The pleiotropic roles of FGLamide allatostatins in the African migratory locust, *Locusta migratoria*

by

Lisa Ann Robertson

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Cell and Systems Biology
University of Toronto

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**Abstract**

The FGLa/ASTs are one family of allatostatin peptides and share an amidated C-terminal sequence (FGL-amide). The inhibitory effect of FGLa/ASTs on juvenile hormone (JH) biosynthesis in *Diploptera punctata* led to their discovery, but there is a lack of allatostatic function across most insect species that suggests this function may not be their primary role. Rather, the FGLa/ASTs are implicated as brain/gut peptides, modulating gut physiology. This thesis demonstrates the pleiotropic nature of FGLa/ASTs in *Locusta migratoria* and emphasizes the role of FGLa/ASTs as brain/gut peptides involved in homeostatic processes.

FGLa/AST-like immunoreactivity (FLI) is associated with the corpus cardiacum (CC) and corpus allatum (CA). FGLa/ASTs increase adipokinetic hormone release from the CC and alter JH biosynthesis from the CA, suggesting roles in energy utilization and in growth and metamorphosis.

Each region of the gut exhibits FLI. The gut is dually innervated: neurons in the abdominal ganglia of the central nervous system (CNS) innervate the posterior gut and some contain FLI, while neurons within the stomatogastric nervous system (STNS) that innervate the anterior gut
do not seem to contain FLI, indicating that source of FLI on the gut are cells within the CNS, which may release FGLa/ASTs at the gut to alter aspects of gut physiology. FGLa/ASTs are involved in peristalsis, neural control of foregut contractions, and ileal K^+ transport. In particular, FGLa/ASTs inhibit contractions of each gut region and also modulate the rhythmic motor output of a central pattern generator within the ventricular ganglion of the STNS. FGLa/ASTs also inhibit ileal K^+ efflux, suggesting a diuretic action and implicating FGLa/ASTs in fluid and ion homeostasis.

This work provides a comprehensive picture of how FGLa/ASTs play an integral role in nutrient processing, energy mobilization, and growth and metamorphosis to contribute to the overall maintenance of homeostasis. This reinforces the role of FGLa/ASTs as brain/gut peptides and emphasizes their involvement in the flexibility of nervous communication and integration of the endocrine system with the CNS to achieve homeostasis.
ACKNOWLEDGMENTS

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Very sincere thanks to my parents. Even if you find it hard to understand what it is that I do, once again you believed in me and supported me unconditionally through another degree. Thank you for your ongoing support of my academic journey; Dad I guess you were right when you said so many years ago that I would be in school forever – you just knew I had a thirst for knowledge that could not be quenched. Mom and Dad, you will never understand how much I treasure you. I love you both so very much.

To my husband Lloyd, thank you for always holding me close and holding my heart in your hands. Life isn’t always easy and love isn’t always easy, but it’s always worth it; I know that now. Thank you for continually boosting my wavering confidence and cheering me on through the hiccups along the way. Thank you for believing in me and my goals and aspirations. You are my rock and I love you so much.

To my children, Keira and Nathan, I thank you from the tips of my toes to the tips of my fingers for being so awesome and helping mommy get through frustrating times when you didn’t even know you were helping me. From light saber duels to dance parties, you made sure I never forgot to have fun, enjoy life, and laugh every day. You are both so young still so I want to give you some advice: Don’t ever doubt yourself or ever give up on your dreams…if you have the motivation and desire to achieve your goals and aspirations then you will. Life will always throw you curve balls along the way, but just breathe, relax, and you’ll overcome the hurdle. I am so proud of both of you already and I am so lucky to be your mom. I love you both so very much. The force is strong in you young padawan learners and may it be with you…always.
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ORGANIZATION OF THE THESIS

Chapter 1 serves as the general introduction to the research chapters of the thesis.

Chapter 2 is published in the Journal of Insect Physiology [54 (2008) 949-958] and is co-authored by Dr. Angela Lange, Jinrui Zhang, and Dr. Stephen Tobe. Jinrui Zhang performed the JH assay.

Chapter 3 is published in the Journal of Insect Physiology [56 (2010) 893-901] and is co-authored by Dr. Angela Lange.

Chapter 4 is published in the Journal of Experimental Biology 215 (2012) 3394-3402] and is co-authored by E. Patricia Rodriguez and Dr. Angela Lange. Patricia performed the proctolin bioassays on foregut and hindgut contraction. The appendix is unpublished and extends the results within this chapter. Dr. Li Zhang performed the MALDI-TOF MS/MS analysis for protein sequence determination.

Chapter 5 is a concise chapter in preparation for publication. This chapter was a collaboration with Dr. Andrew Donini, York University.

Chapter 6 is the general discussion of the thesis. By summarizing and integrating the results of the thesis together, the discussion serves to emphasize the pleiotropic roles of FGLamide allatostatins as brain/gut peptides involved in the regulation of homeostatic processes and in the flexibility and integration of communication within and between the central nervous system, stomatogastric nervous system and endocrine system to ensure proper functioning of target tissue and organs to achieve homeostasis.

Unless otherwise stated, I performed all experimentation and data analysis. Permission was obtained from the publishers to include the published work in this thesis. The published work in this thesis is unedited except for formatting. I wrote all manuscripts and thesis chapters, with valuable input from Dr. Angela Lange and Dr. Ian Orchard.
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LIST OF ABBREVIATIONS

Note: The following abbreviations are defined within the text of each chapter where appropriate.

ACN     Acetonitrile
ALI     Allatostatin-like immunoreactivity
AST     Allatostatin
AKH     Adipokinetic hormone
BSA     Bovine serum albumin
CA      Corpus allatum
cAMP    3′-5′-cyclic adenosine monophosphate
CC      Corpus cardiacum
CCAP    Crustacean cardioactive peptide
CNS     Central nervous system
CPG     Central pattern generator
CTSH    Chloride transport stimulating hormone
ELISA   Enzyme-linked immunosorbent assay
ES      Endocrine system
FGLa/ASTs FGLamide-type allatostatins; cockroach allatostatins; A-type allatostatins
FLI     FGLa/AST-like immunoreactivity
IBMX    3-Isobutyl-1-methylxanthine
ITP     Ion transport peptide
JH      Juvenile hormone
LNC     Lateral neurosecretory cell
LomTK   *Locusta migratoria* tachykinin; locustatachykinin
MALDI-TOF MS/MS Matrix assisted laser desorption ionization time-of-flight tandem mass spectrometry
MIP     myoinhibiting peptide
NCA     Nervus corporis allati
NCC     Nervus corporis cardiaci
NGS     Normal goat serum
NH      Neurohormone
NM      Neuromodulator
NSC     Neurosecretory cell
NSS     Normal sheep serum
NT      Neurotransmitter
PBS     Phosphate buffered saline
RIA     Radioimmunoassay
Scg-AST-6 *Schistocerca gregaria*-AST-6
SIET    Scanning ion-selective electrode technique
SNS, STNS Stomatogastric nervous system
SOG     Subesophageal ganglion
TFA     Trifluoracetic acid
VUM     Ventral unpaired median neuron
CHAPTER 1:
GENERAL INTRODUCTION
All animals face varying environmental conditions and resource availability that challenge survival. Insects are one of the most successful groups of animals, having been able to inhabit a wide variety of ecological niches, each with its own set of unique challenges. To counter these challenges, insects employ a variety of homeostatic control mechanisms, where the regulatory roles of peptides are imperative. Peptides are involved in virtually all physiological processes, including metabolism, growth and development, osmoregulation, reproduction, and behaviour (Goldsworthy and Mordue, 1974; see Gäde et al., 1997). One such peptide family is that of the FGLamide allatostatins (FGLa/ASTs). This thesis will serve to highlight the ways that FGLa/ASTs modulate physiological processes important for homeostasis within the African migratory locust, *Locusta migratoria*. The control of FGLa/ASTs in the synthesis and release of metabolic hormones and hormones involved in growth and metamorphosis is demonstrated. Evidence for additional roles of FGLa/ASTs in feeding and digestion is presented, including the modulation of neurogenic and spontaneous gut contractions involved in peristalsis, inhibition of gut muscle tension and inhibition of K\(^+\) efflux *in vitro* at the hindgut.

Overall, this thesis demonstrates and further supports, that despite being discovered based on their ability to inhibit juvenile hormone (JH) biosynthesis, a more primitive role for the FGLa/ASTs is as brain/gut peptides involved either directly or indirectly in regulating homeostatic processes essential for locust survival. The organization of the locust central nervous system (CNS) and stomatogastric nervous system (STNS) are reviewed briefly. There is then a section regarding the interaction of the CNS with the endocrine system (ES) and how this interaction leads to flexibility in neural and peripheral communication, which is important for the coordination of tissues involved in homeostatic processes. Locust gut morphology and innervation is then reviewed, followed by a brief synopsis of the major ion transporters at the
locust rectum. Following this, a short review of the neuropeptides involved in homeostatic processes (energy mobilization and utilization, growth and development, gut motility and digestion, and water and ion balance) is presented, and the Introduction ends with an outline of the objectives of the thesis.

**THE ORGANIZATION OF THE LOCUST CENTRAL AND STOMATOGASTRIC NERVOUS SYSTEMS**

The nervous system is specialized to use electrical signals in the form of action potentials to transmit information and is thus characterized as a highly specific, high-speed system. The CNS of the locust begins anteriorly with the brain, which consists of 3 fused segments (proto-, deuto-, tritocerebrum). The brain is connected posteriorly to the subesophageal ganglion (SOG) by the circumesophageal connectives. Extending posteriorly from the SOG are connectives that link the SOG to the ganglia of the ventral nerve cord. There are 3 thoracic ganglia (pro-, meso-, metathoracic) and five abdominal ganglia that serve each thoracic and abdominal segment respectively.

In addition to the CNS is the peripheral nervous system, or STNS (Figure 1). The STNS is connected to the brain and SOG and consists of several ganglia and nerve tracts along the locust digestive tract (Hartenstein, 1997). The STNS begins with the frontal ganglion, which is connected anteriorly to the tritocerebral lobes of the brain by the frontal connectives and posteriorly to the hypocerebral ganglion, by the recurrent nerve. Two esophageal nerves extend posteriorly from the hypocerebral ganglion and each ends in a ventricular (ingluvial) ganglion. The ventricular ganglia are bilaterally located on the surface of the posterior foregut and provide the innervation to the anterior gut (foregut and anterior midgut) by way of the gastric nerves (Albrecht, 1953; Bräunig, 2008; Konings et al., 1989).
Figure 1. Schematic illustration of the locust stomatogastric nervous system. Modified from: Robertson et al., 2012.
Nerves

Frontal Connectives  Recurrent Nerve  Esophageal Nerves  Gastric Nerves

Ganglia

Frontal  Hypocerebral  Ventricular (Ingluvial)

Dorsal View

Lateral View
INTERACTION OF THE CENTRAL NERVOUS SYSTEM AND ENDOCRINE SYSTEM

The CNS and the ES release chemical messengers involved in the regulation of physiological processes. Chemical messengers released by the ES are characterized as hormones, while those released by the CNS are characterized as neurotransmitters (NTs), neuromodulators (NMs) or neurohormones (NHs) (Figure 2). A hormone is released by an endocrine cell and travels within the hemolymph to target tissues to modulate their function. Neurohormones are similar to hormones in that they are released into the hemolymph, but are produced and released by specialized neurons called neurosecretory cells (NSCs; see Orchard, 1983). On the other hand, a NT is released by a neuron at a synapse or neuromuscular junction to communicate with the postsynaptic cell, while a NM is released locally onto a target tissue to modulate the activity of the target (see Orchard, 1983; Nässel, 2002). Thus, the effects elicited by NMs range from effects characteristic of NTs (quick and transient) to those of NHs (sustained and longer-lasting) (Figure 2 table).

Briefly, the ES is specialized for long-distance communication and the effects elicited by hormones generally play key roles in the homeostatic regulation of many physiological processes. The major endocrine tissues in insects are the NSCs within the CNS and specialized glands such as the corpus cardiacum (CC) and the corpus allatum (CA). The NSCs form a link between the ES and CNS and deliver neuropeptides that they produce along axons to be released into the hemolymph or at a synapse. In vertebrates, one component of the ES is a series of endocrine cells within the stomach and intestine that produce gastrointestinal hormones. A brain/gut axis also occurs in insects. The endocrine cells of the insect gut are similar in structure to vertebrate intestinal cells and are found in the midgut, which is primarily involved in digestion.
and absorption (Žitňan et al., 1993). These midgut endocrine cells produce regulatory peptides, which are identical to those found in the CNS, indicating the existence of a brain/gut axis in insects where information and feedback flow bidirectionally between the gut and the CNS. Thus, NSCs can act as intermediates between the CNS and ES by releasing neuropeptides in response to stimuli received and analyzed by the CNS, which can lead to the release of hormones from endocrine organs (Chapman, 1998).

Overall, the rapid-acting nature of the CNS and the slower and more sustained nature of the ES complement each other in the integration of physiological and metabolic processes and provide flexibility in communication between the ES and CNS of the insect. The effects of NTs are quick and transient, whereas the effects of NHs (and hormones) are persistent and longer lasting. The effects of NM are variable in their duration of action but are longer than those of NTs and shorter than those of hormones and NHs (Figure 2). Although hormones, NHs, NM, and NT are considered functionally separate classes of chemical messengers, the lines separating them are not always distinct. Thus, flexibility in communication also stems from a particular chemical messenger functioning as a member of one class in one instance, while acting as a member of a different class of messenger in another context. This is exemplified by the peptide proctolin. Proctolin was first suggested as a visceral muscle NT based on its presence within the proctodeal nerve that innervates the hindgut, and its stimulatory effect on neurally-evoked contractions (Brown, 1975; Brown and Starratt, 1975). Similarly, proctolin acts as a co-transmitter with glutamate at the locust oviduct and ovipositor muscles (Lange and Orchard, 1986; Orchard and Lange, 1987; see Lange, 2002). There is also evidence that proctolin functions as a NH in other instances (see Lange, 2002). For example, proctolin is present within processes of the CC and
CA and acts as a releasing factor for hormones produced and released by these organs (Clark et al., 2006).

In addition, some messengers have highly specific functions and influence only a single type of target cell, while other messengers have a variety of effects, depending on the target tissue. Many messengers seemingly function antagonistically, where one messenger has an effect on a system while a different messenger has an opposite effect. For example, the allatoregulatory peptides have opposite effects on the biosynthesis of JH by the CA, where allatostatins inhibit JH biosynthesis and allatotropins stimulate the biosynthesis of JH (see Gäde and Goldsworthy, 2003; Vullings et al., 1999; see Stay, 2000). In addition, myotropins affect insect visceral muscle in a stimulatory or inhibitory fashion. Examples of myostimulatory peptides include proctolin, kinins, and FMRFamide-like peptides (see Bendena et al., 1997). Inhibitory peptides include the myoinhibiting peptides, myosuppressins, and the allatostatins (see Bendena et al., 1997). This antagonistic control can offer one or more of the following advantages. The first is speed, where releasing an antagonistic messenger will oppose the actions of another messenger more quickly than waiting for the effects of a single messenger to dissipate. The second advantage is precision, where a system that depends on the ratio of one messenger to another can be precisely regulated.

The flexibility discussed here is imperative for the coordination of the tissues involved in regulating physiological processes involved in homeostasis. Multicellular organisms, like insects, maintain a relatively constant internal environment that buffers the variations in the external environment, a process known as homeostasis (Cannon, 1929). The French physiologist Claude Bernard (1813-1878) first alluded to homeostasis when he emphasized that the internal environment of an animal is quite different than the external environment surrounding the
animal. He concluded that for animals to become more successful they must become more independent from the environment, and this independence is achieved with increased control over the internal environment (Cannon, 1929). In vertebrates, the interaction of gastrointestinal and urinary epithelia, facilitated sometimes by skin or gills, is responsible for the major portion of water and ion homeostasis. Regulation of body fluids across the cuticle-covered exoskeleton of insects is rare, if not impossible. In insects, the coordinated action of the gut (primarily hindgut) and the Malpighian tubules maintains water and ionic balance. The nature and extent of homeostatic measures depends on the insect’s physiological state and optimal composition of the hemolymph, which in turn may vary with, and be adapted to, the ion content of the insect’s principal diet (Dow, 1981). Thus, solid plant feeders, such as the locust, have to cope with a high $K^+$ and $Mg^{2+}$ content in their hemolymph (Dow, 1981; see Audsley and Weaver, 2009).

The advantage of an organism having a more complex multicellular organization is that different cells and tissues are capable of functional specialization. But there is a potential disadvantage to this complexity – keeping the specialized cells and tissues working correctly and working together. The biochemical reactions and cellular processes of an organism are dependent on its internal conditions and large fluctuations in these internal conditions can affect the physiology of the organism, potentially leading to decreased survival. Biochemical reactions and cellular processes work optimally when important physical and chemical conditions, such as pH, temperature, and salt concentration are maintained within a narrow range. The coordination of these processes is achieved by the interaction of the CNS and ES through neural and/or peripheral communication to elicit an appropriate response(s) in the target tissue(s). Thus, homeostasis may not be achieved without the modulatory role that neuropeptides play in this communication.
**Figure 2.** Classification of chemical messengers and associated message characteristics that lead to flexibility in communication. Adapted and modified from Orchard et al., (2001) and Klowden (2007).
<table>
<thead>
<tr>
<th>Message Property</th>
<th>Neurotransmitter</th>
<th>Neuromodulator</th>
<th>Hormone &amp; Neurohormone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Very private – synaptic release</td>
<td>Somewhat private – local release onto target</td>
<td>Non-private – release into hemolymph</td>
</tr>
<tr>
<td>Speed of Transmission</td>
<td>Fast (msec)</td>
<td>Intermediate (msec, sec)</td>
<td>Slow (sec, min)</td>
</tr>
<tr>
<td>Duration of Effect</td>
<td>Short (msec)</td>
<td>Intermediate (sec, min)</td>
<td>Long (sec, min, wks)</td>
</tr>
</tbody>
</table>
LOCUST GUT MORPHOLOGY

Insects feed on a wide variety of animal, vegetable, and dead organic matter. Some insects are omnivorous, but most insects are specific in what they eat and are restricted to a category of food or even to a particular plant or animal. Thus, feeding and ingestion involves modifications of the mouthparts and appropriate modifications to the rest of the gut in addition to physiological adaptations. Interestingly, despite the wide diversity of insect gut anatomy, the basic enzymes involved in the metabolic pathways remain similar. Thus, the insect gut is flexible and adaptable, which certainly enabled the insects to become so successful in invading such varied habitats and become such a prolific group of animals. The primary function of the gut is to prepare the ingested food for absorption, by transforming it, using both physical and chemical processes, from complex molecules to simple molecules that can pass through the gut epithelial membrane into the hemolymph, making the molecule available for further metabolism (see Treherne, 1967). Like the insect’s exoskeleton, the epithelial lining of the gut serves as a boundary between the body’s internal and external environment and is permeable in nature to allow the absorption of nutrients derived from the breakdown of food (see Treherne, 1967).

The gut of insect herbivores is the most generalized of the insect guts and must deal with food that is generally bulky, with a large non-absorbable component. The alimentary canal of the locust consists of three sections: the foregut, midgut, and hindgut (Figure 3). During embryonic development, the foregut and hindgut are derived from ectoderm and are lined with a thin layer of cuticle, whereas the midgut originates from embryonic endoderm and is void of a cuticular lining (Billingsley and Lehane, 1996; Klowden, 2007).
Foregut

In general, the foregut is divided into the pharynx, esophagus, crop, and proventriculus (Figure 3). The crop serves as a temporary storage area for the food bolus, while the proventriculus serves different roles depending on the food source of the insect. For instance, the proventriculus can act as a grinder to mechanically break up food material due to the presence of teeth-like projections composed of chitin, as is the case for Orthopterans (Biagio et al., 2009) or can also act as a sieve to separate solids and liquids, as exemplified by the honeystopper in bees that separates pollen from nectar (Peng and Marston, 2008). The gastric caecae, located at the junction between the foregut and midgut (Figure 3), produce and secrete digestive enzymes to aid in final chemical digestion (Ferreira and Terra, 1982). Thus, the foregut is responsible for the ingestion, storage, mechanical breakdown, and transport of food to the midgut through the cardiac sphincter.

Midgut

The midgut is predominantly responsible for the chemical breakdown of the food into absorbable nutrients (Billingsley and Lehane, 1996). The locust midgut (and gastric caecae) possesses endocrine cells and nerve processes that contain peptides belonging to a variety of peptide families (Žitňan et al., 1993). It has been suggested that endocrine cells of the gut may communicate with the CNS (Wei et al., 2000; Žitňan et al., 1993). In general, there are two types of endocrine cells, open and closed. The open-type is in direct contact with the midgut lumen by a narrow apical extension, while the closed-type do not extend to the luminal surface (Lange and Orchard, 1998; Montuenga, 1989; Endo and Nishiitsutsuji-Uwo, 1981). Thus, the open-type endocrine cells may function within the brain/gut axis; monitoring the nutrient content of the gut
and acting as an interface between the digestive and endocrine systems, while the closed-type may monitor mechanical tension of the gut wall, providing sensory feedback to the CNS (Montuenga et al., 1996; Fujita et al., 1988). This sensory feedback may then initiate the local release of peptides, such as FGLa/ASTs and proctolin, to alter gut processes such as contractions of the musculature, production of digestive juices/enzymes, or even alter the rate the gut epithelium is replaced (Fujisawa et al., 1993; Lange and Orchard, 1998; Žitňan et al., 1993; Fusé et al., 1999). The released peptides could also act on the nerve terminals, altering the physiological properties of neurotransmission at the gut muscles.

**Hindgut**

The hindgut is generally divided into the ileum, colon, and rectum and together with the Malpighian tubules comprises the excretory system of the insect. The contractions of the hindgut are important in the expulsion of undigested food material from the digestive tract (Nagi and Brown, 1969). In addition, the hindgut and associated Malpighian tubules are responsible for regulating hemolymph volume and composition through the reabsorption and secretion of fluid, ions, and metabolites (see Audsley and Weaver, 2009).
Figure 3. Schematic drawing of the locust gut. Drawn by Zach McLaughlin.
ION TRANSPORTERS OF THE GUT

Ussing and Zerahn (1951) were the first to determine the existence and nature of the electrical potential difference between the inside and outside of an epithelial cell by using isolated frog skin (Ussing and Zerahn, 1999). They correlated this electrical gradient to the active movement of sodium ions, identified later as the Na\(^+\)/K\(^+\)-ATPase (Skou, 1965). Since that time it has been determined that plant and animal cells utilize similar strategies/mechanisms to maintain ionic homeostasis.

The cellular mechanisms of acid-base transport by the locust hindgut are known and reviewed by Phillips et al (1994), and interestingly many of these mechanisms are similar to those in the vertebrate kidney. Specific transport proteins that translocate cations and anions are found on the apical and basolateral cell membranes of the locust gut epithelium (Figure 4). For instance, the basolateral Na\(^+\)/K\(^+\)-ATPase provides the energy to maintain the potential difference across cell membranes by maintaining a high internal [K\(^+\)] and low internal [Na\(^+\)] (Skou, 1965). Some of the transporters involved in proton transport in the locust hindgut include an apical H\(^+\)-V-ATPase and an apical cation/H\(^+\)-exchanger (Figure 4; see Phillips et al., 1994; see Harrison, 2001; see Schooley et al., 2012). For sodium to be absorbed from the gut lumen into the hemolymph, the locust must actively transport Na\(^+\) against an electrochemical gradient to maintain high hemolymph concentrations (Dow, 1981; Chamberlin, 1990). The energy for this active transport is provided by the H\(^+\)-V-ATPase pumps in the apical membrane of the anterior gastric caeca, and by major Na\(^+\)/K\(^+\) exchangers in the basolateral membrane of the rectal cells (Figure 4; Chapman, 1998). Chloride ions are actively removed from the rectal gut lumen by pumps in the apical membrane and pass passively into the hemolymph (Phillips et al., 1988). The apical electrogenic Cl\(^-\) pump is the dominant transepithelial transport mechanism in the
ileum and rectum and creates a negative potential difference across the membrane, enabling Cl\textsuperscript{−} to exit the gut epithelial cells passively through a basolateral anion channel (Figure 4; Hanrahan and Phillips, 1983).

Large, electrogenic net potassium fluxes from hemolymph to gut lumen have been demonstrated in the tobacco hornworm midgut (Harvey and Nedergaard, 1964; Chamberlin, 1990), showing that the midgut contributes to K\textsuperscript{+} excretion in this insect. At another extreme, blood-sucking insects such as *Rhodnius prolixus* have a high-Na\textsuperscript{+} hemolymph and must get rid of excess sodium, chloride and water (Dow, 1986; O’Donnell and Maddrell, 1984; see Maddrell and O’Donnell, 1992).
**Figure 4.** Major ion transporters of locust rectal cells. Adapted from Schooley et al., (2012)
NEURAL CONTROL OF FEEDING AND DIGESTION

For efficient digestion and absorption to occur, peristalsis moves the food bolus through the alimentary canal (Davey, 1964; Miller, 1975). These contractions are myogenic in nature, generated within the muscle rather than as a result of neural stimulation, but this myogenicity can be modulated by nervous input (Donini et al., 2002; Huddart and Oldfield, 1982). The foregut of the locust receives innervation from the STNS by way of the branching of the gastric nerves from the paired ventricular ganglia, forming a nerve plexus over the surface of the foregut and anterior midgut (see Hartenstein, 1997; Albrecht, 1953). The posterior gut, which includes the hindgut and posterior midgut, receives innervation from the CNS. The terminal abdominal ganglion (8th) supplies innervation to the rectum and associated muscles through branches of the 11th sternal nerve (Donini et al., 2002). Anterograde neurobiotin fills of the 11th sternal nerve reveal an extensive network of processes and varicosities on the locust hindgut (Donini et al., 2002).

A central pattern generator (CPG) is a neuronal circuit that produces rhythmic motor patterns that govern rhythmic behaviours such as locomotion and respiration (Bässler, 1986). Central pattern generators tend to be regulated by descending inhibition from the CNS, and when this CNS inhibition is released (through transection in vitro or a modulatory signal), bursts of rhythmic motor output are generated that coordinate with muscle activity, as is seen with the digging and egg-laying CPGs of the locust (Thompson, 1986; Facciponte and Lange, 1992; da Silva and Lange, 2011) and the locust ventilation CPG within the metathoracic ganglion (Bustami and Hustert, 2000).
Recently, CPGs located within the ganglia of the STNS have been implicated in the neural control of gut motility (see Ayali and Lange, 2010). A number of studies have demonstrated the involvement of a frontal ganglion CPG in foregut motor activity, which generates peristaltic waves of contractions of the foregut (Schoofs and Spieß, 2007; Ayali et al., 2002; Zilberstein and Ayali, 2002). In adult locusts, the frontal ganglion controls contractions of the foregut, particularly the pharyngeal region (Ayali et al., 2002; see Ayali, 2004; see Ayali and Zilberstein, 2004). In addition, a CPG within the hypocerebral ganglion is postulated to coordinate foregut contractions and food passage to the midgut (Rand and Ayali, 2010). In a variety of insects muscle activity is abolished and food accumulates in the foregut when the ventricular ganglion’s activity is removed from the foregut, either by severing the recurrent nerve or by removing the ganglion altogether (Hill et al., 1966; Bignell, 1974; Clarke and Grenville, 1960; Lange and Chan, 2008), suggesting the presence of a CPG within the ventricular ganglion.

A variety of neuropeptides have been localized in the STNS, suggesting roles for these peptides in the neural control and modulation of gut contraction, including FGLa/ASTs (see Audsley and Weaver, 2009). Neuromodulation of the frontal ganglion CPG is known (Zilberstein and Ayali, 2002; Zilbertstein et al., 2004) and in the desert locust, FGLa/ASTs modulate the foregut rhythm (Zilberstein et al., 2004). This modulation adds another level of flexibility in neural and peripheral communication in addition to the chemical messenger message characteristics.

