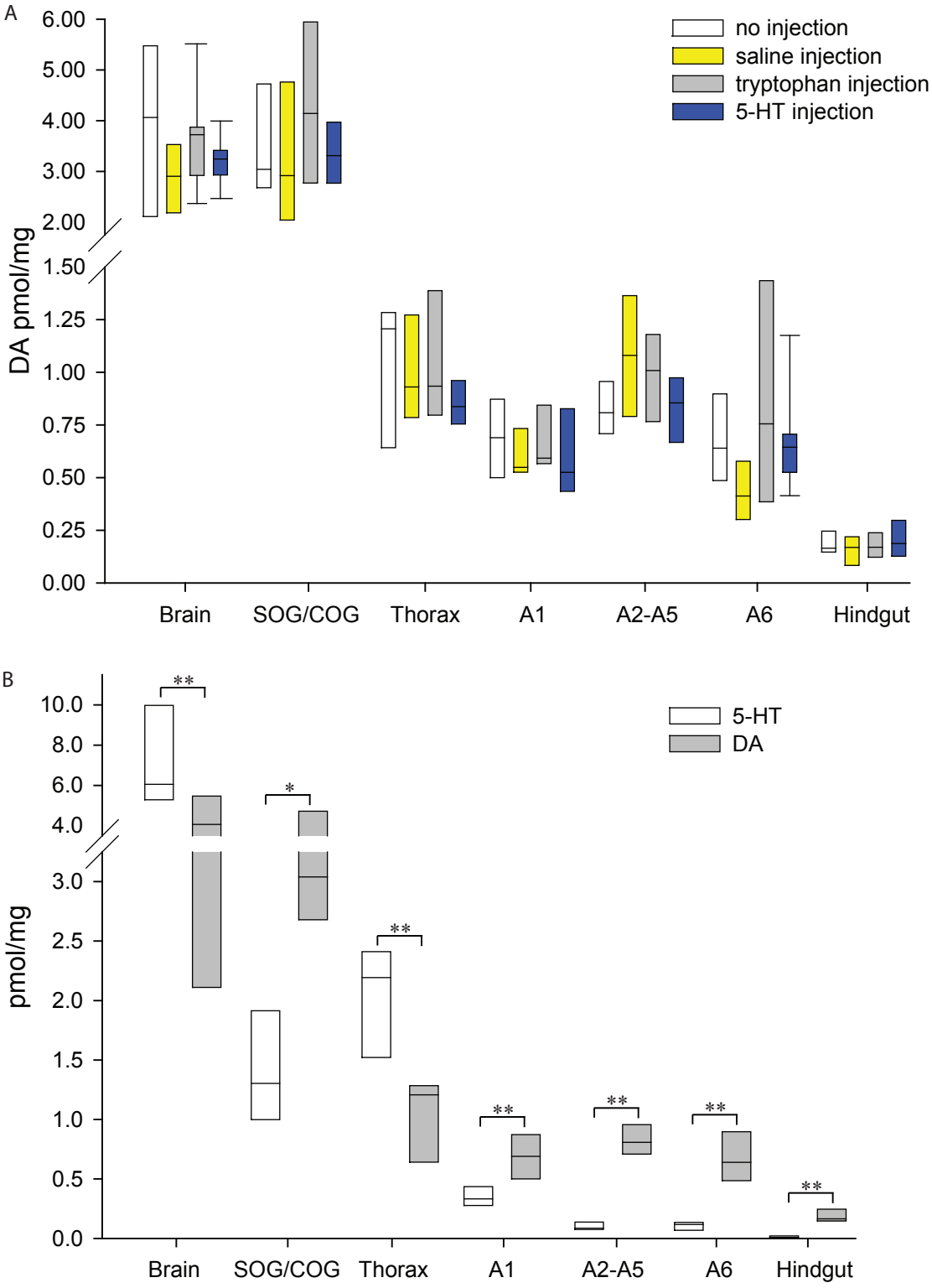

A
DA pmol/mg
6.00
5.00
4.00
3.00
2.00
1.50
1.25
1.00
0.75
0.50
0.25
0.00
no injection
saline injection
tryptophan injection
5-HT injection
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut
B
pmol/mg
10.0
8.0
6.0
4.0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5-HT
DA
**
*
**
**
**
**
**
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut

Table 4.2 Statistics comparing the amounts of DA in different areas of the CNS (KW test, $p<0.0001$, KW statistic=42.31 followed by Dunn's post-test). The brain and CEG/SEG had the most DA, per mg of tissue, followed by the thoracic and A2-A5 ganglia. The A1 and A6 ganglia had the least amount of DA in the CNS and the hindgut had less, per mg of tissue, than what was found in A1 and A6. Light gray, $p<0.05$, medium gray $p<0.01$, dark gray, $p<0.001$.

Table 4.2

Differences in DA levels between specific tissues (saline injected animals)

	CEG/SEG	Thorax	A1	A2-A5	A6	Hindgut
Brain	n.s.	n.s.	P<0.05	n.s.	P<0.01	P<0.001
CEG/SEG		**n.s.**	**P<0.05**	**n.s.**	**P<0.01**	**P<0.001**
Thorax			**n.s.**	**n.s.**	**n.s.**	**n.s.**
A1				**n.s.**	**n.s.**	**n.s.**
A2-A5					**n.s.**	**n.s.**
A6						**n.s.**

5-HT and A2-A5 and A6 had 6 to 7 times as much DA as 5-HT. The hindgut had over 13 times as much DA as 5-HT.

DISCUSSION

Tryptophan injection suggests differences in regulation of 5-HT synthesis

The literature describes two regulatory points in the 5-HT synthetic pathway: availability of tryptophan, and activity of the rate-limiting enzyme tryptophan hydroxylase (TpOH), which converts tryptophan to 5-HTP. Tryptophan availability is thought to be rate-limiting because TpOH is not saturated. Numerous studies have reduced 5-HT levels by restricting intake of dietary tryptophan (Young and Gauthier, 1981). Increasing tryptophan is effective up to concentrations of 200 μM. Levels of tryptophan above 200 μM negatively regulate TpOH (Boadle-Biber, 1993). Here tryptophan was injected into the cardiac sinus of crayfish to reach an approximate hemolymph concentration of 16 μM, which is well below levels that negatively affect TpOH in mammals. This 16 μM of tryptophan is well above tonic levels of 5-HT measured in the hemolymph of marine crustaceans. 1 nM 5-HT has been measured in American lobsters and 0.5 nM 5-HT measured in green crabs (Beltz et al., 1984; Sneddon et al., 2000), The increased tryptophan in the hemolymph led to a near doubling of 5-HTP in the brain and to a 15% increase of 5-HT that did not achieve statistical significance. Other ganglia were largely unaffected, except for A6, where increases in both 5-HTP and 5-HT did not reach significance.

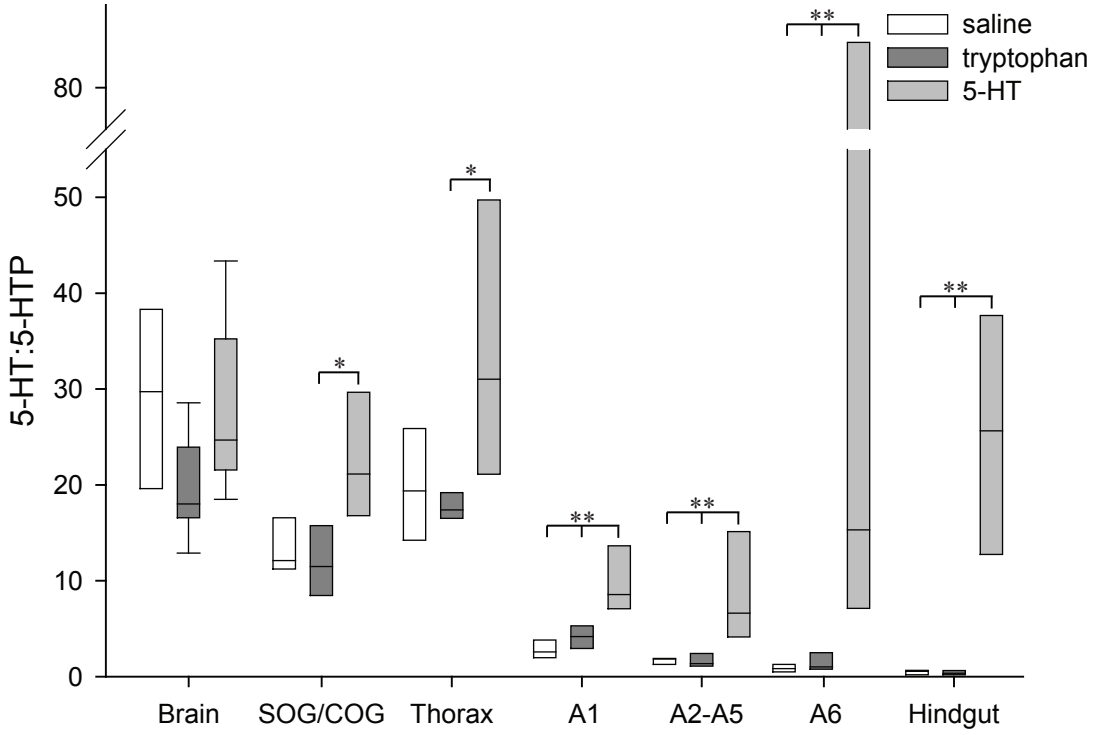
The greater effect of the tryptophan injection on 5-HTP than on 5-HT can be understood in terms of the difference in the size of their pools. In all the CNS, 5-HT is in

much greater concentrations than 5-HTP (Fig. 4.4). The large 5-HT:5-HTP ratio is consistent with immunocytochemical studies in which extensive 5-HT labeling is readily observed while 5-HTP labeling is apparent only in neurons that may synthesize large amounts of 5-HT or when the action of the L-AADC is blocked (Chapter 3). The serotonergic neurons appear to have extensive pools of 5-HT and minimal amounts of 5-HTP. Their tonic activity in crustaceans, as in humans, suggests that tonic release results in a persistent demand on the cells' synthetic and uptake machinery for 5-HT (Heinrich et al., 1999). With a constant throughput of tryptophan to 5-HTP, 5-HTP to 5-HT, and 5-HT to release, the lifetime of 5-HTP should be much shorter than the lifetime of 5-HT, which will spend time before release in intracellular transport or in a storage pool. The additional tryptophan provided by the injection may cause a significant percentage increase in the small 5-HTP content but a much smaller percentage increase in the much larger 5-HT content of the serotonergic neurons, as was observed in the brain. In less active parts of the nervous system, the throughput rate may be less, causing a tryptophan injection to have a smaller effect on both 5-HTP and 5-HT levels. Alternatively, it is possible that the tryptophan injections had their greatest effects on 5-HTP at either end of the nervous system, in fused ganglia that receive massively converging sensory input that is modulated by 5-HT (Sandeman et al., 1995; Edwards et al., 2002; Spitzer et al., 2005).

It may be that a larger tryptophan injection, or injections spaced over time, or injections at different times of day may have achieved a more marked result. 5-HT levels in the brains of adult crayfish do not display significant circadian variation, but do have a major peak at noon, a minor peak at midnight, and troughs at 06:00 and 20:00, at dawn and dusk, respectively (Castanon-Cervantes et al., 1999). The animals in this study were

Fig. 4.4 5-HT:5-HTP following saline, tryptophan, or 5-HT injection. The 5-HT:5-HTP ratio in saline injected crayfish was different in each tissue studied. Tryptophan injection reduced 5-HT:5-HTP in the brain compared to the 5-HT injection ($p=0.022$) and showed a trend following saline injection ($p=0.057$). 5-HT injection significantly increased 5-HT:5-HTP in comparison to saline injection in all tissues but the brain and thorax. KW test: brain, $p=0.0385$, KW statistic $=6.515$; CEG/SEG, $p=0.0091$, KW statistic$=9.409$; TG, $p=0.0433$, KW statistic$=6.281$; A1, $p=0.004$, A2-A5 $p=0.0005$, KW statistic$=15.023$; A6, $p=0.0008$, KW statistic$=14.222$; hindgut, $p=0.0005$, KW statistic$=15.023$. Dunn's post-test results indicated on graph; *=$p<0.05$, **=$p<0.01$.

Fig. 4.4


saline
tryptophan
5-HT
5-HT:5-HTP
80
50
40
30
20
10
0
**
*
*
**
**
**
Brain
SOG/COG
Thorax
A1
A2-A5
A6
Hindgut

sacrificed between 09:15 and 17:15, a period that spans the major peak of 5-HT in the brain.

Uptake of extracellular 5-HT

Extracellular 5-HT can modulate the activity of serotonergic neurons, affect serotonin release at presynaptic terminals, and be taken up by serotonin transporters. Accordingly, I expected that injection of 5-HT would induce uptake and lead to enhanced levels of 5-HT in most regions of the CNS. This occurred everywhere except the brain. The increases were substantial in all parts of the CNS between the brain and A6, but in A6 and in the hindgut they were relatively enormous. A6 contains the somata and the hindgut is the peripheral target of the hindgut neurons (HGNs), which constitute the largest single set of serotonergic neurons in the crayfish CNS (Chapter 3). The HGNs do not appear able to synthesize 5-HT, but they can take it up centrally in A6 and peripherally throughout their extensive projections on the hindgut (Chapter 2 and 3).

The lack of significant uptake in the brain could reflect either a low expression of the serotonin transporter or the possibility that the crayfish brain may have some other mechanism for protecting the brain from circulating hormones. In the arthropod *Drosophila*, a combination of different glial cells was found to function in the CNS as a blood-brain-barrier (Freeman and Doherty, 2006). This lack of increase in 5-HT supports another study that used implanted 5-HT crystals to chronically increase 5-HT levels in the hemolymph. Measurement of 5-HT during the time hemolymph levels of 5-HT were elevated revealed no 5-HT increases in the brain (Panksepp and Huber, 2002).

5-HTP levels are affected by extracellular 5-HT

Feedback from 5-HT to the serotonergic synthetic system appears to produce changes in CNS 5-HTP levels in response to injection of 5-HT. 5-HTP was significantly reduced in A2-A5, a trend for 5-HTP to be reduced occurred in A6, and a trend for 5-HTP to be increased occurred in the brain (Fig. 4.2A). These results suggest that TpOH is negatively regulated by 5-HT in the abdominal nervous system, and L-AADC may be down-regulated in the brain. Chapter 3 showed that superfusion of 5-HT led to reduced 5-HT-ir labeling of the primary 5-HT synthesizing neuron in the A6 ganglion, which supports this result. Both TpOH and L-AADC are down-regulated in vertebrates when 5-HT binds to a 5-HT_1-like autoreceptor which inhibits cAMP (Barnes and Sharp, 1999). An immunocytochemical study of the 5-$HT_{1\alpha}$ receptor in crayfish abdomen indicates that the receptor, which also down-regulates cAMP, coexists with 5-HT varicosities in the neuropil of the abdominal CNS (Spitzer et al., 2005). Results in Chapter 3 showed that conversion of 5-HTP to 5-HT may occur in such varicosities. Because nothing is known of the second messenger systems that regulate the crustacean TpOH or L-AADC enzyme, it is not possible to say whether receptor activation by 5-HT could lead to a downregulation of either enzyme.

Differences in serotonergic systems along the ventral nerve cord

The anterior part of the CNS, including the brain, subesophageal and circumesophageal ganglia, and thoracic ganglia, differs from the posterior part (the abdominal ganglia) and the hindgut in the regulation of 5-HT. This is clear from a comparison of the 5-HT:5-HTP concentration ratios from each group of ganglia (Fig. 4.4). The ratio varies between 15 and 30 in the anterior nervous system (saline injected

preparations), and between 1 and 4 in the posterior nervous system and hindgut. Most of this difference results from differences in the concentration of 5-HT, which is between 5 and 30 times greater in the anterior than in the posterior ganglia, while the concentration of 5-HTP is between 1.3 to 2 times greater (Fig. 4.1). The larger ratio found in the anterior ganglia suggests that they are more active in producing 5-HT than the more caudal ganglia.

The distribution of 5-HT in the crayfish CNS reflects the number of 5-HT neurons found in each of the regions measured. In the brain, all three major neuroanatomical regions, the protocerebrum, deutocerebrum, and tritocerebrum, show extensive 5-HT-ir (Sandeman et al., 1992). In the CEG, there is one pair of 5-HT neurons and a densely labeled neuropil in each hemiganglion, while the SEG has numerous small 5-HT neurons. The thoracic ganglia each have a few large serotonergic neurons and a dense neuropil of immunoreactive processes. While the A1 ganglion has a pair of major synthesizing 5-HT neurons along with five other minor synthesizing neurons, the A2-A5 ganglia only have a small single pair of 5-HT neurons in each ganglion and a much less dense set of serotonergic fibers (Real and Czternasty, 1990). The A6 ganglion has a single prominent 5-HT synthesizing neuron, while the hindgut is innervated by the hindgut neurons that normally display little 5-HT-ir.

The apparent drop in the 5-HT:5-HTP ratio in the brain in response to tryptophan injection reflects the increase in 5-HTP that was not balanced by a proportional increase in 5-HT. The increases in the ratio as a consequence of 5-HT injection reflect the uptake of 5-HT in all ganglia except the brain. Here the abdominal nervous system and hindgut differ greatly from the anterior CNS in the increases of the 5-HT:5-HTP ratio. Uptake

appears to play a much greater role in the abdominal nervous system and hindgut than in the anterior nervous system. This is clear from Fig. 4.5, which shows the change in the 5-HT:5-HTP ratio in abdominal CNS tissues and the hindgut produced by 5-HT injection.

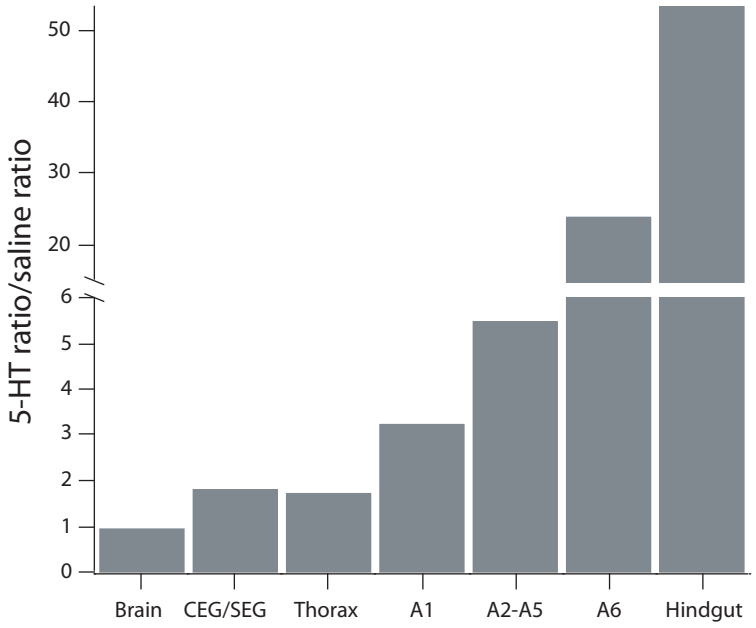
DA distribution is not affected by injection of 5-HT or tryptophan

In *Drosophila*, it has been hypothesized that 5-HT may be synthesized by two different TpOH enzymes, one that is primarily neuronal and exclusively leads to synthesis of 5-HT and one that is peripheral that leads to synthesis of both 5-HT and DA (Coleman and Neckameyer, 2004). Studies of the *Drosophila* tryptophan-phenylalanine hydroxylase (DTPH) enzyme indicated that both 5-HT and DA application enhanced activity of the enzyme. This result suggests that 5-HT application could lead to increases in DA. Furthermore, the two amines share the L-AADC enzyme. Both 5-HTP and L-DOPA could compete for L-AADC, and following tryptophan injection this could lead to lower levels of DA.

