

Neuropeptide Action in Insects and Crustaceans*

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ABSTRACT

Physiological processes are regulated by a diverse array of neuropeptides that coordinate organ systems. The neuropeptides, many of which act through G protein-coupled receptors, affect the levels of cyclic nucleotides (cAMP and cGMP) and Ca²⁺ in target tissues. In this perspective, their roles in molting, osmoregulation, metabolite utilization, and cardiovascular function are highlighted. In decapod crustaceans, inhibitory neuropeptides (molt-inhibiting hormone and crustacean hyperglycemic hormone) suppress the molting gland through cAMP- and cGMP-mediated signaling. In insects, the complex movements during ecdysis are controlled by ecdysis-triggering hormone and a cascade of downstream neuropeptides. Adipokinetic/hypertrehalosemic/hyperprolinemic hormones mobilize energy stores in response to increased locomotory activity. Crustacean cardioacceleratory (cardioactive) peptide, proctolin, and FMRFamide-related peptides act on the heart, accessory pulsatile organs, and excurrent ostia to control hemolymph distribution to tissues. The osmoregulatory challenge of blood gorging in *Rhodnius prolixus* requires the coordinated release of serotonin and diuretic and antidiuretic hormones acting on the midgut and Malpighian tubules. These studies illustrate how multiple neuropeptides allow for flexibility in response to physiological challenges.

Introduction

The functional diversity of neuropeptides in arthropods is astonishing. These relatively small polypeptides, many of which are less than 9 kDa, control a wide range of physiological processes (reviewed in Gäde et al. 1997; Nässel 2002; Gäde and Marco 2006; Nässel and Homberg 2006; Mercier et al. 2007). The action of many arthropod neuropeptides is mediated by binding to G protein-coupled receptors (GPCRs) and involves cyclic nucleotide and Ca²⁺ second messenger systems (reviewed in Hauser et al. 2006, 2008; Mercier et al. 2007; Zitnan et al. 2007; De Loof 2008; Huang et al. 2008). Here we report recent developments in neuropeptide control of molting, osmoregulation, metabolite utilization, and cardiovascular function that illustrates how these neuropeptides can modify the nervous and endocrine systems toward a new physiological/behavioral state.

Cyclic Nucleotides and Neuropeptide Signaling in the Crustacean Molting Gland

Molting in decapod crustaceans is controlled by the eyestalk X-organ/sinus gland complex, which secretes molt-inhibiting hormone (MIH), a neuropeptide that inhibits ecdysteroid production by a pair of Y-organs (YOs) located in the cephalothorax (Skinner 1985; Chang et al. 1993; Lachaise et al. 1993; Gäde and Marco 2006). The effect of the aptly named MIH on YOs has been investigated in many decapod species, including the European shore (green) crab (*Carcinus maenas*), the blackback land crab (*Gecarcinus lateralis*), and the South African spiny lobster (*Jasus lalandii*; Skinner 1985; Chang et al. 1993; Lachaise et al. 1993; Marco et al. 2000; Lee et al. 2007a). Another eyestalk neuropeptide with MIH activity is the crustacean hyperglycemic hormone (CHH), which is so named for its role in elevating glucose levels in the hemolymph (Webster and Keller 1986; Yasuda et al. 1994; Sefiani et al. 1996; Gäde and Marco 2006; Zarubin et al. 2009). CHH may inhibit molting in response to certain environmental stresses (reviewed in Chang 2005). CHH/MIH-like peptides are not as common in insects. The ion transport peptide is the only CHH-like peptide that has been characterized; it functions by stimulating Cl⁻ transport across the hindgut epithelium (Mercier et al. 2007).