**Neuropeptides Involved in Physiological Processes Related to Homeostasis**

Neuropeptides are essential messenger molecules that function as key regulators in physiological processes. There are numerous neuropeptides involved in homeostatic processes, which have
been reviewed extensively (see Nässel 2002; see Altstein and Nässel, 2010; see Nässel and Winther, 2010; see Schooley et al., 2012). The presence of neuropeptides within the innervation projecting to target tissues suggests roles for these peptides in the neural control and modulation of function. Using immunohistochemistry and neurobiotin fills, a variety of peptides have been detected within the ganglia and nerves of the STNS and CNS that innervate visceral tissues such as the gut and oviducts, while in vitro bioassays have been used to demonstrate a physiological role for these peptides in the functioning of target tissues.

**Energy mobilization**

Most insects need carbohydrates as an energy source. Treherne (1958) concluded that glucose absorption in *Schistocerca gregaria* is a passive process that is facilitated by the conversion of glucose into trehalose in the fat body, which is a metabolic tissue that stores lipids, proteins, and glycogen and is involved in glycogenesis and lipid metabolism to regulate hemolymph lipid (and also trehalose) levels (see Canavoso et al., 2001). The first evidence that energy metabolism in the form of lipid breakdown occurs in locusts was provided by Mayer and Candy (1969) and Beenakkers (1969). During short flights, trehalose is utilized as the main energy source, but a switch in energy source concurrent with a rise in hemolymph adipokinetic hormone (AKH) titre occurs at 30 minutes of flight, where lipids become the primary source of energy for the flight muscles (Orchard and Lange, 1983). There are three known isoforms of AKH in *L. migratoria* (Stone et al., 1976; Siegert et al., 1985; Oudejans et al., 1991). The intrinsic NSCs of the CC glandular lobe synthesize, store, and release AKHs into the hemolymph to exert their effects on the fat body (Schooneveld et al., 1983; see Goldsworthy and Wheeler, 1984). The AKHs are present in a number of insect species and have adipokinetic activity, similar in function to mammalian glucagon, regulating the mobilization of lipids and trehalose from the fat body into
the hemolymph to provide usable energy (see Gäde et al., 1997; see Nässel, 2002; Lee and Park, 2004).

There are a number of neuropeptides that modulate AKH release in insects (see Vullings et al., 1999). Two locustatachykinins (Lom-TK I and II) increase the release of AKH from the locust CC in vitro, and crustacean cardioactive peptide (CCAP) also has the same effect (Flanigan and Gäde, 1999; Nässel et al., 1995, 1999; Veelaert et al., 1997). On the other hand, SchistoFLRFamide, a FMRFamide-like peptide, dose-dependently inhibits the release of AKH (Vullings et al., 1998).

**Growth and metamorphosis**

Juvenile hormones are steroid hormones produced and released by the CA to regulate growth and metamorphosis in insects (see Gäde and Goldsworthy, 2003; see Gilbert et al., 2000; see Hartfelder, 2000). Insect molting and metamorphosis are regulated by the interplay between JH and ecdysteroids, where ecdysone and ecdysterone coordinate the process of molting while the JH titre determines the nature of the molt (see Stay and Tobe, 2007). In locusts, JH hemolymph levels remain high during the larval instars, and it is not until the last larval stage that the rate of JH biosynthesis decreases, resulting in a low hemolymph titre (see Gäde et al., 1997). The high JH titre in immature stages allows proper growth and development, while the low JH levels allow the locust to molt into an adult (see Gäde et al., 1997). In addition to the conserved role of JH in larval insects, JH also affects adult reproduction by modulating processes such as oogenesis and vitellogenesis and have also been implicated in caste polymorphisms (see Hartfelder, 2000). Juvenile hormone biosynthesis is modulated by the allatoregulatory peptides. The allatostatins and allatotropins are known to regulate JH biosynthesis and are detected in
NSCs within the brain that project to the corpora allata (Tobe and Stay, 1985; Maestro et al., 1998). The allatotropins stimulate JH biosynthesis, while the allatostatins inhibit the production and release of JH (see Gäde and Goldsworthy, 2003; Vullings et al., 1999).

The allatostatins are a group of three structurally diverse families of neuropeptides (see Stay and Tobe, 2007) and include the FGLa/ASTs (formerly termed the A-type ASTs; cockroach ASTs; see Coast and Schooley, 2011). The FGLa/ASTs share the common Y/FXFGLamide C-terminus, and were originally sequenced from Diploptera punctata (Woodhead et al., 1989; Pratt et al., 1989). Sequences for eight FGLa/ASTs have been determined in the desert locust, S. gregaria (Veelaert et al., 1996) and are identical to those sequences obtained in L. migratoria (Clynen and Schoofs, 2009; Chapter 4 appendix).

Immunohistochemistry has been employed to determine the distribution of FGLa/ASTs within several insect species. FGLa/AST-like immunoreactivity is associated with the brain and retrocerebral complex (the CC and CA) of the cockroach, cricket, and termite (Maestro et al., 1998; Stay et al., 1992; Neuhäuser et al., 1994; Yagi et al., 2005). In the noctuid moth, Helicoverpa armigera, allatostatin-like and allatotropin-like immunoreactive cell bodies within the frontal ganglion send axons within the recurrent nerve that eventually branch over the crop (Duve et al., 1999). Immunohistochemistry has revealed the distribution of FGLa/AST-like peptides in two locust species, L. migratoria and S. gregaria, associated with the CNS, STNS and peripheral targets such as the gut, CC and CA in the form of processes, varicosities, and cell bodies (chapters 2 and 3; Veelaert et al., 1995; Skiebe et al., 2006; Clark et al., 2008; Robertson and Lange, 2010).
Gut motility and digestion

Insect visceral muscle, including the gut, is modulated by a number of neuropeptides that have similar or opposing effects to allow for fine control over the movement of each region. For instance, proctolin and FGLa/ASTs have opposing effects, where proctolin stimulates and FGLa/ASTs inhibit gut contraction (chapter 4; Lange et al., 1995; Lange and Orchard, 1998; Robertson et al., 2012). So by having opposing effects these neuropeptides can regulate gut motility and increase the efficiency with which food travels through the gut, possibly improving the efficiency of nutrient absorption.

Proctolin’s stimulatory action on visceral muscle is well established in several insect species (see Adams and O’Shea, 1983; Lange et al., 1988; Brown and Starratt, 1975; Lange et al., 1993; Gray et al., 2000). In the locust, proctolin increases contraction of each region of the gut (Lange and Orchard, 1998; Gray et al., 2000; Robertson et al., 2012). On the basis of proctolin’s stimulatory action on hindgut muscle, it was proposed that proctolin acts as a neurotransmitter in the gut of *Periplaneta americana* as well as the locust, *S. gregaria* (Brown, 1975; Brown, 1967; Banner et al., 1987), and is a co-transmitter with a more conventional transmitter such as glutamate (Lange and Orchard, 1986; Orchard and Lange, 1987; see Lange, 2002). Proctolin increases circular muscle contraction of the midgut of *L. migratoria* (Lange and Orchard, 1998) and in the desert locust, *S. gregaria*, proctolin increases contraction of the hindgut and the foregut (Gray et al., 2000; Banner et al., 1987).

The FGLa/ASTs inhibit gut muscle contraction in several insect species, including moths, cockroaches, and locusts (chapter 4; Duve et al., 1999; Lange et al., 1995; Robertson et al., 2012). Proctolin-induced contractions of the cockroach hindgut are differentially inhibited by thirteen FGLa/ASTs (Lange et al., 1995) and in the moth, *H. armigera*, peristaltic contractions of
the foregut are inhibited by FGLa/ASTs (Duve et al., 1999). In *H. armigera*, allatotropin antagonizes the effects of FGLa/ASTs on foregut contraction and increases the frequency and amplitude of contractions (Duve et al., 1999).

Efficient digestion and absorption of nutrients relies on the appropriate enzyme being secreted at the appropriate time during the digestive process (Terra et al., 1996). Midgut endocrine cells produce secretions that serve many physiological functions. During feeding, endocrine cells can release contents in response to the onset of feeding or may begin resynthesis or uptake of a peptide after the commencement of feeding, altering the level of immunoreactivity in the endocrine cell. Several neuropeptides are associated with midgut endocrine cells, such as tachykinins, RFamides, and FGLa/ASTs (chapter 3; Zudaire et al., 1998; Žiňan, 1993; Lange and Orchard, 1998; Lange, 2001; Hill and Orchard, 2004). Immunoreactivity of midgut endocrine cells changes in response to feeding (Zudaire et al., 1998; Brown et al., 1986; Jenkins et al., 1989). Neuropeptides have also been implicated in altering digestive enzyme activity in the midgut of the locust. The length of time a locust midgut is exposed to a myosuppressin *in vitro* affects the distribution of two carbohydrases, α-glucosidase and amylase (Hill and Orchard, 2005) and in *D. punctata* leucomyosupressin increases midgut lumen enzyme activity (Fusé et al., 1999).

**Water and ion balance**

Insects employ a variety of strategies to maintain ion and fluid homeostasis to overcome varying environmental stresses and allow them to succeed in a wide array of ecological niches. Insects that live in arid terrestrial environments are challenged with limited intake of water and salts and thus must employ primarily anti-diuretic strategies to prevent salt and water loss. On the other
hand, aquatic and fluid-feeding insects are faced with excess water and ions, and therefore must eliminate excess water and salts by utilizing primarily diuretic strategies.

The regulation of hemolymph composition and volume is dependent on input from the CNS, and this regulation is also subject to modulation by neuropeptides. Experiments utilizing the Ussing chamber or more advanced techniques such as the scanning ion-selective electrode technique (SIET) have determined the effect of various neuropeptides on ion and water transport. An array of factors regulates the excretory tissues, which include the Malpighian tubules, hindgut, and midgut (Coast et al., 2002; Coast, 2009; Farmer et al., 1981; Orchard, 2009; Te Brugge et al., 2002, 2009). Diuretic factors, which stimulate water and salt excretion, include both biogenic amines and peptides. The biogenic amines tyramine and serotonin are especially involved in rapid diuresis that follows ingestion of a bloodmeal in *R. prolixus* (Blumenthal, 2003, 2005; Orchard, 2006, 2009). Several peptide families have also been implicated to have diuretic activity. Such peptide families include the corticotropin-releasing factor (CRF)-related peptides (Baldwin et al., 2001; Kataoka et al., 1989; Patel et al., 1995), kinins (Coast et al., 1990; Holman et al., 1999; Veenstra et al., 1997), calcitonin-like peptides (Coast et al., 2005; Furuya et al., 2000), and the CAPA family of peptides (Davies et al., 1995; Pollock et al., 2004).

Unfortunately, many of these diuretic hormones can not be classified as true diuretic hormones since they have not been shown to be hemolymph-borne at appropriate times. There are only two true diuretic hormones to date, which include *Locusta* DH in locust (Patel et al., 1995) and serotonin in *R. prolixus* (Lange et al., 1989).

As compared to diuretic factors there are far fewer anti-diuretics identified. Anti-diuresis is important since this is the normal physiological state of many terrestrial insects, where insects often only enter a diuretic state following increased water intake due to diet or metabolism
(Coast et al., 2002). In particular, there are three peptides that contribute to anti-diuresis in locusts: chloride transport stimulating hormone (CTSH), ion transport peptide (ITP), and neuroparsins (Fournier and Girardie, 1988; Phillips et al., 1980, 1996; Spring and Phillips, 1980). CTSH leads to reabsorption of fluid and ions by the rectum by acting on ion transport mechanisms via cAMP (Spring and Phillips, 1980; Chamberlin and Phillips, 1988). Ion transport peptide was identified and purified from locust CC (Audsley et al., 1992a; Phillips et al., 1996), and the full-length peptide (72 amino acid residues) was determined following cDNA cloning (Meredith et al., 1996). Ion transport peptide dose-dependently regulates absorption by stimulating ion transport at the locust hindgut (Audsley et al., 1992a, 1992b), by utilizing the second messenger cAMP to stimulate apical cation conductance and an apical electrogenic Cl\(^{-}\) pump and concurrently inhibits apical acid secretion through a second messenger that remains to be elucidated (see Phillips et al., 1998; see Schooley et al., 2012). Data on the role that the neuroparsins play at the locust hindgut is conflicting. The neuroparsins reportedly stimulate fluid reabsorption by *Locusta migratoria* rectal sacs (Fournier and Girardie, 1988), while have no effect on fluid transport or short-circuit current across *Schistocerca gregaria* ileum or rectum (Jeffs and Phillips, 1996).

There are several other accessory peptides that have been implicated in the regulation of salt and water balance. For instance, FGLa/ASTs, neuropeptide F, and proctolin reduce the transepithelial voltage across the anterior midgut of *Aedes aegypti* larvae, indicating modulation of ion transport (Onken et al., 2004). Allatotropin and FLRFamides also inhibit ion transport in *Manduca* midgut, as seen by an inhibition of the short-circuit current (Lee et al., 1998). Specifically in locusts, cAMP elicits an increase in short-circuit current while CC homogenate causes an
increase in short-circuit current and potential difference and a decrease in transepithelial resistance (see Coast et al., 1999; Jeffs and Phillips, 1996).

**Thesis Focus and Objectives**

Advances in peptide isolation and analysis technologies have allowed an explosion in the number of insect neuropeptides that have been isolated and identified. The majority of insect neuropeptides have been identified (and thus named) based on the function for which they were discovered. For instance, proctolin was aptly named due to its excitatory effect on the proctodeum of the cockroach, *P. americana*. With greater than 200 insect neuropeptides currently identified, it has become increasingly clear that insect peptides are frequently pleiotropic, with the role for which they were identified often not their primary role. A great deal of research has been conducted on the effect of FGLa/ASTs on JH release from the CA, the function for which they were identified and isolated, but investigations into additional roles that FGLa/ASTs have on peripheral targets is minimal. Understanding the pleiotropic nature of these peptides is important since FGLa/ASTs act only as true allatostatins in a few insect species. Their real physiological roles cannot be completely understood until all of their effects are identified and placed within a physiological context. Thus, it seems increasingly likely that this allatostatic activity is not the primary function of these peptides. This thesis aims to emphasize the pleiotropic nature of the FGLa/ASTs by illustrating new roles these peptides play peripherally, in the supply of metabolic energy and the control of electrolyte and fluid balance in *Locusta migratoria*. The central hypothesis tested in this thesis is that *FGLa/ASTs are present in L. migratoria and are involved in the physiological functioning of peripheral tissues involved in homeostasis.*
Chapter II is aimed at determining the role that FGLa/ASTs have in the supply of metabolic energy through their effects on the release of AKH and their role in growth and metamorphosis through their effects on JH biosynthesis. Thus, the distribution of FGLa/AST-like immunoreactivity within the CC and CA and the role of FGLa/ASTs in hormone release (AKH from the CC and JH from the CA) in *Locusta* were determined. FGLa/ASTs are extensively distributed in the CC and CA, and members of this family dose-dependently increase the release of AKH from the glandular CC. FGLa/ASTs also alter the release of JH from the CA, which is dependent on peptide concentration and basal rates of JH release.

Chapter III is aimed at determining whether the FGLa/ASTs function in aspects of gut physiology since they have been implicated as brain/gut peptides previously in other insect species. A determination of the innervation of the locust gut, distribution of FGLa/ASTs within the CNS, STNS, and gut of the locust is presented. Results demonstrate that FGLa/AST-like immunoreactivity is associated with cell bodies and processes in all ganglia of the CNS and STNS, in processes on the locust gut, and in midgut endocrine cells. FGLa/AST-like immunoreactivity is contained within some of the identified neurons that innervate the gut, suggesting that these neurons are the source of FGLa/AST-like immunoreactivity associated with the gut, which may be released onto the gut leading to modulation of different physiological functions (such as muscle contraction and nutrient absorption).

In Chapter IV, an examination of the physiological role of FGLa/ASTs in digestion is presented, by assessing the effect of FGLa/ASTs on regional gut muscle contraction. Results are presented that show that FGLa/ASTs have inhibitory effects on gut muscle, where FGLa/ASTs dose-dependently inhibit induced, spontaneous and neurogenic gut contractions and modulates neural output governing foregut contraction. These myoinhibitory peptides oppose other excitatory
peptides, such as proctolin, to increase the efficiency of peristalsis and the movement of the food bolus through the gut, which will increase the efficiency of chemical digestion and the absorption of nutrients and ions.

Chapter V is aimed at determining the regional differences in $K^+$ flux across the locust gut and the role that FGLa/ASTs play in $K^+$ transport since this ion is in high concentration in the herbaceous locust diet. Results presented indicate that the ileum (anterior hindgut) has the highest rate of $K^+$ efflux \textit{in vitro} and FGLa/AST dose-dependently inhibits this efflux of $K^+$. These results suggest that FGLa/ASTs act as diuretics to control fluid and electrolyte balance when the locust is in a fed state.

The general discussion, included in Chapter VI, discusses the results of the research chapters of this thesis and emphasizes the importance of the FGLa/ASTs as pleiotropic peptides that play a part in numerous processes involved in regulating homeostasis. The roles of FGLa/ASTs in the supply of metabolic energy and in fluid and ion balance are highlighted and an emphasis is placed on the suggestion that the primary, or ancestral, role of the FGLa/ASTs may be as brain/gut peptides and contribute to the integration of information from the CNS and ES to ensure proper communication needed to attain a relatively constant internal environment.


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CHAPTER 2:
THE ROLES OF DIPPU-ALLATOSTATIN IN THE MODULATION OF HORMONE RELEASE IN Locusta migratoria
ABSTRACT

Dippu-allatostatins (ASTs) have pleiotropic effects in *Locusta migratoria*. Dippu-ASTs act as releasing factors for adipokinetic hormone I (AKH I) from the corpus cardiacum (CC) and also alter juvenile hormone (JH) biosynthesis and release from the corpus allatum (CA). Dippu-AST-like immunoreactivity is found within lateral neurosecretory cells (LNCs) of the brain and axons within the paired nervi corporis cardiaci II (NCC II) to the CC and the CA, where there are extensive processes and nerve endings over both of these neuroendocrine organs. There was co-localization of Dippu-AST-like and proctolin-like immunoreactivity within these regions. Dippu-ASTs increase the release of AKH I in a dose-dependent manner, with thresholds below $10^{-11}$ M (Dippu-AST 7) and between $10^{-13}$ and $10^{-12}$ M (Dippu-AST 2). Both proctolin and Dippu-AST 2 caused an increase in the cAMP content of the glandular lobe of the CC. Dippu-AST 2 also altered the release of JH from the locust CA, but this effect depended on the concentration of peptide and the basal release rates of the CA. These physiological effects for Dippu-ASTs in *Locusta* have not been shown previously.
Physiological processes such as metabolism and growth are crucial to an insect’s survival, and are regulated by peptide, lipid, and steroid hormones (Gäde et al., 1997). This regulation is brought about through the coordination of the central nervous system (CNS) and the endocrine system. Neurosecretory cells within the CNS make and release neuropeptides and the axons of these cells can terminate within two important neuroendocrine organs, the corpus cardiacum (CC) and the corpus allatum (CA). Three sets of nerves, the nervi corporis cardiaci I, II, and III (NCC I, II, III) connect the CC to the brain and the paired nervus corporis allati (NCA) connects the CC to the CA (Albrecht, 1953; Konings, 1989). Neurosecretory cells within the brain project axons within the NCC I and II to the storage lobe of the CC, where the neuropeptides are stored and then released into the hemolymph (Rademakers, 1977). Neurosecretory cells within the protocerebrum send axons within the NCC III (Konings, 1989) to the glandular lobe where intrinsic cells are responsible for producing adipokinetic hormone (AKH) (Rademakers, 1977). In addition to AKH, other neuropeptides such as ion transport peptide (ITP), diuretic hormones, and crustacean cardioactive peptide (CCAP) have been found to be associated with the CC (Audsley and Weaver, 2003; Audsley et al., 1992; Schoofs et al., 1997; Baggerman et al., 2003). The paired CA are neuroendocrine organs associated with the CC, which produce and release juvenile hormone (JH) into the hemolymph.

The AKHs are members of a structurally diverse and functionally related family of peptides along with the red pigment-concentrating hormones of crustaceans (Gäde, 1990; Gäde et al., 1997). The AKHs are widespread among arthropods, with three known AKH isoforms (AKH I, II, III) occurring within the African migratory locust (Gäde, 1990; Oudejans et al., 1991). These hormones are synthesized and released by intrinsic neurosecretory cells of the glandular lobe of
the CC into the hemolymph. The AKHs have many roles but one main function is to mobilize lipid from the fat body to be used as an energy source for long distance flights (Beenakkers 1969; Mayer and Candy, 1969; Orchard and Lange, 1983). Studies have shown that the second messenger cyclic adenosine monophosphate (cAMP) is involved in the release of AKH from the glandular lobe of the CC (Pannabecker and Orchard, 1986, 1987). Flight is the only known natural stimulus for AKH release, but there is evidence that peptides can act as releasing factors and modulate AKH release. Nässel et al. (1995, 1999) demonstrated that locustatachykinin (LomTK) I and II increase the release of AKH from the CC by rapidly increasing the cAMP content of the glandular lobe of the CC. CCAP has also been shown to increase the release of AKH from the CC \textit{in vitro} in \textit{Locusta migratoria} in a dose-dependent manner (Flanigan and Gäde, 1999; Veelaert et al., 1997). Proctolin was also added to the list of releasing factors for AKH when it was found that proctolin causes a dose-dependent increase in the release of AKH I from the locust CC (Clark et al., 2006b).

The JHs are sesquiterpenoid hormones involved in the regulation of development and reproduction in insects (Gäde and Goldsworthy, 2003). There are three principal juvenile hormones (JH I, II, III) found in insects, with JH III as the most widespread among insects, including \textit{L. migratoria} (Hartfelder, 2000; Stay and Tobe, 2007). JH is not stored, thus intrinsic cells of the CA produce and immediately release JH into the hemolymph (Tobe and Stay, 1985). Modulation of the synthesis and release of JH from the CA occurs by two general groups of neuropeptides; the allatotropins stimulate the production and release of JH while the allatostatins (ASTs) inhibit JH biosynthesis (Gäde and Goldsworthy, 2003; Vullings et al., 1999). Through immunohistochemistry, these neuropeptides were found within neurosecretory cells of the pars intercerebralis and pars lateralis of the brain projecting to the CA (Tobe and Stay, 1985).
Proctolin has also been shown to act as an allatotropin in the locust CA, causing a significant increase in the biosynthesis and release of JH (Clark et al., 2006b).

In insects, there are three families of structurally unrelated ASTs that have been isolated and characterized (Stay and Tobe, 2007). Of interest are the 13 Dippu-ASTs that were originally identified from *Diploptera punctata*, and have the characteristic C-terminal sequence Y/FXFGL-amide (Woodhead et al., 1989; Pratt et al., 1989, 1991). The second family of ASTs was isolated from the cricket *Gryllus bimaculatus* and have a C-terminal sequence W(X₆)W-amide, whereas the third family consists of a small family of non-amidated AST originally isolated from *Manduca sexta* and has the C-terminal sequence PISCF (Kramer et al., 1991; Lorenz et al., 1995). Since their discovery, the Dippu-ASTs have been found to occur in many other insect orders in addition to the Dictyoptera (Stay and Tobe, 2007). Using immunohistochemistry, Dippu-AST-like immunoreactivity was found to be associated with the brain and the retrocerebral complex, especially the CA, of the cockroach, cricket and the termite (Maestro et al., 1998; Stay et al., 1992; Neuhäuser et al., 1994; Yagi et al., 2005). In *L. migratoria*, the occurrence of Dippu-AST-like immunoreactivity was associated with the CNS, CC, CA, and associated nerves (Veelaert et al., 1995). Several Dippu-AST-like immunoreactive cell bodies within the brain stained positively, and in particular three groups of cells send Dippu-AST-like immunoreactive axons within the NCC II and NCA to extend to the storage lobe of the CC and the CA respectively (Veelaert et al., 1995). Despite the abundant Dippu-AST-like staining associated with the CA, Dippu-AST 5 did not have an effect on JH biosynthesis (Veelaert et al., 1995). The distribution of AST I (Dippu-AST 7)-like immunoreactivity in the brain of *Schistocerca gregaria* was studied in detail and the pattern of distribution was similar to that in *L. migratoria* (Vitzthum et al., 1996). In both studies, numerous cell bodies stained positively
for Dippu-AST-like immunoreactivity within several areas of the brain. Lateral neurosecretory cells (LNCs) sent Dippu-AST-like immunoreactive processes within the NCC II to the CC where the processes arborized, and some continued within the NCA to the CA.

The Dippu-ASTs have been shown to act as true allatostatins only in cockroaches, crickets, and termites (Stay, 2000; Stay and Tobe, 2007; Yagi et al., 2005). In addition to their well-known role as suppressors of JH synthesis, the Dippu-ASTs also act as myoinhibitors of visceral musculature. Lange et al. (1995) found that the 13 Dippu-ASTs inhibited both myogenic and proctolin-induced contractions of the hindgut of D. punctata with varying degrees of potency. This study also showed that selected Dippu-ASTs did not have an effect on contraction of D. punctata or L. migratoria oviduct muscle. In addition to Orthopterans, Dippu-ASTs have been shown to have alternative functions in other insects. Dippu-AST 7 was found to inhibit both spontaneous and leucokinin 1-induced contractions of the hindgut of Rhodnius prolixus (Sarkar et al., 2003).

The present study investigates the distribution of Dippu-AST-like immunoreactivity associated with the locust brain and retrocerebral complex. Since the pattern of immunoreactivity for Dippu-AST was similar to that seen for proctolin-like immunoreactivity (Clark et al., 2006a), co-localization of proctolin-like and Dippu-AST-like immunoreactivity was examined. To gain further insight into the functioning of the Dippu-ASTs in L. migratoria, we also determined whether Dippu-AST acts to modulate the release of AKH and JH from the CC and CA, respectively. It was also important to investigate the action of proctolin and Dippu-AST on the production of cAMP in the locust CC since it has been shown previously that the release of AKH occurs through this second messenger (Pannabecker and Orchard, 1986, 1987).
MATERIALS AND METHODS

Animals

Adult locusts were obtained from a colony of L. migratoria housed at the University of Toronto Mississauga, Canada. The locusts were raised in crowded conditions on a 12h light: 12h dark cycle at a temperature of 30 ºC. The colony was fed fresh wheat seedlings and bran.

Chemicals

Synthetic proctolin was purchased from Bachem (Torrance, CA, USA) and was reconstituted in double distilled water to yield 10 µl aliquots of $10^{-3}$ M, which were frozen at -20 ºC. Working dilutions of proctolin were made in physiological saline from the frozen aliquots. The Dippu-ASTs used in the AKH release bioassay were custom synthesized by the Insect Biotech Canada Core Facility (Queen’s University), Kingston, Ont., Canada, or by Research Genetics, Huntsville, AL., USA. Each Dippu-AST was reconstituted in double distilled water to yield a stock solution of $10^{-3}$ M, which was then divided into 10 µl aliquots and stored at -20 ºC and working dilutions were made using saline. Synthetic AKH I was purchased from Pennsula Laboratories (Belmont, CA, USA) and was reconstituted in 1 M glacial acetic acid to yield a stock solution containing 1 µg AKH I per 10 µl. This stock was then divided into 10 µl aliquots and stored at -20 ºC to be used as the standard for the AKH release bioassays. Sigmacote was purchased from Sigma (Oakville, Canada) and was used in the AKH release bioassay to prevent peptide adherence to the wells of the 96-well culture plates and the Eppendorf tips used for collection of the incubation media.
**Immunohistochemistry**

The brain and retrocerebral complex of 3-4 week old adult male and female locusts were dissected in locust saline (150 mM NaCl, 10 nm KCl, 4 mM CaCl₂, 2 mM MgCl₂, 4 mM NaHCO₃, 5 mM HEPES (pH 7.2), 90 mM sucrose, 5 mM trehalose) and then fixed for 1 h at room temperature in 2% paraformaldehyde in Millonig’s buffer (pH 7.0, 0.14 M NaH₂PO₄·H₂O, 0.1 M NaOH, 0.3 mM CaCl₂·2H₂O). After fixation, tissues were washed in phosphate buffered saline (PBS; 0.9% NaCl, pH 7.2) for 2-5 h at room temperature. Tissues were then incubated in PBS containing 4% Triton-X, 2% bovine serum albumin (BSA), and 10% normal goat serum (NGS) at room temperature for 1 h on a stir plate. Tissues were then washed three to four times with PBS at room temperature and then incubated in a rabbit anti-AST I (Dippu-AST 7) IgG fraction purified polyclonal antibody (a gift from Hans-Jürgen Agricola, Friedrich-Schiller Universität, Jena, Germany) diluted 1:1000 in PBS containing 0.4% Triton-X, 2% BSA and 2% NGS on a shaker for 48-72 h at 4 °C. Tissues were washed in PBS for 2-5 h at room temperature on a shaker and then incubated in goat anti-rabbit IgG antibody conjugated to Alexa Fluor 568 F(ab’)₂ fragment (Molecular Probes, Eugene, OR, USA) at a dilution of 1:200 (containing 10% NGS) overnight at 4 °C on a shaker, covered with foil. Tissues were then washed in PBS for 4-18 h and then either run through a forward glycerol series (20, 30, 60, 80%) for 15 minutes each and then mounted in 100% glycerol for single labeling or incubated in rabbit anti-proctolin IgG fraction purified polyclonal antibody (a gift from Hans-Jürgen Agricola, Friedrich-Schiller Universität, Jena, Germany) for double-labeling. The anti-proctolin antibody was used at a dilution of 1:1000 in PBS containing 0.4% Triton-X, 2% BSA and 2% NGS. Tissues were incubated for 48 h at 4 °C and then washed three to four times in PBS. The tissues were then incubated in goat anti-rabbit IgG biotin conjugated to streptavidin Alexa Fluor 488 (Molecular
Probes, Eugene, OR, USA) at a dilution of 1:200 (containing 10% NGS) overnight at 4 °C on a shaker. Tissues were then washed in PBS at room temperature for 4-18 h and then run through a forward glycerol series and mounted in 100% glycerol and left overnight at 4 °C before viewing. A total of 54 preparations, a combination of male and female single and double-labels, were viewed.