This study showed that increases in either tryptophan or 5-HT did not affect levels of DA in crayfish CNS or hindgut. Currently, I do not know whether crayfish have a TpOH-like enzyme with the dual synthetic properties observed in *Drosophila*. Multiple enzymes for 5-HT synthesis are not unique to *Drosophila*. Mice also have two different TpOH enzymes, but neither one has the dual properties of the DPTH enzyme described in *Drosophila* (Walther et al., 2003). In mice, one of the TpOH enzymes is neuronal and the other non-neuronal and peripheral. It is possible that the presence of two TpOH enzymes may be a conserved mechanism to ensure availability of 5-HT and each may have exclusive regulatory sites. The peripheral DPTH enzyme with dual synthetic capabilities

Fig. 4.5 Change in 5-HT:5-HTP in each tissue produced by 5-HT injection. The ratio calculated for each tissue used the average ratio from 5-HT injected animals divided by the average ratio from the corresponding tissue in saline-injected animals.

Fig. 4.5


5-HT ratio/saline ratio
50
40
30
20
6
5
4
3
2
1
0
Brain
CEG/SEG
Thorax
A1
A2-A5
A6
Hindgut

may be far more useful, particularly in animals that do not have a blood brain barrier, in balancing the levels of both 5-HT and DA, both of which are important neurotransmitters and neurohormones in the hindgut as well as other peripheral organs.

In comparison to 5-HT, more DA is found in areas of the crayfish CNS that are associated with the digestive system, CEG/SEG, and abdominal ganglia. In addition there is more DA on the hindgut. In the CNS, single unpaired neurons in A2 through A4, identified as dopaminergic, project to the hindgut, form collaterals, and appear to innervate the hindgut longitudinal muscles (Mercier et al., 1991; Spörhase-Eichmann et al., 1998). Application of 10 to 100 nM DA increased tonus in the hindgut with the maximum effect occurring at 100 nM (Mercier et al., 1991).

Conclusions

The CNS of crayfish differs along its length in both the content of 5-HT and the mechanisms used to maintain that content. An anterior/posterior gradient exists in the concentration of 5-HT found in central ganglia that reflects a corresponding gradient in the number and density of serotonergic neurons. The injection of 5-HT into the ventral sinus uncovered the brain's ability to protect itself from excess levels of 5-HT. While the brain may have fewer 5-HT transporters, it is more likely that it has a type of local blood brain barrier.

In A6 and the hindgut, injection of 5-HT into the ventral sinus led to large increases in 5-HT. The injection of 5-HT, which simulates release of 5-HT from neurosecretory organs, suggests that hormonal release of 5-HT could allow movement of 5-HT from areas that synthesize most of the 5-HT to areas that synthesize very little 5-

HT. Injection of 5-HT showed a trend to decrease 5-HTP in A2-A5 and A6, suggesting that the L-AADC enzyme may be suppressed or downregulated in these ganglia in response to 5-HT. Injection of tryptophan showed a trend to increase 5-HT in A6, where levels of 5-HTP were quite variable. The possibility exists that A6 functions as an opportunistic reservoir for 5-HT, whether it is taken up when it is available in the hemolymph or synthesized and stored as 5-HTP for later conversion to 5-HT.

5

The effects of feeding on levels of 5-HT in crayfish brain, terminal ganglion and hindgut.

For almost 60 years, serotonin (5-hydroxytryptamine, 5-HT) has been known to play a role in digestion. Erspamer (1946) isolated what he called enteramine from the enterochromaffin cells in the guinea pig intestinal mucosa (Erspamer, 1946). Since that time, 5-HT has been shown to regulate feeding behavior and intestinal activity in a variety of different animals (see review, Weiger, 1997): nematodes (Horvitz et al., 1982), mollusks (Kupfermann et al., 1979), mosquitoes (Clark et al., 2000; Moffett et al., 2005), crustaceans (Katz et al., 1989), leech (Lent, 1985), lamprey (Baumgarten et al., 1973) and humans (Gershon, 1999; Simansky, 1996). In these different animals, 5-HT is implicated in a sequential pattern of feeding activity: appetite, biting, salivation, pharyngeal and intestinal peristalsis, mucous secretion, and satiation.

In mammals, almost 95% of 5-HT is found in the gut (Erspamer, 1966). Of that amount, 95% is synthesized and released from enterochromaffin cells, initiating a variety of responses: nausea, vomiting, intestinal secretion, gut contractions, and peristalsis (Gershon, 1999). The other 5% is produced in 5-HT synthesizing neurons. A number of behaviors such as satiety, rate of eating, and amount of food intake are also affected by the 5-HT synthesized in the gut (Simansky, 1996). Numerous 5-HT receptors are found in mammalian gut, one of which, 5-HT_{1P}, is unique to the gut (Fiorica-Howells et al., 1993, Pan and Gershon, 2000). These receptors are responsible for producing numerous and sometimes contradictory responses, contracting and relaxing the gut and stimulating and inhibiting secretions (Gershon, 1999). While 5-HT is only one of many transmitters found in the gut, 5-HT receptors and transporter are considered important enough to target in treating such diverse disorders such as anorexia nervosa, ulcerative colitis, and irritable bowel syndrome (Coates et al., 2004).

In crustaceans, little 5-HT is found in the enteric system. Sites of 5-HT synthesis are in the cephalothoracic region: the pericardial organs located in the ventral pericardial sinus, the second roots of the thoracic ganglia, and the perineural sheath surrounding the ventral nerve cord (Beltz, 1988). Despite the fact that little or no 5-HT is synthesized in the crayfish enteric system, 5-HT has been identified as one of the many neurotransmitters that plays a role in foregut activity (Christie et al., 1995). In crustaceans, individual cells such as the gastropyloric receptor neurons have been identified as providing synaptic release of 5-HT in the foregut of crustaceans (Katz et al., 1989). These neurons, excited by activity of the gastric mill, provide input onto cells that modulate pyloric sac rhythms. Hormonal 5-HT is also used to affect foregut activity. The stomatogastric ganglion is located in the ophthalmic artery and is exposed to 5-HT and numerous amines and peptides that are released from the pericardial organ into the hemolymph. These same aminergic and peptidergic neuromodulators are also distributed to the hindgut via arterioles branching off from the dorsal abdominal artery. The 5-HT released from the pericardial organ modulates arterial valves that direct the flow of the hemolymph through the branching segmental arterioles to the hindgut (Wilkens, 1997).

Superfused 5-HT has clear effects on hindgut motility, initiating peristalsis and both enhancing and reducing contractile force, depending on the concentration of 5-HT and to what region of hindgut 5-HT is applied (Chapter 2). The hindgut shows immunocytochemical labeling for at least two 5-HT receptors, the 5-$HT_{1\alpha}$ and 5- HT_{2B}. The diffuse pattern of the 5-$HT_{1\alpha}$ and 5- $HT_{2\beta}$ receptors on particular regions of the hindgut suggests a role for 5-HT hormonal or paracrine neurotransmission (Chapter 2). Synaptic transmission must also exist since the postglandular region of hindgut, which

initiates peristalsis and produces higher frequency contractions, has 5- $HT_{2\beta}$ receptor plaques that are associated with 5-HT varicosities and plaques are found along both 5-HT-ir and unlabeled fibers (Chapter 2).

Immunocytochemical studies indicate that the hindgut neurons (HGNs) are probably the only source of 5-HT in the gut. Levels of 5-HT vary widely in the HGNs (Musolf and Edwards, 1999; Musolf et al., 2001) and it is possible that levels of 5-HT vary in accordance with feeding or digestive functions. This chapter investigates whether feeding leads to reliable changes in the amount of 5-HT and its precursor, 5-HTP, in hindgut tissue and the terminal ganglion (A6). I used HPLC to measure changes over time in tissue levels of 5-HT and 5-HTP. To determine where cellular increases in 5-HT take place, I used quantitative immunocytochemistry.

Previously, I tested whether constitutive synthesis took place in the HGNs (Chapter 3) and it did not. An unresolved question in Chapter 3 was whether 5-HT could undergo facultative synthesis in the HGNs. If an increase in 5-HT-ir occurs in the HGNs during the digestive process, then this stimulus could be used to test whether facultative synthesis or uptake is responsible for the 5-HT-ir increase. The possibility exists that the variable levels of 5-HT-ir observed in the HGNs indicate that 5-HT synthesis can be turned on or off. To test whether an increase in 5-HT-ir was the result of uptake or synthesis, I tested synthesis as I did in Chapter 3 and blocked L-amino acid decarboxylase (L-AADC) and examined whether 5-HTP-ir accumulated in the HGNs during the 48 hrs following feeding. Positive 5-HTP-ir would indicate that synthesis is turned on in the HGNs during feeding, negative 5-HTP-ir, but positive 5-HT-ir would indicate that an increase in 5-HT-ir during the digestion process may occur through uptake. Since

synthesis does not take place in the HGNs under *in vitro* conditions, finding synthesis under *in vivo* conditions when 5-HT-ir is found to increase would indicate that 5-HT synthesis is facultative.

I also measured changes in the levels of 5-HT and 5-HTP in the brain. The expectation is that blood levels of tryptophan would increase following a meal that contained tryptophan (Young and Gauthier, 1981) and that would lead to an increase in 5-HTP and perhaps to 5-HT. In Chapter 4, I showed that injection of tryptophan gave rise to increased amounts of 5-HTP in the brain, but no increase in 5-HT. However, the response following a meal could differ.

METHODS

Animals and dissection

Crayfish, *Procambarus clarkii*, were acquired from Atchafalaya Biological Supply in Raceland, LA and communally maintained at 22°C in freshwater aquaria exposed to a 12:12 light/dark cycle. Crayfish used for immunocytochemical studies ranged in length from 2.7 to 4.6 cm with an average length of 3.5 cm. Crayfish used for HPLC studies range in length from 4.9 to 6.7 cm with an average length of 5.5 cm. They were fed twice a week. I used 2 groups of crayfish for the quantitative immunocytochemical study and I used 3 groups of crayfish for the HPLC study. Prior to experimental manipulation, crayfish were not fed for 2 weeks. 12 hr before they were fed, 24 crayfish were separated into 6 different tanks. On the day of the experiment all but the unfed group of crayfish were given 2 shrimp pellets each. Dissections were performed on the crayfish that were not fed and then on the others 1, 6, 12, 24, and 48 hr after they

were fed. Dissections followed cold anesthetization, which was done by filling each tank with ice at the designated time to create a freezing ice slurry around the animal. Notes were made on the fullness of stomach (empty, some, full) on hindgut contents (<1/4 full, <3/4 full, and full), the length of animal, and the condition of the animal. An empty stomach was labeled such if it had no food and a full stomach was defined as one that was filled and distended with food. Those that were not empty or full were considered to have some food. Dissection notes on hindgut contents described location of fecal material and what fraction of the hindgut they filled. This information was then used to define the three different groups of hindgut fullness. For the immunocytochemical studies, the CNS and hindgut were dissected out of the crayfish, and for the HPLC studies only the brain, the A6 ganglion and the hindgut were dissected out of the crayfish.

Wholemount immunocytochemistry

The protocol for 5-HT immunoreactivity (5-HT-ir) was derived from Beltz and Kravitz (1983) and is presented in greater detail in Chapter 3. We used anti-5-HT and anti-5-HTP antibodies from Immunostar (Stillwater, MN) at a 1:200 dilution. The secondary fluorescent antibody used with the anti-5-HT was goat anti-rabbit Texas Red and that used with the anti-5-HTP was goat anti-rabbit Alexa 488 (Molecular Probes, Eugene OR) at a 1:100 dilution. Tissue was then dehydrated in an alcohol series, cleared, and mounted in methyl salicylate.

Confocal imaging

A Zeiss LSM 510 confocal microscope was used to produce micrographs of the fluorescent immunolabeled preparations. The wholemount hindgut preparation was imaged using a fluor 5X objective. A projected image was produced using LSM510 3.0 software. This projected image was exported as a tif file into Adobe Photoshop 7.0 and assembled into a photomontage of the entire hindgut. Images of A6 5-HT medial cell, the HGN cell bodies, N7 and the anterior intestinal nerve (AINs), which were used for quantitative analysis were imaged using the 20X objective.

When using the LSM 510 for quantitative analysis, the lasers were allowed to warm up for one hour. An area of the fluorescently labeled preparation that was not of interest to be quantified and that was a similar intensity to the areas of interest that were going to be imaged in a particular session, was used to setup the microscope. The images were setup so that there was very little saturation of the image. These settings: detector gain, amplifier gain and detector offset, remained identical for entire imaging session.

The brightness measurements were calibrated after capturing the images through the use of InSpeck Green, 6 μM diameter calibration beads (Molecular Probes, Eugene, OR): one group had no fluorescence and the other 6 groups of beads had fluorescent intensities that differed by a half a log unit in fluorescent intensities (0 %, 0.3 %, 1.0 %. 3.0 %, 10 %, 30 %, and 100 % intensities). At 24 hr before imaging, the different beads were mixed, mounted on a slide using the supplied mounting medium, coverslipped, and stored in a lightproof container until ready for imaging. Prior to imaging, the bead slide was allowed to warm up to room temperature. The bead slide was imaged prior to

imaging the preparations, in between imaging of each preparation, and at the end of the imaging session.

Images were captured of the HGN cell bodies, N7 and the AINs. As a control, images were captured of the A6 medial 5-HT cell. The total Z axis was determined by setting it above and below what was visible for each of the different areas of interest.

Quantitative immunocytochemistry analysis

The LSM 510 software was used to determine the brightness of the different areas of interest. To adjust for the changes in the sensitivity of the LSM 510 to the fluorescent signal, I used the LSM 510 3.0 software. I outlined 6 beads of at least 3 different fluorescent intensities and measured their brightness and averaged the signal. It was necessary to measure only those beads that did not produce a saturated signal, which limited the range of beads that were used. The average brightness of the beads was plotted on a graph using Sigma Plot 5.0 (SPSS) and this plot was used to generate a nonlinear equation which captured the current sensitivities of the fluorescent microscope. These equations were then used to adjust the brightness measurements of the areas of interest.

To analyze the HGN cell bodies and the A6 medial cell, each Z-section was outlined to include all the 5-HT-ir. Analysis of the A6 medial cell included all of the Z axis images. Analysis of the HGNs used every fifth Z axis section. Analysis of the N7 and AINs was done by locating the brightest areas within the rectal region and defining a cylindrical area of the nerves for brightness analysis. For analysis of all areas, background measurements were determined by choosing an area of the image that

showed no 5-HT-ir and showed the lowest levels of brightness. Brightness measurements were exported into an Excel spreadsheet as the number of pixels at each intensity. The intensity numbers were first transformed using the equations that were derived from the calibration beads. Those pixels below background were then eliminated and the brightness was determined by multiplying the number of pixels at each transformed intensity. The brightness numbers for all of the slices chosen for analysis were then added to get total brightness. Because ICC protocols differed slightly with the 3 different groups of tissues processed, the brightness numbers were normalized within those 3 groups prior to combining all of the data on each area of interest. The normalized values were used for statistical analysis and to produce the box plots.

5-HT quantification

The protocol for measuring 5-HT using HPLC was derived from Fickbohm et al. (2001). A model 528 pump and Coulochem II detector with a flow through model 5011 Analytical Cell (ESA, Inc. MA) made up the HPLC system. A more detailed description of this protocol is in Chapter 2.

On the day samples were analyzed, standard curves were run of 5-HT, 5-HTP, 5-hydroxyindoleaminic acid, and N-methylserotonin (NMS). The amounts of these compounds were determined using software provided by the ESA HPLC system.

Statistics

All values are given as median ± 25/75 inter quartile range. InStat3 for Macintosh (GraphPad Software, San Diego, CA) was used to determine significance among different groups. All analyses were done using nonparametric statistics. I used the

Kruskall Wallis test for comparisons of repeated measures of analysis of variance (ANOVA) followed by Dunn's post-test. Differences were considered significant if $p < 0.05$.

RESULTS

Feeding changes 5-HTP levels but not 5-HT levels

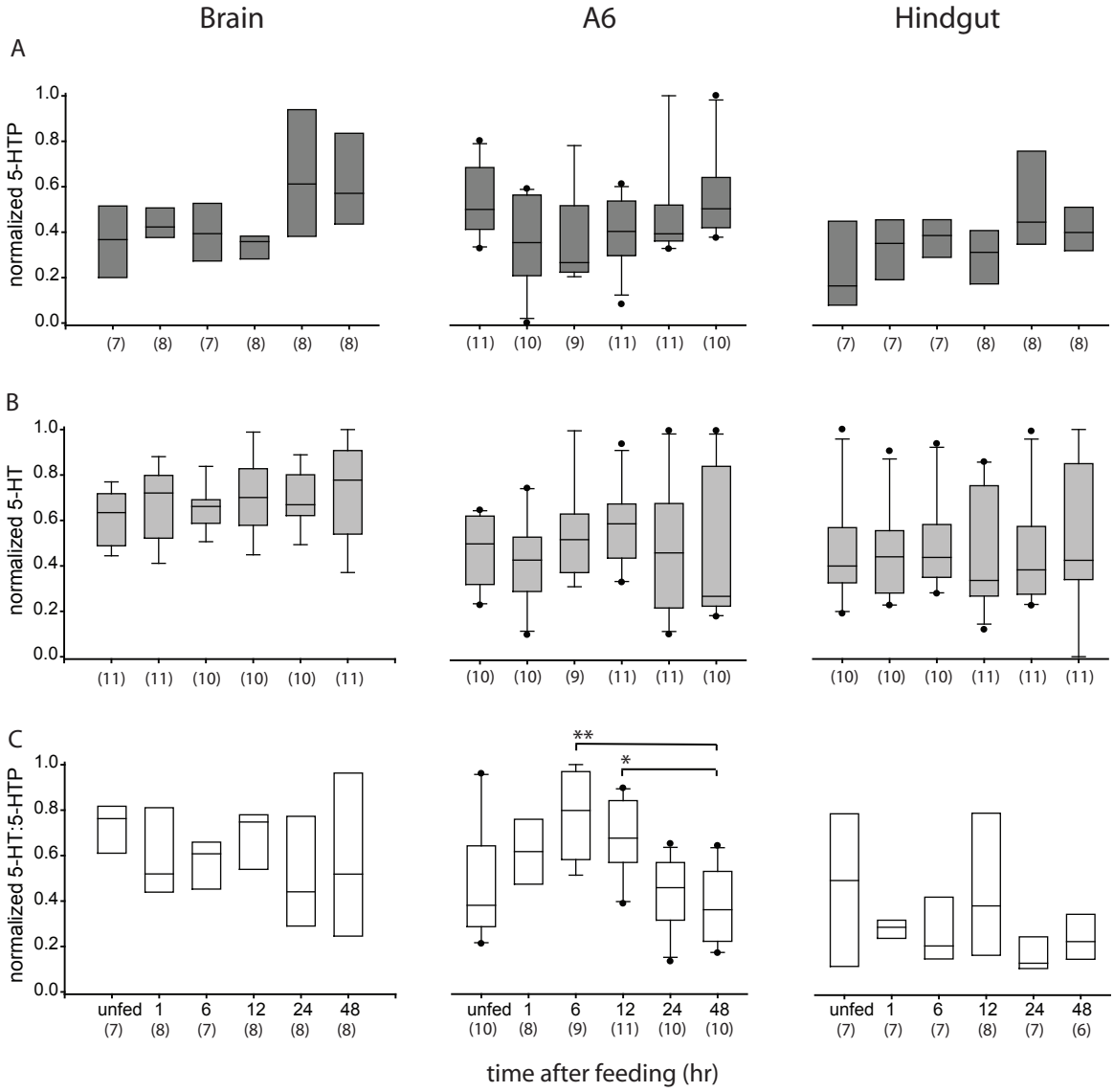
In the brain, food intake correlated with increases in 5-HTP amounts at 24 and 48 hr over levels measured at 12 hr (Fig. 5.1A). In both A6 and the hindgut, there were no significant differences over time (Fig. 5.1A). In A6 5-HTP dropped at 1 hr and levels remained depressed at 6 and 12 hr after feeding (Fig. 5.1A). At 24 to 48 hr after feeding, levels in A6 rebounded to what they were prior to feeding. In the hindgut, 5-HTP levels during the first 12 hr were unchanged from levels measured in unfed crayfish and then trended higher at 24 and 48 hr (Fig. 5.1A).