Although there is consensus that cyclic nucleotides mediate the action of MIH on the YOs in vitro, there is disparity about the relative importance of cAMP and cGMP in different crustacean species (reviewed in Spaziani et al. 1999, 2001; Covi et al. 2009; Nakatsuji et al. 2009). MIH induces an increase in cAMP and cGMP, with subsequent activation of protein kinases in YOs in vitro; a small transient increase in cAMP may precede a larger sustained increase in cGMP (reviewed in Covi et al. 2009). In preliminary experiments (H. G. Marco and S. G. Webster, unpublished data), when YOs from intermolt *J. lalandii* were exposed to a crude extract of sinus glands (i.e., a

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Table 1: Effects of phosphodiesterase inhibitor (IBMX), NO donors (SNAP, SE175), and GC-I agonist (YC-1) on ecdysteroid secretion of *Carcinus maenas* and *Gecarcinus lateralis* Y-organs in vitro

Species/Pharmacological Treatment	Concentration (mM)	Treatment (ng)	Control (ng)	<i>n</i>	Control (%)	<i>P</i>
<i>Carcinus maenas</i> :						
IBMX	.5	30.0 ± 3.8	54.6 ± 7.9	10	55 ^a	.0029
SNAP	1	32.0 ± 5.2	50.7 ± 7.4	22	63 ^a	.0259
SNAP/IBMX	1.0/.5	7.4 ± 3.7	56.9 ± 13.3	13	35 ^a	.0138
SE175	.01	68.7 ± 7.2	67.5 ± 8.6	19	102	.848
SE175/IBMX	.01/.5	7.4 ± 1.9	28.8 ± 6.3	8	26 ^a	.0031
SNAP/IBMX/YC-1	1.0/.5/.1	22.6 ± 3.2	48.7 ± 6.1	14	46 ^a	.0002
<i>Gecarcinus lateralis</i> :						
IBMX	.5	21.5 ± 5.1	24.0 ± 6.3	4	90	.8037
SNAP	1	16.5 ± 1.9	15.8 ± 2.1	12	104	.4811
SNAP/IBMX	1.0/.5	25.0 ± 8.8	21.7 ± 1.1	7	115	.7267
SE175	.01	3.1 ± .5	4.6 ± .9	11	66 ^a	.0413
SE175/IBMX	.01/.5	14.0 ± 1.9	16.7 ± 1.7	11	84	.3405
SNAP/IBMX/YC-1	1.0/.5/.1	7.7 ± 1.5	11.3 ± 2.5	6	68 ^a	.022

Note. Total accumulation of ecdysteroid in medium quantified by radioimmunoassay. Data from Covi et al. (2008). NO, nitric oxide; GC-I, NO-dependent guanylyl cyclase.

^a Significant inhibition.

mixture of CHH and MIH) in the presence of IBMX (an inhibitor of cyclic nucleotide phosphodiesterase), (1) a small (threefold) but significant increase of cAMP was measured in the treated YO ($n = 5$, $P < 0.04$) at 2 min; this increase was transient, and at 10 min, cAMP levels were decreasing but were still significantly higher (twofold) than in nontreated YOs ($n = 5$, $P < 0.02$). (2) The concentration of cGMP increased dramatically (85-fold) in treated YOs compared with nontreated YOs, with a maximal level recorded after 30 min ($n = 7$, $P < 0.00005$), and this was sustained (54-fold increase) over (at least) a further 30 min ($n = 5$, $P < 0.02$). In *G. lateralis* and *C. maenas*, both 8Br-cGMP and 8Br-cAMP inhibit ecdysteroid synthesis in YOs (Saïdi et al. 1994; Covi et al. 2008); in *J. lalandii*, these compounds inhibited YO ecdysteroidogenesis between 53% and 58% ($n = 6$; H. G. Marco, unpublished data). These data indicate that both cyclic nucleotides are involved in MIH signaling in these species. Interestingly, YOs from the two crab species differ in their sensitivity to IBMX (Table 1; Saïdi et al. 1994; Covi et al. 2008), which may reflect differences in the relative importance of phosphodiesterases in maintaining cyclic nucleotide levels.