Preincubation experiments were performed for both proctolin and Dippu-AST. The proctolin and Dippu-AST antisera were preincubated for 24 h with a final concentration of $10^{-5}$ M proctolin or $10^{-5}$ M Dippu-AST 7, respectively. Negative control experiments were also performed, in which no antibody was present in the solution. In total, 14 control experiments were performed (both a combination of male and female single and double-labeled preparations). Pre-incubation of the Dippu-AST 7 and proctolin antibodies and the negative control experiments abolished all staining in the brain and retrocerebral complex.

All preparations were viewed using a Nikon Optiphot-2 Epifluourescence Microscope (Nikon Corporation, Tokyo, Japan). A Zeiss LSM 510 Confocal Laser Microscope (Carl Zeiss, Jena, Germany) was used to acquire confocal images.

**AKH release bioassay**

The glandular lobe of the CC of five 3-4 week old adult female locusts, were dissected and pooled in a culture plate well containing 50 µl of modified locust saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM Hepes, pH 7.2). The five glandular lobes were then rinsed three times with 50 µl of modified locust saline and incubated for 1 h at room temperature on a shaker. This served as the initial incubation. The perfusate was collected in an Eppendorf tube containing 50 µl of 2.5 M acetic acid and the glandular lobes were rinsed three times with
50 µl of modified locust saline. The pooled glandular lobes were then incubated for an additional hour in the presence of 50 µl of peptide. This incubation served as the experimental incubation. For controls, 50 µl of modified locust saline was used instead of peptide in the experimental incubation. There was no significant difference between the initial and experimental saline incubations ($p=0.3$; one-way paired student $t$-test).

Initially, the potency of four Dippu-ASTs (Dippu-AST 2, 7, 12, 13) was assessed to determine if there was a differential effect. Following this experiment, Dippu-AST 7 was used at concentrations of $10^{-11}$ M to $10^{-7}$ M to generate a dose-response curve, and a second dose-response curve was performed using $10^{-13}$ M to $10^{-7}$ M Dippu-AST 2. The AKH I released during the control and experimental incubations was quantified using RP-HPLC with synthetic AKH I as a standard. A Brownlee RP18 Spheri-5 monofunctional C18 column (4.6 mm x 220 mm) (Mandel Scientific, Guelph, Ont., Canada) was attached to a model SP8800 ternary HPLC pump (Spectra-Physics, San Jose, CA.) and to a UV-vis detector (Model SP8450, Spectra-Physics). Absorbance was monitored at 220 nm and 0.05 AUFS. Output was recorded and integrated for peaks using a chromatography integrator (Model SP4470, Spectra-Physics). The peptides were eluted with a solution of 24-36% acetonitrile containing 0.1% TFA (Sigma, Oakville, Ontario, Canada) run at a flow rate of 1 ml min$^{-1}$ for 30 min. Relative release of AKH I was determined by the ratio of AKH I release in the experimental incubation to the release of AKH I in the initial incubation for the same pool of five CCs. A relative release of 1 indicates release equivalent to the initial incubation.
**cAMP radioimmunoassay**

The glandular lobe of the CC of 10-15 day old virgin female locusts was dissected and pooled in a dish containing modified locust saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 10 mM Heps, pH 7.2). Once all dissections were completed, one glandular lobe was placed in an eppendorf tube containing 80 µl of saline (sample tubes) or 90 µl of saline (control tubes). Then 10 µl of 5 x 10⁻⁴ M 3-isobutyl-1-methylxanthine (IBMX) was added to all of the tubes and then 10 µl of either proctolin or Dippu-AST 2 was added to the appropriate eppendorf tubes and incubated for 10 min at room temperature. The assay was stopped using 500 µl boiling 0.05 M sodium acetate buffer (pH 6.2; 0.5 ml of 1N acetic acid, 19.5 ml of 1M sodium acetate, 380 ml of distilled water), followed by boiling for 5 min. After boiling, the tubes were sonicated and centrifuged for 15 min at 10,000 × g. The supernatant was then removed and stored at 4 °C for radioimmunoassay (RIA).

To quantify the amount of cAMP produced, an aliquot of the supernatant was assayed in duplicate for cyclic AMP using a modified commercially available RIA kit (Perkin Elmer, Woodbridge, ON, Canada). The supernatant was then aspirated and radioactivity was counted using a Gamma 4000 Counter (Beckmann, Irvine, CA, USA).

**JH assay for locust CA**

The protocol for the JH radiochemical assay was followed as previously reported (Clark et al., 2006b). The CA of 7-10 day old adult virgin females were dissected and preincubated in radioactive control medium for 1 h. The medium was removed and discarded and replaced with 50 µl fresh radioactive control medium. The CA were then incubated for 3 h on a shaker, at which time the medium was removed and placed in culture tubes for later extraction. This was
followed by an additional 3 h incubation in 50 µl of labeled medium. This medium was then removed and placed in culture tubes for later extraction with isooctane.

**RESULTS**

**Immunohistochemistry**

*Single-Label for Dippu-allatostatin-like immunoreactivity*

The locust brain contains Dippu-AST-like immunoreactivity within cell bodies, processes and extensive neuropile areas (Fig. 1). Most cells within the brain occurred in bilateral pairs or groupings. Male and female preparations were analyzed and there was no difference in the pattern of Dippu-AST-like immunoreactivity between the two sexes. Extensive neuropile areas were found within the protocerebrum and the tritocerebrum. Within the protocerebrum, 80-100 small (approximately 24-32 µm in diameter) cells stained faintly for Dippu-AST-like immunoreactivity (filled arrowheads; Fig. 1A). Larger (approximately 32-40 µm diameter) Dippu-AST-like immunoreactive cell bodies were found in the midline of the protocerebrum (open arrowheads; Fig. 1A).

One group of cells that were of particular interest included the LNCs that are located within the protocerebrum (Figs. 1A and 2A). These cells (8-12 per bilateral group – approximately 24-32 µm in diameter) send axons within NCC II to the CC where the axons from these cells branched extensively over the storage lobe (Figs. 1B and 2B). These processes had varicosities and blebs, indicative of release sites for neurohormones. The staining was restricted to the storage lobe and did not enter the glandular lobe of the CC, suggesting that Dippu-AST may act as a neurohormone or as a neuromodulator or releasing factor for neurohormones released from the CC. Four to five Dippu-AST-like immunoreactive processes continued within the NCA to the
CA, where these processes travel deep within the CA and branch extensively (Figs. 1C and 2C). These processes had varicose or bleb-like endings, again suggestive of neurohormonal release sites and a modulatory role for Dippu-AST at the CA. Dippu-AST-like processes within the CA continued within the NCA II (Figs. 1C and 2).

**Double-labeling of allatostatin-like and proctolin-like immunoreactivity**

Following the single-labeled immunohistochemical analysis of the distribution of Dippu-AST-like immunoreactivity, it was noticed that the pattern and localization of Dippu-AST-like immunoreactivity was very similar to the localization of proctolin-like immunoreactivity within the brain and retrocerebral complex of the locust (Clark et al., 2006a). To determine if there was co-localization of these two peptides, a double-labeling procedure was employed. The results revealed some co-localization of proctolin-like and Dippu-AST-like immunoreactivity within the brain and retrocerebral complex (Fig. 2G-I). Within the LNCs, seven or eight of the cell bodies in each bilateral group stained positively for both peptides (yellow-orange colouration; Fig. 2G). As a consequence of the overlap of some of the cells, not all cells are visualized in the confocal image. The axons projecting from the LNCs within the NCC II toward the CC were also labeled for both Dippu-AST-like and proctolin-like immunoreactivity (Fig. 2G). As shown in Fig. 2G, at least six of these axons revealed co-localization, whereas at least two of the axons labeled for proctolin-like immunoreactivity only and at least one stained only for Dippu-AST-like immunoreactivity.

The immunoreactive processes associated with the storage lobe of the CC also exhibited co-localization of proctolin-like and Dippu-AST-like immunoreactivity (Fig. 2H). The co-localized processes were confined to the central portion of the storage lobe, as indicated by the processes stained in a yellow color in Fig. 2H. The axons around the periphery of the storage lobe were
mostly singly labeled for Dippu-AST-like immunoreactivity. Few proctolin-like immunoreactive processes were observed. Co-localization of the two peptides continued within the axons of the NCA and into the CA (Fig. 2I). The NCA contained four to six Dippu-AST-like immunoreactive axons and four to six proctolin-like immunoreactive axons. Of these axons, at least three were co-localized and at least two of these showed only proctolin-like immunoreactivity. Most of the processes within the CA exhibited co-localization. Co-localization of Dippu-AST-like and proctolin-like immunoreactivity continued within the axons of the NCA II (Fig. 2I).

**AKH release**

As a consequence of the presence of Dippu-AST-like immunoreactivity associated with the CC, AKH release was examined to determine a role for Dippu-ASTs at the CC. Initially, four members of the Dippu-AST family were chosen to ascertain if there was a differential effect based on the unique sequence of each Dippu-AST. Dippu-AST 7 (APSGAQRLYGFL-NH2) is the most well-known and characterized cockroach AST. Dippu-AST 12 (PFNGL-NH2) has the shortest sequence (six amino acid residues), the sequence of Dippu-AST 13 (IPMYDFGI-NH2) ends in an isoleucine rather than a leucine, and Dippu-AST 2 (AYSYVSEYKRLPYNFGLYLGFL-NH2) has the longest amino acid sequence consisting of 18 amino acids. All four of these cockroach ASTs significantly increased the relative release of AKH I from the glandular lobe of the CC (as determined by quantification using RP-HPLC) at a dose of 10^{-8} M (Fig. 3). Dippu-AST 2 was the most effective at stimulating the release of AKH I from the CC (approximately three times the release of AKH I above control levels). Both Dippu-AST 7 and Dippu-AST 13
resulted in a doubling of AKH I release from the CC. The least effective cockroach AST was Dippu-AST 12, which increased release by about 50%.

Dose-response curves for the two most effective Dippu-ASTs in releasing AKH I, Dippu-AST 2 and 7, were generated (Fig. 4). For each Dippu-AST, the release of AKH I was dependent on the concentration of Dippu-AST tested. In both dose-response curves, a relative release of 1 indicates that the experimental release does not differ from that of the initial release of AKH I from the isolated glandular lobe of the CC. Dippu-AST 7 increased the relative release of AKH I from the CC but this trend was not significant (Fig. 4A). A greater than 2.5-fold increase in AKH I release was noted with a dose of $10^{-10}$ M Dippu-AST 7. Threshold for release occurred below $10^{-11}$ M, whereas maximum release of AKH I occurred at a dose greater than $10^{-7}$ M Dippu-AST-7. Dippu-AST 2 treatment also resulted in an increase in the relative release of AKH I in a dose-dependent manner, but with a lower threshold ($10^{-12}$-$10^{-13}$ M Dippu-AST 2) than Dippu-AST 7 (Fig. 4B). Maximum effect was observed at $10^{-11}$ M Dippu-AST 2, with a greater than four-fold increase in the release of AKH I.

**cAMP assay for proctolin and Dippu-allatostatin**

Both proctolin and Dippu-AST 2 increased the cAMP content of the glandular lobe of the CC (Fig. 5). At a dose of $10^{-9}$ M proctolin, the cAMP content of the CC increased significantly (by approximately five-fold) from a control level of $0.07 \pm 0.02$ pmol cAMP per CC to $0.35 \pm 0.11$ pmol per CC (Fig. 5A). Proctolin also significantly increased the cAMP content of the CC at a dose of $10^{-7}$ M but to a lesser extent (approximately a three-fold increase).

Dippu-AST 2 treatment resulted in a significant increase in the cAMP content of the CC at a dose of $10^{-11}$ M (Fig. 5B). At this dose, Dippu-AST 2 caused approximately a two-fold increase
of cAMP in the CC. A dose of 10^{-13} M was also tested but this dose did not significantly increase the content of cAMP. These results are consistent with the dose-response curve for Dippu-AST 2, in which 10^{-11} M showed maximal effect.

**JH assay for Dippu-AST 2**

Dippu-ASTs modulate the release of JH in *L. migratoria* (Fig. 6). Individual CA showed variable basal rates of JH release and therefore the data were grouped into four categories for the Dippu-AST 2 dose-response curves in Fig. 6: low (<10 pmol h^{-1} per CA), medium (10-35 pmol h^{-1} per CA), high (>35 pmol h^{-1} per CA) and all (a combination of all data). This analysis was done to determine if the effect of Dippu-AST 2 on JH release is dependent on the level of activity of the CA. Combination of all data reveals that there is no significance between the control and stimulated CA at any dose tested (Fig. 6A). However, Dippu-AST 2 stimulated the release of JH from low activity CA (thus apparently acting as an allatotropin) (Fig. 6B). Dippu-AST 2 did not have a significant effect on CA of medium activity (Fig. 6C). Interestingly, Dippu-AST 2 showed allatostatic activity in the high basal release group (Fig. 6D). For this group, Dippu-AST 2 significantly decreased the release of JH from the locust CA at all doses tested.

Dippu-AST 7 was also tested for its ability to modulate JH release at two doses, 10^{-6} M and 10^{-7} M (data not shown). As with Dippu-AST 2, Dippu-AST 7 stimulated JH release from low activity CA at both doses tested. Interestingly, Dippu-AST 7 did not significantly affect JH release in either medium- or high-release CA (data not shown).
Figure 1. Dippu-allatostatin-like immunoreactivity associated with the brain and retrocerebral complex of the locust. (A) Immunoreactivity was found within lateral neurosecretory cells (LNC) that send processes within the paired nervi corporis cardiaci II (NCC II). Immunoreactivity was also found within numerous small cells (filled arrowheads) and larger medial cells (open arrowheads). (B) Close-up of the storage lobe of the corpora cardiaca (CC) that receives immunoreactive processes from the NCC II. Note: the numerous bleb-like endings (arrows). (C) Close-up of the corpus allatum (CA). Immunoreactive processes from the CC continue within the paired nervi corporis allata (NCA) and project to, and branch within, the CA. Scale bars = 100 µm.
Figure 2. Co-localization of Dippu-AST-like and proctolin-like immunoreactivity. (A-C) Dippu-AST-like immunoreactivity within the brain and retrocerebral complex. (A) Dippu-AST-like immunoreactive lateral neurosecretory cells (LNC) and axons within the nervus corporis cardiaci II (NCC II). (B) The storage lobe of the corpus cardiacum (CC) showing Dippu-AST-like immunoreactive processes. (C) Dippu-AST-like immunoreactivity within axons of the nervus corporis allata (NCA) that enter and branch within the corpus allatum (CA) and continue within the NCA II. (D-F) Proctolin-like immunoreactivity associated with the locust brain and retrocerebral complex: (D) LNCs and processes within the NCC II displaying proctolin-like immunoreactivity; (E) proctolin-like immunoreactive processes associated with the storage lobe of the CC; (F) Axons within the NCA that enter the CA display proctolin-like immunoreactivity. (G-I) Co-localization of Dippu-AST-like and proctolin-like immunoreactivity: (G) LNCs and axons within the NCC II display co-localization of these two peptides; (H) co-localization of immunoreactivity within the storage lobe of the CC; (I) co-localization of Dippu-AST-like and proctolin-like immunoreactivity within axons of the NCA and processes within the CA. Scale bars = 100 µm.
Figure 3. Release of AKH I from the glandular lobe of the CC in response to Dippu-ASTs at a
dose of $10^{-8}$ M. Dippu-AST significantly increased the relative release of AKH I (Dippu-AST 7,
$p = 0.03$; Dippu-AST 12, $p = 0.004$; Dippu-AST 13, $p = 0.01$; Dippu-AST 2, $p = 0.005$; one-
tailed paired $t$-test). Values are mean ± S.E. of 4-7 preparations. *$p<0.05$, **$p \leq 0.005$. 
Figure 4. Effect of Dippu-ASTs on AKH I release from the glandular lobe of locust CC. (A) AKH I release is increased by incubation of CC glandular lobes with Dippu-AST 7 ($p=0.06$, d.f.=47, F=2.02; one-way ANOVA). (B) Dippu-AST 2-induced release of AKH I in locust CC ($p=0.036$, d.f.=25, F=2.87; one-way ANOVA). Different letters indicate significant difference between $10^{-12}$ and $10^{-11}$ M Dippu-AST 2 ($p<0.05$; Tukey’s multiple comparison test). Relative release of AKH I represents the percentage of AKH I released during the experimental incubation (in the presence of Dippu-AST 7 or 2), compared to the initial incubation. Values are expressed as mean ± S.E. of 4-5 preparations for Dippu-AST 7 and of 3-6 preparations for Dippu-AST 2.
Figure 5. cAMP content of the locust glandular lobe following exposure to proctolin or Dippu-AST 2. (A) Proctolin significantly increased cAMP content at both doses tested ($10^{-9}$ M $p = 0.0231$; $10^{-7}$ M $p = 0.0183$; one-tailed paired $t$-test). (B) Dippu-AST 2 significantly increased cAMP content at $10^{-11}$ M ($p = 0.0465$; one-tailed paired $t$-test) but not at $10^{-13}$ M ($p=0.3$; one-tailed paired $t$-test). Histogram bars represent mean ± S.E. of 10 and 5 determinations for Dippu-AST and proctolin, respectively; *$p<0.05$.
Figure 6. Effect of Dippu-AST 2 on JH biosynthesis. (A) Data from all preparations. There was no significant difference between the control and stimulated CA at any dose. (B) Data from CA that displayed low basal JH release (\(<10\) pmol h\(^{-1}\) per CA). Dippu-AST 2 significantly increased the release of JH at 10\(^{-8}\) M (\(*p<0.0001\) one-way ANOVA, \(p<0.005\) Tukey’s multiple comparison test) and 10\(^{-6}\) M (\(**p<0.0001\) one-way ANOVA, \(p<0.01\) Tukey’s multiple comparison test). (C) Data obtained from CA showing medium rates of basal release of JH (10-35 pmol h\(^{-1}\) per CA). There was no significant effect at any dose (\(p=0.2\); one-way ANOVA). (D) Data obtained from preparations that displayed high basal JH release (\(>35\) pmol h\(^{-1}\) per CA). All doses tested significantly decreased the release of JH from basal release rates (\(p<0.0001\) one-way ANOVA, \(*p<0.05, **p<0.01, ***p<0.001,\) respectively, Tukey’s multiple comparison test). Grey bars show control levels of JH release and black bars indicate Dippu-AST 2-induced JH release. Values are expressed as mean ± S.E. of 5-25 individual CA.
DISCUSSION

The present study shows that Dippu-AST 7 (AST I)-like immunoreactivity is distributed within cell bodies and processes of the brain and in neural processes within the retrocerebral complex of *L. migratoria*. The identity of these endogenous AST-like peptide(s) is currently unknown, but they will probably have sequence similarity to the Dippu-ASTs. AST-like peptides have been isolated and characterized from another locust species, *S. gregaria* (Veelaert et al., 1996). The cDNA for these AST-like peptides has also been sequenced and found to contain peptide sequences for ten schistostatins, each with the characteristic Y/FXFGLeamide of the cockroach ASTs (Vanden Broeck et al., 1996).

Using immunohistochemistry, Dippu-AST-like peptides have previously been reported in the LNCs, CC, and CA of cockroaches, crickets, a single termite species, and locusts (Stay et al., 1992; Neuhäuser et al., 1994; Stay and Tobe, 2007, Yagi et al., 2005; Veelaert et al., 1995; Vitzthum et al., 1998; Maestro et al., 1998). The present study showed Dippu-AST-like immunoreactivity to be associated with LNCs that project axons within the NCC II to the CC, in which the processes arborize extensively. Dippu-AST-like immunoreactive processes then continued within the NCA to the CA, in which processes extensively branch. The pattern and distribution of Dippu-AST-like immunoreactivity was similar to previous reports in two locust species, *L. migratoria* and *S. gregaria* (Veelaert et al., 1995; Vitzthum et al., 1996). Veelaert et al. (1995) described Dippu-AST 2 (AST 5)-like immunoreactivity within three groups of cells in the pars lateralis of the protocerebrum that send axons within the NCC II to the CC. The present study supports and extends these findings, describing additional numerous small cells within the protocerebrum. In *S. gregaria*, Dippu-AST 7-like immunoreactivity was visualized in the brain within LNCs and small cells just dorsal to the protocerebral bridge that give rise to the columnar
fiber system I (ASC1 neurons) (Vitzthum et al., 1996). The ASC1 cells are in a similar location to the numerous small cells found in this study.

The Dippu-AST-like immunoreactivity found within the CC and the CA suggests that Dippu-ASTs may act as neurohormones, neurotransmitters, or neuromodulators within these important neuroendocrine organs. The ASTs are pleiotropic peptides, and most well known for their inhibitory effect on juvenile hormone release from the insect CA (Lorenz et al., 1995; Neuhäuser et al., 1994; Stay and Tobe, 2007; Woodhead et al., 1989; Yagi et al., 2005). In addition, the Dippu-ASTs also act on visceral cardiac muscle inhibiting spontaneous contractions (Lange et al., 1995; Gäde and Goldsworthy, 2003), and also stimulate the activity of specific carbohydrate-metabolizing enzymes in the gut (Gäde and Goldsworthy, 2003).

The present study is the first to implicate Dippu-ASTs as modulators of AKH release and of JH production and release in *L. migratoria*. Dippu-AST-like immunoreactivity was found to be restricted to the storage lobe of the CC in both *L. migratoria* and *S. gregaria* (present study; Veelaert et al., 1995; Vitzthum et al., 1996). The pattern of Dippu-AST-like immunoreactivity was found to be similar to the pattern of proctolin-like immunoreactivity found in the locust (Clark et al., 2006a). Proctolin (RYLPT) was the first insect neuropeptide to be fully characterized and sequenced based on its association with the hindgut of the cockroach *Periplaneta americana* (Brown, 1975; Brown and Starratt, 1975; Starratt and Brown, 1975).

Proctolin is extensively distributed within arthropods, with many physiological effects such as potent stimulation of visceral and skeletal muscle and release as a co-transmitter with glutamate at the neuromuscular junction (Adams and O’Shea, 1983; Nässel, 2002). Co-localization of proctolin and Dippu-AST-like peptides was observed within the LNCs, the CC, and the CA. Dippu-AST-like immunoreactivity was also found within processes associated with the CA of *L.*
*migratoria* and *S. gregaria* (Veelaert et al., 1995; Vitzthum et al., 1996) although Dippu-AST did not inhibit or stimulate JH release from the CA (Veelaert et al., 1995). However, these previous studies did not differentiate between the effects of the Dippu-ASTs on CA of differing biosynthetic activity (i.e., low rate vs. high rate of basal JH release). Thus, when this differentiation is done in the present study, Dippu-AST 2 stimulates the release of JH from CAs that have a low basal release while it inhibits JH release in high basal release CAs. Therefore, Dippu-AST 2 appears to act as an allatotropin on low release CAs but as an AST on high release CAs.

Adult age and the physiological stage of females with respect to the gonadotrophic cycle affect the sensitivity of the CA to Dippu-ASTs (Stay and Tobe, 2007). This phenomenon has been studied principally in the cockroach *D. punctata* (Pratt et al., 1990; Pratt et al., 1991; Stay et al., 1991). Stay et al. (1991) found that the sensitivity of CA related more to the stage of development of the CA rather than to the rate of JH biosynthesis, and that in general, CA with low activity are more sensitive to Dippu-AST than those showing high basal activity. A similar result has been reported for proctolin, which increases the release of JH from the CA depending on the basal activity of the CA (Clark et al., 2006b). Therefore, CA of low biosynthetic activity were stimulated more than CA that released JH at high rates (Clark et al., 2006b). In the crayfish *Procamburus clarkii*, this dual action of Dippu-AST 2, as well as Dippu-AST 5 and 7, was also observed (Kwok et al., 2005). The mandibular organ (MO) of crustaceans is the crustacean homologue of the insect CA. Both produce sequiterpenoids; in crustaceans, the final products are farnesoic acid (FA) and methyl farnesoate (MF) and in insects, these compounds are converted to JH III through methylation and epoxidation (Tobe and Bendena, 1999). Kwok et al. (2005) found that MO showed variable basal rates of MF release and Dippu-AST 2, 5 and 7.
significantly stimulated the release of MF from MO with low basal rates of MF release. However, in MO with high basal rates of MF release, the Dippu-ASTs did not alter FA or MF release (Kwok et al., 2005).

Interactions between the Dippu-ASTs and other peptides and their effects on JH release have not been extensively studied and the results depend, to a large extent, on the developmental stage of the animals (Stay and Tobe, 2007). For example, in D. punctata, RFamides have been shown to co-localize with Dippu-ASTs in LNCs and nerves innervating the CA, and these peptides function to attenuate the inhibition of JH biosynthesis by Dippu-AST (Stay et al., 2003). In the future, it would be useful to determine the interaction of Dippu-AST and proctolin on locust CA since the locust CA with low biosynthetic activity seem to be more sensitive to proctolin, eliciting a greater stimulation of JH release than Dippu-AST 2 at the same dose. In addition, understanding of the interaction of these two peptides on locust CA showing high basal activity should be instructive, since proctolin stimulates the release of JH (Clark et al., 2006b), while Dippu-AST 2 inhibits it.

Proctolin acts as a releasing factor for AKH in the locust (Clark et al., 2006b), and the present study demonstrates that Dippu-ASTs also act as releasing factors for AKH I in a dose-dependent manner. Pannabecker and Orchard (1986, 1987) found that cAMP mediates the release of AKH. Both Dippu-AST 2 and proctolin significantly increased the content of the second messenger, cAMP, of the glandular lobe of the locust CC indicating that these two peptides mediate their effects through this second messenger. Nässel et al. (1999) also found that locustatachykinins increase the cAMP level within the glandular lobe of the CC as well as increase the release of AKH I in a dose-dependent manner. These actions have not been previously reported for Dippu-AST.
AKH I release is modulated by neuropeptides such as CCAP (Veelaert et al., 1997) and locustatachykinin (Nässel et al., 1995, 1999). In comparison to proctolin, Dippu-AST 7 and Dippu-AST 2 are more effective at releasing AKH I from the locust glandular lobe of the CC, with Dippu-AST 2 being more effective than Dippu-AST 7. Previous research on cockroach hindgut has shown that the 13 Dippu-ASTs differed in effectiveness (Lange et al., 1995). Lange et al. (1995) demonstrated that Dippu-AST 12 was the least effective cockroach AST in inhibition of both myogenic and proctolin-induced contractions. Dippu-AST 13 ranked third in inhibiting proctolin-induced contractions of the cockroach hindgut, Dippu-AST 7 was ranked fourth and Dippu-AST 2 was ninth (Lange et al., 1995). In the present study, Dippu-AST 12 was also the least effective in stimulating AKH I release from the CC whereas Dippu-AST 2 was the most effective.