Measurements of 5-HT over time did not significantly vary in brain, A6, or hindgut following food intake (Fig. 5.1B). In the brain, 5-HT exhibited a shallow rise over time with mean levels greatest at 48 hr (Fig. 5.1B). In A6, there was a slight drop 1 hr after feeding; levels then peaked at 12 hr and decreased over the next 24 hr (Fig 5.1B). In the hindgut, mean 5-HT levels remained constant (Fig. 5.1B).

I calculated the ratio of 5-HT to 5-HTP in individual animals and then took the median of that ratio to describe the dynamics of synthesis and uptake. Results in Chapter 4 showed that injected 5-HT significantly increased 5-HT:5-HTP in abdominal ganglia, indicating increased uptake while injected tryptophan significantly decreased the ratio in

Fig. 5.1 5-HTP and 5-HT levels and 5-HT:5-HTP in brain, A6, and hindgut in unfed animals and 1, 6, 12, 24, and 48 hr after feeding. The 5-HT:5-HTP ratio was calculated for individual animals before determining median values. (A) Normalized 5-HTP levels in the brain, A6 and the hindgut. In the brain, there was a significant difference among the time periods with no pairwise significant differences (Kruskal-Wallis (K-W) test p= 0.043, KW statistic = 11.458; Dunn's post-test n.s.). In A6, 5-HTP levels show a trend to be depressed at 6 and 12 hr in comparison to unfed crayfish and those fed 48 hr earlier (K-W test p=0.0518, KW statistic = 10.981). In hindgut, there were no significant differences in measured levels of 5-HTP (K-W, p=0.2027, KW statistic = 7.251). (B) Normalized 5-HT levels in brain, A6, and hindgut. There were no significant differences in 5-HT levels in the brain (K-W test p=0.6039, KW statistic = 3.629), A6 (K-W test p=0.5201, KW statistic = 4.206), or hindgut (K-W test p=0.8802, KW statistic = 1.768). (C) 5-HT:5-HTP levels in brain, A6, and hindgut. 5-HT:5-HTP ratio in the brain and hindgut did not significantly differ (Brain: K-W test p=0.4597, KW statistic = 0.4597; hindgut: K-W test p=0.1045, KW statistic= 9.116). 5-HT:5-HTP in A6 was significantly decreased at 48 hr compared to the elevated ratios at 6 hr and 12 hr (K-W test p=0.0021, KW statistic = 18.776). Box plots show median values and upper 75% and lower 25% limits. Error bars indicate the upper 90% and lower 10%; dots represent the outliers; * = $p<0.05$, ** = $p<0.01$.

Fig. 5.1


Brain
A6
Hindgut
A
B
C
normalized 5-HTP
normalized 5-HT
normalized 5-HT:5-HTP
1.0
0.8
0.6
0.4
0.2
0.0
(7) (8) (7) (8) (8) (8)
(11) (10) (9) (11) (11) (10)
(7) (7) (7) (8) (8) (8)
(11) (11) (10) (10) (10) (11)
(10) (10) (9) (11) (11) (10)
(10) (10) (10) (11) (11) (11)
**
*
unfed 1 6 12 24 48
(7) (8) (7) (8) (8) (8)
unfed 1 6 12 24 48
(10) (8) (9) (11) (10) (10)
unfed 1 6 12 24 48
(7) (7) (7) (8) (7) (6)
time after feeding (hr)

the brain, signaling decreased conversion of 5-HTP to 5-HT. Following food intake, 5-HT:5-HTP in the brain was lower than in unfed crayfish except at 12 hr (Fig. 5.1C). A6 had a significantly higher mean ratio at 6 and 12 hr compared to 48 hr, reflecting the slight increases in 5-HT along with suppressed levels of 5-HTP (Fig. 5.1C). In the hindgut, the mean pattern of 5-HT:5-HTP following food intake was similar to that measured in the brain with the ratio decreased except at 12 hr (Fig. 5.1C); there was no significant difference among the time periods tested, but there was a very modest decrease in 5-HT:5-HTP over the 48 hr.

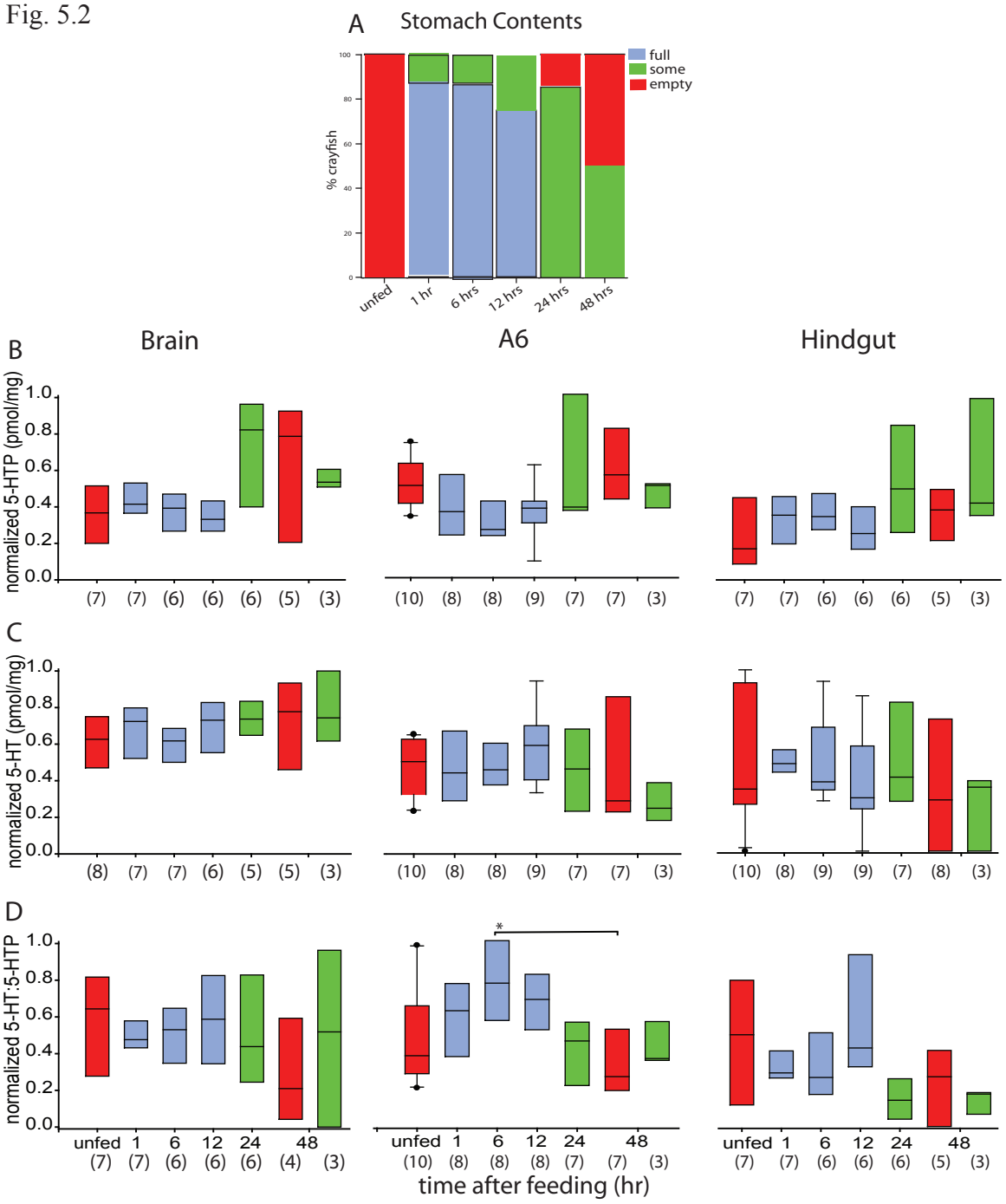
Effects of stomach contents on 5-HT, 5-HTP, and 5-HT:5-HTP

An examination of stomach contents in all groups showed that the unfed crayfish did have empty stomachs and that the stomachs were full or had some food for up to 12 hr following placement of the shrimp pellets in the aquaria (Fig. 5.2A). At 24 hr, levels of food in the stomach decreased and by 48 hours half of the crayfish had empty stomachs again. To examine how stomach contents correlated with 5-HTP, 5-HT, and 5-HT:5-HTP, I used the same time periods and formed subgroups of crayfish that had empty, full or some stomach contents (Fig. 5.2A). I analyzed and graphed subgroups that had more than two animals.

In the brain and hindgut, degree of stomach fullness did not appear to correlate with 5-HTP levels (Fig. 5.2B). In A6, a full stomach, particularly at 6 hr correlated with low levels of 5-HTP that then trended upward at 48 hr in crayfish that had empty stomachs (Fig. 5.2B). As in the analysis of 5-HT changes over time, stomach contents did not correlate with changes in 5-HT levels in brain, A6, or hindgut (Fig 5.2C). An

Fig. 5.2 Effect of stomach contents on 5-HT and 5-HTP levels. (A) Fullness of stomach over time. (B) Stomach fullness correlated with changes in 5-HTP levels in A6, although pairwise comparisons using Dunn's Multiple Comparison's test showed no significant difference (K-W test: brain p=0.0791, KW statistic = 11.316; A6 p=0.0284, KW statistic = 14.114; hindgut p=0.3381 KW statistic = 6.817. In A6, a full stomach during the first 12 hr after feeding depressed 5-HTP from crayfish that had an empty stomach prior to feeding and 48 hr after feeding. (C) Stomach fullness did not affect 5-HT levels in brain, A6, or hindgut (K-W test: brain p=0.4940 KW statistic = 5.397; A6 p=0.5622, KW statistic = 4.867; hindgut p=0.3938, KW statistic = 6.268). (D) Stomach fullness correlated with changes in 5-HT:5-HTP in A6 and the hindgut (K-W test: brain p=0.7259, KW statistic = 3.635; A6 p=0.0098, KW statistic = 16.874; hindgut p=0.0432, KW statistic = 12.989, Dunn's post-test n.s.). The ratio from crayfish that had a full stomach 6 hr after feeding was enhanced vs. unfed crayfish that had an empty stomach. While Dunn's post-test showed no significant difference, crayfish that had full stomachs 12 hr after feeding appeared to have a higher ratio than those crayfish at 48 hr that had some food in their stomachs.

Fig. 5.2


A
Stomach Contents
full
some
empty
% crayfish
unfed
1 hr
6 hrs
12 hrs
24 hrs
48 hrs
B
Brain
A6
Hindgut
normalized 5-HTP (pmol/mg)
C
normalized 5-HT (pmol/mg)
D
normalized 5-HT:5-HTP
time after feeding (hr)

examination of the 5-HT:5-HTP ratio in A6 showed that there was a significant difference between those crayfish that had a full stomach at 6 hr and those that had an empty one at 48 hr. While the 5-HT:5-HTP values differed significantly in hindgut, no pairwise significant differences occurred. The 5-HT:5-HTP values were highest in unfed crayfish with an empty stomach. Stomach contents had no impact on 5-HT:5-HTP in the brain (Fig. 5.2D).

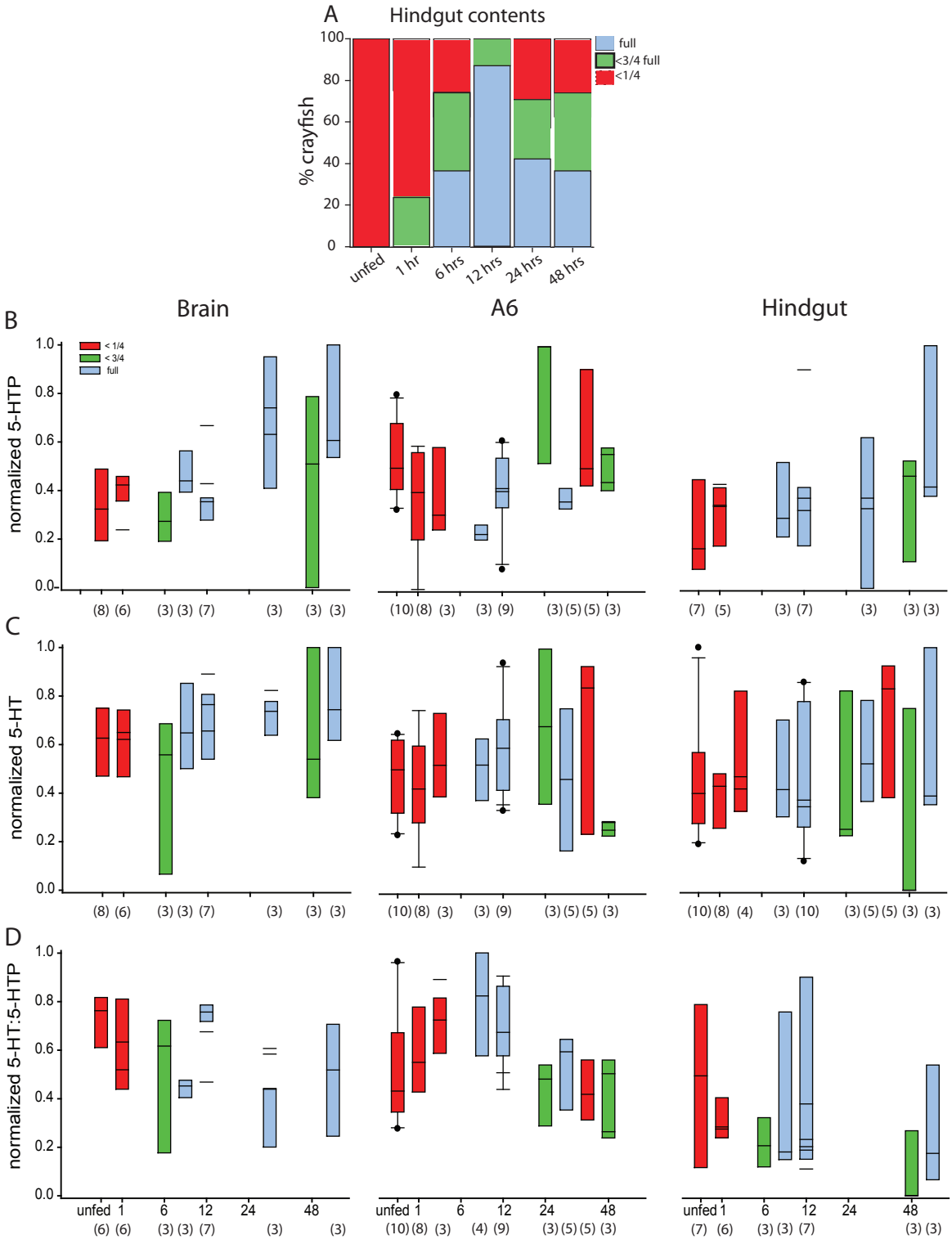
Effects of hindgut contents on 5-HT, 5-HTP, and 5-HT:5-HTP

To examine how hindgut contents affected 5-HT and 5-HTP, I looked at the periods of time when the hindgut was < 1/4 full, < 3/4 full, and full. Half of the unfed crayfish had some fecal material in the hindgut despite receiving no food for two weeks and after 48 hr none of the crayfish sampled had an empty hindgut (Fig. 5.3A). At 6 hours 37.5% hindguts had become full and at 12 hours 87.5% of the hindguts were full (Fig. 5.3A). After 12 hours the amount of fecal material in the hindgut declined. Differences in hindgut fullness could be attributed to individual differences in motility of the alimentary tract or differences in the length of time used to consume the shrimp pellets. Some shrimp pellets may not have been immediately consumed because at 24 hours, 43% of the hindguts were full and at 48 hours 37.5% were full (Fig. 5.3A).

There were no significant differences in 5-HTP levels in the brain, A6, or hindgut in response to hindgut fullness at different times (Fig. 5.3B). There was a trend for higher levels of 5-HTP in the brain when hindguts were full at 24 and 48 hours than at other times, regardless of hindgut fullness. There was also a trend for lower levels of 5-HTP in

Fig. 5.3 Effect of hindgut contents on 5-HT and 5-HTP levels. (A) Fullness of the hindgut over time. (B) Hindgut fullness did not correlate with changes in 5-HTP levels in brain, A6, or hindgut (K-W test: brain p=0.0545, KW statistic = 19.385; A6 p=0.0520, KW statistic = 19.541; hindgut p=0.3887, KW statistic = 11.673). In the brain, levels of 5-HTP were elevated in those crayfish that had full hindguts at 24 and 48 hours over crayfish that were unfed, had <1/4 full stomach at 1 hr and had a full hindgut at 6 and 12 hr. In A6, a full hindgut showed a trend in having lower levels of 5-HTP. (C) Hindgut fullness does not affect 5-HT levels in brain, A6, or hindgut (K-W test; brain p=0.5045, KW statistic = 10.290; A6 p=0.2683, KW statistic = 13.395; hindgut p=0.7299, KW statistic = 7.814). (D) Hindgut fullness correlated with changes in 5-HT:5-HTP in A6 (K-W test: brain p=0.5501 KW statistic = 9.782; A6 p=0.0450, KW statistic = 20.027; hindgut p=0.5159, KW statistic = 10.162). In A6, there may be a correlation between full hindguts at 6 and12 hr that have an elevated 5-HT:5-HTP over all hindguts at 24-48 hours, regardless of fullness (Dunn's post-test n.s.).