There are two components to ecdysteroidogenesis that are regulated by cyclic nucleotides, namely, constitutive and facultative synthesis (reviewed in Covi et al. 2009). Constitutive synthesis, which is regulated by gene expression and protein synthesis, is a function of YO synthetic capacity and has a response time of minutes to hours; increased constitutive synthesis occurs during premolt or in response to eyestalk ablation (reviewed in Skinner 1985; Lachaise 1993). In contrast, facultative synthesis is regulated allosterically by the phosphorylation of ecdysteroidogenic enzymes and has a response time of minutes. Figure 1 presents a schematic representation of the

probable signaling pathways of CHH and MIH in YOs: (1) cAMP primarily inhibits constitutive synthesis but also inhibits facultative synthesis, either directly or indirectly by way of cGMP; and (2) cGMP inhibits facultative synthesis but not constitutive synthesis (see Covi et al. 2009 for references).

It has been proposed that the inhibition of facultative synthesis by MIH is mediated by a cAMP-dependent activation of nitric oxide synthase (NOS) and NO-dependent guanylyl cyclase (GC-I), both of which are expressed in YOs (Kim et al. 2004; Lee and Mykles 2006; Lee et al. 2007a, 2007b). Moreover, YO activation by eyestalk ablation coincides with the phosphorylation of NOS (Lee and Mykles 2006). In mammals, phosphorylation reduces NOS activity, whereas dephosphorylation by calcineurin stimulates NOS activity (Kone 2001). By implication then, the inactivation of NOS by phosphorylation would be necessary for ecdysteroid synthesis. YO activation also is coincident with increased mRNA levels of GC-I catalytic subunit, an NO-insensitive guanylyl cyclase (GC-III), and ecdysone receptor, whereas GC-II mRNA level is unchanged (S. G. Lee et al. 2007a). These data suggest that YOs can modulate responses to eyestalk neuropeptides by altering GC-I and GC-III expression.

GC-I agonists can inhibit YO ecdysteroidogenesis in blackback land and green crabs. NO donors (SNAP and SE175) inhibit YO ecdysteroid secretion, but *G. lateralis* and *C. maenas* differ in their sensitivities to these compounds (Table 1; Covi et al. 2008). SNAP inhibits *C. maenas* YO but not *G. lateralis* YO, whereas SE175 inhibits *G. lateralis* YO but not *C. maenas* YO. The difference in YO sensitivity to NO donors can be attributed to differences in the mechanism for NO production by SNAP and SE175 and differences in NO-dependent chemical transformations (e.g., oxidation, nitration, S-nitrosation, and

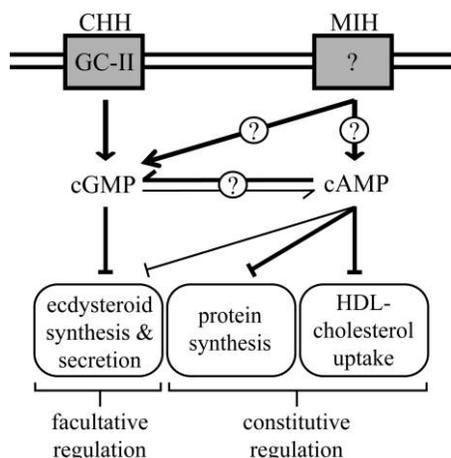


Figure 1. Cyclic nucleotide action in the decapod Y-organ (YO). The neuropeptides, crustacean hyperglycemic hormone (CHH), and molt-inhibiting hormone (MIH) bind to independent receptors on the YO plasma membrane. It appears that CHH binds to a receptor guanylyl cyclase (GC-II) and induces an increase in cGMP, the primary result of which is inhibition of facultative ecdysteroidogenesis. MIH induces an elevation of cGMP and possibly cAMP, but its receptor and link(s) to cyclic nucleotide levels are not conclusively determined. The primary effect of cAMP is the inhibition of constitutive synthesis (protein synthesis and cholesterol uptake); cAMP also inhibits facultative synthesis. Thickness of lines indicates relative strength of action. From Covi et al. (2009).