Why are there so many factors capable of releasing AKH from the CC of the locust? One answer might stem from the fact that the delivery of nutrients needs to be integrated into the physiological state of an animal. Therefore, the ability of peptides, such as proctolin and Dippu-ASTs, either released within the CC or into the hemolymph, to stimulate AKH release, ensures a sufficient supply of nutrients from the fat body to the tissues. This would be important under conditions of stress or increased metabolic demand such as egg-laying, ecdysis, or flight. Moshitzky and Applebaum (1990) demonstrated that AKH I inhibited vitellogenin production in vitro by minced fat bodies of gravid female locusts. The same study suggested functional independence in terms of the utilization of fuel reserves and the synthesis of vitellogenin, providing a continual supply of fuel reserves while at the same time a continual synthesis of proteins necessary for egg development. In terms of the regulation of JH production by the CA, the ability of Dippu-ASTs and proctolin to alter biosynthetic activity, based on initial levels of
activity, suggests a level of integration more complex than originally thought. These peptides may autoregulate the level of JH production so that in low activity CA, the peptides stimulate JH production, whereas in higher activity CA Dippu-AST-like peptides inhibit JH production. It is likely that there are other peptides that also modulate JH biosynthesis in locusts, which act in concert with ASTs and proctolin, since Dippu-ASTs are unable to completely inhibit JH production. It is also significant that transection of the NCA in adult locusts results in a rapid decay in JH biosynthesis, indicating that the CA may need constant stimulation by peptides for sustained biosynthetic activity (Tobe et al., 1977; Girardie et al., 1982).
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CHAPTER 3:
NEURAL SUBSTRATE AND ALLATOSTATIN-LIKE INNERVATION OF THE GUT OF Locusta migratoria
**ABSTRACT**

Allatostatin-like immunoreactivity (ALI) is widely distributed in processes and varicosities on the fore-, mid-, and hindgut of the locust, and within midgut open-type endocrine-like cells. ALI is also observed in cells and processes in all ganglia of the central nervous system (CNS) and the stomatogastric nervous system (SNS). Ventral unpaired median neurons (VUMs) contained ALI within abdominal ganglia IV-VII. Neurobiotin retrograde fills of the branches of the 11th sternal nerve that innervate the hindgut revealed 2-4 VUMs in abdominal ganglia IV-VIIth, which also contain ALI. The VIIIth abdominal ganglion contained 3 ventral medial groups of neurons that filled with neurobiotin and contained ALI. The co-localization of ALI in the identified neurons suggests that these cells are the source of ALI on the hindgut. A retrograde fill of the nerves of the ingluvial ganglia that innervate the foregut revealed numerous neurons within the frontal ganglion and an extensive neuropile in the hypocerebral ganglion, but there seems to be no apparent co-localization of neurobiotin and ALI in these neurons, indicating the source of ALI on the foregut comes via the brain, through the SNS.
**INTRODUCTION**

The locust gut is divided into three regions, based not only on embryonic origin but also on physiological function. The foregut and hindgut originate from ectoderm and are lined with cuticle that is shed during ecdysis, while the midgut is derived from endoderm and is not cuticular in nature (Billingsley and Lehane, 1996; Chapman, 1998). The foregut is involved with the ingestion, mechanical breakdown, storage and passage of food, while the midgut secretes digestive enzymes, absorbs nutrients, and propels the remains to the hindgut which is primarily responsible for osmoregulation and expulsion of faeces and urine (Chapman, 1998).

In insects, the central nervous system (CNS) and the enteric or stomatogastric nervous system (SNS) innervate the gut. The anterior regions of the gut are innervated by the SNS (Penzlin, 1985; Albrecht, 1953); a series of three peripheral ganglia associated with visceral functioning and feeding (Hartenstein, 1997; Ayali and Lange, 2010). The SNS includes the frontal ganglion, which is connected to the tritocerebrum of the brain by the paired frontal connectives. The recurrent nerve extends from the frontal ganglion to the hypocerebral ganglion, which is linked by two esophageal nerves to a pair of ingluvial ganglia that are located bilaterally on the foregut wall and nerves from each ingluvial ganglion extend over the surface of the foregut (Albrecht, 1953; Konings et al., 1989; Hartenstein, 1997; Stern et al., 2007).

Recently, Bräunig (2008) has shown through neurobiotin retrograde fills of the frontal connective of 4th instar Locusta migratoria that neurons within the CNS project within the frontal connectives toward the SNS, suggesting that the innervation of the gut is complex and that the tritocerebrum of the brain may act as an area of communication between the CNS and SNS. Early work by Clarke and Grenville (1960) on the nervous control of locust foregut
contractions suggested that each ganglion of the SNS has effects on foregut contractions, but it is
the ingluvial ganglion itself that controls foregut contractions, since contractions of the foregut
are completely abolished upon severing the nerves arising from the ingluvial ganglia. Lange and
Chan (2008) also suggest that the ingluvial ganglia are involved in foregut contraction, since
removing the ganglia ceased foregut contractions. Since then it has been shown that the frontal
ganglion plays a key role in feeding behaviour, whereby removal of the frontal ganglion
decreases feeding activity and prevents the crop from emptying its contents (Hill et al., 1966;
Bignell, 1973). The locust frontal ganglion contains a central pattern generator that controls
foregut motor patterns that control a portion of the foregut (Ayali and Lange, 2010; Ayali et al.,
2002; Zilberstein and Ayali, 2002). A rhythmic motor pattern is recorded from the nerves of the
frontal ganglion that coordinate with peristaltic movements of the foregut muscles, and this
rhythm increases as the foregut fills (Zilberstein and Ayali, 2002; Ayali and Lange, 2010).

The CNS innervates the posterior regions of the locust gut through branches of the 11th sternal
nerve that originates from the VIIIth abdominal ganglion (Donini et al., 2002). This neural
innervation influences the contraction of the gut musculature. For example, Nagai and Brown
(1969) reported that neural stimulation of the longitudinal muscles of the rectum of the
cockroach resulted in muscle contraction that was associated with the discharge of rectal
contents.

Adaptability to the environment and proper functioning of the gut is key to an insect’s survival.
Not only is this plasticity and functionality of the gut controlled by nervous input, but it is also
modulated by neuroactive chemicals such as biogenic amines and peptides. The allatostatins
(ASTs) are a family of peptides that have been shown to have effects on insect visceral muscle
but were originally isolated and purified from the cockroach, Diploptera punctata (Stay et al.,
1994; Pratt et al., 1989; Woodhead et al., 1989) based on their ability to inhibit the production and release of juvenile hormone from the corpora allata (Stay et al., 1994; Tobe and Stay, 1985). Lange et al (1993) reported that Dippu-AST 7 (also called allatostatin 1) inhibits myogenic and proctolin-induced contractions of the hindgut of D. punctata. ASTs have also been shown to modulate contractions of the foregut (Duve et al., 1995) and the midgut (Fusé et al., 1999). A number of peptides have been localized to midgut endocrine cells, suggesting that these peptides are involved in digestive processes (Lange and Orchard, 1998; Žitňan et al., 1993; Veenstra, 2009). For example, Dippu-AST 7 stimulates carbohydrase activity in the cockroach midgut lumen (Fusé et al., 1999). Expression of an allatostatin receptor gene (DAR-2) in the gut of Drosophila melanogaster and identification of a putative AST receptor in the cockroach midgut suggests that the inhibitory effect of AST-like peptides is physiologically-relevant and is mediated by a G protein-coupled receptor (Lenz et al., 2001; Bowser and Tobe, 2000).

The ASTs have been detected using immunohistochemistry and AST-like immunoreactivity is widely distributed in the CNS, within axons in nerves that innervate visceral muscle (including the gut), as well as within midgut endocrine cells of multiple insect species (Stay, 2000). In particular, AST-like immunoreactivity has been shown to be associated with the midgut and hindgut of D. punctata (Yu et al., 1995; Lange et al., 1993). The presence of AST-like immunoreactivity within nerves that innervate the viscera, as well as within extensive neuropile regions in ganglia of the CNS, suggests a role for these peptides as neurotransmitters and/or neuromodulators both centrally and peripherally (Hoffman et al., 1999). A hormonal role for ASTs is also suggested by the presence of these peptides within the hemolymph (Hoffman et al., 1999).
Understanding the neural control of digestion, the association of peptides with the gut, and the role that these peptides play in gut functioning is important in understanding the physiology of digestion. The purpose of this study is to identify the neurons within the SNS and CNS that innervate the locust gut, and to determine which of these identified neurons contain AST-like peptides and thus may be the source of AST-like immunoreactivity (ALI) associated with the gut.

**MATERIALS AND METHODS**

**Animals**

Male and female adult *L. migratoria*, two to three weeks old, were used for all experimentation. Locusts were housed in a long-kept colony at the University of Toronto Mississauga, Canada. The colony was fed fresh wheat seedlings and bran and raised in crowded conditions at 30 °C on a 12:12 light cycle.

**Chemicals**

Neurobiotin was purchased from Vector Laboratories Inc (Burlingame, CA, USA). The monoclonal mouse anti-biotin cy3 antibody was purchased from Sigma (Oakville, Ont., Canada). The allatostatin 1 (also called *Dipppu*-AST 7 APSGAQRLYGFGL-NH₂) antibody was a kind gift from Hans-Jürgen Agricola (Jena, Germany). The AST 1 peptide used in the preadsorption control experiments for the immunohistochemistry procedure was custom synthesized by the Insect Biotech Canada Core Facility (Queen’s University, Kingston, Ont., Canada) or by Research Genetics (Huntsville, AL, USA). AST 1 was reconstituted in double distilled water to
yield a stock solution of $10^{-3}$ M, which was divided into 10 µL aliquots and stored at -20 °C until needed.

**Immunohistochemistry**

The CNS, SNS, and gut were dissected in locust physiological saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 2 mM MgCl, 4 mM NaHCO$_3$, 5 mM HEPES, 90 mM sucrose, 5 mM trehalose, pH 7.2) and then fixed in 2% or 4% paraformaldehyde in Millonig’s buffer (0.14 M NaH$_2$PO$_4$·H$_2$O, 0.1 M NaOH, 0.3 mM CaCl$_2$·2H$_2$O, pH 7.2) either for 1 h at room temperature or overnight at 4 °C. After fixation, preparations were washed with phosphate buffered saline (PBS; 0.9% NaCl, pH 7.2) for 2 to 5 h and then incubated for 1 h in PBS containing 4% Triton-X, 2% bovine serum albumin (BSA), and 10% normal sheep serum (NSS) at room temperature. Preparations were then washed with PBS and incubated for 2-4 days at 4 °C in rabbit anti-AST 1 (Dippu-AST 7) IgG fraction purified polyclonal antibody at a dilution of 1:1000 in PBS that contained 0.4% Triton-X, 2% BSA and 2% NSS.

Preparations were then washed with PBS and incubated overnight in affinity purified goat anti-rabbit antibody conjugated to cy3 at a dilution of 1:600 in PBS containing 2% NSS. For preparations that were double-labeled with neurobiotin (see procedure below), affinity purified goat anti-rabbit antibody conjugated to FITC (1:600) was used. Preparations were then washed with PBS, run through an ethanol series, and cleared with a glycerol series. Preparations were mounted in 100% glycerol and were viewed using an epifluorescence microscope (Nikon Optiphot-2, Nikon Corporation, Tokyo, Japan) and drawings were made with a camera lucida attachment. Images were taken using a confocal microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany). For single cy3-labeled preparations, images were taken using the 20x/1.0 objective.
A 543 nm laser line was utilized with a 560-615 nm band pass filter. The oil immersion objective (63×/1.4) was utilized for high-magnification images of the gastric ceacal endocrine-like cells. For double-labelling, the laser lines and filter combinations utilized were 488nm with a 505-530 nm band pass filter for the FITC-labeled AST-like immunoreactivity and 543 nm laser with a 560-615 nm band pass filter for the cy3-labeled neurobiotin filled preparations. Double-labeled images were taken using the 20×/1.0 objective.

In total, 31 CNS preparations were examined, as well as 10 SNS preparations and 30 whole gut preparations. Controls were performed in which the AST 1 antiserum was pre-incubated with $10^{-5}$ M synthetic AST 1 for 24 h. Fourteen pre-absorption control experiments were performed: 4 SNS preparations, 6 CNS preparations, and 4 gut preparations. Pre-absorption of AST 1 antibody with synthetic AST 1 abolished all staining within the SNS, CNS, and gut preparations.

A competitive ELISA was used to determine the specificity of the Dippu-AST 7 (Dip- AST I) antibody used in this study (Vitzthum et al., 1996). Dip-AST I, II, III, IV, and B2 (Dippu-AST 7, 9, 8, 5, and 2 respectively) were recognized by the antibody, while the antibody was two orders more sensitive to Dippu-AST 7 than the other allatostatins tested. A non-competitive ELISA was used to detect cross-reactivity with peptides outside of the allatostatin family. The antibody does not cross-react with crustacean cardioactive peptide, proctolin, corazonin, FMRFamide, locustatachykinin II, leucomyosuppressin, and perisulfakinin (Vitzthum et al., 1996).

**Neurobiotin retrograde filling**

Male and female locusts were dissected under physiological saline. For the SNS, branches of the nerves extending from one ingluvial ganglion were cut as close to the foregut wall as possible. Branches of the 11th sternal nerve of the VIIIth abdominal ganglion were cut as close to the
hindgut as possible. These cut nerves were placed in a well made with petroleum jelly that contained distilled water and secured in place by a petroleum jelly bridge. The rest of the nervous system was placed in an adjacent petroleum jelly well filled with physiological saline. Distilled water was placed in the well containing the cut nerve endings for approx 5 min to allow the nerve to swell allowing the uptake of neurobiotin to occur more readily. The distilled water was then removed and replaced with 5% neurobiotin tracer to immerse the cut nerve endings. Preparations were then left to incubate for 1-2 days at 4 °C. During this incubation period the saline in the nervous system well was replaced at least once a day to ensure that degradation of the preparations was minimal. The dish containing the preparations was covered and paper soaked with distilled water was used to ensure that moisture was kept high in the dish and that the preparations did not dehydrate.

After incubation in neurobiotin, the preparations were fixed in 2% or 4% paraformaldehyde overnight at 4 °C. Preparations were then washed with phosphate-buffered saline and incubated for 1 h in detergent (1% Triton-X in PBS). Preparations were then washed in PBS and incubated for 2 h in block (10% normal sheep serum in PBS). After this incubation the preparations were then incubated in 1:600 cy3-conjugated monoclonal mouse anti-biotin antibody for 1-2 days at 4 °C on a spin wheel wrapped in foil. Preparations were then washed with PBS and dehydrated with ethanol (70% and 100% for 15 minutes each). A glycerol series was then performed to clear the preparations and they were then mounted in 100% glycerol for viewing. In total, 37 preparations were examined: 29 CNS and 8 SNS, where 22 of the CNS preparations and 4 of the SNS preparations were double-labeled with neurobiotin and for ALI.

Preparations were viewed with an epifluorescence microscope (Nikon Optiphot-2, Nikon Corporation, Tokyo, Japan) and images were taken using a confocal microscope (Zeiss LSM
510, Carl Zeiss, Jena, Germany). Drawings of the preparations were completed on the epifluorescence microscope using a camera lucida attachment.

**RESULTS**

**Alimentary canal**

All three regions of the locust gut (foregut, midgut, and hindgut) contain AST-like immunoreactive axons and processes in different patterns (Fig. 1). AST-like immunoreactive axons can be traced within the nerves of the pair of ingluvial ganglia located on the foregut (Fig. 1B) and can be seen to project to the foregut. Upon reaching the foregut the AST-like immunoreactive axons branch and give rise to an irregular pattern of AST-like immunoreactive processes and varicosities (Fig. 1B). AST-like immunoreactive processes extend the length of the midgut and form an irregular latticework pattern with associated varicosities (Fig. 1C). Interestingly, midgut endocrine-like cells were found to contain ALI (Fig. 1C and C1). Hundreds of these endocrine-like cells are scattered throughout the midgut, with a higher density in the anterior midgut. These endocrine-like cells are teardrop shaped and have an apical process that extends toward the lumen of the midgut (Fig. 1C1). In addition, the gastric caeca also contain AST-like immunoreactive endocrine-like cells and processes (Fig. 1C2). The endocrine-like cells associated with the gastric caeca are smaller than those in the midgut and increase in abundance toward the tip of the caeca. The processes within the gastric caeca are fine and increasingly branch toward the tip of the caeca. The hindgut contains ALI within processes and varicosities that arise from AST-like immunoreactive axons within the 11th sternal nerve from the VIIIth abdominal ganglion. The rectum (posterior hindgut) contains a network of processes and varicosities that contain ALI that extend to the colon (middle portion of hindgut).
where six main longitudinal nerve tracts containing AST-like immunoreactive processes arise and project anteriorly into the ileum (anterior hindgut) to the pyloric sphincter, where the Malpighian tubules insert on the hindgut (Fig. 1D). The AST-like immunoreactive axons within these longitudinal nerve tracts give rise to numerous fine lateral processes and varicosities that contain ALI. At the pyloric sphincter, the AST-like immunoreactive processes within the main nerve tracts branch extensively and these processes project anteriorly to give rise to the latticework pattern of AST-like immunoreactive processes associated with the midgut.

**Stomatogastric nervous system**

*AST-like immunoreactivity*

Fig. 2A is a schematic representation of the SNS and its location relative to the locust gut. Each ganglion of the SNS contains ALI within cell bodies, processes, and an extensive neuropile region (Fig. 2B1-B3). The frontal connectives contain 10-15 AST-like immunoreactive axons that pass through the neuropile of the frontal ganglion (Fig. 2B1). The frontal ganglion also contains ALI in 15-30 cell bodies of varying sizes (10-40 μm in diameter). Between 5 and 15 AST-like immunoreactive axons are present in the recurrent nerve and ALI is seen within processes of the neuropile of the hypocerebral ganglion (Fig. 2B2). The hypocerebral ganglion contains 10-20 small AST-like immunoreactive cell bodies (15-25 μm in diameter). AST-like immunoreactive axons project within each of the esophageal nerves to the paired ingluvial ganglia (10-20 axons per nerve; Fig. 2B2 and B3). An extensive neuropile region is seen within the ingluvial ganglia, and 10-25 axons containing ALI project to the foregut within each of the three ingluvial nerves (Fig. 2B3). Each ingluvial ganglion contains 20-25 AST-like immunoreactive cell bodies (15-30 μm in diameter) located around the periphery of the ganglion.
Neurons within the SNS that innervate the gut

The nerves of one ingluvial ganglion were backfilled with neurobiotin in order to trace the source of neurons projecting to the foregut. Due to the brightness of the neurobiotin staining, cells or processes could not be visualized within the ingluvial ganglion but it can be postulated that, at the very least, that neurobiotin-filled axons pass through the ingluvial ganglion to fill neuronal cell bodies and processes within the rest of the SNS. Approximately 10-20 axons were seen within each of the esophageal nerves that extend between the ingluvial ganglia and the hypocerebral ganglion (Fig. 2C2). Some of the axons passed directly through the hypocerebral ganglion while others have neuronal cell bodies situated within the hypocerebral ganglion. The hypocerebral ganglion contains a smaller number of neuronal cell bodies (approximately 15-30), which range in size from 20-25 µm in diameter (Fig. 2C2). Approximately 20 axons were revealed within the recurrent nerve (Fig. 2C1 and C2), which form the neuropile within the frontal ganglion or continued through the frontal ganglion to the frontal connectives where 10-20 axons were revealed (Fig. C1). The frontal ganglion contains 40-50 neurobiotin-filled neuronal cell bodies that range in size from 25 to 50 µm in diameter (Fig. 2C1). In two preparations the neurobiotin travelled within axons of the frontal connectives to produce a small neuropile within each tritocerebral lobe. These axons extended to the protocerebrum to fill a group of 6-10 medially located small neuronal cell bodies (not shown).

Double-labeling of SNS neurons with ALI

None of the neuronal cell bodies in the SNS that filled with neurobiotin stain for ALI (Fig. 5C). This indicates that the ALI that is present on the foregut arises from neurons with their cell bodies located in the CNS. In the frontal ganglion, the cell bodies that contain ALI are mostly located in the posterior portion of the ganglion (Fig. 5A), while the neurons that were backfilled
with neurobiotin are mostly located in the anterior portion of the ganglion (Fig. 5B). Within the hypocerebral ganglion few cells consistently stained for ALI and these did not fill with neurobiotin.

**Central nervous system**

*AST-like immunoreactivity*

AST-like immunoreactivity was found in neurons of the brain (Clark et al., 2008) and in neurons within all ganglia of the ventral nerve cord. The cell bodies and processes that consistently stained with ALI in the VIIth and VIIIth abdominal ganglia in all of the preparations were mapped using camera lucida (Fig. 3A). Any cells that stained faintly or inconsistently were not mapped here. The cells within the VIIth and VIIIth abdominal ganglia that stain positively for ALI were in groups located bilaterally or medially (Figs. 3A and 4A, B). The majority of the cells that stain positively for ALI within each ganglion are located ventrally (Fig. 3A, filled cell bodies). Both ganglia contain an extensive neuropile of processes that stain for ALI (Figs. 3A and 4A, B).

Within the VIIth abdominal ganglion, two large ventral median cell bodies stain positively for ALI (Fig. 4A). ALI was also present within a bilateral group of 4 cells that have axons within the sternal nerve and fine AST-like immunoreactive axons are also seen within the tergal nerves (Fig. 4A). At least six AST-like immunoreactive processes project to the VIIIth abdominal ganglion through the connectives. The VIIIth abdominal ganglion contains ALI within two clusters of ventrally-located medial cell bodies (Figs 3A and 4B, groups a and b). Two bilaterally located groups of 5-7 cell bodies that stained positively for ALI are also seen within the ganglion (Figs. 3 and 4B). Two to four AST-like immunoreactive processes project to visceral targets within the 11th sternal nerve and the 10th sternal nerve, respectively (Fig. 4B).
**Neurons within the CNS that innervate the gut**

After backfilling branches of one of the 11th sternal nerves (the nerve branches that innervate the hindgut) of the VIIIth abdominal ganglion, neuronal cell bodies were filled within all of the abdominal ganglia. The neurobiotin did not travel anteriorly beyond the abdominal ganglia to the thoracic ganglia. A composite camera lucida drawing of these neuronal cell bodies and associated axons is shown in Fig. 3B, where the VIIth abdominal ganglion is drawn as a representative of the neurons filled in abdominal ganglia IV-VII. As shown in Figs. 3B and 4D, the VIIIth abdominal ganglion contains three ventral medial clusters of neurons with cell body diameters of 40 µm or more (groups a, b and c, Fig. 4D) that filled with neurobiotin. In addition, four ventral neurons are located contralaterally to the nerve that was filled and the axons project medially toward the neuropile or to the lateral margin of the ganglion. Three dorsal neurons located ipsilateral to the filled nerve also filled with neurobiotin and axons from these cell bodies extend toward the midline of the ganglion into the neuropile region (Fig. 4D). At least six axons were visualized within the 11th sternal nerve that was filled (right 11th sternal nerve, Fig. 3B). Three axons leave the neuropile to project toward the hindgut within the 11th sternal nerve that was contralateral to the nerve that was filled with neurobiotin (left 11th sternal nerve, Fig. 4D).

The VIIth abdominal ganglion contains 2 (occasionally 4) large ventral unpaired median (VUM) neurons (cell body diameter at least 40 µm), which recur in all anterior abdominal ganglia (Figs. 3B and 4C). At least two axons pass directly from the VIIIth abdominal ganglion, through the VIIth abdominal ganglion, ipsilateral to the nerve that was filled (Fig. 3B). Axons were revealed within the anterior and posterior connectives contralateral to the nerve that was filled (Fig. 4C).
Double-labeling of CNS neurons with ALI

Some neurons within the CNS that fill with neurobiotin and therefore innervate the hindgut also appear to contain AST-like peptides (Figs. 4E and 5F, I). In the VIIth abdominal ganglion, the VUM neurons that innervate the gut also contain ALI (Fig. 5F) Within the VIIIth abdominal ganglion, two groups of neurons within the ventral medial neuron groups that filled with neurobiotin co-localize with ALI (groups a and b, Figs. 4E and 5I).
Figure 1. AST-like immunoreactivity associated with the locust gut. (A) Schematic representation of the 3 regions of the locust gut (foregut, midgut, hindgut). The location of the gastric caeca denotes the border between the fore- and midgut, while the location of the Malpighian tubules delineate the midgut and hindgut. (B) Processes within the foregut arise from the nerves from the ingluvial ganglia (open arrowhead). These processes branch extensively and end in varicosities (closed arrowhead). (C) Latticework of processes and varicosities (closed arrowhead) within the midgut. Endocrine-like cells were also found to contain ALI (open arrowhead). (C1) Close-up of the midgut endocrine-like cells showing an apical extension (open arrowhead). (C2) A gastric caecum showing ALI within endocrine-like cells (open arrowhead) and fine processes (closed arrowhead). (D) Main longitudinal axonal tracts with lateral projections and varicosities (closed arrowhead) of the hindgut. Scale bars = 100 µm.
**Figure 2.** AST-like immunoreactivity (B) and neurobiotin retrograde fill (C) of the stomatogastric nervous system (SNS). (A) Diagram showing the location of the SNS relative to the gut. (B) Immunoreactivity associated with the SNS. All ganglia are oriented so anterior is to the top and posterior toward bottom of each image. (B1) Immunoreactivity within the frontal ganglion, showing processes within the frontal connectives (double arrow) and cell bodies (closed arrowhead). (B2) Immunoreactivity associated with the hypocerebral ganglion, where AST-like immunoreactive processes are present within the recurrent nerve (single arrow) and the esophageal nerves (double arrow). (B3) The ingluvial ganglion contains ALI in an extensive neuropile, axons in the esophageal nerve (single arrow), and the ingluvial nerves (closed arrowheads). ALI is also seen within cell bodies (open arrowheads). (C) Retrograde neurobiotin fills of the SNS ganglia through the ingluvial nerves of the ingluvial ganglion. (C1) Filled axons are seen within the frontal connectives (double arrow) and within the recurrent nerve (open arrowhead). Numerous cell bodies were filled with neurobiotin (closed arrowhead). (C2) Axons within the recurrent nerve (open arrowhead), passing through the hypocerebral ganglion and into the paired esophageal nerves (double arrow) fill with neurobiotin, as well as several neuronal cell bodies within the hypocerebral ganglion (closed arrowhead).
Figure 3. Composite camera lucida drawings of the VII\textsuperscript{th} and VIII\textsuperscript{th} abdominal ganglia with ALI (A) and neurobiotin fills (B). Black cell bodies are located ventrally and open cell bodies are located dorsally. (B) Retrograde neurobiotin fill of the right 11\textsuperscript{th} sternal nerve of the VII\textsuperscript{th} abdominal ganglion (arrow). Black cell bodies are located on the ventral surface and open cell bodies are located on the dorsal surface. Scale bars = 100 μm.
Figure 4. Co-localization of CNS neurons that fill with neurobiotin and also stain for ALI. (A) ALI associated with the VII
th abdominal ganglion in ventral unpaired median neurons (open arrowhead) and bilaterally occurring clusters of cells that send processes within the sternal nerves (closed arrowhead). Processes project to the VIII
th abdominal ganglion through the posterior connectives (double arrow). (B) The VIII
th abdominal ganglion showing ALI within ventral unpaired medial cell bodies (groups a and b) and bilateral groups of cell bodies (closed arrowhead). (C) Ventral unpaired median neurons (open arrowhead) filled with neurobiotin after retrograde filling of the 11
th sternal nerve of the VIII
th abdominal ganglion. Axons were seen within the contralateral connectives to the nerve that was filled (closed arrowhead). (D) Neurons identified within the VIII
th abdominal ganglion occur in three ventral medial groups of neurons (groups a, b and c) and within neurons that are located contralateral to the nerve that was filled (closed arrowhead). The right 11
th sternal nerve was filled (double headed arrow) and axons are seen within the contralateral sternal nerve (single arrow). (E) Ventral neurons identified within the VII
th and VIII
th abdominal ganglia that filled with neurobiotin also contain AST-like immunoreactivity (black cells). Scale bars = 100 µm.
**Figure 5.** Double-labels of ALI and neurobiotin-filled neurons and processes in the SNS and CNS ganglia. (A-C) Frontal ganglion. (A) ALI within the frontal ganglion. (B) Neurobiotin-filled neuronal cell bodies. (C) Merge showing the lack of co-localization. (D-F) VII\textsuperscript{th} abdominal ganglion. (D) ALI within VUM cell bodies (hatched circle). (E) VUM neuronal cell bodies containing neurobiotin (hatched circle). (F) Merge showing co-localization within the VUM neurons (hatched circle). Note that the ALI in this preparation was weak and therefore only one VUM neuron showed double-label staining. (G-I) Posterior portion of the VIII\textsuperscript{th} abdominal ganglion. (G) ALI within posterior VUM cell bodies. (H) VUM neuronal cell bodies stained with neurobiotin. (I) Merge showing co-localization within VUM neurons (hatched circle). Scale bars = 100 µm.
**DISCUSSION**

This study shows that ALI is associated with all regions of the locust gut and that ALI is localized to cell bodies and processes within the ganglia of the SNS and CNS. Through neurobiotin retrograde fills of the nerves that innervate the foregut and hindgut, the neural substrate of the locust gut was determined. These neurons can release peptide onto the gut, so double-labeling of neurobiotin with ALI was performed.