Fig. 5.5


A
Hindgut contents
% crayfish
full
<3/4 full
<1/4
unfed
1 hr
6 hrs
12 hrs
24 hrs
48 hrs
Brain
A6
Hindgut
B
C
D
normalized 5-HTP
normalized 5-HT
normalized 5-HT:5-HTP
< 1/4
< 3/4
full

in A6 when crayfish had full hindguts at 6, 12, 24 and 48 hr. Levels of 5-HTP varied little in the hindgut in the 12 hr after feeding but showed great variability at 24 to 48 hr.

5-HT levels did not show a correlation in brain, A6, or the hindgut in response to hindgut fullness (Fig. 5.5C). Median 5-HT levels were highest in the brain at 24 and 48 hr regardless of hindgut fullness. In A6 and the hindgut, median levels of 5-HT were greatest when the hindgut was full at 6 and 12 hr, however a full hindgut at 24 and 48 hr correlated with low 5-HT levels in A6 and high levels in the hindgut.

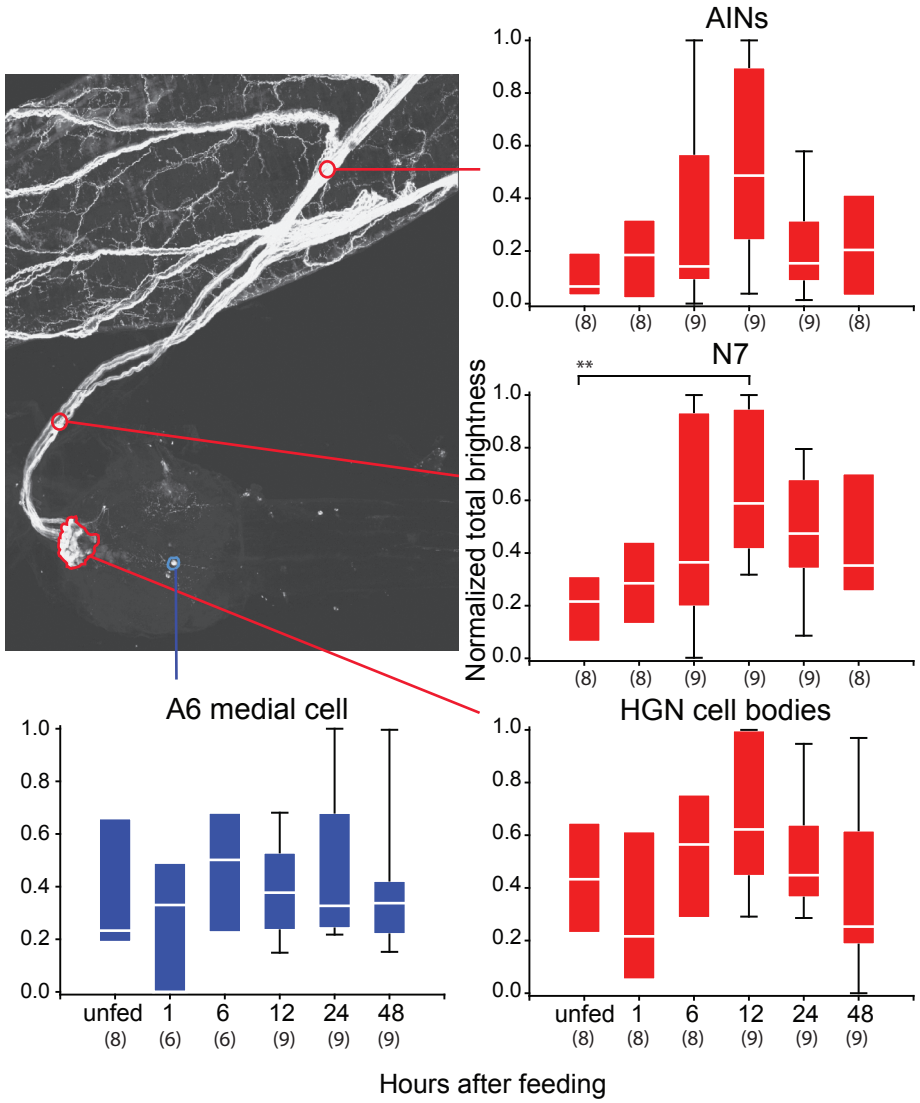
Median 5-HT:5-HTP in the brain appeared to be unaffected by gut contents (Fig. 5.5D). At 24 and 48 hr the median ratio decreased from the first 12 hr reflecting the increase in 5-HTP measured at that time. In A6, fullness of the hindgut did not correlate with specific values; the ratio was significantly higher at 6-12 hr when the hindgut was full than the ratio measured at 24 and 48 hr when the hindgut was also full. In the hindgut, median 5-HT:5-HTP was highest in the unfed animals.

Quantitative 5-HT-ir shows an increase in 5-HT-ir in HGNs following feeding

I measured the intensity of 5-HT-ir in the HGN cell bodies, their axons in N7, and their axon collaterals in the anterior intestinal nerves (AINs) using the same time periods as above: before feeding, 1, 6, 12, 24, and 48 hr after feeding (Fig. 5.4). Total 5-HT-ir was measured in the HGN cell bodies and defined cylindrical areas of N7 and the AINs were used to quantify 5-HT-ir. As a comparison, total 5-HT-ir of the A6 medial cell was measured at the same time periods. In the HGN cell bodies, N7 and AINs, the intensity of 5-HT-ir was greatest 12 hours after feeding (Fig. 5.4B-D). The intensity of 5-HT-ir was

Fig. 5.4 Brightness of 5-HT-ir in the HGNs following food intake. (A) The HGNs reside in the distal region of the A6 ganglion and project through N7 to the hindgut where they form the AINs which project anterior and the PINs, which project towards the anus. Outlined areas indicate where 5-HT-ir was quantified. (B) Median normalized brightness of 5-HT-ir in the AINs peaks at 12 hr and drops off during the next 36 hr (K-W test p=0.0828, KW statistic = 9.745). (C) Median normalized brightness of 5-HT-ir in N7 significantly increases at 12 hr from unfed crayfish (K-W test p=0.0115, KW statistic = 14.734) (D) Median normalized brightness of 5-HT-ir in the HGNs drops at 1 hr and then rises to a peak at 12 hr before falling again (K-W test p=0.0855, KW statistic = 9.659) (E) Median brightness of 5-HT-ir in the 5-HT medial cell drops at 1 hr and peaks at 6 hr before declining in brightness (K-W test p=0.7430, KW statistic = 2.720).

Fig. 5.4


AINs
N7
**
Normalized total brightness
A6 medial cell
HGN cell bodies
1.0
0.8
0.6
0.4
0.2
0.0
unfed 1 6 12 24 48
(8) (6) (6) (9) (9) (9)
(8) (8) (9) (9) (9) (8)
Hours after feeding

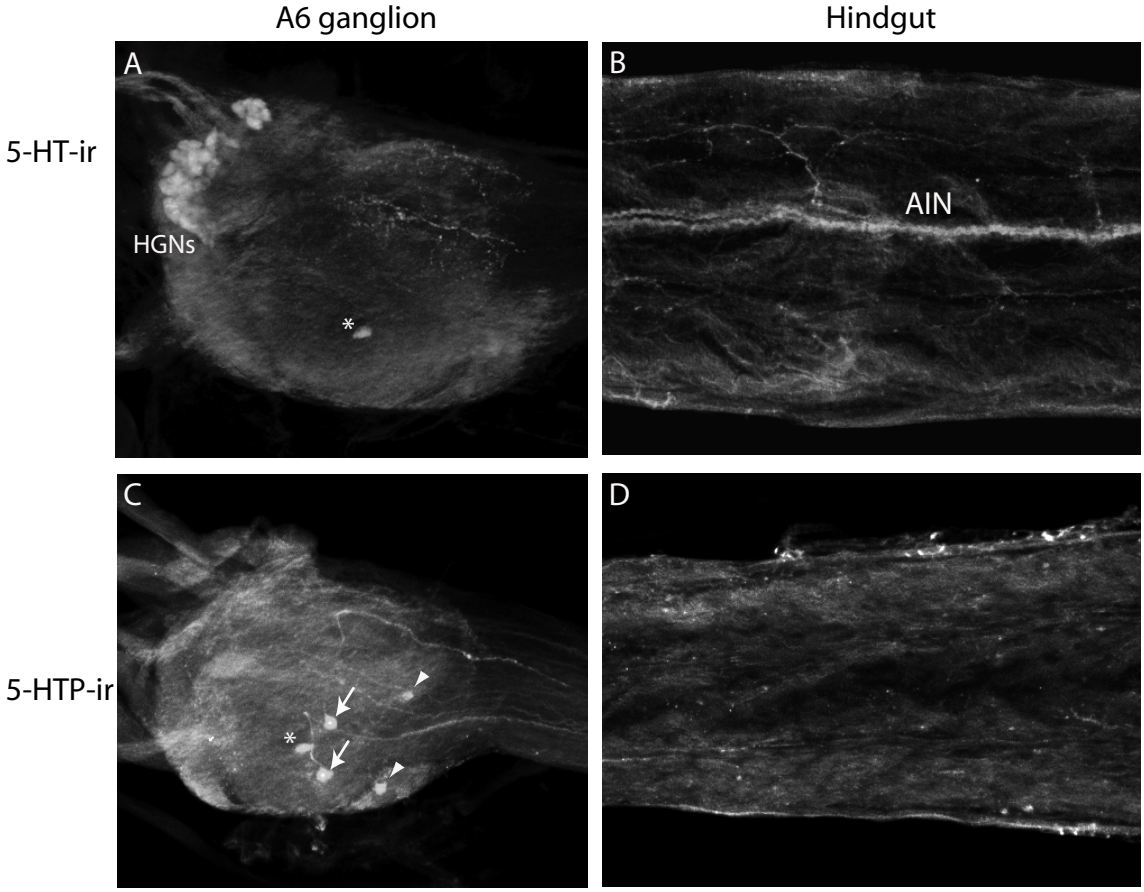
significantly increased at 12 hr in HGN axons in N7 over that measured in unfed crayfish. The 5-HT-ir intensities of N7 and the AINs were lowest in unfed crayfish (Fig. 5.4B,C). In the AINs, the intensity steadily increased from levels measured in unfed crayfish up to 12 hr after feeding and sharply decreased 24 and 48 hr after feeding (Fig. 5.4B). Intensity of 5-HT-ir in N7 followed the same pattern of increase but then showed less of a decrease at 24 and 48 hr after the peak in 5-HT-ir intensities at 12 hr (Fig. 5.4C). In the HGN cell bodies, intensity of 5-HT-ir decreased 1 hr after feeding, increased at 6 and 12 hr and then decreased at 24 and 48 hr (Fig. 5.4D). In the A6 medial cell 5-HT-ir also decreased at 1 hr, peaked at 6 hr and decreased at 12, 24, and 48 hr (Fig. 5.4E).

Feeding does not lead to 5-HT synthesis in the HGNs

Feeding appeared to be a natural stimulus that led to increased 5-HT-ir in the HGN cell bodies and axons and so provided a means to test whether 5-HT is normally synthesized in these cells after feeding. Crayfish were placed in aquaria with either distilled water or water with the L-AADC inhibitor NSD1015. Results in Chapter 3 showed that use of NSD1015 led to a buildup of 5-HTP-ir in cells that presumably had the tryptophan hydroxylase enzyme, the rate limiting enzyme in the 5-HT synthetic pathway. Two groups of crayfish fed 12 hours and 48 hours earlier were examined for labeling of 5-HT (n = 4) and 5-HTP (n = 6). In the 4 tested using 5-HT-ir, the HGN cell bodies showed moderate to intense 5-HT-ir, but under the same conditions the other group fed in distilled water showed no 5-HTP-ir (0 of 6) (Fig 5.5A). Both N7 and the AINs also showed 5-HT-ir (N7, 3 of 4; AINs 4 of 4) (Fig. 5.5B). Fine fibers on the hindgut longitudinal muscles showed 5-HT-ir in 3 of 4 preparations. 5-HTP-ir was not observed in N7, the AINs, or in fibers on the hindgut longitudinal muscles. Crayfish that

Fig. 5.5 Lack of facultative synthesis of 5-HT in the HGNs. (A) 5-HT-ir in crayfish exposed to NSD-1015, shows HGN cell bodies in A6, and HGN projections in N7, and the 5-HT medial cell (*). (B) 5-HT-ir on the hindgut shows one branch of the AINs and projections to the longitudinal muscle bundles. (C) 5-HTP-ir in crayfish exposed to the the L-AADC inhibitor, NSD-1015 shows build up of 5-HTP in the 5-HT medial cell (*), the lateral anterior cells (arrowheads), and the lateral medial 5-HT neurons (arrows). There is no 5-HTP-ir of the HGN cell bodies or N7. (D) 5-HTP-ir shows no labeling of the HGN axon projections or their collaterals.

Fig. 5.5



were fed in the water without L-AADC labeled for 5-HT but did not label for 5-HTP (data not shown).

The labeling of previously identified synthesizing neurons in A6 (Fig. 3.4) with 5-HTP-ir was an indication that NSD-1015 was effective in blocking conversion of 5-HT to 5-HTP (Fig. 5.5C, D). The primary synthesizing neuron, the A6 medial cell, was labeled with 5-HTP-ir in all preparations that were exposed to NSD1015 (3 of 3). Two preparations showed additional 5-HTP-ir cell bodies; one preparation showed 5-HTP-ir of the pair of lateral anterior neurons identified in Chapter 3 and another showed those neurons and a pair of medial lateral neurons that appear homologous to the large 5-HT neurons in the first abdominal ganglion (Fig. 5.5C).

DISCUSSION

Effects of feeding on levels of 5-HT and 5-HTP in A6 and hindgut

Results presented in previous chapters indicate that 5-HT plays a role in hindgut activity, in particular it may be important in initiating peristalsis. Here I tested whether feeding can influence the amount of 5-HT that is taken up by the HGNs and thus influences the amount of 5-HT in A6 and hindgut tissue as measured by HPLC. This experimental setup also allowed me to test whether 5-HT undergoes facultative synthesis or is instead taken up from extracellular sources. My results indicate that following food intake, 5-HT levels changed very little in the A6 ganglion and hindgut. 5-HT-ir results, however, indicated that there are significant increases of 5-HT in N7, through which the HGN axons project.

In response to feeding, there was significant variation in A6 5-HTP levels. While Dunn's test was unable to determine pairwise differences, the crayfish that had full stomachs or hindguts had lower 5-HTP levels when compared to levels measured in animals that had empty or partially full stomachs or hindguts. A decrease was measured in animals over the first 12 hr following feeding, a time when both hindgut and stomach were full. While it is difficult to determine whether the decrease in 5-HTP occurs over time or in response to stomach fullness, the more significant results in response to stomach fullness suggests that, in A6, fullness of stomach may be a more significant marker for changes in 5-HTP levels. The reduction in 5-HTP levels could indicate that synthesis of 5-HT is increased along with release. 5-HTP could be rapidly converted to 5-HT in A6 by synthesizing neurons identified in Chapter 3. The unchanging levels of 5-HT found in all three tissues suggests that pools of 5-HTP are stored ready for rapid conversion to 5-HT for release and to maintain homeostatic levels of 5-HT. Another interpretation is that TpOH, the enzyme that converts tryptophan to 5-HTP is downregulated, leading to reduced synthesis of 5-HTP. This result in A6 and hindgut is in contrast to what is happening in the brain where 5-HTP levels show a step-like increase at 24 and 48 hr, similar to the increase measured when 5-HT was injected into the hemolymph (Fig. 4.2).

The putative alterations in 5-HT synthesis may arise from hormonal signals or sensory signals from the hindgut to A6. 5-HT levels in the hemolymph may be higher in crayfish that have a full hindgut and this hormonal 5-HT may act on 5-HT autoreceptors leading to reduced 5-HT synthesis (Boadle-Biber, 1993). Injection of 5-HT into the hemolymph lowered 5-HTP in abdominal ganglia (Fig. 4.2) and application of 5-HT

reduced 5-HT-ir in a 5-HT synthesizing neuron (Table 3.1). Sensory feedback may be provided by small bipolar neurons embedded in the longitudinal folds of the hindgut, which were revealed following backfills of N7 to the hindgut (Musolf and Edwards, 1999). These neurons may interact with the 5-HT neurons in A6 to regulate synthesis.

Regulation of 5-HT in hindgut is likely different than in A6. Levels of 5-HTP in the hindgut were not associated with stomach or hindgut fullness, but did show a trend to increase following food intake, with peak levels measured at 24 to 48 hr. The possibility exists that 5-HTP levels may increase as tryptophan is released into the blood following digestion, however, injection of tryptophan into crayfish hemolymph had no effect on 5-HTP levels in hindgut (Fig. 4.1). Because there appears to be no synthesis occurring of 5-HTP in hindgut, the possibility exists that 5-HTP is released by other cells and taken up by the HGNs. The HGNs on the gut take up 5-HTP and readily convert it to 5-HTP (Fig. 3. 5). Levels of 5-HT in hindgut did not significantly differ over time or in response to stomach or hindgut fullness (Fig. 5.1, 5.2, 5.3).

While A6 and the hindgut are two very different tissues, they do have the HGNs in common. The HGN cell bodies and dendrites are located in A6 and the axons are on the hindgut (Elekes et al., 1988). Quantitative 5-HT-ir indicated that 5-HT-ir increased from levels measured in unfed crayfish and peaked in the HGN cell bodies and axons at 12 hr. The intensity of 5-HT-ir in the cell bodies differed from that measured in axons at 1 hour following feeding. At this time, 5-HT-ir decreased in the HGN cell bodies and a similar decrease was measured in the 5-HT synthesizing neuron in A6. The possibility exists that in both the HGNs and the A6 5-HT neuron, there is fast axonal transport of 5-HT from the cell bodies to the terminal endings on the hindgut and the A6 neuropil,

respectively. In addition, the A6 synthesizing neuron may be releasing 5-HT in the A6 ganglion, depleting its stores.

Effects of feeding on brain levels of 5-HT and 5-HTP

Results in Chapter 4 showed that injection of tryptophan into the hemolymph led to significant increases in levels of 5-HTP in the brain. Feeding appeared to produce similar effects. At 24 and 48 hr after feeding, 5-HTP levels rose in the brain. It is possible that digestion of shrimp pellets elevated blood levels of tryptophan, which increased brain 5-HTP. The measured increases in 5-HTP appeared to be associated with the number of hours following feeding rather than fullness of the stomach or hindgut. A full hindgut was not consistently associated with more 5-HTP in the brain; a full hindgut at 24 and 48 hr led to more 5-HTP but a full hindgut at 6 and 12 hr did not. This is consistent with the fact that the 5-HT from the brain is likely not used in hindgut functions.

Uptake and not facultative synthesis supplies the HGNs with 5-HT

There is no evidence that 5-HT is synthesized in hindgut neurons or paraneurons such as enteroendocrine cells. Previously, I showed that constitutive synthesis of 5-HT did not occur in the HGNs. Application of tryptophan did not lead to 5-HT-ir or 5-HTP-ir in the HGNs nor did tryptophan injection lead to significant increases in 5-HT or 5-HTP as measured by HPLC (Fig. 3.4, 4.1). Here, I showed that facultative synthesis also did not occur. Blocking of the conversion of 5-HTP to 5-HT at a time when 5-HT-ir was increasing led to no buildup of 5-HTP-ir in the HGNs (Fig. 5.6).

5-HT is important in modulating motility of crayfish hindgut. 5-HT enhances the force and frequency of contractions all along the hindgut length, initiates peristalsis, and eventually leads to a prolonged reduction in force and frequency of contractions (Chapter 2). I found that 5-HT concentrations of 100 nM most reliably initiated peristalsis (Table 2.1) and that higher concentrations were needed for more pronounced increases in force and frequency (Fig. 2.8). These concentrations are considerably above 1.0 nM in American lobster hemolymph and 0.5 nM in green crab hemolymph (Beltz et al., 1984; Sneddon et al., 2000). The lack of 5-HT synthesis in any neurons or paraneurons associated with the hindgut indicates that there must be other ways to bring more concentrated amounts of 5-HT to the hindgut.

Uptake of 5-HT appears to be critical in crayfish hindgut motility. Without the uptake and concentration of 5-HT in the HGNs, it is possible that peristalsis and other changes in hindgut motility may not occur. I propose that 5-HT is released from neurosecretory sites such as the pericardial organ, the second nerve roots of the thoracic ganglia, and the perineural sheath surrounding the ventral nerve cord (Beltz, 1988). The 5-HT is distributed through the dorsal abdominal artery that supplies the hindgut and when it reaches the hindgut the HGN axons take up the 5-HT. Injection of 5-HT into the hemolymph leads to a huge increase of 5-HT in hindgut and A6 tissue (Fig. 4.2). Immunocytochemical evidence indicates that uptake occurs in the HGNs (Chapters 2 and 3). While there is no direct evidence of release, it is possible that stimulation of the HGNs could lead to release of accumulated 5-HT. The release of 5-HT from the HGNs could initiate peristalsis and modulate contractile properties along the length of the hindgut. The uptake of 5-HT also serves to shape and inactivate the serotonergic signal. In

addition, because of the large capacity of the HGNs for 5-HT, these neurons may act as the abdominal 5-HT sink, storing and releasing or metabolizing excess 5-HT. HPLC evidence does not show increased uptake of 5-HT in response to food intake, however, it is possible that uptake and release are occurring simultaneously, thereby maintaining constant low levels of 5-HT in the gut.

Uptake of 5-HT is also critical in mammalian gut. 5-HT is released from the enterochromaffin cells in the mucosal layer of the gut and primarily taken up by enteric serotonergic neurons, platelets, mast cells, and crypt epithelial cells (Costa et al., 1982; Furness and Costa, 1982; Gershon, 1999) The rapid uptake of 5-HT by serotonin transporters prevents desensitization of 5-HT receptors that are expressed on sensory neurons. If desensitization of 5-HT receptors occurs, the peristaltic reflex is abolished (Wade et al., 1996). The excess 5-HT that is released and taken up by platelets and mast cells is transported through the vascular system and functions in hemostasis, producing vasoconstriction, and acting as a chemical signal in platelet aggregation. The crypt epithelial cells use the 5-HT they take up as a proliferative signal (Tutton and Barkla, 1984). 5-HT also stimulates the crypt epithelial cells, regulating aspects of Cl^- secretion in the jejunum and distal colon (Budhoo and Kellum, 1994; Siriwardena, et al., 1993).

The organization of the crustacean enteric systems is quite different than the mammalian system. One major difference is that mammals have a closed circulatory system and crustaceans have an open circulatory system. In mammals, the enteric system produces 95% of the organism's 5-HT (Erspamer, 1966), while in crustaceans the hindgut appears to produce no 5-HT. In mammals, the vascular system relies on the enteric system for its supply of 5-HT. In crustaceans, the enteric system relies on neurosecretion

of 5-HT into the hemolymph for its supply of 5-HT. Both rely on 5-HT to produce hindgut peristalsis and to modulate hindgut contractility and both rely on uptake of 5-HT for proper functioning of the gut.

6

General Conclusion

The hypothesis that 5-HT acts as a borrowed transmitter in the HGNs motivated many of the aims in this dissertation. While other experiments need to be done, such as tracking applied 5-HT into the HGNs to determine that it is released, this dissertation provides a compelling case that borrowing of 5-HT could occur and that released 5-HT may be important in water uptake and in eliminating waste. To establish a borrowed transmitter I determined that 5-HT produced effects when applied to the hindgut, that 5-HT was not synthesized by the HGNs but instead was acquired from extracellular sources, that there was a possible source of 5-HT for the HGNs, and that acquisition of 5-HT occurred within a behaviorally relevant context, feeding and digestion.

While the pursuit of 5-HT as a borrowed transmitter was a primary aim, other important findings emerged from this research. I found that the hindgut was a heterogeneous organ in form and function and I experimentally determined that 5-HT produced numerous and variable effects on the contractile properties of the hindgut. This latter result suggests that 5-HT plays a role in coordinating the processing of fecal material in the hindgut. In addition, I supported the idea that the crayfish brain has a type of blood brain barrier to protect it from circulating levels of 5-HT in the crayfish open circulatory system. This barrier is not evident in the rest of the crayfish CNS. I also showed that 5-HT sources in the thoracic and abdominal areas of the crayfish CNS respond differently to increases in tryptophan, 5-HTP, and 5-HT. In addition to the HGNs, I described phenotypical differences in neurons that are 5-HT-ir. I presented evidence of neurons that synthesized but did not take up 5-HT, neurons that synthesized 5-HT but did not typically show 5-HT-ir, and neurons that had the decarboxylating enzyme but not the hydroxylating enzyme necessary to make 5-HT.

The following is a brief summary of my results, an overview of other neurohomones and neurotransmittes found on cruastacean hindgut, a discussion on accumulated and borrowed 5-HT, and a discussion on why borrowing may be a particularly effective way for an organism with an open circulatory system to use 5-HT efficiently.

Results Summary

A careful observation of the gross morphology of the hindgut shows that it is a heterogenous organ (Fig. 2.1). While this heterogeneity has been described in regards to differences in the cuticular spines and variation in circular and longitudinal muscle (Chisaka et al., 1999), this dissertation focused on differences in innervation and contractile properties. The crayfish hindgut performs a number of functions and I propose that different regions along its length are specialized for particular functions. The fecal pellet needs to be compacted, nutrients that are produced by bacterial enzymatic activity need to be reabsorbed, water and salts need to be reabsorbed, and finally the fecal string needs to be eliminated (Vogt, 2002).

Results from Chapter 2 of this dissertation showed that contractile activity differed along the length of the hindgut; contraction frequency increased on an anterior to posterior axis and force decreased along the same axis (Fig. 2.3). While all regions of the hindgut produced torsional contractions, only the middle and caudal segments produced contractions that were capable of moving a wax bead and the caudal segment was the only one that initiated an anterior moving peristaltic wave (Fig. 2.6). The antiperistaltic

wave was observed in a hindgut swollen with food and it was observed regularly when 5-HT was applied to the hindgut.

Antiperistalsis was primarily observed in crayfish hindgut, although there appeared to be segmentation occurring as well, which has been described as a local churning action produced by alternating anterior and posterior moving peristaltic waves (Lovett and Felder, 1990). Winlow and Laverack (1972b, 1972c) described posterior moving peristaltic waves in the gut of *Homarus gammarus* that resulted from stimulation of the esophagus. Antiperistalsis, along with the rhythmic opening of the anal vent, has been described as anal swallowing (Muramoto, 1981) and anal drinking (Fox, 1952). The function of anal swallowing is clearly important during ecdysis when the crayfish takes up large amounts of water to produce the necessary hydrostatic pressure to split the exoskeleton and to provide a temporary hydroskeleton while the new exoskeleton hardens (Chung et al., 1999). It may also function to wash nutrients produced by bacteria into the midgut. Finally, antiperistalsis could function in osmoregulation; aquatic birds use antiperistalsis to take urine up into their colon to reclaim ions (Hughes et al., 1999).

5-HT had numerous effects on crayfish hindgut in addition to initiating contractions and antiperistalsis. The effects were concentration dependent and they varied in different hindgut segments. Superfusion of 300 nM 5-HT to rostral hindgut increased the frequency of contractions, particularly small amplitude contractions, while application of 300 nM 5-HT decreased contraction force in middle hindgut segments and power in all frequencies of the power spectrum (Fig. 2.7). Superfusion of 300 nM 5-HT clearly did not function to move the fecal string along in the hindgut, but instead produced contractions that could slow the propulsion of the fecal string, allowing for

compaction of fecal material, absorption of nutrients or reabsorption of ions (Fig. 2.7). Application of 1 μM 5-HT to the middle hindgut restored force and increased total power and power in all frequencies of the power spectrum (Fig. 2.7). The different responses of middle hindgut to the two concentrations of 5-HT suggested that the middle hindgut functioned as a switch in propelling the fecal string through the hindgut. A significant increase in total power and power in all the frequency ranges was also recorded in the caudal hindgut in response to 1 μM 5-HT, which suggests that at this concentration caudal hindgut assisted the middle hindgut in moving the fecal string through the hindgut. The caudal hindgut also assisted the middle hindgut by producing peristaltic waves that facilitated defecation. The peristaltic waves were periodic and short-lived; they reached peak power 1000 s following application (Fig. 2.8) and then the power of contractions waned. A washout of 300 nM and 1 μM 5-HT significantly reduced total power of contractions in rostral and caudal segments (Fig. 2.7).

I found evidence of 5-HT and the 5-$HT_{1\alpha}$ and the 5-$HT_{2\beta}$ receptors on the hindgut (Fig. 2.5). An initial immunocytochemical study showed no 5-HT-ir on the hindgut. However, superfusing the hindgut with 5-HT led to robust 5-HT-ir and injecting 5-HT into crayfish hemolymph led to a large increase in measured amounts of 5-HT (Fig. 3.2). The diffuse distribution of both 5-HT receptors and the evidence that 30 nM 5-HT did decrease contraction frequency in middle hindgut suggests that hormonal levels of 5-HT can produce changes in hindgut contractility, however, the more propulsive changes in contractions required higher concentrations of 5-HT that would need to be released as a paracrine or synaptic signal. The apposition of 5-HT-ir and 5-$HT_{2\beta}$ir suggests that synaptic or paracrine release likely occurs (Fig. 2.5).

I presented evidence that strongly suggests that 5-HT is not synthesized in the HGNs but instead is taken up from extracellular sources. The HGNs do not have the ability to convert tryptophan to 5-HTP, but they do have the ability to convert 5-HTP to 5-HT (Chapter 3, Fig. 3.4). They also appear to have very effective and specific serotonin transporters that are engaged in taking up large quantities of 5-HT (Fig. 3.2). The results from Chapter 3 strongly support the idea that 5-HT is not synthesized by the HGNs, but is accumulated by them and the results from Chapter 2 support the hypothesis that the accumulated 5-HT could be used to affect hindgut contractions.

Chapter 4 demonstrated that increasing levels of 5-HT in the hemolymph can lead to an increase of 5-HT in the hindgut (Fig. 4.2) and Chapter 3 indicated that this 5-HT can be accumulated in the HGNs (Fig. 3.2). The experiments in Chapter 5 attempted to demonstrate that increases in 5-HT levels in the hindgut and 5-HT-ir in the HGNs would occur within a relevant behavioral context, feeding and digestion. Chapter 2 supports the hypothesis that 5-HT could be important in processing and eliminating fecal material. In response to feeding, 5-HT-ir in N7 increased (Fig. 5.6) and in response to a full stomach, the 5-HT:5-HTP ratio in A6 and hindgut increased at 6-12 and 24-48 hr following food intake (Fig. 5.4D), which is also the time when the hindgut was filling or was filled with fecal material (Fig. 5.5A). The increased ratio supports a scenario where the hindgut is accumulating rather than synthesizing 5-HT. The more important result, which supports the idea that the HGNs accumulate rather than synthesize 5-HT, is that 5-HT is not facultatively synthesized in the HGNs at a time when 5-HT-ir is increasing. Blocking the conversion of 5-HTP to 5-HT showed that there was no buildup of 5-HTP-ir in the HGN

cell bodies or axons, indicating that 5-HT is present in the HGNs at 48 hr after feeding (Fig. 5.7).

The above results appear to support the following narrative. During feeding, 5-HT is released into the hemolymph from neurosecretory sites such as the pericardial organ, the perineural sheath, the nerve root plexuses, and perhaps the gastropyloric neurons. This 5-HT is distributed through the arterial system and initially affects foregut activity. The 5-HT also acts on valves in the dorsal abdominal artery that direct the flow of hemolymph to the hindgut, where hormonal levels (~30 nM) of 5-HT inhibit frequency of contractions in middle hindgut and where the HGNs axons can take up 5-HT. The 5-HT is accumulated by the HGNs for a period of 6-12 hr during which the food has been processed and has started entering into the hindgut as fecal pellets. Initial processing of the fecal pellets occurs in rostral hindgut where bacterial enzymes can help reclaim essential nutrients and compaction of the fecal pellet takes place. Release of lower concentrations of the accumulated 5-HT (300 nM) supports this pause in the movement of the fecal string through the hindgut, allowing for compaction of the fecal material in rostral hindgut. As more 5-HT is accumulated, a higher concentration of 5-HT is released by the HGNs, leading to an increase in contractile activity in the middle and caudal hindgut and stimulation of the antiperistaltic wave in the caudal hindgut. This leads to elimination of the fecal string. Following the movement of the fecal string, hindgut activity is reduced and the processing of the next bolus of fecal material begins.

Alternatively, the HGNs could function as stretch receptors and release 5-HT centrally, much as the gastropyloric receptor (GPR) cells do in the foregut (Katz and Harris-Warrick, 1989). The GPRs are proprioceptors that monitor stretch of the gastric

mill muscles and that project to the stomatogastric ganglion where they, through the release of 5-HT, recruit gastric mill neurons into the pyloric motor pattern (Katz and Harris-Warrick, 1991). While the GPRs, which have cell bodies in the periphery, are morphologically more likely to be a sensory neuron, there is evidence that crustaceans do have neurons like the HGNs that have central cell bodies that resemble motor neurons but have dendritic arbors that monitor the telson and uropods. These primary sensory neurons produce afferent input in the form of non-graded signals to the terminal ganglion hippid crabs (Paul and Wilson, 1994). The HGNs appear to have limited central input. While there is a small neuropil located within the cluster of cells, backfills of N7 show very few central projections (Elekes, et al., 1988). The HGNs do have numerous *en passant* synapses with collaterals in N7, where they could be modulating motor output from other centrally located neurons in abdominal ganglia (Elekes, et al., 1988). The more rapid retrograde movement of 5-HT (Fig. 3.3) and the increased intensity of 5-HT-ir in N7 (Fig. 5.6) following food intake, may provide some support for this hypothesis. The effects of different concentrations of 5-HT on different regions of the hindgut (Fig. 2.7), however, were produced without central input and are the result of 5-HT effects on the 5-HT receptors present on hindgut. Experimental preparations that include central input to the hindgut and that test only central 5-HT effects, would need to be done to determine if 5-HT is also acting centrally to affect hindgut activity. In addition, it would also be important to identify a central circuit that has input to the hindgut.

Crayfish hindgut neurotransmitters and neuromodulators

Numerous other amines and peptides produce changes in the contractile activity of the crustacean hindgut, most of which are excitatory and enhance contractile force and frequency. One family of peptides, the allostatins, has been identified as having an inhibitory effect on the contractile force and frequency of the hindgut (Dircksen et al., 1999). Most of these peptides act as neurohormones as evidenced by either the low concentrations with which they are physiologically effective or by the fact that, while not present on the hindgut, they still produce physiological effects. In crustacean hindgut, ICC evidence exists for the peptides crustacean hyperglycemic hormone (CHH), proctolin, FMRF-amide (Appendix, Fig.1), crustacean cardioactive peptide (CCAP) (Appendix, Fig.2), and the orcokinins (Webster et al., 2000; Mercier et al., 1997; Audehm et al., 1993; Stangier et al., 1992; Dircksen et al., 2000). ICC and HPLC evidence also indicates that dopamine (DA) is present in the hindgut (Elofsson et al., 1978; Mercier et al., 1991; Chapter 4). Physiological evidence indicates that all of the above peptides and dopamine can function as neurohormones on crustacean hindgut (Webster et al., 2000; Mercier et al., 1997; Stangier and Keller, 1990; Dircksen et al., 2000; Mercier et al., 1991). DA and FMRF-amide may also act as neurotransmitters. Other neurotransmitters that have excitatory effects are those typically associated with vertebrate and arthropod neuromuscular junctions: acetylcholine and glutamate (Jones, 1962; Wrong et al., 2003).

It is likely that 5-HT acts synergistically or perhaps permissively with a number of neurohormones and neurotransmitters to effect changes in force and frequency. The orcokinins appear to be colocalized with 5-HT in the HGNs in the crayfish *Orconectes*

limosus (Dircksen et al., 2000) and FMRFamide-ir is found in a subset of the HGNs in *Procambarus clarkii*, the intestinal nerve, and in fibers that innervate the circular muscles of the rectum and the longitudinal muscles of the entire hindgut (Appendix, Fig.1). CCAP-ir is also found in A6 but not in the HGNs, however, there is a prominent neurohemal release area associated with N7 and the rectum (Appendix, Fig.2). The abundance of neurohormones and neurotransmitters on the hindgut suggests that, as in crustacean foregut (Christie et al., 1995), the interactions are complex and thus no neurohormone or neurotransmitter is functioning alone to produce the different effects on hindgut that have been described. Furthermore, it is likely that not only do different neurotransmitters act together synergistically, but that different concentrations of a peptide or amine have different and sometimes paradoxical effects.

5-HT as an accumulated or borrowed neurotransmitter

The above narrative describes 5-HT as a borrowed neurotransmitter. While the evidence for 5-HT to function as a borrowed neurotransmitter in other systems also is not complete, there are numerous references in the literature that suggest that accumulation of 5-HT, a necessary first step in borrowing, occurs (Schutte, 1994; Lebrand et al., 1996; Upton et al., 1999). Neurons that accumulate 5-HT that is endogenously produced by the organism can be grouped into two categories, transient accumulating 5-HT neurons and persistant accumulating 5-HT neurons. The HGNs fall under the latter category. Typically the neurons lack some or all of the synthetic enzymes, can accumulate 5-HT and often have a transporter that actively transports 5-HT into vesicles for release.

Nonvesicular release of amines, however, can occur by reversal of the plasma membrane transporter that takes up the specific amine (Falkenburger et al., 2001).