The distribution of ASTs has been described in the CNS and SNS of several insect species. For instance, ALI is present in the brain, stomatogastric ganglia, and retrocerebral complex of the cockroach, cricket, locust, termite, and most recently the honeybee (Clark et al., 2008; Kreissl et al., 2010; Maestro et al., 1998; Stay et al., 1992; Neuhäuser et al., 1994; Yagi et al., 2005) and ALI is present in the frontal ganglion of *Lacanobia oleracea* (Duve et al., 2000). ALI does not seem to co-localize with neurobiotin in neuronal cell bodies within the SNS that innervate the foregut, indicating that the source of ALI on the foregut comes from neurons with cell bodies in the brain. Candidate neurons may be the small group of cells located within the protocerebrum that were filled with neurobiotin (Bräunig, 2008; this study). AST-like immunoreactive cell bodies are located within the protocerebral lobes of locust brain (Clark et al., 2008), in a similar location to those identified in the neurobiotin backfills in the present study. This suggests that the neuronal cell bodies in the protocerebrum may contain ALI and thus be the source of ALI extending over the foregut. The SNS would then act as a conduit for the immunoreactive axons from the brain innervating the foregut. Thus, the AST-like immunoreactive neurons that are within the SNS do not appear to project to the foregut and must be considered essentially interneurons. Perhaps these integrate and co-ordinate pattern generation for the SNS.
In *L. migratoria* and *Schistocerca gregaria*, Dippu-AST 2-like immunoreactivity is associated with the thoracic ganglia but not with the VII\textsuperscript{th} and VIII\textsuperscript{th} abdominal ganglia (Veelaert et al., 1995). Skiebe et al. (2006) used an antibody against Dippu-AST 7 and, unlike Veelaert et al. (1995), found ALI within cells, processes, and neuropile regions within all of the abdominal ganglia, including the VII\textsuperscript{th} and VIII\textsuperscript{th}. The location of cells within these ganglia is consistent with the cells described in this study. Of interest are the VUMs that recur within all of the abdominal ganglia. These VUMs also contain ALI, indicating these cells may be the source of ALI on the hindgut. Cells of a similar size and location to the VUMs identified here have been found to have axons in the oviducal nerve of the VII\textsuperscript{th} abdominal ganglion (Kalogianni and Pflüger, 1992). Some of these efferent VUMs, as well as dorsal unpaired median neurons within the abdominal ganglia, are octopaminergic (Stevenson et al., 1994; Lange and Orchard, 1986), suggesting that AST-like peptides within this class of cells may be released as a co-transmitter with octopamine onto the oviducts, but also onto the hindgut. This is interesting since both octopamine (Huddart and Oldfield, 1982) and Dippu-AST (Lange et al., 1993, 1995) have been shown to be inhibitory on hindgut muscle contractions. These cells may also be involved in activities that require repetition within successive segments, such as peristalsis of the gut.

In addition to the ALI associated with the SNS and CNS, all regions of the locust gut exhibit ALI, suggesting that AST-like peptides are involved in gut functioning. AST-like immunoreactivity has been found to be associated with the gut of other insects, including the cockroach (Maestro et al., 1998), the blood-sucking bug *Rhodnius prolixus* (Sarkar et al., 2003), and the earwig (Rankin et al., 1998). ASTs have been found to inhibit myogenic and proctolin-induced contractions of the gut (Lange et al., 1993; Duve et al., 1995; Fusé et al., 1999). Thus,
by opposing the effect of myostimulatory peptides, AST can help regulate gut motility and the movement of the food bolus through the gut, increasing the efficiency of digestion.

Based on the ALI contained within endocrine-like cells, AST-like peptides may serve other physiological functions. Endocrine-like cells are distributed throughout the midgut epithelium, indicating that the gut is an endocrine organ in its own right (Žitňan et al., 1993). In general there are two types of endocrine cells, open and closed, and it is the open-type that were found to contain ALI within this study. The open-type cells are in direct contact with the midgut lumen by a narrow extension of the apical end of the cell (Endo et al., 1981; Fujita and Kobayashi, 1977). Since these cells have contact with the gut lumen, they may serve as an interface between the digestive and endocrine systems, by assessing nutrient content (Fujita and Kobayashi, 1977). The existence of ALI within midgut endocrine-like cells suggests that these peptides have multiple physiological effects relating to digestion. Not only do these peptides affect gut contractility, but also regulate enzyme secretion (Fusé et al., 1999). Feeding state alters the peptide content of these endocrine-like cells. For example, FMRFamide-like and tachykinin-like immunoreactive content of midgut endocrine-like cells changes with feeding state in L. migratoria (Lange, 2001). This decrease in content may be due to release, decreased synthesis, or increased turnover of the peptide (Lange, 2001). AST-like peptides are released into the hemolymph from the cockroach midgut (Yu et al., 1995), indicating that the ASTs are acting as endocrine hormones during feeding.

Whereas the SNS of crustaceans is a well studied model for neural functioning and pattern generation, the insect SNS has received far less attention (Stein, 2009; Marder and Bucher, 2007; Böhm et al., 2001). Most research on the SNS of insects is in the field of developmental biology, where it is used as a model for embryological cell migration, due to the resemblance of the insect
SNS with the autonomic nervous system of vertebrates (Hartenstein 1997; Copenhaver 2007). Connections are made between the CNS and SNS within the protocerebrum and tritocerebrum of the brain, where neurons and associated axons originate and project through the SNS. Bräunig (2008) recently completed a retrograde fill of one frontal connective and determined that approximately 250 neurons within the CNS project within the frontal connective toward the SNS, with 70 of these neurons located within the brain; more than had been found previously (Aubele and Klemm, 1977).

The present study extends these findings by completing a retrograde neurobiotin fill of the SNS to determine the neural substrate of the foregut. In two preparations, the neurobiotin was capable of filling neurons within the brain that are in a similar location to a group of neurons within the protocerebrum filled by Bräunig (2008). Bräunig (2008) also filled neurons within the subesophageal ganglion and thoracic ganglia that projected within the SNS and that may ultimately innervate the locust foregut. This is quite interesting since a central pattern generator that controls motor rhythms of the foregut exists within the frontal ganglion, while the SOG is responsible for coordinating the mouthparts, and the metathoracic ganglion is responsible for the ventilatory motor pattern (Rand et al., 2008; Ayali, 2004; Ayali et al., 2002; Zilberstein and Ayali, 2002; Bräunig, 2008; Rast and Bräunig, 2001; Ayali and Lange, 2010).

The central pattern generator controlling foregut movement situated within the frontal ganglion can be influenced not only by the ventilatory rhythm that resides within the metathoracic ganglion, but also by neuromodulators like ASTs (Bräunig, 2008; Zilberstein et al., 2004; Ayali and Lange, 2010). It has also recently been shown that the frontal ganglion motor pattern integrates with the motor pattern from the SOG in the absence of sensory input to coordinate food passage (Rand et al., 2008). Thus, the neurons filled with neurobiotin within this study and
Bräunig (2008) may work together to coordinate the aforementioned motor patterns for feeding and gut motility. Since ALI is associated with all regions of the gut and within all ganglia of the SNS, this peptide family may be involved in neuromodulation of the motor patterns within the relevant ganglia and in the modulation of the gut contraction itself.
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CHAPTER 4:
THE NEURAL AND PEPTIDERGIC CONTROL OF GUT CONTRACTION IN *Locusta migratoria*: THE EFFECT OF AN FGLα/AST
ABSTRACT

The regulation of insect gut physiology is complex and involves the interactions of a number of mechanisms, including the neural regulation of gut contraction by altering neural input and the modulation of gut contractions by neuropeptides directly affecting the muscle. The FGLa-type allatostatins (FGLa/ASTs) are known brain/gut peptides with numerous physiological roles, including modulation of gut contraction and neural input. To further investigate the pleiotropic roles of FGLa/AST peptides in *Locusta migratoria*, we have examined the role of a locust FGLa/AST (Scg-AST) in the gut. Proctolin and Scg-AST have opposing effects on gut contraction, where proctolin dose-dependently increases gut muscle tension, while Scg-AST inhibits both muscle tension and spontaneous and neurogenic contractions in a dose-dependent manner. Results from neurophysiological recordings indicate that there may be a central pattern generator (CPG) within the ventricular ganglia regulated by descending inhibition, and the addition of Scg-AST dose-dependently modulates this ventricular ganglion CPG. This work provides a comprehensive picture of how FGLa/ASTs may modulate and coordinate each region of the locust gut, and shows that FGLa/ASTs have both central effects, on the ventricular ganglion CPG, and peripheral effects on the gut muscle. Overall, this study shows how FGLa/ASTs contribute to the complex regulation and fine tuning of gut contraction.
INTRODUCTION

Movement of the food bolus posteriorly through the digestive tract is achieved by peristalsis, the coordinated contraction of the circular and longitudinal muscles that are present in each region of the gut (Davey, 1964; Miller, 1975). These peristaltic contractions are myogenic in nature, but can be modified by neural input. In the locust, foregut and hindgut myogenic contractions are modulated by input from the nervous system through the innervation from the stomatogastric nervous system (STNS) and last abdominal ganglion of the central nervous system (CNS), respectively (Albrecht, 1953; Donini et al., 2002; Möhl, 1972; Huddart and Oldfield, 1982). The frontal ganglion is involved in the control of movements of the anterior foregut or pharynx, and is the major source of innervations to the dilator muscles (Albrecht, 1953; Zilberstein and Ayali, 2002; Bräunig, 2008). Nerves arising from the paired ventricular (ingluvial) ganglia located bilaterally on the foregut directly supply the innervation to the posterior foregut, while the 11th sternal nerve of the eighth abdominal ganglion innervates the hindgut (Robertson and Lange, 2010; Donini et al., 2002). The midgut receives innervation from both the STNS and the CNS, which results in an extensive nerve plexus over the entire midgut (Robertson and Lange, 2010; Donini et al., 2002; Albrecht, 1953).

In the locust, the ganglia of the STNS (frontal ganglion, hypocerebral ganglion and ventricular ganglia) control movement of the foregut (Fig. 1) (see Ayali and Lange, 2010). A central pattern generator (CPG) within the frontal ganglion controls motor patterns that result in coordinated peristaltic movements of the pharynx and muscles involved in swallowing (Ayali and Lange, 2010; Ayali et al., 2002; Zilberstein and Ayali, 2002). Cutting the nerves from the frontal ganglion to the gut decreases feeding activity and prevents the foregut from emptying its contents, suggesting the frontal ganglion plays an important role in feeding (Hill et al., 1966;
Bignell, 1973). Recently, a CPG within the hypocerebral ganglion has been identified, which may interact with the frontal ganglion CPG to coordinate foregut contractions and crop emptying (Rand and Ayali, 2010). Neural activity within cells of the ventricular ganglion also directly control foregut contractions. For example, when the gastric nerves arising from the ventricular ganglia are severed from the foregut, muscle contractions are eliminated (Clarke and Grenville, 1960). Foregut contractions persist when only the ventricular ganglia are left attached to the gut and are abolished when the ganglia are completely removed from the gut (Lange and Chan, 2008).

The detection of several myoactive peptides within cell bodies of the STNS and CNS, and especially within the innervation to each of the gut regions suggests that peptides are involved in the neuromodulation of feeding and digestion, or in the control of gut motility (see Gäde et al., 1997; Robertson and Lange, 2010; Wei et al., 2000). For example, proctolin-like immunoreactivity is associated with cell bodies of the ganglia of the STNS and CNS, as well as within the nerves that innervate the gut (Clark et al., 2006a). The allatostatin (AST) family referred to as FGLa/ASTs (previously known as A-type or cockroach type ASTs ending in the amino acid sequence FGLamide) (see Coast and Schooley, 2011) are pleiotropic, and based on their distribution in the locust CNS and STNS and innervation to the gut it has been suggested that FGLa/ASTs also regulate digestive functions (Robertson and Lange, 2010).

Several insect peptides have been found to control insect gut contraction in many insect species, including the allatoregulatory hormones, myoinhibiting peptides (MIPs), proctolin, and FMRFamide-related peptides (see Audsley and Weaver, 2009; see Spit et al., 2012). The allatostatins and allatotropins have opposing effects on gut contraction. The FGLa/ASTs inhibit spontaneous and proctolin-induced gut contraction (Lange et al., 1993; Lange et al., 1995; Duve
et al., 1995; Sarkar et al., 2003; Fusé et al., 1999). The MIPs (also known as B-type ASTs) were originally named because of their inhibition of hindgut and oviducal contractions in the locust (Schoofs et al., 1991) and members of this family inhibit gut contraction in other insect species, including cockroaches (Predel et al., 2001; see Audsley and Weaver, 2009). While the FGLa/ASTs inhibit gut contraction, the allatotropins stimulate gut contraction in several insect species including moths (see Spit et al., 2012). Proctolin was isolated based on its excitation of hindgut muscle contraction in *Periplaneta americana* (Starratt and Brown, 1975). Since its discovery, proctolin has been found to have a stimulatory effect on the gut of several insect species including the locust midgut and foregut (Lange et al., 1988; Banner et al., 1987). Lastly, the FMRFamide-related peptides referred to as myosuppressins have an inhibitory effect on contraction of the gut regions, including the foregut and midgut of the locust (Banner and Osborne, 1989; Lange and Orchard, 1998).

The regulation of feeding in insects is complex, involving CPGs and sensory feedback, distension of the alimentary canal, nutrient effects, and the modulation of activity by neuropeptides and hormones (Wei et al., 2000; see Audsley and Weaver, 2009). One aim of the current study was to determine how one member of the FGLa/AST family (*Schistocerca gregaria* AST-6, Scg-AST-6) isolated from another locust, may modify the activity of the neurons in the ventricular ganglia that lead to the neurogenic contractions of the foregut. This particular FGLa/AST was chosen because it was isolated and sequenced from locust brain extracts (Veelaert et al., 1996) and the members of the FGLa/AST family show similarity in the C-terminal sequence and thus all have similar actions on visceral tissues, albeit with varying degrees of effectiveness (Lange et al., 1993). In addition, the modulatory role that neuropeptides play in the regulation of feeding and digestion is still poorly understood, and thus another aim of
this study was to elucidate further the role of FGLa/ASTs (in particular Scg-AST-6) in gut physiology. This is important since it is the digestion and metabolism of nutrients from food that provides the energy for important physiological processes such as growth, flight and reproduction. As we have shown previously that proctolin and FGLa/ASTs act as releasing factors for adipokinetic hormone I and juvenile hormone, both of which are metabolically important peptides (Clark et al., 2006b; Clark et al., 2008), it is important to understand how proctolin and FGLa/ASTs may also affect gut physiology, and thus directly affect homeostasis and the physiology of the insect. Using a variety of techniques we show that the FGLa/AST peptide family influences foregut contractions in two ways; by modulating the CPG, which controls the timing and intensity of foregut contractions, and also in a complementary fashion by a direct action on the muscle to inhibit contraction.

**MATERIALS AND METHODS**

**Animals**

All experiments were conducted on mature 2-3 week old adult male *Locusta migratorioides* (Fairmaire and Reiche 1849). Locusts were housed in crowded conditions and were kept on a 12 h:12 h light:dark regime at 30°C at 50% humidity. The locusts were fed fresh wheat seedlings and bran, supplemented with carrots.

**Chemicals**

Scg-AST-6 (ARPYSFGL-NH₂) was custom synthesized by the Insect Biotech Canada Core Facility (Queen’s University, Kingston, ON, Canada) and proctolin was obtained from Bachem (Torrance, CA, USA). Peptides were reconstituted in double-distilled water to yield a 10⁻³ M
stock solution, which was divided into 10 µL aliquots and frozen at -20°C until needed.

Immediately prior to use, working dilutions of each peptide were made in physiological saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 4 mM NaHCO₃, 5 mM pH 7.2 HEPES, 90 mM sucrose, and 5 mM trehalose).

**Muscle contraction assays**

The locust gut was dissected under physiological saline. To isolate the foregut, the gut was bisected at the cardiac valve (where the gastric caeca attach to the gut), and to isolate the hindgut the bisection was made just anterior to the pyloric valve at the point where the Malpighian tubules attach to the gut. Bisection at the cardiac valve and the pyloric valve also isolated the midgut. Once the appropriate region of the locust gut was dissected, one end was pinned securely to a Sylgard-coated dish using minuten pins and fine thread was tied tightly around the other end of the gut and then attached to a Grass FT 03 force transducer (Grass Medical Instruments, Quincy, MA, USA). The force transducer was connected to an amplifier and contractions were monitored on a flatbed chart recorder.

All preparations were maintained in either 400, 600 or 800 µl of saline (depending on the size of the preparation) and the peptide was added by removing half of the volume of saline and replacing it with the same volume of saline containing peptide at twice the desired final concentration. Each preparation was washed extensively with saline between peptide applications. The effect of Scg-AST-6 on neurogenic contractions of the foregut was determined by leaving the ventricular ganglia attached to the foregut, but disconnected from the rest of the STNS.
Neurophysiology

The foregut was dissected out, as above, and bathed in 600 or 800 µl of saline in a Sylgard-coated dish. For extracellular nerve recordings using glass suction electrodes, the gut was dissected such that one ventricular ganglion and associated nerves was accessible. All nerve recordings were amplified and filtered (low band-pass filter 300 Hz and high band-pass filter 500 Hz) using an AM Systems model 1700 differential AC amplifier (Everett, WA, USA). In some preparations the muscle was attached to a force transducer to simultaneously monitor muscle contraction (see above). Extracellular nerve recordings and muscle contractions were displayed, stored and analysed using a Powerlab acquisition system (ADI Instruments, Colorado Springs, CO, USA) and LabChart 6 Pro. Analysis of nerve recordings was made for 2 min prior to and 2 min after application of peptide, and included the measurement of the following variables: burst duration, interburst interval, cycle period, number of action potentials per burst and frequency of action potentials per burst.

Statistics

The data is reported as mean ± s.e.m. A one-tailed paired t-test was used to assess the difference between groups. Significance for all statistical tests was \( P<0.05 \).

RESULTS

Muscle contraction assays

Proctolin

Proctolin was stimulatory on the foregut and hindgut, causing dose-dependent increases in basal tension that were reversible with washing (Fig. 2A; Fig. 3A). The thresholds for contraction
occurred at $10^{-10}$ M for the foregut and between $10^{-11}$ M and $10^{-10}$ M for the hindgut (Fig. 2B; Fig. 3B). The maximum increase in basal tension for both of these gut regions occurred at $10^{-6}$ M proctolin (Fig. 2B; Fig. 3B).

**Scg-AST-6**

Our studies examined the effect of Scg-AST-6 on contraction of the locust gut. Scg-AST-6 inhibited contractions of all regions of the locust gut (Figs 4 to 7).

**Foregut**

The inhibitory effect of Scg-AST-6 was determined in two ways: directly on a proctolin-induced contraction when the ventricular ganglia were removed from the foregut, and on the neurogenic contractions produced by the ventricular ganglia that innervate the foregut muscle when the ganglia were left attached. To assess the inhibitory effect of Scg-AST-6 on proctolin-induced contractions of the foregut, a standard dose of $10^{-9}$ M proctolin was chosen, which produced a contraction that was approximately 40% maximal (see Fig. 2B). Scg-AST-6 dose-dependently inhibited the proctolin-induced contraction of the foregut (Fig. 4). Maximum inhibition of the $10^{-9}$ M proctolin-induced contraction occurred at $10^{-6}$ M Scg-AST-6, where Scg-AST-6 inhibited the proctolin-induced contraction by approximately 55% (Fig. 4B).

When the ventricular ganglia and associated nerves were left attached to the foregut, the foregut muscle revealed a rhythmic pattern of neurogenic contractions (Fig. 5A) that were not seen when the ganglia were removed (see Fig. 4A). Scg-AST-6 reduced the frequency and amplitude of the neurogenic contractions of the foregut in a dose-dependent manner (Fig. 5). The inhibitory effect on frequency and amplitude was reversible with washing and the rhythmic pattern of contractions returned to saline levels after 4 min. Maximum inhibition of contraction frequency
occurred at $10^{-6}$ M (Fig. 5B), where the neurogenic contractions of the foregut were completely abolished in 80% of the preparations (4 out of 5 preparations).

**Midgut**

The isolated midgut possesses spontaneous contractions (Fig. 6A). Scg-AST-6 caused a dose-dependent reduction of midgut basal tension that was reversible upon washing (Fig. 6B). Maximum relaxation of the midgut was measured at $5 \times 10^{-6}$ M Scg-AST-6. Scg-AST-6 also led to an inhibition of the frequency of spontaneous contractions (Fig. 6A).

**Hindgut**

The locust hindgut was not spontaneously active; thus, the inhibitory effect of Scg-AST-6 was assessed on a contraction induced by $10^{-8}$ M proctolin (Fig. 7). Scg-AST-6 caused a dose-dependent inhibition of the proctolin-induced contraction, which was reversible with washing (Fig. 7A). Scg-AST-6 was not capable of fully inhibiting the $10^{-8}$ M proctolin-induced contraction of the hindgut. Scg-AST-6 caused maximum inhibition at $10^{-7}$ M, resulting in an approximately 60% decrease in the proctolin-induced contraction (Fig. 7B). Scg-AST-6 appeared to increase the time it took to reach the maximum contraction induced by proctolin, with $10^{-7}$ M Scg-AST-6 causing a significant delay (Fig. 7C).

**Neurophysiology**

When the ventricular ganglia were left connected to the rest of the STNS and brain there were no apparent bursts of action potentials recorded from the gastric nerves that innervate the foregut muscle (Fig. 8A). Upon isolation of the ventricular ganglia by transecting the oesophageal nerves (which connect the ventricular ganglia to the hypocerebral ganglion; see Fig. 1), bursts of action potentials were seen in the gastric nerves of the ventricular ganglia. These bursts
contained a variety of sizes of action potentials (Fig. 8B). The onset and pattern of bursting activity was variable, which may be related to the degree of fullness of the foregut. For guts that were empty or partially full, either the bursting motor patterns were not exhibited or the bursting was intermittent, whereas guts that were full exhibited bursts that were more coordinated. Thus, all experiments were conducted on preparations where the foregut was full of fresh food.

When simultaneous neurophysiological recordings and foregut muscle contraction assays were performed it was seen that the bursts of action potentials were coordinated with foregut contractions (Fig. 9). In saline, the cycle period was fairly constant and coordinated 1:1 with foregut contractions, which were all of similar force (Fig. 9A). At a dose of $10^{-8}$ M Scg-AST-6, the burst duration and interburst interval significantly decreased, leading to a shorter and more irregular cycle period (Fig. 9B; Fig. 10A). The contraction frequency also increased concurrent with the decrease in cycle frequency but the magnitude of the contractions was more variable (Fig. 9B). After washing, the bursting pattern maintained a shorter cycle period and a greater frequency of foregut contractions (Fig. 9C). After the addition of $10^{-6}$ M Scg-AST-6 the foregut muscle underwent an initial period of irregularity, with bursts of varying length and frequency of action potentials coordinated with irregular contractions. This was followed by a period where bursting was abolished and where no contractions of the foregut were seen. Over time, the bursting pattern gradually reappeared, as did foregut contractions (Fig. 9D). On examining preparations where bursting persisted (20% of the preparations), trends in the data suggest that the burst duration decreased slightly as compared to saline, while the interburst interval and cycle period increased (Fig. 10B). In addition, Scg-AST-6 caused a dose-dependent decrease in the number of action potentials per burst (Fig. 11A) and a dose-dependent decrease in the frequency of action potentials within a burst (Fig. 11B).
Figure 1. Schematic representation of the locust stomatogastric nervous system (STNS). The STNS is situated on the dorsolateral surface of the gut. Two frontal connectives join the frontal ganglion to the brain. The hypocerebral ganglion is connected anteriorly to the frontal ganglion by the recurrent nerve and posteriorly to the paired ventricular ganglia by the oesophageal nerves. Each ventricular ganglion gives rise to 3 gastric nerves that arborize to form a network of processes over the posterior foregut and the gastric caecae. These ceacal processes then form a plexus over the anterior midgut, which can extend to the midgut-hindgut boundary.
Figure 2. Proctolin stimulates locust foregut contraction. (A) Sample trace showing the effect of increasing concentrations of proctolin on basal tension. The phasic contractions (vertical deflections) superimposed on the basal tension change are also induced by proctolin. Upward pointing arrowheads indicate application of proctolin and downward pointing arrowheads indicate the beginning of the saline wash. (B) Dose-response curve illustrating the dose-dependent increase in basal tonus of the foregut upon application of proctolin (values are means ± s.e.m. of 4-6 preparations).
Figure 3. Proctolin stimulates locust hindgut contraction. (A) Sample trace illustrating the dose-dependent increase in basal tension of the hindgut. Proctolin also causes phasic contractions, seen as vertical deflections extending from the tension curve. Upward pointing arrowheads indicate when proctolin was applied and downward pointing arrowheads indicate when the saline wash began. (B) Dose-response curve illustrating the dose-dependent increase in hindgut basal tension. Points represent the means ± s.e.m. of 4-7 preparations.
Figure 4. Scg-AST-6 inhibits proctolin-induced contractions of the foregut. (A) Sample trace showing that Scg-AST-6 (AST) dose-dependently inhibits a standard contraction produced by $10^{-9}$ M proctolin (Proc). Arrowheads pointing upwards indicate when peptide(s) was applied and arrowheads pointing downwards indicate when the saline wash commenced. (B) Dose-response curve showing the dose-dependent decrease in proctolin-induced contraction of the foregut. Values are plotted as the mean ± s.e.m. of 3-5 preparations.
**Figure 5.** Scg-AST-6 inhibits the frequency of neurogenic contractions of the foregut. (A) Sample trace showing the effect of increasing the concentration of Scg-AST-6. Upward pointing arrowheads indicate when Scg-AST-6 was applied, while downward pointing arrowheads indicate when the saline wash commenced. (B) Dose-response curve illustrating the dose-dependent decrease in the frequency of foregut neurogenic contractions (plotted as a percentage of the number of contractions in saline). Values are shown as the mean ± s.e.m. of 4-6 preparations.
Figure 6. Scg-AST-6 decreases midgut basal tension. (A) Sample trace illustrating the inhibitory effect of Scg-AST-6 on muscle tone. Vertical deflections indicate spontaneous contractions, which were also inhibited in a dose-dependent manner by Scg-AST-6. (B) Dose-response curve showing the dose-dependent decrease in midgut muscle tension (plotted as a percentage of the maximum inhibition). Points are means±s.e.m. of 3-5 preparations.
Figure 7. Scg-AST-6 inhibits proctolin-induced contraction of the hindgut. (A) Sample trace showing the inhibition of the effects of a standard $10^{-8}$ M dose of proctolin (Proc) by increasing doses of Scg-AST-6 (AST). Arrowheads pointing upwards indicate when peptide was applied, while arrowheads pointing downwards indicate when peptide was washed off with saline. (B) Dose-response curve illustrating the dose-dependent decrease in the percentage of proctolin-induced contraction. Points represent the mean ± s.e.m. of 4-7 preparations. (C) Scg-AST-6 dose-dependently delays the time to reach maximum proctolin-induced contraction compared to a standard contraction produced by $10^{-8}$ M proctolin. Bars represent the mean ± s.e.m. of 4-7 preparations. This delay to reach maximum contraction is statistically significant for $10^{-7}$ M Scg-AST-6 (inset, *$P<0.05$; one-tailed t-test, $P<0.05$).
Figure 8. Extracellular recordings from a gastric nerve of the ventricular ganglion that innervates the foregut of Locusta migratoria. (A) Extracellular recordings with an intact STNS. Note that there is no apparent bursting activity. (B) Extracellular recordings after the transection of the oesophageal nerves to isolate the ventricular ganglia from the STNS. Note the bursting activity. Lines below the trace indicate the duration of each burst. This is a representative trace of the bursting motor pattern seen (N=20).
Figure 9. Coordination of bursting motor patterns with foregut contraction. (A) Extracellular recordings (top trace) and foregut contractions (bottom trace) recorded in saline are coordinated. (B) Recordings after the addition of $10^{-8}$ M Scg-AST-6. (C) Recordings after a saline wash. Note that cycle period decreases. (D) Recordings made after the addition of $10^{-6}$ M Scg-AST-6. Note the irregular activity and absence of a repeating pattern. These are representative traces ($N=10$).
Figure 10. The effect of Seg-AST-6 on neurophysiological pattern characteristics. (A) Seg-AST-6 at $10^{-8}$ M significantly decreased burst duration, interburst interval and cycle period relative to saline (* $P=0.03$, **$P=0.01$; one-tailed paired $t$-test, $P<0.05$ indicates significance). Bars represent means + s.e.m. of 7 preparations. (B) There was no significant effect of $10^{-6}$ M Seg-AST-6 on burst duration, interburst interval and cycle period relative to saline for preparations where bursting persisted (one-tailed paired $t$-test, $P<0.05$ indicates significance). Bars represent means + s.e.m. of 10 preparations.
Figure 11. Dose-dependent effect of Sgc-AST-6 on (A) the number of action potentials per burst and (B) the frequency of action potentials within a burst. Bars represent the mean ± s.e.m. of 7 preparations for $10^{-8}$ M and 10 preparations for $10^{-6}$ M Sgc-AST-6. *$P<0.05$; **$P=0.002$, ***$P=0.007$; one-tailed paired $t$-test, $P<0.05$. 
DISCUSSION