Transient expression of 5-HT accumulating neurons primarily occurs in developing organisms and in nervous tissue that undergoes continual neurogenesis. During development, 5-HT participates in cell migration of neuronal and nonneuronal tissue (Persico et al., 2001) and in neuronal differentiation (Lauder and Krebs, 1978) (Whitaker-Azmitia et al., 1996; Fiorica-Howells et al., 2000). Neuronal differentiation involves autoregulation of 5-HT neurons (Whitaker-Azmitia et al., 1996) and other neurotransmitter-producing neurons. In mice, projection neurons from retina to the tectum and thalamus and projection neurons of the somatosensory thalamocortical systems in mice accumulate 5-HT during development of cortical maps (Lebrand et al., 1996; Hansson et al., 1998). Normal formation of these sensory maps is accompanied by transient expression of 5-HT-ir along with transient expression of SERT. This 5-HT appears early in development and evidence exists that too much 5-HT during this developmental time period can have deleterious effects on sensory map formation (Salichon et al., 2001). Experiments that eliminated synthesis of 5-HT in the CNS produced more subtle changes in the sensory maps (Persico et al., 2001). These studies seem to suggest that formation of sensory maps rely more on sequestering 5-HT rather than releasing 5-HT.

Transient accumulation of 5-HT may regulate the proliferation and differentiation of olfactory projection neurons in lobster (Beltz et al., 2001). 5-HT is transiently taken up by newborn projection neurons a few hours after their birth (Beltz et al., 2001). Unlike in mammalian studies, reducing levels of 5-HT through the use of 5-7 dihydroxytryptamine

leads to aberrant growth of the projection neurons (Sullivan et al., 2000). In lobsters, reduction of 5-HT does not alter the glomerular organization of the olfactory and accessory lobe, but it does diminish the size of the glomeruli (Benton et al., 1994; Benton et al., 1997).

Persistent accumulation is described primarily in three vertebrate neuronal tissues, retina, hypothalamus and pituitary, and in perivascular neurons. The accumulation of 5-HT in 3 populations of retinal neurons has been studied for over 25 years and it offers the most complete look at 5-HT accumulation. It is from this literature that Schutte and Witkovsky (1990) first used the concept of borrowing to explain the appearance of 5-HT in frog retinal neurons. In their study, large soma amacrine cells that synthesized 5-HT appeared to be the source of 5-HT that was also detected in small soma amacrine cells and OFF-center bipolar cells. Schutte (1994) later presented ICC evidence that the large soma amacrine cells were the only ones that had the enzymes for synthesizing 5-HT. In a study of frog tectum, 5-HT was found to be accumulated by lamina 6 piriform projection neurons (Bieger and Neuman, 1984). Evidence that these neurons did not synthesize 5-HT was their inability to convert applied tryptophan to 5-HT. These studies in retina and tectum show accumulation but do not indicate whether the 5-HT is used to produce meaningful effects.

Most of the studies of 5-HT accumulation in mammalian retina are in rabbits where different amacrine cells have been identified as 5-HT accumulating neurons. These neurons primarily use GABA as a neurotransmitter (Massey et al., 1992; Fletcher and Wässle, 1999), however, both the 5-HT_{3A} and the 5-HT_{7} receptors are found in mammalian retina, with the 5-HT_{3A} localized to rod photoreceptor terminals that are

contacted by the amacrine cells (Chidlow et al., 1998; Pootanakit and Brunken, 2001). Again, too little is known about whether the particular cells that accumulate 5-HT release it to act on the identified 5-HT receptors. Furthermore, high affinity uptake of tryptophan occurs in retina and it is possible that the low levels of 5-HT that are normally found in mammalian retina may be the result of facultative synthesis of 5-HT (Mitchell and Redburn, 1985).

Several studies have examined 5-HT accumulation in pituitary (Saland et al., 1988;Vanhatalo and Soinila, 1994; Vanhatalo et al., 1995) and hypothalamus (Beaudet and Descarries, 1979; Frankfurt et al., 1981; Iturriza, 1989; Vanhatalo and Soinila, 1996; Vanhatalo and Soinila, 1998). In pituitary, DA neurons took up 5-HT through the DA transporter (Vanhatalo and Soinila, 1994). This study boosted 5-HT levels in the pituitary intermediate lobe (IL) by incubating the IL explant in 1 to 10 uM 5-HT. However, it is not clear whether 5-HT naturally occurs in this area since IL supposedly does not have 5-HT projections from other areas of the brain and, because it is avascular, does not have access to 5-HT from blood platelets. In a subsequent paper, Vanhatalo and Soinila (1995) determined that 5-HT could be released through classic exocytotic mechanisms and via reversal of the DA transporter.

In the hypothalamus, developmental studies show that there is transient synthesis and uptake of 5-HT in hypothalamic neurons (Ugrumov et al., 1986; 1989). Recent studies show that in adults, neurons in this region may retain their ability to engage in high affinity uptake of 5-HT (Beaudet and Descarries, 1979; Frankfurt et al., 1981; Iturriza, 1989), however, it is unclear whether the nerve processes are from TpOH-ir neurons or not. Increasing 5-HT levels through application of tryptophan led to an

increase of 5-HT-ir in the dorsomedial nucleus (DMN) of hypothalamus (Vanhatalo and Soinila, 1998). Use of the SERT antagonist fluoxetine diminished some 5-HT-ir in nerve fibers and eliminated 5-HT-ir in somata. The authors speculated that involvement of both 5-HT and DMN in the regulation of food intake supported a physiological function for 5-HT. In support of their hypothesis, they cite evidence that 5-HT levels in the accumulating neurons are carefully regulated by DA and noradrenaline (Vanhatalo and Soinila, 1998; Iturriza, 1989).

5-HT acts as a vasoconstrictor in the vascular system. Typically it is released from platelets and mast cells, although 5-HT neurons are found on some arteries and arterioles and in the pia-arachnoid structures in the dura mater. The accumulation of 5-HT into perivascular neurons appears to have two sources, the blood and cerebral spinal fluid. There is evidence that catecholamine (CA) neurons arising from the superior cervical ganglion may participate in accumulating 5-HT to perhaps control vascular levels of 5-HT. These neurons, along with serotonergic neurons that may also arise from the superior cervical ganglion (Cohen et al., 1992), have abundant terminals on cerebral arteries, in particular the middle cerebral artery. The accumulation of 5-HT appears to be via uptake through a CA transporter (Jackowski et al., 1988). Blocking this transporter with desmethylimipramine, a CA uptake antagonist that is ineffective on the SERT protein found on 5-HT neurons in the raphe nucleus, led to an absence of 5-HT-ir. Whether the 5-HT taken up by the CA transporter is released with norepinephrine to modulate arterial contractions is unknown.

Serotonin is a vasoconstrictor that acts on 5-HT_2 (Medgett et al., 1984; Takahashi et al., 2000) and 5-HT_{1B} receptors located on the muscular walls of the temporal and

brachial arteries (de Hoon et al., 2000; Bouchelet et al., 2000). A recent report suggests that this 5-HT stimulated vasoconstriction is more effective when applied to arterioles from the CSF side of the blood vessel rather than luminally (Takahashi et al., 2000; Vanhatalo and Soinila, 1994). This may enable it to take advantage of 5-HT that is released into CSF via 5-HT fibers found in the pia-arachnoid membranes.

The cerebral blood vessels in dura mater are innervated by sympathetic and sensory neurons. The CA sympathetic neurons appear to be accumulating 5-HT from mast cells, which form connections as little as 20 nm apart with sympathetic nerve endings found within the same basement membrane (Stanley et al., 1993). In contrast to other vascularized regions of the brain, there is no evidence of neuronal TpOH-ir in the dura mater (Stanley et al., 1993). 5-HT1B/1D receptors, howeve,r are found on the sensory neurons in dura mater and are known to affect levels of the neuropeptide, calcitonin gene related peptide (CGRP), a peptide implicated in degranulation of mast cells. While there are receptors for 5-HT, it is not known whether the 5-HT released from mast cells acts on the 5-HT receptors or whether the CA sympathetic neurons can also release the 5-HT they accumulated.

5-HT is the most common neurotransmitter to be described as accumulated and possibly borrowed. Transient accumulation appears to be important in developing cortical maps. The role of persistent accumulation is less clear. In retina it appears to be involved in sensory signaling, but because of the amount of endogenously synthesized 5-HT in retina it is difficult to separate out whether accumulated 5-HT is reused. In pituitary and hypothalamus it still is unclear how borrowed 5-HT would function and whether borrowing occurs under normal physiological conditions. Perivascular accumulation of 5-

HT provides more complete evidence that 5-HT may be borrowed and these studies are most relevant to my study of the HGNs. In vertebrates, much of the 5-HT that is accumulated and later released from platelets originates from the 5-HT synthesizing enterochromaffin cells in the gut, which do not express SERT or engage in reuptake of the released 5-HT. 5-HT appears to move from an endocrine source to mast cells and platelets in the vascular system to sympathetic neurons. 5-HT also functions within this system, producing vasoconstriction, although it is difficult to distinguish whether the 5-HT is coming from mast cells or has been borrowed and released by CA neurons. In crayfish, the movement of 5-HT may start with hormonal release of 5-HT from the perineural sheath and the pericardial organ and uptake by the HGNs. This dissertation shows that injected 5-HT is accumulated by the HGNs and that 5-HT does have functional effects on the hindgut. How 5-HT travels in the hemolymph is not known. Formed elements in the hemolymph may function as carriers and/or utilizers of 5-HT as well, however, there does not appear to be a crustacean equivalent of platelets or mast cells (Martin and Hose, 1995).

Why would a neuron accumulate or borrow 5-HT? The example above suggests that accumulating 5-HT may be a very economical and efficient mechanism to acquire a neurotransmitter. This may be more important for indoleamines such as 5-HT that rely on an organism's diet to provide the essential amino acid tryptophan from which 5-HT is synthesized. While high levels of tryptophan do not lead to unlimited synthesis of 5-HT (Chapter 4), reducing intake of tryptophan containing foods can lower levels of 5-HT (Young and Gauthier, 1981). In addition, the types of food eaten also regulate availability of tryptophan. A high carbohydrate meal can facilitate entry of tryptophan into the brain

by 54% over a high protein meal (Wurtman et al., 2003). If supplies of a neurotransmitter are dependent on the vagaries of diet, then it may be important that mechanisms are in place to not only recycle the neurotransmitter locally but also to distribute it. It is probably not a coincidence that the bulk of 5-HT synthesized by vertebrates and crustaceans occurs in cells that are proximate to where tryptophan containing foods are finally digested down to individual amino acids. In vertebrates this would occur in the small intestine and in crustaceans it would occur in the digestive gland that surrounds the pericardial organ and lies dorsal to the perineural sheath. Both the intestine and pericardial organ engage in broadcast release of 5-HT which is then transported by the arteries to more distant sites. The use of 5-HT in gut motility and secretion and the recycling of the same 5-HT for use in the vascular system appears to be a very efficient way to use and conserve a neurotransmitter that relies on dietary intake of its precursor amino acid.

Another reason for accumulating 5-HT is to shape the 5-HT signal and regulate where it produces effects. 5-HT is implicated in an enormous variety of developmental, physiological, and behavioral processes. While 5-HT acts directly on numerous particular targets, 5-HT is also thought to function as a “master control neurotransmitter”, providing indirect input through interactions with other neurotransmitter systems (Lesch and Mossner, 1998). The involvement of 5-HT in a variety of processes and its interactions with other neurotransmitters points to the importance of regulating 5-HT supplies, concentration, and distribution. In crayfish, broadcast release of 5-HT in the blood would have widespread effects on a number of systems in addition to hindgut motility; foregut activity, escape behavior, stress responses, and a number of motor circuits (Beltz et al.,

1984; Katz et al., 1989; Yeh et al, 1997; Chang et al., 1999; Nagayama, 2002) Interestingly, the brain appears to be protected from this release of 5-HT in the blood (Chapter 4).

Organization of 5-HT sources

The organization of 5-HT synthesizing sources appears to be partly a function of the synthesizing cells' or organs' proximity to abundant supplies of tryptophan and where it is used. Whether an organism has an open or closed circulatory system may also play a role in where 5-HT is synthesized and used. Vertebrates have two separate TpOH enzymes to synthesize 5-HT. One is found in the periphery in the enterochromaffin cells of the GI tract and the other is found in the CNS and peripheral neuronal tissue. Over 95% of the 5-HT synthesized by vertebrates is found in the gastrointestinal system and this 5-HT is used to promote gastrointestinal motility and is broadcast and picked up by platelets for use in the vascular system as well (Cote et al., 1994). Neurons of the GI tract also take up 5-HT released by the enterochromaffin cells, however these same accumulating 5-HT neurons also synthesize 5-HT using another TpOH, neuronal TpOH (Walther and Bader, 2003). Neuronal 5-HT is primarily found and used in the CNS. This dichotomous arrangement of 5-HT synthetic machinery and supplies may have arisen as a result of the blood-brain barrier.

Organisms such as arthropods and mollusks have an open circulatory system comprised of heart(s), arteries, and hemal sinuses. There is no venous system to return blood to heart(s). Blood is delivered to the different organs through the arteries and collects in blood cavities called hemocoels (Maynard, 1960). Muscular movement and, in

some organisms, accessory hearts assist in the diffusion of blood to the gills and back to the heart. This organization may provide a different rationale for the organization and regional regulation of 5-HT. In arthropods and mollusks, broadcast release of 5-HT occurs outside the GI tract and is more associated with a neuroendocrine system. However, unlike in vertebrate animals, this hormonally released 5-HT is able to affect most of the CNS with the exception of the brain. This leads to the question of how an open circulatory system operates to regulate 5-HT. Massive release of 5-HT as seen in the vertebrate gut would overwhelm the more accessible CNS and introduce problems with cross talk between the different organ systems and neuronal circuits that respond to 5-HT. The HGNs appear to function as a way to rapidly reduce levels of 5-HT in the blood and also acquire the 5-HT necessary to promote hindgut motility. Future research should test whether the organization of 5-HT sources and sinks in crayfish could apply to other organisms with an open circulatory system.

Literature Cited

Alexandrowicz, J. S. (1909). Zur Kenntnis des sympathischen Nervensystems der Crustaceae. *Jena Z. Naturw.* **45**, 395-444.

Audehm, U., Trube, A., and Dircksen, H. (1993). Patterns and projections of crustacean cardioactive-peptide-immunoreactive neurones of the terminal ganglion of crayfish. *Cell Tissue Res.* **272**, 473-485.

Barnes, N. M. and Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharm.* **38**, 1083-1152.

Basu, A. C and Kravitz, E. A. (2003). Morphology and monoaminergic modulation of Crustacean Hyperglycemic Hormone-like immunoreactive neurons in the lobster nervous system. *J Neurocytol*. **32**, 253-263.

Baumgarten, H. G., Bjorklund, A., Lachenmayer, L., Nobin, A. and Rosengren, E. (1973). Evidence for the existence of serotonin-, dopamine-, and noradrenaline-containing neurons in the gut of Lampetra fluviatilis. *Z Zellforsch Mikrosk Anat* **141**, 33-54.

Bayliss, W. M. and Starling, E. H. (1899). The movement and innervation of the small intestine. *J. Physiol.* **24**, 99-143.

Beaudet, A. and Descarries, L. (1979). Radioautographic characterization of a serotonin-accumulating nerve cell group in adult rat hypothalamus. *Brain Res.* **160**, 231-243.

Beltz, B. S. (1988). Crustacean neurohormones. *Endocrinology and Selected Invertebrate Types*, 235-258.

Beltz, B. S. (1999). Distribution and functional anatomy of amine-containing neurons in decapod crustaceans. *Microsc. Res. Tech.* **44,** 105-120.

Beltz, B. S., Benton, J. L. and Sullivan, J. M. (2001). Transient uptake of serotonin by newborn olfactory projection neurons. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 12730-12735.

Beltz, B. S., Eisen, J. S., Flamm, R., Harris-Warrick, R. M., Hooper, S. L. and Marder, E. (1984). Serotonergic innervation and modulation of the stomatogastric ganglion of three decapod crustaceans (*Panulirus interruptus, Homarus americanus* and *Cancer irroratus*). *J. Exp. Biol.* **109,** 35-54.

Beltz, B. S. and Kravitz, E. A. (1983). Mapping of serotonin-like immunoreactivity in the lobster nervous system. *J. Neurosci.* **3**, 585-602.

Bieger, D. and Neuman, R. S. (1984). Selective accumulation of hydroxytryptamines by frog tectal neurons. *Neuroscience* **12**, 1167-1177.

Blakely, R. D., De Felice, L. J. and Hartzell, H. C. (1994). Molecular physiology of norepinephrine and serotonin transporters. *J. Exp. Biol.* **196**, 263-81:263-281.

Boadle-Biber, M. C. (1993). Regulation of serotonin synthesis. *Prog. Biophys. Mol. Biol.* **60**, 1-15.

Bouchelet, I., Case, B., Olivier, A. and Hamel, E. (2000). No contractile effect for 5-HT1D and 5-HT1F receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br. J. Pharmacol.* **129**, 501-508.

Brenner, T. L. and Wilkens, J. L. (2001). Physiology and excitation-contraction coupling in the intestinal muscle of the crayfish *Procambarus clarkii*. *J. Comp. Physiol. [B]* **171**, 613-621.

Brown, P. B. (1995) Physiological adaptations in the gastrointestinal tract of crayfish. *American Zoologist*, **35**, 20-27.

Budhoo, M. R. and Kellum, J. M. (1994). Evidence for a 5-HT4 receptor pathway mediating chloride secretion in the rat distal colon. *J. Surg. Res.* **57**, 44-48.

Bulbring, E. and Lin, R. C. Y. (1958). The effect of intraluminal application of 5-hydroxytryptamine and 5-hydroxytryptophan on peristalsis; the local production of 5-HT and its release in relation to intraluminal pressure and propulsive activity. *J. Physiol.***140**, 381-407.

Bungart, D., Dircksen, H. and Keller, R. (1994). Quantitative determination and distribution of the myotropic neuropeptide orcokinin in the nervous system of astacidean crustaceans. *Peptides* **15**, 393-400.

Bunin, M. A. and Wightman, R. M. (1999). Paracrine neurotransmission in the CNS: involvement of 5-HT. *Trends Neurosci.*, **22**, 377-382.

Castonon-Cervantes, O, Batelle, B. and Fanjul-Moles, M.L. (1999). Rhythmic changes in the serotonin content of the brain and eyestalk of crayfish during development. *J. Exp. Biol.* **202**, 2823-2830.

Chang, E. S., Chang, S. A., Beltz, B. S. and Kravitz, E. A. (1999). Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the subesophageal ganglion and thoracic second roots. *J. Comp. Neurol.* **414**, 50-56.

Chang, E. S., Chang, S. A., Keller, R., Reddy, P., Sr., Snyder, M. J. and Spees, J. L. (1999). Quantification of stress in lobsters: Crustacean hyperglycemic hormone, stress proteins, and gene expression. *American Zoologist* **39**, 487-495.

Chen, J. J., Li, Z., Pan, H., Murphy, D. L., Tamir, H., Koepsell, H. and Gershon, M. D. (2001). Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. *J. Neurosci.* **21**, 6348-6361.

Chidlow, G., Le Corre, S. and Osborne, N. N. (1998). Localization of 5-hydroxytryptamine1A and 5-hydroxytryptamine7 receptors in rabbit ocular and brain tissues. *Neurosci.* **7**, 675-689.

Chisaka, H., Uen, M. and Futaesaku, Y. (1999) Spines in the hindgut of the crayfish *Procambarus clarkii* (Decapoda): their distribution and correlation with hindgut muscles. *J. Crust. Biol.* **19**, 337-343.