The distribution of FGLa/AST-like immunoreactivity in *L. migratoria* suggests these peptides have diverse physiological functions (Clark et al., 2008). Recently, FGLa/AST innervation to the locust gut was described, indicating the source of FGLa/AST-like peptides associated with the foregut to be cell bodies within the brain, and the hindgut to be cell bodies within the eighth abdominal ganglion (Robertson and Lange, 2010). These cells may release FGLa/AST-like peptides locally as neurotransmitters or neuromodulators onto the gut tissue or into the hemolymph to act as neurohormones. The results from this study show that proctolin can stimulate muscle contraction of the foregut and hindgut of the locust and that Scg-AST-6 can act directly on the foregut and hindgut muscle to inhibit proctolin-induced contractions. Scg-AST-6 can also decrease tonus of midgut tissue. This myoinhibitory property of FGLa/ASTs is well documented in a number of insect species. For example, FGLa/ASTs inhibit peristaltic contractions of the foregut in *Calliphora vomitoria*, *S. gregaria*, and *Leucophaea maderae* (Duve and Thorpe, 1994; Zilberstein et al., 2004; Duve et al., 1995), proctolin-induced contractions of the midgut in *Diploptera punctata* (Fusé et al., 1999), and spontaneous and proctolin-induced contractions of the hindgut in *D. punctata* and *S. gregaria* (Lange et al., 1993, 1995; Veelaert et al., 1996). Other myoinhibitory effects of FGLa/ASTs include inhibition of spontaneous oviducal contractions in *S. gregaria* (Veelaert et al., 1996) and modulation of the cardiac rhythm in *Blattella germanica* (Vilaplana et al., 1999). However, we now also report that FGLa/ASTs indirectly inhibit foregut contractions via altering activity from a CPG located in the ventricular ganglia and thereby inhibiting neurogenic contractions.

A motor pattern controlling the contractions of the foregut is generated by the isolated ventricular ganglia, as shown by the coordination of the bursts of action potentials recorded from
the nerves arising from the ventricular ganglion with contraction of the foregut. This confirms the work (Lange and Chan (2008); Clarke and Grenville (1960)) predicting that a CPG within the ventricular ganglia directs foregut contraction. This CPG is probably under descending inhibitory control, as transection of the oesophageal nerves activates the CPG, leading to a rhythmic motor pattern. In the intact locust, a distinct food passage rhythmic motor pattern associated with the start of feeding has been recorded from the frontal ganglion nerves (see Ayali and Lange, 2010). This motor pattern, which is also under descending inhibitory control, coordinates with peristaltic contractions of the foregut and increases in cycle frequency as the foregut and crop distend, and ceases when the gut is fully stretched and full (Zilberstein and Ayali, 2002). A similar observation was made in the current study, where bursts of action potentials from the ventricular ganglia gastric nerves were not observed or were intermittent when the foregut was empty or minimally distended, but were observed when the foregut was full of food. Neuromodulation of foregut rhythm from the frontal ganglion by peptides or amines has been documented (Zilberstein and Ayali, 2002; Zilberstein et al., 2004). Neurophysiological studies have previously suggested that FGLa/ASTs modulate the foregut rhythm in vitro and in vivo in the desert locust (Zilberstein et al., 2004). The effect of FGLa/AST on the characteristics of the rhythm from the frontal ganglion were complex, with low doses of FGLa/AST caused an excitatory effect on the motor pattern while $10^{-6}$ M Scg-AST-6 caused complete inhibition of both nerve and muscle activity (Zilberstein et al., 2004). In the current study, neuromodulation of the foregut motor pattern arising from the ventricular ganglion by Scg-AST-6 indicates a similar control to that shown by Zilberstein and colleagues (Zilberstein et al., 2004). A low dose of Scg-AST-6 ($10^{-8}$ M) caused a decrease in cycle period due to a significant decrease in the interval between bursts, thereby leading to an increase in the frequency of contractions, whereas at $10^{-6}$ M the neural and muscle activity were completely
abolished or greatly reduced. In preparations where neural and muscle activity were still detected at $10^{-6}$ M Scg-AST-6, the rhythm was altered such that there was a decrease in burst duration and frequency of action potentials within the burst. These changes lead to less forceful contractions of the foregut muscle. Thus, FGLa/ASTs control foregut movement at two levels; first, by influencing the motor patterns from the ventricular ganglia, and second, by direct inhibitory action on the foregut muscles. Interestingly, there may be an added complexity in that the frequency of foregut contractions increased after washing out the allatostatin. Thus, there may be a ‘post-inhibitory rebound’ effect whereby the CPG becomes more robust following the removal of the peptide. Perhaps this is a true physiological effect allowing for even greater fine tuning depending upon the context of events.

To aid in the passage of food from one region to another or to increase the mixing of the food within a specific region, the motility of the various regions of the gut must be altered. The FGLa/ASTs can inhibit contractions, while proctolin can stimulate contractions. Thus, proctolin may be released to increase the motility of the muscle. This is reasonable since proctolin was originally discovered based on its myotropic effect on cockroach hindgut (Brown and Starratt, 1975), and has since been implicated in the regulation of feeding (see Audsley and Weaver, 2009). When peristaltic contractions need to be reduced or abolished completely, FGLa/ASTs may be released to decrease the contractile activity of the gut region to allow for more efficient absorption of nutrients and absorption of water by increasing the time the food bolus remains in the midgut or hindgut. FGLa/ASTs may also act on the cardiac and pyloric sphincters to relax them, allowing the passage of food into the next gut region more easily.

We also show here that Scg-AST-6 inhibits midgut muscle contraction by lowering basal tonus. Previous studies have indicated a role of FGLa/ASTs in midgut physiology. For example,
FGLa/AST content of the cockroach midgut changes with nutritional status. In starved and dehydrated cockroaches there is an initial increase in midgut FGLa/AST content, which decreases as the duration of the nutritional stress increases (Yu et al., 1995). During periods of water and food deprivation, FGLa/ASTs may be released into the haemolymph to modulate local midgut contraction, thus decreasing metabolic activity and increasing the time the food bolus remains in this region, allowing increased time for chemical digestion and the absorption of nutrients. This is in line with the suggestion that these peptides function as neurotransmitters/neuromodulators at the locust gut, based on the presence of networks of FGLa/AST-like immunoreactive processes and varicosities on the surface of foregut, midgut, and hindgut (Robertson and Lange, 2010). In addition, FGLa/AST-like peptides have been detected in the haemolymph and are released from the cockroach midgut into the haemolymph during feeding, acting as endocrine hormones (Woodhead et al., 1993; Yu et al., 1995).

As FGLa/ASTs modulate hindgut muscle contractility, the action of FGLa/ASTs may also be related to excretion. It is possible that FGLa/ASTs can affect both muscle contractility and reabsorption because FGLa/ASTs have been shown to alter ion transport in other insect species (Onken et al., 2004). Future work will examine the role that FGLa/ASTs play in locust gut ion transport to further elucidate the physiological roles that this peptide family plays in digestion.

Here, we show that the FGLa/ASTs are important in the regulation of gut muscle activity, by modulating not only the neural input but also, directly, the gut muscle tissue. FGLa/ASTs modulate each region of the gut, coordinating the functioning of each region. It would appear that a physiological function in the gut is a common role for FGLa/ASTs in insects (see Audsley and Weaver, 2009; Lange et al., 1995; Duve and Thorpe, 1994) (see also current study). The ancestral role for the FGLa/ASTs may be as brain/gut peptides, as a myoinhibitory role for
visceral muscle contraction is so widespread for the FGLa/ASTs, and because the FGLa/ASTs do not function as true allatostatins in most species of insects (see Gäde, 2002). FGLa/ASTs and proctolin are pleiotropic and involved in several physiological processes in animals, so it is not surprising that the control of the contractile activity of insect gut musculature is modulated by these peptides. By having opposing effects on gut contraction, proctolin and FGLa/ASTs can regulate gut motility to increase the efficiency of nutrient absorption and the passage of food along the alimentary canal.


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Möhl, B. (1972). The control of foregut movements by the stomatogastric nervous system in the European house cricket Acheta domesticus L. J. Comp. Physiol. 80, 1-28


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APPENDIX A:

Isolation and sequencing of native *Locusta* FGLa/ASTs and the effect of a native FGLa/AST, Locmi-FGLa/AST-2, on foregut and hindgut contractions
ARISING from the previous chapters within this thesis where FGLa/ASTs from *Diploptera punctata* and *Schistocerca gregaria* were used to determine physiological effects on *Locusta migratoria* tissues, we wished to sequence endogenous FGLa/ASTs in *L. migratoria* and determine their physiological effects on gut contraction. Previously, Clynen and Schoofs (2009) reported partial sequences for four Locmi-FGLa/ASTs, and here we confirm and extend those findings by determining the sequence of two of these FGLa/ASTs. In addition, the amino acid sequence of a novel *Locusta* FGLa/AST, Locmi-FGLa/AST-2, was determined to be LPVYNFGL-NH$_2$. Locmi-FGLa/AST-2 dose-dependently inhibited foregut neurogenic contractions and proctolin-induced contractions of the hindgut, but was not as effective as Scg-AST-6.
INTRODUCTION

The insect allatostatins (ASTs) were originally discovered based on their ability to inhibit juvenile hormone synthesis and release from the corpora allata of *Diploptera punctata*, hence their name (see Bendena et al., 1997, 1999; Woodhead et al., 1989). Additional ASTs were discovered and characterized in a variety of insects, leading to the grouping of the ASTs into 3 families based on C-terminal sequence similarity (see Stay and Tobe, 2007; Bendena et al., 1999). The largest AST family is the of the FGLa/ASTs (previously named cockroach or A-type ASTs) that share the C-terminally amidated Y/FXFGL sequence (see Coast and Schooley, 2011; Pratt et al., 1989).

Many studies utilizing immunohistochemistry and peptide isolation have shown that FGLa/ASTs are widely distributed within the central and stomatogastric nervous systems (CNS and STNS respectively) within interneurons and extensive neuropile regions, as well as in motor neurons that project to visceral and skeletal muscle, neurohemal organs and endocrine cells (chapter 2 and 3; see Bendena et al., 1997; Robertson and Lange, 2010; Clark et al., 2008). FGLa/ASTs are also widely distributed within crustacean CNS and STNS as well as the mandibular organs (Skiebe, 1999; Duve et al., 2002; Duve et al., 1997; Kwok et al., 2005). This widespread distribution is interesting since FGLa/ASTs are present within insect species where these peptides do not have an allatostatic function. Thus, the widespread distribution of FGLa/ASTs coupled with the widespread myoinhibitory role on visceral muscle suggests that the ancestral function of FGLa/ASTs may not be inhibition of juvenile hormone (JH) synthesis, for which they were discovered, but is likely as brain/gut peptides involved in gut physiology.
In locusts, as well as several other insect species, FGLa/ASTs are known for their gut myoinhibitory effects (chapter 4; Veelaert et al., 1996a; Robertson et al., 2012). In addition, neurophysiological studies indicate that FGLa/ASTs are involved in the neuromodulation of feeding and digestion, where they modulate the frontal ganglion and ventricular ganglion rhythmic motor patterns (chapter 4; Zilberstein et al., 2004; Robertson et al., 2012). FGLa/ASTs have also been implicated in the modulation of JH biosynthesis and adipokinetic hormone I release in the locust (chapter 2; Clark et al., 2008).

Recently, Clynen and Schoofs (2009) completed a peptidomic survey of *Locusta migratoria* neural and endocrine tissue and partially identified four FGLa/AST peptides, named Lom-AST-4, 5, 9, and 10. These sequences are identical to those sequenced in the desert locust, *Schistocerca gregaria* (Veelaert et al., 1996a). Isolation of the first gene for the FGLa/ASTs occurred in the cockroach *D. punctata*, and an FGLa/AST gene is now known to occur in a wide variety of insects and crustaceans (Donly et al., 1993; see Stay and Tobe, 2007; Yin et al., 2006; see Zandawala et al., 2012). The FGLa/AST gene codes for 13 to 14 peptides that vary in the N-terminal sequences (Donly et al., 1993; see Stay and Tobe, 2007).

In this study we confirm and extend the findings of Clynen and Schoofs (2009) and describe the purification of three *L. migratoria* FGLa/AST peptides from methanolic brain/retrocerebral complex extracts. The effect of Locmi-FGLa/AST-2 on neurogenic contractions of the foregut and hindgut proctolin-induced contractions is described and compared to the effect of Seg-AST-6 on these tissues.
MATERIALS AND METHODS

Animals

Experiments were carried out on reproductively mature Locusta migratoria, taken from a long-standing colony at the University of Toronto Mississauga (Mississauga, ON, Canada). Locusts were housed in crowded conditions at 30ºC and 50% humidity. The colony was kept on a 12h light: 12h dark cycle and the diet consisted of fresh wheat seedlings and bran, supplemented with carrots. For peptide sequencing, male and female locusts were used, while for muscle bioassays only male locusts were utilized.

Chemicals

Locmi-FGLa/AST-2 (LPVYNFGL-NH₂) was synthesized by Bio Basic (Markham, Ontario, Canada). For gut muscle contraction assays, Locmi-AST-2 was reconstituted in double distilled water, yielding a 10⁻³ M stock solution that was divided into aliquots and frozen at -20ºC. Immediately prior to use, working dilutions of the peptide were made in physiological saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 4 mM NaHCO₃, 5 mM pH 7.2 HEPES, 90 mM sucrose, and 5 mM trehalose) using a 10 µL aliquot of the stock solution.

Tissue extraction and purification for protein sequencing

The brain and retrocerebral complex of 100-200 adult L. migratoria were dissected in physiological saline and immediately placed in an ice-cold mixture of methanol: acetic acid: distilled water (90:9:1 by volume). Samples were then sonicated and centrifuged at 4ºC for 20 minutes at 10 000 r.p.m. The supernatant was concentrated using a speed-vac concentrator.
(Savant, Farmingdale, NY, USA) and then diluted in 1 mL 0.1% trifluoroacetic acid (TFA), and applied to a Sep-Pak C\textsubscript{18} cartridge to remove salts and other impurities. Before application of the solution, the cartridge was equilibrated with 8 mL each of methanol, double distilled water, double distilled water containing 0.1% TFA, and 2 mL of 0.1% TFA in water containing 10 µg of bovine serum albumin (BSA; Sigma-Aldrich). Eluents from the column were collected using 30% acetonitrile (ACN) containing 0.1% TFA, 50% ACN containing 0.1% TFA, and 90% ACN containing 0.1% TFA. The collected extracts were then dried using a speed-vac concentrator for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

**Mass Spectrometry for protein sequencing**

The samples were analyzed by MALDI-TOF MS (Q Star; Applied Biosystems Inc, Sciex, Concord, ON, Canada) at the Advanced Protein Technology Centre (Hospital for Sick Kids, Toronto, ON, Canada). One microlitre of sample was diluted in half by matrix solution (20 mg/ml of dihydroxybenzoic acid in 50% acetonitrile) and then spotted on a MALDI plate and allowed to dry. Spectra were recorded in reflection mode in the mass range of 800 to 3000 \( m/z \). The peaks of interest were subjected to further analysis using tandem MS (MALDI-TOF MS/MS) to determine the amino acid sequence of each peptide using an Applied Biosystems Procise Model 494 sequencer (Foster City, CA, USA).

**Muscle contraction assays**

For full details regarding the dissection and set-up of the foregut and hindgut contraction assays, please refer to the methodology within chapter 4. Briefly, the foregut or hindgut was isolated and then one end was secured to a Sylgard-coated dish using minuten pins, while fine thread was tied tightly around the other end of the gut region. This thread was tied to a Grass FT 03 force...
transducer (Grass Medical Instruments, Quincy, MA, USA), which was connected to an amplifier. Amplified contractions were then monitored on a flatbed chart recorder.

All preparations were maintained in 600 µl or 800 µl of saline. To obtain the final concentration of Locmi-FGLa/AST-2 bathing the preparation, half of the volume of saline was removed and replaced with the same volume of saline containing twice the desired final concentration of Locmi-FGLa/AST-2. Each preparation was washed extensively with saline between peptide applications. The effect of Locmi-FGLa/AST-2 on neurogenic contractions of the foregut and proctolin-induced hindgut contractions were examined and compared to the results for Scg-AST-6.

**RESULTS**

**FGLa/AST sequence determination**

Locusta FGLa/ASTs were isolated from adult brain and retrocerebral complex tissue using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Analysis revealed several peaks that were further analyzed with tandem MS to determine the amino acid sequence. A typical example is shown in Figure 1, where one of the peaks, 921.5 (Figure 1A), was subsequently sequenced and the amino acid sequence was confirmed to be LPVYNFGLa (Figure 1B). Complete sequences for 3 L. migratoria FGLa/AST peptides were determined (Table 1).

**Muscle contraction assays for Locmi-FGLa/AST-2**

Having sequenced a native L. migratoria FGLa/AST, Locmi-FGLa/AST-2, it was tested on foregut neurogenic contractions and proctolin-induced contractions of the hindgut (Figure 2).
Locmi-FGLα/AST-2 dose-dependently inhibited the frequency of neurogenic foregut contractions (Figure 2A). Locmi-FGLα/AST-2 also dose-dependently inhibited proctolin-induced contractions of the hindgut (Figure 2B). It is suggested by these results that Locmi-FGLα/AST-2 is not as potent as Scg-AST-6 at inhibiting gut contraction.
Figure 1. Sequencing of Locmi-FGLa/AST-2 from adult *L. migratoria*. (A) MALDI-TOF MS spectrum showing the presence of LPVYNFGLa (theoretical m/z of 921.5). (B) The sequence was deduced using tandem MS. Prominent b-type fragment ions are labeled. M, mass number; Z, atomic number.
Table 1. FGLa/AST peptide sequences from 100-200 *L. migratoria* brain preparations.
<table>
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</table>

⁺Partial sequences first published by Clynen and Schoofs (2009) and confirmed here
**Figure 2.** Locmi-FGLa/AST-2 inhibits foregut and hindgut contractions. (A) Locmi-FGLa/AST-2 (black bars) was less effective than Scg-AST-6 (grey bars) at inhibiting foregut neurogenic contractions.  (B) Comparison of the effectiveness of Scg-AST-6 (grey bars) and Locmi-FGLa/AST-2 (black bars) on inhibiting proctolin-induced contractions of the hindgut. Black bars represent the mean ± s.e. of 5 preparations; grey bars represent the results from Figure 4 (foregut) and Figure 6 (hindgut) in chapter 4 for the two doses indicated.
We have isolated and sequenced three Locmi-FGLa/ASTs from the brain and retrocerebral complex of adult *Locusta migratoria*. Previously, Clynen and Schoofs (2009) identified four FGLa/AST peptides in *L. migratoria*, named Lom-AST-4, 5, 9, and 10. The presence of leucine and isoleucine were, however, ambiguous at positions 3 and 8 for Lom-AST-5 and at position 11 for Lom-AST-10. We confirm here that these residues are leucine, extending the findings of Clynen and Schoofs (2009) and definitively classifying these peptides as FGLa/AST peptides. In addition, a third FGLa/AST, named Locmi-FGLa/AST-2, was identified for the first time in *L. migratoria*. Locmi-FGLa/AST-2 is a septapeptide with the C-terminally amidated amino acid sequence, LPVYNFGL-NH$_2$. The amino acid sequence for Locmi-FGLa/AST-2 has been identified in a number of insect species and is 100% conserved across these species in which it was examined (see Zandawala et al., 2012). This amino acid sequence conservation indicates evolutionary significance for this FGLa/AST; perhaps this FGLa/AST is the ancestral member to the other FGLa/AST peptides identified in this family. The three FGLa/AST peptide sequences obtained in the current study are identical to those sequenced previously in the desert locust, *S. gregaria* (Veelaert et al., 1996a, 1996b; Clynen and Schoofs, 2009). There have been members of the FGLa/AST family identified in *S. gregaria* and in *L. migratoria*, and so it would not be surprising to find additional members of the FGLa/AST family in *L. migratoria*.

In *Locusta*, FGLa/AST-like immunoreactivity is associated with the CNS, STNS, within nerves that innervate the gut, and the gut itself (chapter 3; Robertson and Lange, 2010). Each region of the locust gut exhibits a different pattern of FGLa/AST-like immunoreactive processes, in addition to FGLa/AST-like immunoreactive midgut endocrine cells. This indicates that these peptides are involved in digestive processes such as gut muscle contraction, transport of ions, or
even hormone release. Gut contractions during feeding are important for the peristaltic movement of the food bolus through the gut, as well as to mechanically break down the gut contents to provide a large surface area for chemical digestion achieved by enzymes. The tension of the gut muscle as well as peristaltic contractions can be modulated by neuropeptides. The locust gut is sensitive to proctolin and to FGLa/ASTs, where proctolin stimulates gut contraction in a dose-dependent manner, while Sgc-AST-6 and Locmi-FGLa/AST-2 dose-dependently inhibit proctolin-induced gut contraction (chapter 4; Robertson et al., 2012). With opposing effects, excitatory and inhibitory neuropeptides can increase the efficiency of digestion and absorption of nutrients and water by modulating the contractility and tonus of the gut musculature and subsequently the movement of the food bolus through the gut.

Sgc-AST-6 and Locmi-FGLa/AST-2 differ in their effectiveness at inhibiting gut contraction. Variation in FGLa/AST peptide sequence has previously accounted for differences in effectiveness or potency of FGLa/ASTs on cockroach hindgut muscle activity and JH biosynthesis (Lange et al., 1995; see Stay et al., 1994). These data suggest that responsiveness to these peptides may reside at the receptor level within the tissues and not at the level of gene translation. This is despite that the preproallatostatin appears to be produced in the brain and gut (see Lange et al., 1995; Donly et al., 1993), and that the preprohormone is processed such that all of the FGLa/AST peptides are expressed simultaneously (see Stay and Tobe, 2007).

The mode of action of the FGLa/ASTs at the gut has not been examined. Their ability to inhibit neurogenic and proctolin-induced contractions indicates that they not only have an action directly on the muscle but also beyond the receptor activation level that leads to this muscular action. Alternate avenues for action include effects on ion channels, second-messenger systems, and the alteration of the activity of neurons that control gut muscle contraction.
Neurophysiological studies indicate that FGLa/ASTs modulate peristaltic contractions of the foregut by altering the central pattern generator (CPG) within the frontal ganglion of the desert locust (Zilberstein et al., 2004) and the CPG within the ventricular ganglion (chapter 4; Robertson et al., 2012). Seg-AST-6 modulates the foregut motor pattern arising from the ventricular ganglia and subsequent foregut contraction in a complex manner, such that at $10^{-8}$ M Seg-AST-6 caused an increase in the frequency of foregut contractions due to a decrease in cycle period, while at $10^{-6}$ M Seg-AST-6 abolished or greatly decreased neural and muscle activity. Since both Seg-AST-6 and Locmi-FGLa/AST-2 have similar effects on foregut neurogenic contractions, it would not be surprising if Locmi-FGLa/AST-2 also affected the electrical activity of ventricular ganglion neurons to modulate the CPG controlling foregut contraction.

The FGLa/AST family has been found in most insect species studied to date, and although these peptides have multiple physiological functions, the high degree of conservation of this gene and its peptide products across arthropods indicates that FGLa/ASTs modulate or control essential life processes. This study has once again implicated FGLa/ASTs in the modulation of what is arguably one of the most essential life processes – feeding and digestion.
REFERENCES


CHAPTER 5:
THE EFFECT OF AN FGLa/AST ON K⁺ TRANSPORT BY THE LOCUST (*Locusta migratoria*) GUT: A NOVEL APPLICATION OF THE SCANNING ION-SELECTIVE ELECTRODE TECHNIQUE
The Scanning Ion-Selective Electrode Technique (SIET) was utilized for the first time in *Locusta migratoria* to characterize K\(^+\) transport along the digestive tract and to determine the effect of Scg-AST-6 (*Schistocerca gregaria* AST-6; ARPYSFGL-NH\(_2\)) on K\(^+\) transport. SIET was used to measure [K\(^+\)] gradients adjacent to the basolateral membrane of the gut, which were then used to calculate the flux of K\(^+\). Regional differences in K\(^+\) fluxes across the gut were evident, where the average rate of K\(^+\) efflux was the greatest at the anterior ileum and lowest at the colon. Since the anterior ileum had the highest rate of K\(^+\) efflux, this region was chosen to determine the effect of Scg-AST-6 on K\(^+\) transport. There was a dose-dependent inhibition of K\(^+\) efflux between 10\(^{-14}\) M and 10\(^{-7}\) M Scg-AST-6. The greatest inhibition of K\(^+\) efflux occurred at 10\(^{-10}\) M Scg-AST 6, while threshold was below 10\(^{-14}\) M. These results suggest that FGLa/ASTs may be acting as diuretics, likely involved in the control of hemolymph water levels during and post feeding.
INTRODUCTION

The ability of insects to regulate water and ion homeostasis in varying environmental conditions is fundamental to their survival and depends on the coordinated activities of many tissues. The organs involved in ionoregulation and osmoregulation in insects are primarily the hindgut and Malpighian tubules, while the midgut is responsible for digestion and the absorption of nutrients and ions (Chapman, 1998). The Malpighian tubules produce the primary urine by actively secreting ions into their lumen from the hemolymph, with water following passively by osmosis (see Schooley et al., 2012). This primary urine is then modified as it passes posteriorly through the hindgut by reabsorption of ions from the hindgut lumen into the hemolymph, again with water following passively. This reabsorptive process is important to terrestrial insects, especially those that live in hot and dry conditions, like locusts. Insects have a high surface to volume ratio, which makes them vulnerable to dessication, and this process in combination with a variety of other adaptations, such as a cuticle covered by hydrocarbons, minimizes water loss.