Christie, A. E., Skiebe, P. and Marder, E. (1995). Matrix of neuromodulators in neurosecretory structures of the crab Cancer borealis. *J. Exp. Biol.* **198 (Pt 12)**, 2431-2439.

Chung, J. S., Dircksen, H. and Webster, S. G. (1999). A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13103-13107.

Clark, M. C., Dever, T. E., Dever, J. J., Xu, P., Rehder, V., Sosa, M. A. and Baro, D. J. (2004). Arthropod 5-HT2 receptors: a neurohormonal receptor in decapod crustaceans that displays agonist independent activity resulting from an evolutionary alteration to the DRY motif. *J. Neurosci.* **24,** 3421-3435.

Clark, T. M., Koch, A. and Moffett, D. F. (2000). The electrical properties of the anterior stomach of the larval mosquito (*Aedes aegypti*). *J. Exp. Biol.* **203 Pt 6**, 1093-1101.

Coates, M. D., Mahoney, C. R., Linden, D. R., Sampson, J. E., Chen, J., Blaszyk, H., Crowell, M. D., Sharkey, K. A., Gershon, M. D., Mawe, G. M. et al. (2004). Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* **126**, 1657-64.

Cohen, Z., Bovento, G., Lacombe, P., Seylaz, J., MacKenzie, E.T. and Hamel, E. (1992). Cerebrovascular nerve fibers immunoreactive for tryptophan-5- hydroxylase in the rat: distribution, putative origin and comparison with sympathetic noradrenergic nerves. *Brain Res.* **598**, 203-214.

Coleman, C. M. and Neckameyer, W. S. (2004). Substrate regulation of serotonin and dopamine synthesis in *Drosophila*. *Invert. Neurosci.* **5**, 85-96.

Costa, J. L., Kirk, K. L. and Stark, H. (1982). Uptake of 6-fluoro-5-hydroxytryptamine and 4,6-difluoro-5-hydroxytryptamine into releasable and non-releasable compartments of human platelets. *Br J Pharmacol* **75**, 237-42.

Cote, F., Fligny, C., Fromes, Y., Mallet, J. and Vodjdani, G. (2004). Recent advances in understanding serotonin regulation of cardiovascular function. *Trends Mol. Med.* **10**, 232-238.

de Hoon, J. N., Willigers, J. M., Troost, J., Struijker-Boudier, H. A. and Van Bortel, L. M. (2000). Vascular effects of 5-HT1B/1D-receptor agonists in patients with migraine headaches. *Clin. Pharmacol. Ther.* **68**, 418-426.

Dircksen, H., Burdzik, S., Sauter, A. and Keller, R. (2000). Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. *J. Exp. Biol.* **203**, 2807-2818.

Dircksen, H., Skiebe, P., Abel, B., Agricola, H., Buchner, K., Muren, J.E. and Nassel, D. R. (1999). Structure, distribution, and biological activity of novel members of the allatostatin family in the crayfish *Orconectes limosus*. *Peptides* **20**, 695-712.

Dubbels, R. and Elofsson, R. (1989). N-acetylation of arylalylamines (serotonin and tryptamine) in the crayfish brain. *Comp. Biochem. Physiol. C*, **93 (2),** 307-312.

Duerr, J. S., Gaskin, J. and Rand, R. B. (2001). Identified neurons in *C. elegans* coexpress vesicular transporters for acetylcholine and monoamines. *Am. J. Physiol. Cell Physiol.* **280**, C1616-1622.

Ebara, A. (1969). Spontaneous activity of crayfish intestine. *Annot. Zool. Jap.* **42**, 169-175.

Edwards, D. H., Yeh, S. R., Musolf, B. E., Antonsen, B. L. and Krasne, F. B. (2002) Metamodulation of the crayfish escape circuit. *Brains, Behavior, and Evolution* **60**, 360-369.

Ehinger, B. and Floren, I. (1976). Indoleamine-accumulating neurons in the retina of rabbit, cat and goldfish. *Cell Tissue Res.* **175**, 37-48.

Elekes, K., Florey, E. and Cahill, M. A. (1988) Morphology and central synaptic connections of the efferent neurons innervating the crayfish hindgut. *Cell Tissue Res.* **254**, 369-379.

Elofsson, R., Elekes, K. and Myhrberg, H. E. (1978). Catecholaminergic innervation of muscles in the hindgut of crustaceans. An ultrastructural study. *Cell Tissue Res.* **189**, 257-266.

Erspamer, V. (1946). Pharmacological study of indolealkylamines in toad venom. VII. Enteramines and indolealkylamines in toad venom. *Arch. Sci. Biol. Napoli* **31**, 86-95.

Erspamer V. (1966) Handbook of Experimental Pharmacology: 5-Hydroxytryptamine and Related Indolealkylamines. New York: Springer-Verlag.

Erspamer V. and Asero, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature* **169**, 800-801.

Escamilla-Chimal, E. G., Hiriart, M., Sanchez-Soto, M.C. and Fanjul-Moles, M. L. (2002). Serotonin modulation of CHH secretion by isolated cells of the crayfish retina and optic lobe. *Gen. Comp. Endocrinol.* **125**, 283-290.

Factor J. R. (1995) The digestive system. In *Biology of the Lobster Homarus americanus* (ed. J. R. Factor), pp 395-440. San Diego: Academic Press.

Falkenburger, B. H., Barstow, K. L. and Mintz, I. M. (2001). Dendrodendritic inhibition through reversal of dopamine transport. *Science* **293**: 2465-2470.

Fickbohm, D. J., Lynn-Bullock, C. P., Spitzer, N., Caldwell, H. K. and Katz, P. S. (2001). Localization and quantification of 5-hydroxytryptophan and serotonin in the central nervous systems of *Tritonia* and *Aplysia*. *J. Comp. Neurol*. **437**:91-105.

Fiorica-Howells, E., Wade, P. R. and Gershon, M. D. (1993). Serotonin-induced increase in cAMP in ganglia isolated from the myenteric plexus of the guinea pig small intestine: mediation by a novel 5-HT receptor. *Synapse* **13**: 339-349.

Fiorica-Howells, E., Maroteaux, L. and Gershon, M. D. (2000). Serotonin and the 5-HT(2B) receptor in the development of enteric neurons. *J. Neurosci.* **20**, 294-305.

Fletcher, E. L. and Wässle, H. (1999). Indoleamine-accumulating amacrine cells are presynaptic to rod bipolar cells through $GABA_C$ receptors. *J. Comp. Neurol.* **413,** 155-167.

Fox, H. M. (1952). Anal and oral intake of water by Crustacea. *J. Exp. Biol.* **29**, 583-599.

Frankfurt, M., Lauder, J. M. and Azmitia, E. C. (1981). The immunocytochemical localization of serotonergic neurons in the rat hypothalamus. *Neurosci. Lett.* **24,** 227-232.

Freeman, M. R. and Doherty, J. (2006) Glial cell biology in *Drosophila* and vertebrates. *Trends Neurosci.* **29**, 82-90.

Furness, J. B. and Costa, M. (1982). Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea-pig small intestine. *Neuroscience* **7**, 341-9.

Galligan, J. J. (2004) 5-hydroxytryptamine, ulcerative colitis, and irritable bowel syndrome: molecular connections. *Gastroenterology* **26**, 1897-1899.

Gershon, M. D. (1999). Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment. Pharmacol. Ther.* **13 (2)**, 15-30.

Grider, John E. (1994). CGRP in the sensory pathway mediating peristaltic reflex. *Am. J. Physiol* . **266**, 1139-1145.

Grider, John E. (2003). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J. Pharmacol. Exp. Ther*. **307**, 460-467.

Hansen, M. B. (2003). Neurohumoral control of gastrointestinal motility. *Physiol. Res.* **52**, 1-30.

Hansson, S. R., Mezey, E. and Hoffman, B. J. (1998). Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non- serotonergic neurons. *Neuroscience* **83,** 1185-1201.

Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J. E. and Evans, P. D. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. *Science* **216**, 1012-1014.

Hughes, M. R., Bennett, D. C., Sullivan, T. M. and Hwang, H. (1999). Retrograde movement of urine into the gut of salt water acclimated Mallards (*Anas platyrhynchos*). *Can. J. Zool.* **77,** 342–346.

Huxley, T. H. (1880). *The Crayfish: An Introduction to Zoology*. New York: D. Appleton & Co.

Iturriza, F. C. (1989). Two kinds of cells in grafts of pituitary pars intermedia and their probably dependence on dopamine. *Neuroendocrinology*, **49**, 1-6.

Jackowski, A., Crockard, A. and Burnstock G. (1988). Ultrastructure of serotonin-containing nerve fibres in the middle cerebral artery of the rat and evidence for its localization within catecholamine-containing nerve fibres by immunoelectron microscopy. *Brain Res.* **443**, 159-165.

Johansen, P. A., Jennings, I., Cotton, R. G. and Kuhn, D. M. (1996) Phosphorylation and activation of tryptophan hydroxylase by exogenous protein kinase A. *J. Neurochem.* **66**, 817-823.

Johansson, A. S. and Schreiner, B. (1965). *L. Gen. comp. Endocr.* Neurosecretory cells in the ventral ganglia of the lobster, *Homarus vulgaris* L. **5**, 558-567.

Johnson, B. R. and Harris-Warrick, R. M. (1990). Aminergic modulation of graded synaptic transmission in the lobster stomatogastric ganglion. *J. Neurosci.* **10**, 2066-2076.

Jones, H. C. (1962). The action of L-glutamic acid and of structurally related compounds on the hind gut of the crayfish. *J. Physiol.* **164,** 295-300.

Katz, P. S., Eigg, M. H. and Harris-Warrick, R. M. (1989). Serotonergic/cholinergic muscle receptor cells in the crab stomatogastric nervous system. I. Identification and characterization of the gastropyloric receptor cells. *J. Neurophysiol.* **62**, 558-570.

Katz, P. S. and Harris-Warrick, R. M. (1990). Neuromodulation of the crab pyloric central pattern generator by serotonergic/cholinergic proprioceptive afferents. *J. Neurosci.* **10**, 1495-1512.

Katz, P. S. and Harris-Warrick, R. M. (1991) Recruitment of crab gastric mill neurons into the pyloric motor pattern by mechanosensory afferent stimulation. *J. Neurophysiol.* **65**, 1442-1451.

Kennedy, M. B. (1978). Products of biogenic amine metabolism in the lobster: sulfate conjugates. *J. Neurochem.* 30:315-320.

Kondoh, Y. and Hisada, M. (1986). Neuroanatomy of the terminal (sixth abdominal) ganglion of the crayfish, *Procambarus clarkii* (Girard). *Cell Tissue Res*. **243,** 273-288.

Kulkarni, G. K. and Fingerman, M. (1992). Quantitative analysis by reverse phase high performance liquid chromatography of 5-hydroxytryptamine in the central nervous system of the red swamp crayfish, *Procambarus clarkii*. *Biological Bulletin* **182,** 341-347.

Kupfermann, I., Cohen, J. L., Mandelbaum, D. E., Schonberg, M., Susswein, A. J. and Weiss, K. R. (1979). Functional role of serotonergic neuromodulation in *Aplysia*. *Fed. Proc.* **38**, 2095-2102.

Lauder, J. M. and Krebs, H. (1978). Serotonin as a differentiation signal in early neurogenesis. *Dev. Neurosci.* **1**, 15-30.

Lebrand, C., Cases, O., Adelbrecht, C., Doye, A., Alvarez, C., El Mestikawy, S., Seif, I. and Gaspar, P. (1996). Transient uptake and storage of serotonin in developing thalamic neurons. *Neuron* **17**, 823-835.

Lee, C.Y., Yang, P. F. and Zou, H. S. (2001). Serotonergic regulation of crustacean hyperglycemic hormone secretion in the crayfish, *Procambarus clarkii. Physiol. Biochem. Zool.* **74**, 376-382.

Lent, C. M. (1985). Serotonergic modulation of the feeding behavior of the medicinal leech. *Brain Res. Bull.* **14**, 643-655.

Lovett, D. L. and Felder, D. L. (1990). Ontogeny of Kinematics in the Gut of the White Shrimp *Penaeus setiferus* (Decapoda: Penaeidae). *J. Crust. Biol.* **10**, 53-68.

Martin, G. G. and Hose, J. E. (1995). Circulation, the blood and disease. In *Biology of the Lobster* Homarus americanus (Factor, J. R., ed.), pp 465-495. New York: Academic Press.

Massey, S. C., Mills, S. L. and Marc, R. E. (1992). All indoleamine-accumulating cells in the rabbit retina contain GABA. *J. Comp. Neurol.* **322**, 275-291.

Maynard, D. M. (1960). Circulation and heart function. In *The Physiology of Crustacea* (Waterman, T. H., ed.), pp 161-226. New York: Academic Press.

Medgett, I. C., Fearn, H. J. and Rand, M. J. (1984). Serotonin enhances sympathetic vasoconstrictor responses in rat isolated perfused tail artery by activation of postjunctional serotonin- 2 receptors. *Clin. Exp. Pharmacol. Physiol.* **11**, 343-346.

Mercier, A. J., Lange A. B., TeBrugge, V. and Orchard, I. (1997). Evidence for proctolin-like and RFamide-like neuropeptides associated with the hindgut of the crayfish *Procambarus clarkii*. *Canadian Journal of Zoology*. **75**, 1208-1225.

Mercier, A. J. and Lee, J. (2002). Differential effects of neuropeptides on circular and longitudinal muscles of the crayfish hindgut. *Peptides* **23**, 1751-1757.

Mercier, A. J., Orchard, I. and Schmoeckel, A. (1991). Catecholaminergic neurons supplying the hindgut of the crayfish *Procambarus clarkii*. *Can. J. of Zool*. **69**, 2778-2785.

Mickénienė, L. (1999). Bacterial flora in the digestive tract of native and alien species of crayfish in Lithuania. *Freshwater Crayfish* **12**, 279-287.

Miller, F. R. (1910). On the rhythmical contractility of the anal musculature of the crayfish and lobster. *J. Physiol.* **40**, 431-444.

Mitchell, C. K. and Redburn, D. A. (1985). Analysis of pre- and postsynaptic factors of the serotonin system in rabbit retina. *J. Cell Biol.* **100**, 64-73.

Mockus, S. M. and Vrana K. E. (1998). Advances in the molecular characterization of tryptophan hydroxylase. *J. Mol. Neurosci.* **10**, 163-179.

Moffett, S. B. and Moffett, D. F. (2005). Comparison of immunoreactivity to serotonin, FMRFamide and SCPb in the gut and visceral nervous system of larvae, pupae and adults of the yellow fever mosquito Aedes aegypti. *J Insect Sci* **5**, 20.

Muramoto, A. (1977). Neural control of rhythmic anal contraction in the crayfish. *Comp. Biochem. Physiol* **56A**, 551-557.

Muramoto, A. (1981). Effects of eyestalk extracts and ecdysterone on water intake through the anus of the crayfish. *Comp. Biochem. Physiol.* **69A**, 197-203.

Musolf, B. E., Antonsen, B. L., Spitzer, N., and Edwards, D. H. (2005). Differential distribution of the 5-$HT_{1\alpha}$ and the 5-$HT_{1\beta}$ receptors on crayfish hindgut. Program No. 754.10. *2005 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2004. http://sfn.scholarone.com/itin2005/index.html.

Musolf, B. E., Craft, C. and Edwards, D. H. (2001). Crayfish hindgut neurons "borrow" serotonin following food ingestion. *Soc. Neurosci. Abstr.* **27**, 942.10.

Musolf, B. E. and Edwards, D. H. (1999). Sensory stimulation regulates intensity of 5-HT-ir crayfish hindgut neurons. *Soc. Neurosci. Abstr.* **25**, 678.13.

Musolf, B. E. and Edwards, D. H. (2004). Serotonin modulates contractions of crayfish hindgut Program No. 314.15. *2004 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2004. Online.

Musolf, B. E. and Edwards, D. H. (2003) Regulation of serotonin varies in different areas of the crayfish CNS. Program No. 270.7. *2003 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2003. Online.

Nagayama, T. (2002). Serotonergic modulation of nonspiking local interneurones in the terminal abdominal ganglion of the crayfish. *J. Exp. Biol.* **205**, 3067-3076.

Niacaris, T. and Avery, L. (2003). Serotonin regulates repolarization of the *C. elegans* pharyngeal muscle. *J. Exp. Biol.* **206**, 223-231.

O'Gara, B. A., Illuzzi, F. A., Chung, M., Portnoy, A. D., Fraga, K. and Frieman, V. B. (1999). Serotonin induces four pharmacologically separable contractile responses in the pharynx of the leech *Hirudo medicinalis*. *Gen. Pharmacol.* **32**, 669-681.

Orlov, J. (1926). Die innervation des darmes des flusskrebses. *Z. Mikr. Anat. Forsch.* **4**, 101-148.

Pan, H and Gershon, M. D. (2000). Activation of intrinsic afferent pathways in submucosal ganglia of the guinea pig small intestine. *J. Neurosci.* **20**, 3295-3309.

Panksepp, J. B. and Huber, R. (2002). Chronic alterations in serotonin function: dynamic neurochemical properties in agonistic behavior of the crayfish, *Orconectes rusticus*. *J. Neurobiol.* **50**, 276-90.

Paul, D. H. and Wilson, L. J. (1994) Replacement of an inherited stretch receptor by a newly evolved stretch receptor in hippid sand crab. *J. Comp. Neurol.* **350**, 150-160.

Persico, A. M., Mengual, E., Moessner, R., Hall, F. S., Revay, R .S., Sora, I., Arellano, J., DeFelipe, J., Gimenez-Amaya, J. M., Conciatori, M., Marino, R., Baldi, A., Cabib, S., Pascucci, T., Uhl, G. R., Murphy, D. L., Lesch, K. P., Keller, F., Hall, S. F. (2001). Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. *J. Neurosci.* **21**, 6862-6873.

Pootanakit, K. and Brunken, W. J. (2001). Identification of 5-HT(3A) and 5-HT(3B) receptor subunits in mammalian retinae: potential pre-synaptic modulators of photoreceptors. *Brain Res.* **896**, 77-85.

Prosser, C. L., Nystrom R. A. and Nagai, T. (1965). Electrical and mechanical activity in intestinal muscles of several invertebrate animals. *Biochem. Physiol.* **14**, 53-70.

Rapport, M. M., Green, A. A. and Page, J. H. (1948a). Partial purification of the vasoconstrictor in beef serum. *J. Biol. Chem.* **174**, 735-741.

Rapport, M. M., Green, A. A. and Page, J. H. (1948b). Crystalline serotonin. *Science* **24,** 329-330.

Real, D. and Czternasty, G. (1990). Mapping of serotonin-like immunoreactivity in the ventral nerve cord of crayfish. *Brain. Res.* **521**, 203-212.

Richards, K. S., Simon, D. J., Pulver, S. R., Beltz, B. S. and Marder, E. (2003). Serotonin in the developing stomatogastric system of the lobster, *Homarus americanus*. *J. Neurobiol.* **54**, 380-392.

Saland, L. C., Wallace, J. A., Samora, A. and Gutierrez, L. (1988). Co-localization of tyrosine hydroxylase (TH)- and serotonin (5-HT)-immunoreactive innervation in the rat pituitary gland. *Neurosci. Lett.* **94**, 39-45.

Salichon, N., Gaspar, P., Upton, A. L., Picaud, S., Hanoun, N., Hamon, M., De Maeyer, E. E., Murphy, D. L., Mossner, R., Lesch, K. P., Hen, R. and Seif, I. (2001). Excessive Activation of Serotonin (5-HT) 1B Receptors Disrupts the Formation of Sensory Maps in Monoamine Oxidase A and 5-HT Transporter Knock-Out Mice. *J. Neurosci.* **21**, 884-896.

Sandeman, D., Beltz, B. and Sandeman, R. (1995). Crayfish brain interneurons that converge with serotonin giant cells in acessory lobe glomeruli. *J. Comp. Neurol.* **352**, 263-273.

Sandeman, R. E. and Sandeman, D. C. (1987). Serotonin-like immunoreactivity of giant olfactory interneurons in the crayfish brain. *Brain Res.* **403**, 371-374.

Sandeman, D., Sandeman, R., Derby, C. D. and Schmidt, M. (1992). Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature of homologous structures. *Biol. Bull.* **183**, 304-326.

Schroeter, S., Levey, A. I. and Blakely, R. D. (1997). Polarized expression of the antidepressant-sensitive serotonin transporter in epinephrine-synthesizing chromaffin cells of the rat adrenal gland. *Mol. Cell Neurosci.* **9**, 170-184.

Schutte, M. (1994). Serotonergic and serotonin-synthesizing cells of the *Xenopus* retina. *Int. J. Neurosci.* **78**, 67-73.

Schutte, M. and Witkovsky, P. (1990). Serotonin-like immunoreactivity in the retina of the clawed frog *Xenopus laevis*. *J. Neurocytol.* **19**, 504-518.

Simansky, K. J. (1996). Serotonergic control of the organization of feeding and satiety. *Behav.Brain Res.* **73**, 37-42.

Siriwardena, A. K., Budhoo, M. R., Smith, E. P. and Kellum, J. M. (1993). A 5-HT3 receptor agonist induces neurally mediated chloride transport in rat distal colon. *J. Surg. Res.* **55**, 55-59.

Sneddon, L. U., Taylor, A. C., Huntingford, F. A. and Watson, D. G. (2000). Agonistic behaviour and biogenic amines in shore crabs *Carcinus maenas*. *J. Exp. Biol.* **203**, 537-545.

Spitzer, N., Antonsen, B. L. and Edwards, D. H. (2005). Immunocytochemical mapping and quantification of expression of a putative type 1 serotonin receptor in the crayfish nervous system. *J. Comp. Neurol.* **484**, 261-282.

Sporhase-Eichmann, U., Musolf, B. E. and Edwards, D. H. (1998). Dopamine- and octopamine-immunoreactive neurons in the central nervous system of the crayfish. *Soc. Neurosci. Abstr.* **24**, 731.16.

Stangier, J., Hilbich, C., Burdzik, S. and Keller, R. (1992). Orcokinin: a novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. *Peptides* **13**, 859-864.

Stangier, J. and Keller, R. (1990). Occurrence of crustacean cardioactive peptide (CCAP) in the nervous system of the crayfish, *Orconectes limosus*. In *Frontiers in Crustacean Neurobiology*. (ed. K. Wiese, W.-D. Krenz, J. Tautz, H. Reichert and B. Mulloney), pp. 394-400. Basel: Birkhauser.

Stanley, M. I., Berger, R. J., Zuccarello, M. and Keller, J. T. (1993). Serotonin (5-HT) fibers of the rat dura mater: 5-HT-positive, but not authentic serotoninergic, tryptophan hydroxylase-like fibers. *Neurosci. Lett.* **162**, 89-92.

Sullivan, J. M., Benton, J. L. and Beltz, B. S. (2000). Serotonin depletion *in vivo* inhibits the branching of olfactory projection neurons in the lobster deutocerebrum. *J. Neurosci.* **20**, 7716-7721.

Takahashi, R., Sakai, T., Furuyama, Y., Kondo, Y., Inoue, C. N., Onuma, S. and Iinuma, K. (2000). The vasocontractive action of norepinephrine and serotonin in deep arterioles of rat cerebral gray matter. *Tohoku. J. Exp. Med.* **190**, 129-142.

Tao-Cheng, J. H. and Zhou, F. C. (1999). Differential polarization of serotonin transporters in axons versus soma-dendrites: an immunogold electron microscopy study. *Neuroscience* **94**, 821-830.

Tierney, A. J., Godleski, M. S. and Rattananont, P. (1999). Serotonin-like immunoreactivity in the stomatogastric nervous systems of crayfishes from four genera. *Cell Tissue Res.* **295**, 537-551.

To, T. H., Brenner, T. L., Cavey, M. J. and Wilkens, J. L. (2004). Histological organization of the intestine in the crayfish *Procambarus clarkii*. *Acta Zoologica.* **85**, 119-130.

Tutton, P. J. and Barkla, D. H. (1988). Steroid hormones as regulators of the proliferative activity of normal and neoplastic intestinal epithelial cells. *Anticancer Res.* **8**, 451-456.

Ugrumov, M. V., Proshlyakova, E. V. and Sapronova, A. (1989). Development of the hypothalamic 5-hydroxytryptamine system during ontogenesis in rats: uptake and release of 5-hydroxytryptamine in vitro. *Neurosci.* **32**, 127-131.

Ugrumov, M. V., Taxi, J., Mitskevich, M. S. and Tramu, G. (1986). Development of the hypothalamic serotoninergic system during ontogenesis in rats. Immunocytochemical and radioautographic study. *Brain Res.* **395**, 75-84.

Upton, A. L., Salichon, N., Lebrand, C., Ravary, A., Blakely, R., Seif, I., and Gaspar, P. (1999). Excess of serotonin (5-HT) alters the segregation of ispilateral and contralateral retinal projections in monoamine oxidase A knock-out mice: possible role of 5-HT uptake in retinal ganglion cells during development. *J. Neurosci.* **19**, 7007-7024.

van Harreveld, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. Exp. Biol. Med.* **34**, 428-432.

Vaney, D. I., Nelson, J. C. and Pow, D. V. (1998). Neurotransmitter coupling through gap junctions in the retina. *J. Neurosci.* **18**, 10594-10602.

Vanhatalo, S. and Soinila, S. (1994). Pharmacological characterization of serotonin synthesis and uptake suggest a false transmitter role for serotonin in the pituitary intermediate lobe. *Neurosci. Res.* **21**, 143-149.

Vanhatalo, S. and Soinila, S. (1995). Release of false transmitter serotonin from the dopaminergic nerve terminals of the rat pituitary intermediate lobe. *Neurosci. Res.* **22**, 367-374.

Vanhatalo S. and Soinila, S. (1996). Superfluous expression of tryptophan hydroxylase in the zona incerta dopaminergic neurones. *Neuroreport* **7**, 2889-2892.

Vanhatalo S. and Soinila, S. (1998.) Serotonin is not synthesized, but specifically transported in the neurons of the hypothalamic dorsomedial nucleus. *Euro. J. of Neurosci.* **10**, 1930-1935.

Vanhatalo, S., Soinila, S., Kaartinen, K. and Back, N. (1995). Colocalization of dopamine and serotonin in the rat pituitary gland and in the nuclei innervating it. *Brain Res*. **669**, 275-284.

Verney, C., Lebrand, C. and Gaspar, P. (2002). Changing distribution of monoaminergic markers in the developing human cerebral cortex with special emphasis on the serotonin transporter. Anat Rec 267:87-93.

Vogt, G. (2002). Functional anatomy. In *Biology of Freshwater Crayfish* (ed. D. M. Holdich), pp 53-146. Oxford: Blackwell Science.

Wade, P. R., Chen, J., Jaffe, B., Kassem, I. S., Blakely, R. D. and Gershon, M. D. (1996). Localization and function of a 5-HT transporter in crypt epithelia of the gastrointestinal tract. *J.Neurosci.* **16**, 2352-2364.

Walther, D. J. and Bader, M. (2003). A unique central tryptophan hydroxylase isoform. *Biochem. Pharmacol.* **66**, 1673-1680.

Webster, S. G., Dircksen, H., and Chung, J. S. (2000). Endocrine cells in the gut of the shore crab Carcinus maenas immunoreactive to crustacean hyperglycaemic hormone and its precursor- related peptide. *Cell Tissue Res.* **300**, 193-205.

Weiger, W. A. (1997). Serotonergic modulation of behaviour: A phylogenetic overview. **72**, 61-95.

Weihe, E. and Eiden, L. E. (2000). Chemical neuroanatomy of the vesicular amine transporters. *FASEB J* **14**, 2435-2449.

Wilkens, J. (1997). Possible mechanisms of control of vascular resistance in the lobster *Homarus americanus*. *J. Exp. Biol.* **200**, 487-493.

Winlow, W. and Laverack, M. S. (1972a). The control of hindgut motility in the lobster *Homarus gammarus* (L.) 3. Structure of the sixth abdominal ganglion (6 A.G.) and associated ablation and microelectrode studies. *Mar. Behav. Physiol.* **1**, 93-121.

Winlow, W. and Laverack, M. S. (1972b). The control of hindgut motility in the lobster, *Homarus gammarus* (L.) 2. Motor output. *Mar. Behav. Physiol.* **1**, 29-47.

Winlow, W. and Laverack, M. S. (1972c). The control of hindgut motility in the lobster, *Homarus gammarus* (L.) 1. Ananlysis of hindgut movements and receptor activity. *Mar. Behav. Physiol.* **1**, 1-27.

Whitaker-Azmitia, P. M., Druse, M., Walker, P. and Lauder, J. M. (1996). Serotonin as a developmental signal. *Behav. Brain Res.* **73**, 19-29.

Wrong, A. D., Sammahin M., Richardson, R. and Mercier A. J. (2003). Pharmacological properties of L-glutamate receptors associated with the crayfish hindgut. *J Comp Physiol A* **189**, 371-378.

Wurtman, R. J., Wurtman, J. J., Regan, M. M., McDermott, J. M., Tsay, R. H. and Breu, J. J. (2003). Effects of normal meals rich in carbohydrates or proteins on plasma tryptophan and tyrosine ratios. *Am. J. Clin. Nutr.* **77**, 128-132.

Yeh, S. R., Musolf, B. E. and Edwards, D. H. (1997). Neuronal adaptations to changes in the social dominance status of crayfish. *J. Neurosci.* **17**, 697-708.

Young, E. A., Neff, N. H. and Hadjiconstantinou, M. (1994). Phorbol ester administration transiently increases aromatic L-amino acid decarboxylase activity of the mouse striatum and midbrain. *J. Neurochem.* **63**, 694-697.

Young, S. N. and Gauthier, S. (1981). Tryptophan availability and the control of 5-hydroxytryptamine and tryptamine synthesis in human CNS. *Adv. Exp. Med. Biol.* **133**, 221-230.

Zhou, F. C., Tao-Cheng, J. H., Segu, L., Patel, T. and Wang, Y. (1998). Serotonin transporters are located on the axons beyond the synaptic junctions: anatomical and functional evidence. *Brain Res.* **805**, 241-254.

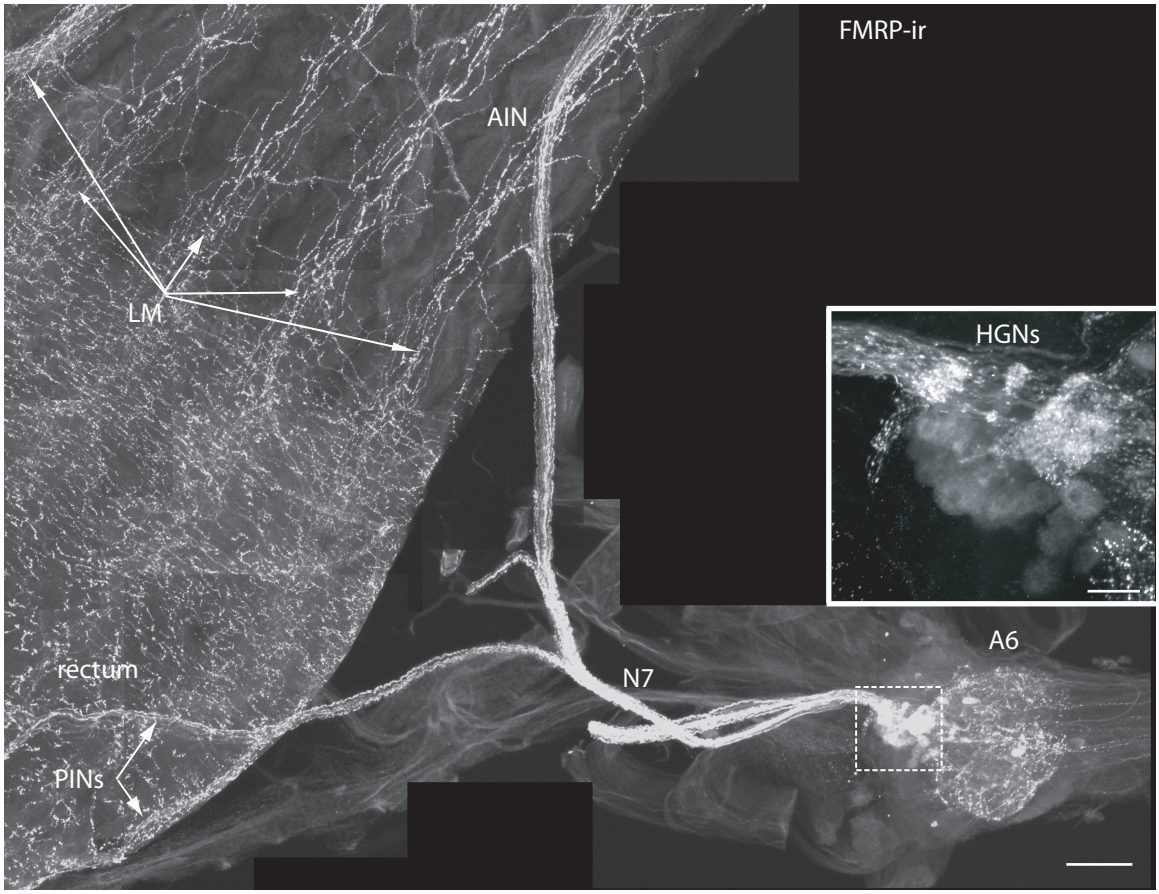
Zhu, M.Y., Juorio, A. V., Paterson, I. A. and Boulton, A. A. (1994) Regulation of aromatic L-amino acid decarboxylase in rat striatal synaptosomes: effects of dopamine receptor agonists and antagonists. *Br. J. Pharmacol*. **112**, 23-30.

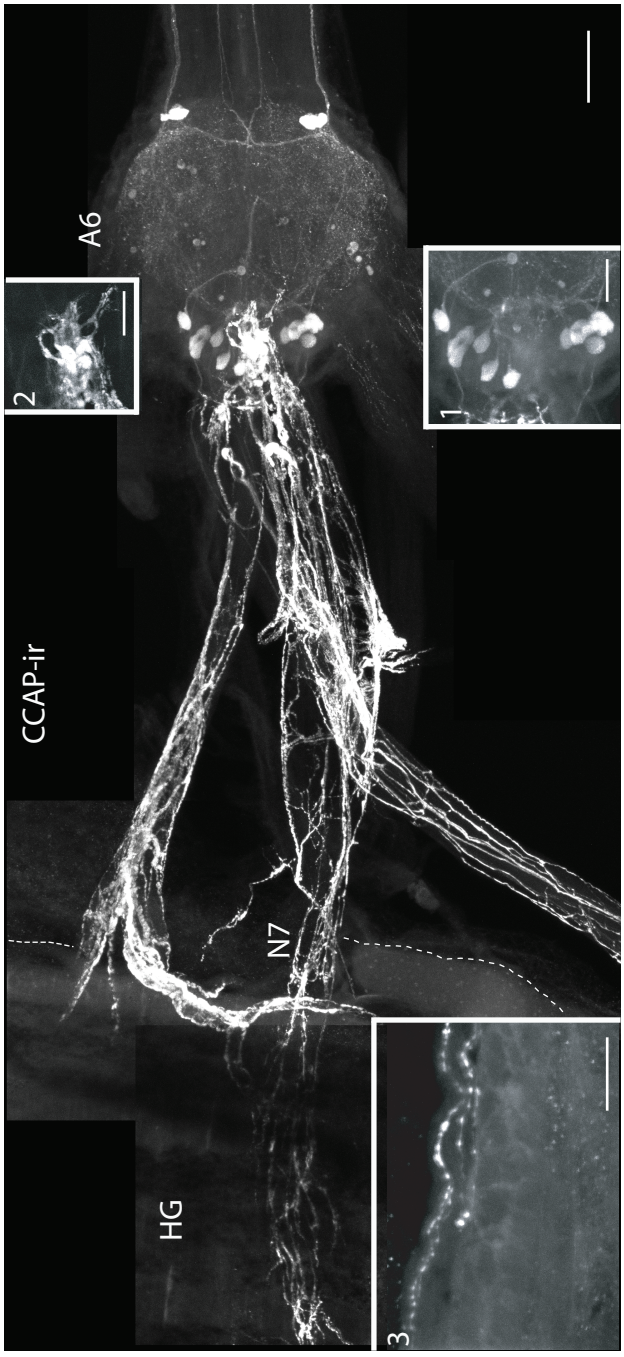
Appendix

FMRF-amide and custacean cardioactive peptide immunoreactivity in HGNs

Appendix, Fig. 1 FMRFamide-ir in A6 and the hindgut. FMRFamide-ir exhibits a pattern of innervation that is more profuse than the 5-HT-ir seen in the HGNs. In addition to the HGN subset that shows FMRFamide-ir, there is an additional cluster of cells that are located lateral to the HGN cluster. These neurons appear to innervate the rectal circular muscles where numerous fine fibers show FMRF-amide-ir. This pattern of innervation on the circular muscles of the hindgut is distinctly different than the 5-HT-ir in that location. FMRFamide-ir is also localized in the perineural sheath near A5 and A6, suggesting hormonal release occurs there.

Appendix Fig. 1



Appendix, Fig. 2 CCAP-ir in A6 and the hindgut. CCAP-ir indicates that there is a large neurohemal release area that arises from the medial posterior area of A6 and extends along N7. This neurohemal release area projects to the hindgut and along N5 and N6 to the telson and anal musculature. (Inset 1) Numerous cell bodies showed intense CCAP-ir in the posterior region of A6, however, the cell bodies were larger and more laterally placed in the ganglion than the HGNs (Inset 2) Four cells were found dorsal to the HGNs, close to where N7 exited the ganglion and where HGN axons ramify. (Inset 3) The axons of the CCAP-ir neurons projected to the periphery and formed a web of neurosecretory release sites that surrounded N7 and paired nerves 5 and 6. A limited number of labeled fibers along the hindgut sheath showed CCAP-ir. No CCAP-ir was found on the muscles or in the intestinal nerves of the hindgut.

Appendix Fig. 2