Maintenance of an appropriate hemolymph composition and volume in terrestrial insects depends on the controlled and selective reabsorption of ions, water, and metabolites. Hemolymph composition can vary with, and be adapted to, the ion content of an insect’s principal diet (Dow, 1986). Thus, solid plant feeders, such as the locust, have to cope with excess K\(^+\) and Mg\(^{2+}\) content in their hemolymph (Dow, 1986; Dow et al., 1984). The cellular mechanisms of ion (and fluid) transport by the locust Malpighian tubules and hindgut are well described and reviewed by Phillips et al. (1994) and Schooley et al. (2012). Interestingly, many of the mechanisms are similar to the kidney tubules of vertebrates. Malpighian tubule secretion is driven by the active transport of KCl and NaCl into the tubule lumen, which draws water into the lumen by osmosis. The primary urine then enters the hindgut, where hemolymph composition in
the desert locust, *Schistocerca gregaria*, can also be regulated by selective reabsorption in the rectum (Phillips, 1977). The hindgut contents are concentrated through active chloride absorption by an apical electrogenic $\mathrm{Cl}^-$ pump, which allows passive movements of cations such as $\mathrm{Na}^+$ and $\mathrm{K}^+$ into the hemolymph, allowing water conservation (Phillips et al., 1986). There is also active $\mathrm{H}^+$ secretion due to a V-ATPase (Phillips et al., 1986; see Harvey et al., 1983; see Klein, 1992). Within the rectal cells, $\mathrm{Na}^+/\mathrm{K}^+$ exchangers in the basolateral membrane transport $\mathrm{K}^+$ into/out of the locust hindgut (Chapman, 1998; see Coast, 2001). In the locust, studies examining the transport of specific ions are limited; thus one aim of this study is to characterize the regional transport of $\mathrm{K}^+$ across the gut, an ion in high concentration in the locust diet.

A number of neural factors alter fluid and/or ion transport processes of the excretory system in various insect species. Specifically, neuropeptides have been studied to determine their influence on transport across the gut and Malpighian tubules of various insects. Diuretic hormones stimulate Malpighian tubule secretion, while peptides that act at the hindgut to stimulate fluid and ion reabsorption from the primary urine are antidiuretic hormones (see Schooley et al., 2012). Three antidiuretic hormones that promote fluid reabsorption have been isolated or characterized from the corpus cardiacum (CC) of locusts and act at different regions of the hindgut: neuroparsins, ion transport peptide (ITP), and chloride transport stimulating hormone (CTSH) (Phillips et al., 1988, 1986). Briefly, two neuroparsins were isolated and sequenced from the CC of *Locusta migratoria* (Girardie et al., 1989, 1990). Chloride transport stimulating hormone was characterized by its ability to increase short-circuit current in $S. \ gregaria$ rectal tissue using an Ussing chamber (Phillips et al., 1980), while ITP stimulates ion transport in the locust ileum in a dose-dependent manner, as seen by an increase in short-circuit current (Audsley et al., 1992a; Audsley et al., 1992b). Hemolymph composition is also altered
due to hormonal regulation of midgut transport in several insect species. For instance, FGLa/ASTs, neuropeptide F, and proctolin decrease the transepithelial voltage across the anterior midgut of *Aedes aegypti*, indicating an alteration in the transport of ions (Onken et al., 2004). In addition, allatotropin and extended FLRFamides were found to inhibit ion transport across the midgut of *Manduca sexta* (Lee et al., 1998).

Electrophysiological concepts and experimental techniques have played a pivotal role in defining the transport and permeability properties of the gut. The scanning ion-selective electrode technique (SIET) allows for the measurement of [ion] gradients at localized positions along the length of transporting epithelia, permitting an assessment of ion gradients adjacent to the gut. In this study, SIET was utilized for the first time in a locust species to characterize *in vitro* K\(^+\) transport along an intact locust gut and to examine the effects of a locust FGLa/AST (*Schistocerca gregaria* AST-6; Seg-AST-6) on K\(^+\) efflux across the ileum.

**MATERIALS AND METHODS**

**Animals**

*Locusta migratoria* were raised in a long-standing colony at the University of Toronto Mississauga. The colony was kept on a 12h light: 12h dark cycle at 30ºC in high humidity.

Locusts were kept in crowded conditions and were fed fresh wheat seedlings and bran *ad libitum*. Two- to four-week old adult male animals were used. Experiments were completed on fed animals, one to ten hours post feeding, and all locusts used contained fresh food within the hindgut region of the digestive system.
Construction of ion-selective microelectrodes

To measure $[K^+]$ at the basolateral surface of the locust gut, $K^+$-selective microelectrodes were constructed. Glass capillary tubes (TW 150-4, World Precision Instruments, Sarasota, Florida, USA) were pulled on a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, California, USA) into micropipettes. The micropipettes were then baked at 300°C for 30-40 minutes and then vapour-silanized with dimethyltrimethylsilylamine (Fluka, Bachs, Switzerland) for one hour and then cooled before use. To create the $K^+$ microelectrode, the micropipette was backfilled with 100 mM KCl and then frontloaded with $K^+$ ionophore (potassium ionophore I cocktail B; Fluka, Bachs Switzerland). The microelectrode was left to condition for at least 15 minutes before use and was calibrated, before and after each preparation, using 300 mM KCl and 30 mM KCl (with 270 mM NaCl) standard solutions.

Scanning Ion-selective Electrode Technique

The SIET system used in this study was as follows, and has been described previously (Rheault and O’Donnell, 2001; 2004). Briefly, the $K^+$ microelectrode was fit onto an Ag/AgCl wire electrode holder, which was attached to the headstage. The headstage was attached to an IPA-2 Ion/Polarographic amplifier (Applicable Electronics, Forestdale, Massachusetts), where the gain was set at 100×. A reference electrode was made by filling a capillary tube with 3 M KCl containing 3% agar. The reference electrode was mounted to the headstage with an electrode holder containing a silver pellet and filled with 3 M KCl. The reference electrode was allowed to rest within the saline bath surrounding the locust gut preparation.
Measurement of [K$^+$] gradients

An *in vitro* preparation for the measurement of [K$^+$] gradients at the basal surface of the locust gut was developed as follows. The locust gut was dissected out and placed in a Sylgard-coated recording dish in 1000 µl of physiological saline (150 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 2 mM MgCl$_2$, 4 mM NaHCO$_3$, 5 mM HEPES, 90 mM sucrose, 5 mM trehalose; pH 7.2). Once the preparation was positioned appropriately, the microelectrode was moved to a target site that was 20-30 µm away from the gut and a voltage was recorded. After recording the voltage at the target site, the microelectrode was moved 100 µm away to obtain a second voltage reading. The sampling protocol used a wait time of four seconds after microelectrode movement and a recording time of one second. At each target site this sampling protocol was repeated four times. The voltage difference between the two sites was used to calculate a voltage gradient by the Automated Scanning Electrode Technique software (ASET; Science Wares, East Falmouth, Massachusetts) and the gradient reported for each of the sites was an average of the four readings taken. To obtain background voltage readings, the K$^+$ microelectrode was moved 1920-2560 µm away from the preparation, and the same sampling protocol was employed. These background voltage signals were then subtracted from those recorded adjacent to the locust gut. To achieve reliable voltage readings it was important that the gut did not move very much; therefore minuten pins were used to limit movement of the region where readings were being taken. The preparation was visually monitored during every recording and those measurements that were made while the gut moved were discarded.
Characterization of $K^+$ fluxes along the whole gut

Using the sampling protocol above, voltage recordings were made along the whole locust gut to determine if regional differences exist for $K^+$ transport. Six regions were chosen: posterior foregut, anterior lobe of the gastric caeca, the middle of the midgut, the anterior ileum, the middle of the colon, and the middle of the rectum. For each region, three target sites were chosen, each 640 $\mu$m apart, encompassing an area of each region approximately 1920 $\mu$m in length.

Effect of Scg-AST-6 on $K^+$ transport

Scg-AST-6 (Schistocerca gregaria AST-6; ARPYSFGL-NH$_2$) was custom synthesized by the Insect Biotech Canada Core Facility (Queen’s University, Kingston, ONT, CAN) or by Research Genetics (Huntsville, AL, USA). Scg-AST-6 was reconstituted in double distilled water to yield a $10^{-3}$ M stock solution. Aliquots were made and stored at -20°C until needed. Doses of Scg-AST-6 were made fresh from a 10 $\mu$l aliquot and applied to the preparation at room temperature.

To determine the effect of Scg-AST-6 on $K^+$ transport, baseline voltage recordings were completed for each of six target sites on the anterior ileum using the sampling protocol above. Afterward, a saline change was completed and the bath was mixed manually with a pipette for 30 seconds. After a two-minute wait period, voltage recordings were then obtained as above for the baseline recordings. A dose of Scg-AST-6 was then added to the preparation, the bath was mixed for 30 seconds, and voltage recordings were completed at the six sites after a 2-minute wait period. The preparation was then washed for 5 minutes and voltage recordings were obtained after a two-minute wait to determine the reversibility of the allatostatin effect. Background voltage recordings were taken after the baseline, saline, and wash voltage readings.
Controls

Time-course controls were completed to ensure that the K$^+$ gradients did not run down over the duration of the experiments. The experimental protocol was the same as explained above, but a saline change was completed without adding a dose of Scg-AST-6. Also, to ensure that Scg-AST-6 did not affect the electrode, the electrode was calibrated in the calibration solutions alone and then calibrated in the same calibration solutions containing Scg-AST-6. It was found that the peptide did not affect the electrode.

Calculation of K$^+$ fluxes

The voltage gradients obtained from the ASET software program were converted into concentration gradients using the equation:

$$\Delta C = C_b \times 10^{\Delta V/S} - C_b \quad (1)$$

where $\Delta C$ is the concentration gradient between the two points measured at the locust gut ($\mu$mol·cm$^{-3}$); $C_b$ is the background K$^+$ concentration ($\mu$mol·cm$^{-3}$); $\Delta V$ is the voltage gradient ($\mu$V) obtained from the ASET software; and S is the Nernst slope of the electrode. The concentration gradient was then converted into a K$^+$ flux using Fick’s law of diffusion:

$$J = D(\Delta C)/\Delta X \quad (2)$$

where J is the net flux of K$^+$ in pmol·cm$^{-2}$·sec$^{-1}$; D is the diffusion coefficient of K$^+$ (1.92E-05 cm$^2$/s); $\Delta C$ is the concentration gradient ($\mu$mol·cm$^{-3}$); and $\Delta X$ is the distance between the two points measured in micrometers. All necessary unit conversions were made accordingly.
Statistics

Data are expressed as mean ± standard error. A paired student t-test was used to determine significance between the baseline and experimental K\(^+\) flux for each dose of Scg-AST-6. For the time length controls, a one-way ANOVA was used to determine significance.

RESULTS

Time Length Controls

To ensure that the change in measured [K\(^+\)] gradients was not due to the run-down of the [K\(^+\)] gradient over the course of experimentation, time length controls were conducted. As shown in Figure 1, the average rate of K\(^+\) efflux (movement of K\(^+\) from the gut lumen to the saline bath) did not significantly decrease over the duration of the experiment, which was between 1 and 1.5 hours (Figure 1; one-way ANOVA; p=0.8872). Individual preparations did vary in the rate of K\(^+\) efflux, where two of the six preparations had higher than average rates. In these preparations, baseline K\(^+\) fluxes were greater than 10 000 pmol/cm\(^2\)/sec and remained above average for the duration of the trial. Interestingly, there were differences between recently fed and starved locusts with regard to the rate of K\(^+\) efflux from the gut. The rate of K\(^+\) efflux in recently fed locusts (4819±1601 pmol/cm\(^2\)/sec) was more than double that of locusts starved for 24 hours (1412±309 pmol/cm\(^2\)/sec) (Food data not shown). Therefore, K\(^+\) flux was only measured on recently fed locusts that had food within the gut.

Characterization of K\(^+\) fluxes along the gut

Regional differences in K\(^+\) flux along the locust gut were evident (Figure 2, 3). Six regions along the length of the locust gut were chosen for measurement: posterior foregut, gastric caecum,
midgut, anterior ileum, colon, and rectum (Figure 2A). Localized K⁺ flux and average regional flux of K⁺ are illustrated with arrows. The direction of the arrow indicates the direction of movement of K⁺ ions while the length of the arrow reflects the magnitude of the flux. The anterior ileum had a significantly higher average rate of K⁺ efflux than the other gut regions, which were not significantly different from one another (Figure 3; p=0.001 one-way ANOVA), indicating that K⁺ is highly absorbed into the hemolymph in the ileal area. Like the ileum, the midgut consistently moved K⁺ out of the gut lumen, and had the second highest average rate of K⁺ efflux (1926±914 pmol/cm²/sec). The posterior foregut had the third highest average rate of K⁺ efflux of 1549±466 pmol/cm²/sec and also consistently moved K⁺ out of the gut lumen.

The remaining gut regions exhibited inconsistency in the direction of K⁺ movement across the gut basolateral membrane (Figure 2, 3). For instance, the colon had the lowest rate of K⁺ efflux (445±216 pmol/cm²/sec), but some preparations transported K⁺ out of the lumen while others moved K⁺ into the lumen. The rectum also exhibited site-specific alterations in the direction of K⁺ transport and had an average rate of K⁺ efflux of 1494±505 pmol/cm²/sec. The average rate of K⁺ efflux was 1039±380 pmol/cm²/sec at the gastric caecae, and interestingly, as the electrode was moved from the tip of the gastric caecum toward the junction with the gut, there was an increase in the flux of K⁺ (Figure 2B).

**The effect of Scg-AST-6 on K⁺ transport**

Since the anterior ileum showed the highest rate of K⁺ efflux from the gut, this area was chosen to determine the effect of Scg-AST-6 on K⁺ transport. There was variability in the measured baseline [K⁺] gradients, causing variation in the mean rate of baseline K⁺ efflux. The rate of baseline K⁺ movement out of the gut ranged from 4272±640.6 pmol/cm²/sec to 9851±2932
pmol/cm²/sec (Figure 4A). Scg-AST-6 significantly decreased the baseline $K^+$ efflux in a dose-dependent manner (Figure 4; *$p<0.05$, **$p<0.01$ one-tailed student t-test). A dose of $10^{-10}$ M caused the greatest inhibition ($57.18±7.3\%$), while threshold for inhibition occurred below $10^{-14}$ M (Figure 4). Partial recovery of the dose-dependent inhibitory effect of Scg-AST-6 was achieved upon washing (data not shown), indicating that the effects of the peptide are difficult to reverse.
**Figure 1.** Rate of $K^+$ efflux from the anterior ileum. Baseline indicates voltage readings taken immediately after the preparation is set up. Scan 1, scan 2, and scan 3 correspond to approximately 20 minutes, 40 minutes, and one hour after baseline readings were completed. These scans were completed 2 minutes after a saline wash was completed (to correspond to the saline change, addition of Scg-AST-6 dose, and wash in the experimental protocol). The average $K^+$ efflux of six preparations (filled circles) does not significantly change over the duration of the experimental protocol ($p=0.8872$; one-way ANOVA; $p<0.05$ indicates significance). Average values are expressed as mean±S.E. of the 6 individual preparations shown.
Figure 2. Locust gut schematic illustrating average regional differences in K$^+$ efflux. (A) Mean rate of K$^+$ efflux at each gut region tested. (B) Site-specific K$^+$ efflux increases from the tip of the gastric caecum toward the junction with the gut. Red arrows indicate the mean of 4 to 6 individual gut preparations and the direction of the arrows indicate the direction of movement of K across the gut basolateral membrane.
**Figure 3.** Regional differences in $K^+$ efflux along the basolateral surface of the locust gut. Mean rate of $K^+$ efflux at the ileum was significantly higher than the other gut regions, where different letters indicate significance ($p=0.001$; one-way ANOVA with Tukey’s post-hoc test; significance $p<0.05$). Values are expressed as mean±S.E. of 4 to 6 individual gut preparations.
**Figure 4.** Effect of Scg-AST-6 on the efflux of K⁺ from the anterior ileum. (A) Baseline K⁺ efflux is significantly decreased by Scg-AST-6 (*p<0.05; **p<0.01 one-tailed student t-test between the baseline and Scg-AST-6 values for each dose; p<0.05 indicates significance). (B) Dose-dependent inhibition of K⁺ absorption from the gut lumen. Values are expressed as mean±S.E. of 6-9 individual gut preparations.
DISCUSSION

In the present study, the scanning ion-selective electrode technique (SIET) was utilized to measure spatial patterns of K\textsuperscript{+} flux adjacent to the basolateral membrane of the isolated locust gut. Regional differences in the efflux of K\textsuperscript{+} exist, where the chief site of K\textsuperscript{+} efflux was found to be the anterior ileum. The baseline rate of K\textsuperscript{+} efflux from the ileal lumen did not significantly change during the course of the experiment. This is supported by the results of Goh and Phillips (1978) on everted cannulated sac of the locust rectum, where the absorption rate of water and ions (Na\textsuperscript{+}, Cl\textsuperscript{-}, K\textsuperscript{+}) did not significantly change after the first hour of experimentation and remained steady for four hours (Goh and Phillips, 1978). Thus, the inhibition in K\textsuperscript{+} efflux from the anterior ileum seen upon bath application of Scg-AST-6 indicates the effect was due to the peptide and not from the run-down of the K\textsuperscript{+} gradient.

The regional differences in K\textsuperscript{+} efflux were expected and presumably relate to the morphology and/or function of each region. For instance, developmentally, the foregut arises from ectoderm and is lined with a relatively impermeable cuticle (Chapman, 1998). The hindgut also originates from ectoderm, but the cuticular lining of the hindgut is more permeable (Klowden, 2007), allowing greater K\textsuperscript{+} efflux from this region relative to the foregut. This also relates to the primary functions of the hindgut, which are osmoregulation and the uptake of ions. The midgut, on the other hand, is endodermal in origin and does not possess a cuticular lining, allowing for absorption of digested nutrients and ions in this region (Billingsley and Lehane, 1996; Chapman, 1998).

In previous reports on locust ion transport, the net flux of ions was determined by utilizing an Ussing chamber. Although this technique has several advantages, there are, however, some
drawbacks that need to be considered. This technique is invasive, utilizing a piece of gut tissue that has been stretched. This could lead to issues of increased ion transport due to increased surface area from stretching or as a result of leakage due to membrane damage. The Ussing chamber is also incapable of measuring ion flux at very specific locations. Thus, in the present study SIET was used to non-invasively measure $K^+$ flux at specific locations adjacent to the basolateral surface of an intact adult male locust gut. Unlike traditional ion-selective microelectrodes that are used to measure intra- or extracellular ion activity, SIET is capable of detecting extracellular activity and flux at specific locations and measures net transepithelial ion flux (calculated using Fick’s Law), representing a combination of transcellular and paracellular ion transport. There are factors that may affect the measured flux values. For instance, the tissue being measured should be relatively immobile since movements of the tissue can disrupt the unstirred layer adjacent to the transporting epithelium. This can adversely affect the ion gradient and in turn the calculated fluxes by potentially increasing ion efflux from the tissue.

The scanning ion-selective electrode technique has been used to successfully detect the movement of various ions, including $H^+$, $K^+$, $Na^+$, $Ca^{2+}$, $Cl^-$ and ammonia, as well as toxic compounds like salicylate in a variety of animal and plant transporting epithelia (Shih et al., 2008; see Tong et al., 2007; Nawata et al., 2010; Del Duca et al., 2011; Rheault and O’Donnell, 2004; O’Donnell and Rheault, 2005). The application of SIET to specifically measure $K^+$ flux in insects has been reported in studies of cockroach blood-brain barrier (Kocmarek and O’Donnell, 2011), the Malpighian tubules of *D. melanogaster* (Rheault and O’Donnell, 2004 and 2001), and the anal papillae of the midge (Nguyen and Donini, 2010). The present study utilized SIET for the first time to detect $K^+$ flux at an intact locust gut preparation and examined the effect of a locust FGLa/AST (Scg-AST-6) on $K^+$ transport. Scg-AST-6 inhibited $K^+$ efflux across the
anterior ileum in a dose-dependent manner. FGLa-ASTs have previously been implicated in the modulation of ion transport in insects. In larval A. aegypti, FGLa/ASTs decreased the transepithelial voltage of the isolated gut (anterior stomach or midgut) indicating a modulatory effect on ion transport (Onken et al., 2004).

Malpighian tubule fluid (the primary urine) enters into the gut at the junction of the midgut and hindgut and this primary urine then flows posteriory into the hindgut (Dow, 1981). Phillips and colleagues have performed much of the work on reabsorption in the hindgut and the associated solute and water transport processes of the ileum and rectum (two regions of the hindgut) of the desert locust, S. gregaria (Phillips et al., 1986; Phillips et al., 1996). For both the ileum and rectum, the driving force for the uptake of ions from the lumen into the hemolymph is an apical (luminal) membrane electrogenic Cl\(^-\) pump (Phillips et al., 1996), which allows the passive uptake of K\(^+\) and Na\(^+\) from the lumen. Channels on the basolateral membrane allow K\(^+\) and Cl\(^-\) to exit the gut membrane and be absorbed into the hemolymph, while Na\(^+\) leaves via the Na\(^+\)/K\(^+\)-ATPase (see Figure 4 chapter 1). Ion transport peptide acts via cAMP to stimulate the apical electrogenic Cl\(^-\) pump in the ileum and also opens conductances for Na\(^+\) and K\(^+\) at the apical membrane (King et al., 1999). This results in an increase in the uptake of salts (KCl and NaCl) and fluid reabsorption. Overall, the action of FGLa/ASTs may be either on the transporters or pumps themselves or perhaps these peptides may inhibit the action of ITP at the ileum. To discriminate specific source, inhibitors will be utilized in future studies to determine the effect that Scg-AST-6 has on the transporters involved in K\(^+\) transport. In the caterpillar of the moth M. sexta large electrogenic net K\(^+\) fluxes from the hemolymph to the gut lumen have been recorded, suggesting the midgut is also involved in K\(^+\) excretion (Harvey and Nedergaard, 1964;
Chamberlin, 1990; Zeiske, 1992). Thus, one may anticipate that the action of FGLa/ASTs may not be restricted to the hindgut but may affect midgut $K^+$ absorption as well.

Feeding is believed to act as a stimulus for diuretic hormone (DH) release, and this has been directly demonstrated for *L. migratoria* diuretic hormone (Locmi-DH) (Patel et al., 1995) as well as serotonin, which acts as a DH in *Rhodnius prolixus* (Lange et al., 1989; Maddrell et al., 1991). Interestingly, the concentration of Locmi-DH in the hemolymph of recently fed locusts is not sufficient to stimulate maximal Malpighian tubule secretion (Audsley et al., 1997). In addition, in recently fed insects the rate of fluid and ion recycling between the Malpighian tubules, hindgut, and hemolymph is increased (Phillips and Audsley, 1995). At first it would seem counter-intuitive to increase Malpighian tubule secretion only to recover the fluid by reabsorption at the hindgut. However, this increase in fluid recycling increases the rate of clearance of solutes from the hemolymph (especially toxic compounds), allowing these substances to be concentrated for excretion and minimize water loss. The experiments performed in this study were completed on recently fed locusts; therefore, they would have an increased $K^+$ load as well as excess fluid to deal with. Thus, in this state, FGLa/ASTs may be released to act as diuretic hormones, in conjunction with Locmi-DH, to allow for the excess $K^+$ and fluid to be excreted and for toxic substances to be eliminated. There are extensive FGLa/AST-like immunoreactive processes and varicosities associated with each region of the locust gut, as well as within open-type endocrine cells of the midgut (chapters 2 and 3; Clark et al., 2008; Robertson and Lange, 2010). Additionally, FGLa/AST-like immunoreactivity is associated with processes within the corpus cardiacum and corpora allata, suggesting these peptides may be released from neurohemal structures into the hemolymph to act distantly as
neurohormones or from the midgut endocrine cells to function locally as neuromodulators (chapter 2; Clark et al., 2008).

Life on land takes ionoregulation to the extreme due to the intermittent access to ions from food and water, compounded by the need to conserve water. The ability of insects to conserve water under dessicating conditions may be the primary reason for the evolutionary success of terrestrial insects. Terrestrial plant feeding insects such as the locust must cope with varying $K^+$ content in the hemolymph (Dow, 1986). One way for locusts to cope is through modulation of ionoregulation by peptides, such as the FGLa/ASTs. Overall this work provides new insights into the functions of the FGLa/ASTs and the role of these peptides in the modulation of digestion in this insect pest species.
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CHAPTER 6:
GENERAL DISCUSSION
The overarching objective of physiological research is to gain knowledge and understanding of the intricate network of systems in the body that synchronize their activities to achieve homeostasis and increase survivability of the animal. To facilitate the optimal functioning of cellular processes, insects must maintain a relatively constant internal environment. As discussed throughout the chapters of this thesis, a variety of neuropeptides regulate the physiological processes related to homeostasis, and regulation of these processes allows insects to adapt to, and live in, a variety of ecological niches. Specifically, the purpose of the research included in this thesis is to emphasize the pleiotropic nature of the FGLamide peptides and suggest that perhaps a more primitive role for the FGLa/ASTs may be as brain/gut peptides involved in regulating the physiological processes essential for homeostasis.

In particular, throughout the thesis I infer physiological function based on the distribution of FGLa/AST-like immunoreactivity and of the neural substrate of the gut. How the FGLa/ASTs affect peripheral targets depends on whether the peptide is acting as a neurotransmitter (NT), neuromodulator (NM) or neurohormone (NH), which is inferred from FGLa/AST-like peptide distribution coupled to identification of the neural substrate of the peripheral target (gut). I suggest a neuromodulatory role for the FGLa/ASTs in the regulation of hormone release important for energy utilization (adipokinetic hormone; AKH) and growth and metamorphosis (juvenile hormone; JH). I also suggest a neuromodulatory and/or neurohormonal and/or neurotransmitter role for FGLa/ASTs in regulating two aspects of gut physiology - peristalsis and ion transport. Throughout this discussion I will discuss how the FGLa/ASTs contribute to the flexibility in neural communication that is important in the coordination of homeostasis in a variety of tissues. Thus, the results presented in this thesis and discussed here support the
hypothesis that FGLa/ASTs are present in *Locusta migratoria* and involved in the physiological regulation of peripheral tissues and organs involved in homeostasis.

**THE CENTRAL AND PERIPHERAL DISTRIBUTION OF FGLa/AST-LIKE PEPTIDES SUGGEST PHYSIOLOGICAL FUNCTION**

The FGLa/ASTs have been isolated from a wide variety of insects (Stay and Tobe, 2007). For example, FGLa/AST-like immunoreactivity is often found in neurons in the central nervous system (CNS), and processes in the corpus cardiacum (CC) and corpus allatum (CA), as is the case for cockroaches, termites, crickets, as well as locusts (Chapters 2 and 3; Maestro et al., 1998; Stay et al., 1992; Neuhäuser et al., 1994; Yagi et al., 2005; Veelaert et al., 1995; Vitzthum et al., 1996; Clark et al., 2008; Robertson and Lange, 2010). In Chapters 2 and 3, immunohistochemistry and neurobiotin retrograde fills were utilized to determine the FGLa/AST distribution on the gut and the neural substrate of the gut. The FGLa/AST-like distribution was widespread; identified both centrally within neurons of the CNS and stomatogastric nervous system (STNS) and associated axons as well as peripherally within processes on two neurohaemal organs, the CC and CA, and the gut (Chapters 2 and 3; Clark et al., 2008; Robertson and Lange, 2010). This localization suggests that these peptides have actions within the CNS, but may be delivered to the targets via the hemolymph and act as peripheral NHs or NMs, or directly within the innervation to the tissue and act as NTs as well as have multiple physiological functions that must be integrated.
ENERGY UTILIZATION: A NEUROTRANSMITTER/NEUROMODULATOR ROLE FOR FGLa/ASTs

For homeostasis to occur there must be integration in neural communication as well as integration between the CNS and the endocrine system (ES). Neurosecretory cells (NSCs) within the brain, lateral neurosecretory cells (LNCs) in this case, must integrate their activity with that of the rest of the CNS to modulate AKH release in line with the physiological state of the locust. Not only must their activity be turned on and off, but the activity of the LNCs must also be regulated with regard to the rate of neuropeptide release. Critical titres of neuropeptide must be maintained or reached at the appropriate time to signal a physiological event. For instance, after flight commences AKH is released from the glandular lobe of the CC (Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983a,b) to mobilize lipid from the fat body to increase hemolymph lipid concentration to be utilized as an energy source, especially for long distance flights (Beenakkers, 1969; Mayer and Candy, 1969). Clearly, the initial stimulus is associated with flight, but the mechanism is not known. Since trehalose is used as the initial energy source for flight, perhaps the lowering hemolymph trehalose concentration stimulates the release of AKH (Flanigan and Gäde, 1999). This is supported by experiments where injection of trehalose inhibits the usual rise in hemolymph lipid associated with flight (Houben and Beenakkers, 1973; Cheeseman et al., 1976).

The nervous control of AKH release is by way of three sets of nerves, the nervus corporis cardaci I, II and III (NCCI, II, III) (Albrecht, 1953; Konings et al., 1989). Axons from LNCs of the protocerebrum project within the NCCII to the storage and glandular lobes of the CC, and specifically synapse on intrinsic NSCs of the glandular lobe of the CC, which produce AKH
(Figure 1; Rademakers, 1977; Konings et al., 1989; see Dierderen et al., 2002) and electrical stimulation of the NCCII in vitro leads to release of AKH from the glandular CC (Orchard and Loughton, 1981; Orchard and Lange, 1983a). An additional source of regulatory input is from neuropeptides, which may indirectly or directly alter the activity of the NSCs within the CC.

There is a variety of neuropeptides associated with the CC, including ion transport peptide (ITP), SchistoFLRFamide-like peptides, locustatachynkinin I (Locmi-TKI), proctolin, and FGLa/ASTs (Chapter 2; Nässel et al., 1995; Vullings et al., 1998; Audsley et al., 1992; Clark et al., 2006b; Clark and Lange, 2005; Clark et al., 2008). The association of FGLa/AST-like peptides with the CC suggests that FGLa/ASTs act as NHs or NMs at this neurosecretory organ. FGLa/AST-like immunoreactivity is found within LNCs with cell bodies in the protocerebrum that project axons within the NCCII to arborize on and within the storage lobe of the CC (Chapter 2; Clark et al., 2008). These processes also possess varicosities, which are indicative of peptide release sites. The localization of FGLa/AST peptides in processes and varicosities on the surface of the storage lobe of the CC suggests that these peptides may be released locally onto the glandular lobe of the CC to act as NMs to alter the release of AKH from the intrinsic AKH-producing NSCs of the glandular CC. The FGLa/AST-containing LNCs may release FGLa/ASTs at synapses with intrinsic NSCs of the storage lobe to alter the activity of the intrinsic NSC, as suggested by the branching of FGLa/AST-like immunoreactive processes within the storage CC. In addition, the FGLa/ASTs associated with the storage CC could be released into the hemolymph to act as NHs, travelling to peripheral tissues such as the gut to alter the functioning of the tissue. Proctolin has a similar distribution to FGLa/AST-like immunoreactivity (Chapter 2; Clark et al., 2006a; Clark et al., 2008). It is interesting that there is some colocalization of proctolin and FGLa/ASTs within the LNCs, axons within the NCCII and in processes associated
with the storage lobe of the CC (Chapter 2; Clark et al., 2008). This suggests that these peptides may be released together or separately, as NTs, NM or NHs, to allow finer regulation of AKH release and functioning of peripheral target tissues like the gut.

FGLa/ASTs are involved in the regulation of energy utilization by acting as releasing factors for AKH, which is in line with the FGLa/AST-like immunoreactivity associated with the CC and its associated nerves (Figure 1; Chapter 2; Clark et al., 2008). This stimulatory role for FGLa/ASTs is interesting since these peptides are often inhibitory in their actions on target tissues. Proctolin has a similar effect as the FGLa/ASTs on the CC, where proctolin also acts as a releasing factor for AKH (Clark et al., 2006b). Some additional neuropeptides act as releasing factors for AKH. For example, LomTKI and II and crustacean cardioactive peptide (CCAP) increase AKH release in a dose-dependent manner (Nässel et al., 1995, 1999; Flanigan and Gäde, 1999; Veelaert et al., 1997). Thus, the FGLa/ASTs associated with the CC storage lobe may alter the release of one or more of these other releasing factors, indirectly affecting AKH release from the intrinsic NSCs of the glandular lobe of the CC.

Thus, flexibility in the control of AKH release stems from the interaction between neural and peptidergic modulation of the intrinsic NSCs of the glandular CC. Numerous factors capable of releasing AKH are important because the delivery of nutrients needs to be integrated into the physiological state of the animal. Therefore, neuropeptides like the FGLa/ASTs and proctolin add greater flexibility in neural communication and integration between the CNS and ES depending on how they are delivered to peripheral targets. Thus, FGLa/ASTs could be released into the hemolymph to act as NHs to affect the functioning of peripheral targets such as the gut, or could be released locally onto the CC to act as NM or released within the CC at synapses with intrinsic NSCs to act as NT to modulate AKH release from the CC. This flexibility and
integration ensures that a sufficient supply of nutrients and energy are mobilized from the fat body to the tissues. This is especially important in conditions of stress or high metabolic demand such as egg-laying, ecdysis, or flight.
Figure 1. A model based on research within this thesis illustrating new roles of FGLα/ASTs in the mobilization and utilization of energy and in growth and metamorphosis. Drawing of the retrocerebral complex and associated nerves innervating each organ is a schematic and is not proportional and is modified from Konings et al., (1989). AO = aorta; CA = corpus allatum (paired); gCC = glandular lobe of the CC (surrounding the aorta); sCC = storage lobe of the CC; FG = frontal ganglion; HCG = hypocerebral ganglion; LNCs = lateral neurosecretory cells; NCA = nervus corporis allata; NCCI = nervus corporis cardiaci I; NCCII = nervus corporis cardiaci II; SOG = subesophageal ganglion; AKH = adipokinetic hormone; JH = juvenile hormone.
Growth & Metamorphosis

- Modulation dependent on basal CA activity

Energy Utilization

Releasing Factor
- ↑ AKH release
  - ↑ cAMP content of glandular lobe

- Lipid mobilization from fat body post-digestion
  - ↑ lipid in hemolymph

- Allatostatic
  - ↓ JH biosynthesis
  - ↓ JH titre
  - allows molt into adult

- Allatotropic
  - ↑ JH biosynthesis
  - ↑ JH titre
  - allows molt into next instar
GROWTH AND METAMORPHOSIS: FGLa/ASTs AS NEUROTRANSMITTERS OR NEUROMODULATORS

The CA is innervated by NSCs within the brain and CC that project axons within the nervus corporis allata (NCA) and cardiostomatogastric nerves (NCA II) from the hypocerebral ganglion of the STNS (Figure 1; see Hartfelder, 2000; Konings et al., 1989; Tobe and Stay, 1985). Electrical stimulation of the median NSCs within the brain increases JH biosynthesis (Tobe, 1982). There are also neuropeptides associated with the CA. For instance, through immunohistochemistry the association of FGLa/AST-like peptides and allatotropin-like peptides with the CA was determined in Blattella germanica (Maestro et al., 1998), where allatostatins inhibit JH biosynthesis and allatotropins stimulate JH biosynthesis (see Gäde and Goldsworthy, 2003; Vullings et al., 1999). Proctolin is also within NSCs of the brain, within axons of the NCA and is associated with the CA in the form of processes and varicosities (Chapter 2; Clark et al., 2006a, b; Clark et al., 2008). FGLa/AST-like immunoreactivity is also found within NSCs of the locust brain and in axons projecting within the NCA, which appear to enter and branch within the CA (Chapter 2; Clark et al., 2008). The hypocerebral ganglion and associated nerves also possess FGLa/AST-like immunoreactivity and proctolin-like immunoreactivity (Chapter 2, 3; Clark et al., 2008; Robertson and Lange, 2010; Clark et al., 2006b).

As discussed above with the CC, the presence of FGLa/AST-like peptides within the cell bodies of neurons in the brain that project their axons to the CA and end in varicosities, suggests that these peptides may be released as NMs or NTs to alter CA activity or released as NHs to alter peripheral tissue functioning. This is supported by the dual role that FGLa/ASTs play at the CA, where they seem to act as allatotropins on locust CA with low basal activity while act as allatostatins on CA with high basal activity (Figure 1; Chapter 2; Clark et al., 2008).
biphasic action for the FGLa/ASTs has been shown previously in the crustacean mandibular organ (Kwok et al., 2005). The ability of the FGLa/ASTs to alter the biosynthetic activity of the CA based on the level of basal activity suggests a level of integration more complex than once thought. Perhaps the FGLa/ASTs have a regulatory function, where the same dose of FGLa/AST has a different effect based on the activity level of the CA. During growth and metamorphosis, the JH titre determines whether the insect will molt into a juvenile (nymph, instar) or into the adult form; thus, the titre of JH must be precisely regulated to ensure that the molt is correct (see Gilbert et al., 2000; see Hartfelder, 2000; see Stay and Tobe, 2007).

The regulation of the CA by neuropeptides may be necessary to allow for integration of information from different sources and lead to finer control of the regulation of JH release. The number of neuropeptides involved in the regulation of JH release from the CA can allow for finer regulation of CA activity and JH biosynthesis. For instance, since FGLa/ASTs could not completely inhibit JH biosynthesis (Chapter 2; Clark et al., 2008), it is likely that other neuropeptides work with the FGLa/ASTs to modulate JH biosynthesis. Proctolin and FGLa/ASTs have a similar effect on low basal activity CA (Chapter 2; Clark et al., 2008), where they stimulate JH release, but on CA with high basal rates of JH release proctolin’s action remains allatotropic and the FGLa/ASTs become allatostatic (Chapter 2; Clark et al., 2006b; Clark et al., 2008). It is interesting that there is some colocalization of proctolin and FGLa/ASTs within the axons of the NCA and in processes of the CA, suggesting that these peptides may be released together or separately as suggested above with CC regulation, adding further flexibility in neural communication and integration between the CNS and ES.
NEURAL CONTROL OF FEEDING AND DIGESTION: FGLa/AST AS NEUROTRANSMITTERS/NEUROMODULATORS

The innervation to the gut is complex, where neurons with cell bodies situated within the CNS project axons within branches of the 11th sternal nerve of the VIIIth abdominal ganglion to innervate the hindgut and form part of a nerve plexus over the posterior midgut (Chapter 3; Donini et al., 2002; Robertson and Lange, 2010). Of interest are the recurring ventral unpaired median neurons (VUMs) that are identified to innervate the hindgut and posterior midgut and contain FGLa/AST-like peptides (Chapter 3; Robertson and Lange, 2010). Some of these efferent VUMs are octopaminergic, suggesting that FGLa/ASTs may be released as NTs (co-transmitters) with octopamine at the hindgut (Stevenson et al., 1994; Lange and Orchard, 1986). This is in line with the inhibitory effect of octopamine (Huddart and Oldfield, 1982) and FGLa/ASTs on hindgut of insects (Chapter 4; Lange et al., 1993; 1995; Robertson et al., 2012).

In addition, due to the recurring nature of these cells, they may be involved in activities that require successive repetition such as gut peristalsis. Thus, these VUM cells may release FGLa/ASTs as NTs or NMs onto the hindgut and posterior midgut muscle to modify and coordinate gut contraction of each region, affecting gut peristalsis and the movement of the food bolus through the gut. The FGLa/ASTs inhibit contractions (Chapter 4; Robertson et al., 2012), relaxing the muscle, and allowing the food bolus to remain within a gut region for longer periods of time (Figure 2). This would then potentially increase the ability for nutrient and water reabsorption. In addition, neural stimulation of cockroach rectal longitudinal muscles results in the expulsion of feces (Nagi and Brown, 1969). Thus, it can be postulated that inhibitory or stimulatory signals transmitted through the midgut nerve plexus may also be involved in the regulation of excretory processes.
The midgut nerve plexus is interesting since it receives dual input (from the CNS and the STNS) suggesting integration and coordination of information between the CNS and STNS. Neural information from the CNS and STNS must be coordinated for proper functioning of the midgut related to contraction. Results of neurobiotin retrograde fills of the gastric nerves of the ventricular ganglion revealed a small neuropile within the tritocerebral lobe, whose origin is cells located within the protocerebrum of the brain (Chapter 3; Robertson and Lange, 2010). Thus, connections are made between the CNS and STNS within the protocerebrum and tritocerebrum of the brain, areas where the neural input from the CNS and STNS can be integrated and coordinated for proper midgut contraction.

The neural substrate of the locust foregut and anterior midgut is neurons with cell bodies within the ganglia of the STNS (frontal, hypocerebral and paired ventricular ganglia) (Chapter 3; Robertson and Lange, 2010), and each ganglion contains a central pattern generator (CPG) that directs foregut contraction (Chapter 4; see Ayali and Lange, 2010; Ayali et al., 2002; Zilberstein and Ayali, 2002; Rand and Ayali, 2010; Robertson et al., 2012). These CPGs may interact to coordinate and fine-tune gut contraction and peristalsis, allowing for more efficient digestion and nutrient processing (Rand and Ayali, 2010; Robertson et al., 2012). In addition to the neurons within the STNS, Bräunig (2008) and this thesis (Chapter 3; Robertson and Lange, 2010) also found additional neurons with cell bodies within the brain, subesophageal ganglion (SOG) and thoracic ganglia that project axons within the frontal connectives to enter the STNS and ultimately innervate the foregut and anterior regions of the midgut. This is interesting since there is a CPG within the SOG that coordinates the mouthparts during feeding, while the metathoracic ganglion contains the ventilatory CPG responsible for the ventilatory motor pattern (Rand et al., 2008; Ayali, 2004; Ayali et al., 2002; Zilberstein and Ayali, 2002; Bräunig, 2008; Rast and
Bräunig, 2001; see Ayali and Lange, 2010). There is already evidence of integration between the frontal ganglion motor pattern controlling foregut (pharyngeal) contraction and the SOG motor pattern governing mouthpart coordination (Rand et al., 2008), therefore, all of the aforementioned CPGs may integrate to enable coordination of breathing, ingestion, swallowing, and gut peristalsis.

The FGLa/AST-like immunoreactive cells within the STNS ganglia are not the same as the identified neurons that innervate the foregut and anterior midgut (Chapter 3; Robertson and Lange, 2010). This indicates that these FGLa/AST-containing cells may be considered interneurons and perhaps integrate and coordinate pattern generation for the STNS and hence are involved in CPG modulation. This is supported by the biphasic modulation of the ventricular ganglion motor program by FGLa/ASTs (Figure 2; Chapter 4; Robertson et al., 2012). Prior to this study, little information was known about the role of the ventricular ganglion in the neural control of foregut contraction. The neuromodulation of the ventricular ganglion CPG by FGLa/ASTs is complex, where low concentrations excite the motor pattern and stimulate foregut contraction, while high doses can lead to complete cessation of neural and muscular activity (Figure 2; Chapter 4: Robertson et al., 2012). The coordination of the CPGs associated with each STNS ganglion indicates levels of flexibility and fine control of the foregut motor pattern and ultimately contraction of the foregut that are not fully known or understood. It is still largely unknown how the CPGs in each of the STNS ganglia are coordinated and how the modulation of the motor output by the FGLa/ASTs simultaneously affects the frontal and hypocerebral ganglion CPGs. Research suggests interaction between the CPGs within the frontal and hypocerebral ganglia to coordinate foregut contraction and crop emptying (Rand and Ayali,
2010). It would be interesting to determine if these peptides interact with other peptides to modulate the CPGs to further fine-tune the regulation of gut contraction.
Figure 2. A model based on research in this thesis examining the pleiotropic roles of FGLa/ASTs related to the control of nutrient processing in *Locusta migratoria*. Gut drawing by Zach McLaughlin.
Gut Motility & Digestion

**Foregut**
- inhibit contractions:
  - spontaneous
  - neurogenic
  - proctolin-induced

**Midgut**
- decrease basal tension
- inhibit contractions:
  - spontaneous
  - proctolin-induced

**Hindgut**
- inhibit proctolin-induced contraction
- increase time to reach maximum proctolin-induced contraction
- relax muscle
- less forceful contraction
- decreased peristalsis
- increased time for passage of food
- allows for more efficient digestion of food
- allows for more efficient absorption of nutrients

Food

Neural Control

**Ventricular CPG modulation**
- $10^{-8}$ M
  - ↓ burst duration
  - ↓ interburst interval
  - ↓ cycle period
- $10^{-6}$ M
  - ↓ burst duration
  - ↑ interburst interval
  - ↑ cycle period

Water & Ion Balance

**Inhibition of K⁺ transport**
- decreased K⁺ efflux *in vitro*
- decreased K⁺ absorption
- increased K⁺ excretion
- increased H₂O reabsorption/loss
- diuretic action

- ↑ frequency of action potentials within a burst
- more frequent contractions
GUT MOTILITY AND DIGESTION: FGLa/ASTs as Neurotransmitters, Neuromodulators or Neurohormones

Most FGLa/AST members are myotropic but few have been shown to be truly allatostatic. It has been suggested that a more primitive role for these peptides are as brain/gut peptides based on their localization in the brain and gut coupled with effects on gut physiology, and through evolution were perhaps co-opted to affect JH biosynthesis in a limited number of insect species. The FGLa/AST-like immunoreactivity associated with each region of the gut within processes and varicosities suggests that FGLa/ASTs act as NTs or NMs to affect aspects of gut physiology or as NHs to affect the functioning of additional peripheral targets. The FGLa/AST-like immunoreactivity in midgut endocrine cells suggests an additional role of FGLa/ASTs as hormones.

The FGLa/AST-containing endocrine cells are of the open type, which are in direct contact with the gut lumen by an apical extension (Endo and Nishiitsutsuji-Uwo, 1981; Fujita and Kobayashi, 1977). This contact may allow assessment of the luminal contents and nutrients and thus serve as an interface between the gut and endocrine system (Fujita and Kobayashi, 1977) – forming part of a brain/gut axis that allows information flow and feedback bidirectionally between the gut and CNS. In cockroaches, FGLa/ASTs are released from the midgut into the hemolymph and act as endocrine hormones during feeding and digestion (Yu et al., 1995; Woodhead et al., 1993), providing support for a hormonal role for FGLa/ASTs. The FGLa/ASTs may also be released from endocrine cells to affect processes crucial for digestion, such as regulation of enzyme secretion (Fusé et al., 1999). Other neuropeptides have also been shown to regulate enzyme
secretion, such as myosuppressins (Fusé et al., 1999), suggesting further complexity and integration of communication and function of target tissues to achieve homeostasis.

The FGLa/ASTs not only have central effects by altering neural input via ventricular CPG modulation but also have peripheral effects on the gut muscle of each region (Figure 2; Chapter 4; Robertson et al., 2012). The motility of each gut region must be altered to increase the mixing of the food within that region but also to aid in food passage from one region to the next. Thus, the FGLa/ASTs may be released to increase the efficiency of digestion and nutrient and water absorption by relaxing the gut muscle by inhibiting muscle contraction, allowing the bolus of food to remain within a gut region for a prolonged period of time (Figure 2). It has been suggested that the midgut circular muscles do not have neuromuscular junctions associated with them (Anderson and Cochrane, 1978). Thus, the decrease in basal tonus induced by FGLa/ASTs would suggest that the midgut longitudinal muscles are relaxed (Figure 2; Chapter 4; Robertson et al., 2012). Therefore, the FGLa/ASTs may be released from the midgut endocrine cells into the hemolymph to function as hormones or may be released locally onto the gut to act as NMs on this region of the gut.

The FGLa/ASTs may also be released to affect excretory processes at the hindgut since hindgut proctolin-induced contractions are attenuated by FGLa/ASTs and K⁺ excretion is increased after feeding (Figure 2; Chapters 4 and 5; Robertson et al., 2012). Thus, it seems that FGLa/ASTs have multiple yet simultaneous functions at one or more gut regions that must be coordinated and integrated with the physiological state of the insect to enable proper gut functioning and thus the provision of energy for utilization in physiological processes. The midgut endocrine cells, functioning within the brain/gut axis, may provide this coordination and integration. How the FGLa/ASTs arrive at the gut determines whether these peptides function as NTs, NMs, NHs or
hormones, which also increases the flexibility in neural communication and integration required for homeostatic regulation of physiological processes.

The proper functioning of the locust gut is key to survival, allowing adaptation to food source and environment. The plasticity and functionality of the gut is controlled by neural input, hormonal input and modulation by neuropeptides. By having opposing effects on gut contraction, myostimulators like proctolin and myoinhibitors like the FGLa/ASTs help coordinate and finely tune the regulation of gut motility and the movement of food through the gut. This allows for an increase in the efficiency with which digestion and absorption of nutrients occur, providing energy to be utilized in other physiological processes within the insect. Thus, the regulation of feeding and digestion is complex, stemming from the interaction of CPGs, sensory feedback, nutrient effects, and modulation by neuropeptides.

**WATER AND ION BALANCE: FGLa/ASTs AS NEUROMODULATORS AND NEUROHORMONES**

Insects generally regulate the composition of their hemolymph and body water content over a normal homeostatic range in a variety of aquatic and terrestrial environments. This homeostasis is achieved and influenced by physiological processes such as feeding behaviour, fluid and ion secretion and/or absorption, and elimination. The ability to regulate water and ion homeostasis is fundamental to survival and depends on the coordination of the activities of several tissues.

The excretory system in locusts is comprised of the Malpighian tubules and hindgut, and factors that regulate excretion in insects include peptide families as well as biogenic amines (Coast et al., 2002; Coast, 2009; Orchard, 2009). As outlined above, the inhibitory effect on hindgut contractions elicited by the FGLa/ASTs may be related to an excretory function, where
relaxation of the muscle may allow for more efficient reabsorption of ions and water in this region by increasing the time the food remains in the hindgut (Figure 2; Chapter 4; Robertson et al., 2012). The FGLa/ASTs may also act on the cardiac (between the foregut and midgut) or pyloric (between the midgut and hindgut) sphincters to relax them to allow the food to pass to the next region more easily.

The FGLa/ASTs are implicated to act as diuretics after feeding, where they inhibit K\(^+\) reabsorption (increase K\(^+\) excretion) at the ileum (anterior hindgut) but the mechanism for this inhibition is not yet known (Figure 2; Chapter 4; Robertson et al., 2012). Ion uptake can be activated by increasing ion transporter expression or ion transporter activation, while ion loss can be minimized by decreasing paracellular permeability and suppressing active ion secretion through transporter internalization, protein inactivation by phosphorylation, or suppression of ion transporter expression, where FGLa/ASTs could potentially affect each of these processes. Perhaps the FGLa/ASTs affect transporter dynamics, such as inhibiting the apical electrogenic Cl\(^-\) pump directly, decreasing the driving force for uptake of cations from the lumen into the hemolymph (see Figure 4 Chapter 1). But the FGLa/ASTs must be absorbed through the gut membrane to reach this transporter or be released into the hemolymph from neurohaemal release sites or neurohaemal organs like the CC and CA. Another candidate transporter that may be inhibited could be the Na\(^+\)/K\(^+\)-ATPase located on the basolateral membrane of the gut. But perhaps the FGLa/ASTs inhibit the effect of neuropeptides that act as antidiuretics and stimulate hindgut ion and water reabsorption, such as ITP or the neuroparsins. Salt and water uptake from the locust ileum is stimulated by ITP via cAMP (Phillips et al., 1998) as seen by a stimulation of short circuit current. This stimulation is Cl\(^-\) dependent, where movement of Cl\(^-\) from lumen to hemolymph is increased (Phillips et al., 1998). Chloride exits the cell to the hemolymph
passively through a channel, which allows cations (Na\(^+\), K\(^+\)) to follow passively through separate channels (see Schooley et al., 2012; Phillips et al., 1998). Potassium exits the cell passively into the hemolymph through a channel in the basolateral membrane, while Na\(^+\) is actively removed from the cell via the Na\(^+\)/K\(^+\)-ATPase. Thus, active salt transport drives fluid reabsorption from the locust ileum, which is stimulated fourfold by ITP and cAMP (Phillips et al., 1998; see Schooley et al., 2012). Thus, the inhibitory effect on K\(^+\) efflux \textit{in vitro} by FGLa/AST (Chapter 5) suggests that these peptides decrease K\(^+\) reabsorption (increase K\(^+\) excretion) at the ileum, which may be due to modulation of the action of ITP. Since water follows passively, FGLa/ASTs are implicated as diuretics after feeding to rid the excess K\(^+\) and water obtained from the food. Future experiments examining the interaction of neuropeptides on ion and water transport would be interesting and offer information about the role of neuropeptides in communication and integration of information to achieve homeostasis.

**BRINGING IT ALL TOGETHER**

The occurrence of FGLa/ASTs in several species of insects, even in those species where these peptides do not exhibit allatostatic activity, and their widespread distribution in neural and nonneural tissue, coupled with the widespread myoinhibitory role at the gut and the neuromodulation of feeding and digestion, suggests that perhaps the ancestral role for FGLa/ASTs are as brain/gut peptides (see Gäde and Hoffman, 2005; see Bendena et al., 1999; Robertson and Lange, 2010). Lending support to this argument is the conservation in the C-terminal peptide sequence (FGLa) seen across insects and crustaceans (see Stay and Tobe, 2007). There is also one FGLa/AST peptide sequence (LPVYNFGLa) that is 100% conserved in the species in which it has been examined, which was also sequenced in \textit{L. migratoria} (appendix Chapter 4). The high degree of conservation of the C-terminal sequence alludes to the
importance of FGLa/ASTs in the modulation and control of essential life processes important in homeostasis and the ultimate survival of the insect. The FGLa/ASTs exhibit both central and peripheral effects, as evidenced by the effect on the ventricular ganglion CPG and the effects of the FGLa/ASTs on the CC, CA, and the gut muscle itself. These multiple effects at multiple targets must coordinate to allow proper functioning of physiological processes, and how the FGLa/ASTs contribute to the complex regulation and fine-tuning of the peripheral targets involved in homeostasis emphasizes the flexibility in neural communication to achieve homeostasis.

Understanding the neural control of endocrine hormone release and the neural control of digestion, as well as the association of neuropeptides with these peripheral targets and the role they play in endocrine and gut function is important in understanding the interplay/interaction between the ES and CNS and the flexibility these interactions lend to communication. Thus FGLa/ASTs may have duality in function at peripheral tissues; on modulation of CPGs and neural input to indirectly inhibit contraction by altering the CPG to affect the timing and intensity of contraction or perhaps to even alter the timing and amount of hormone released from the CC or CA, in addition to having direct action on the target tissue itself, such as the direct action of the FGLa/ASTs to inhibit contractions and attenuate contractions stimulated by other neuropeptides such as proctolin.

**Some Future Directions**

The identification of additional native *Locusta* FGLa/ASTs is an important step in better understanding how these peptides affect physiological processes in this locust species. Since members of this peptide family are involved, either directly or indirectly, in homeostasis,
identifying and understanding the members is important in understanding the survivability of this important pest species. FGLa/AST receptors have been identified in other insect species including *Drosophila*, 2 cockroach species and *Bombyx mori* (Birgül et al., 1999; Bowser and Tobe, 2000; Lenz et al., 2000a, 2000b; Secher et al., 2001; Lungchukiet et al., 2008). Thus, future studies could focus specifically on elucidating the receptor distribution on peripheral targets as well as interaction dynamics between the receptor and peptide in *Locusta*. The localization of these receptors may provide clues that help identify the signaling cascade utilized. Members of this peptide family are involved in locust gut peristalsis, but the second messenger pathway for this effect has not yet been elucidated. Future work may attempt to deduce the pathway(s) involved in this effect. This would be important since the FGLa/ASTs have inhibitory effects to other known gut contraction stimulants. Understanding the antagonistic nature of their interactions is important in elucidating the fine control over this important tissue.
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