S-nitrosylation; reviewed in Ridnour et al. 2004; Hess et al. 2005). Inclusion of IBMX enhances inhibition of ecdysteroidogenesis by NO donor in *C. maenas* YOs but has no effect on secretion for *G. lateralis* (Table 1). Although YC-1, an NO-independent activator of GC-I, used alone has no effect in either species, secretion in both species is inhibited by a combination of SNAP, IBMX, and YC-1 (Table 1). This is not unexpected, since NO is required for activation of GC-I by YC-1 (Russwurm et al. 2002; Ott et al. 2004) and endogenous NO production in the YO would be low in the absence of MIH. These data indicate that an NO-sensitive guanylyl cyclase could regulate YO ecdysteroidogenesis. Elevation of NO levels could also control constitutive synthesis by regulating gene expression through Ras/ERK signaling (Imayavaramban et al. 2007), since S-nitrosation can activate Ras and other monomeric GTPases under certain conditions (Raines et al. 2007). It is unclear whether physiologically relevant levels of NO elicit the same responses.

The CHH receptor appears to be a membrane (class II) guanylyl cyclase (Goy 1990; Chung and Webster 2006; Fanjul-Moles 2006). In *C. maenas*, CHH increases cGMP levels in the YO (cAMP was not measured; Chung and Webster 2003). CHH increases cGMP, but not cAMP, in gill and midgut; it elicits a large increase in cGMP and a small increase in cAMP in hindgut (Chung and Webster 2006). High-affinity CHH receptors are present in membrane preparations from YO, hepatopancreas, heart, gill, hindgut, and epidermis (Kummer and Keller 1993; Webster 1993; Chung and Webster 2006). CHH is about 10- to 20-fold less effective as MIH in inhibiting YO ecdysteroidogenesis (Webster and Keller 1986; Chung and Webster 2003),

suggesting that molt inhibition is secondary to its primary function in controlling glucose metabolism in crustacean tissues (Webster and Keller 1986; Yasuda et al. 1994; Sefiani et al. 1996; Chung et al. 1998). There appears to be a single GC-II gene that is expressed as three alternatively spliced isoforms (Lee et al. 2007b). All tissues express at least one isoform, which is consistent with its function as the CHH receptor (Liu et al. 2004; Zheng et al. 2006; Lee et al. 2007a). The wide tissue distribution argues against the notion that the MIH receptor is also a GC-II. Because high-affinity MIH receptors occur only in YO membranes (Webster 1993; Asazuma et al. 2005), one would expect the GC-II to be highly or exclusively expressed in the YO; this is not what is observed. However, a recent study showed that an antibody directed against the extracellular domain of blue crab GC-II blocked MIH repression of YO ecdysteroidogenesis in vitro, suggesting that GC-II is a MIH receptor (Zheng et al. 2008).

In summary, a working hypothesis is that both CHH and MIH inhibit facultative ecdysteroidogenesis by increasing cGMP but through different mechanisms (Fig. 1). Crab YO membranes have distinct receptors for MIH and CHH (Webster 1993; Chung and Webster 2003), and this is supported by structural differences in the surface properties of the two neuropeptides (Katayama et al. 2003). It is likely that MIH and CHH signaling pathways are highly conserved and, therefore, should be the same in all decapods. This is supported by cross activities of MIHs and CHHs among species (Covi et al. 2009; Zarubin et al. 2009). cAMP and cGMP have distinct but overlapping functions in the inhibition of ecdysteroidogenesis by MIH and CHH (Fig. 1). CHH action is most likely mediated directly by production of cGMP by a membrane receptor GC that inhibits facultative synthesis. In contrast, data indicate that both cAMP and cGMP are involved in MIH signaling, in which cAMP initiates a cascade that is amplified by NOS and GC-I.

Neuropeptide Regulation of Insect Ecdysis

Molting is a recurrent event in the life history of arthropods during which complex interplays between ecdysteroid and peptide signals orchestrate transition to the next stage. It begins when ecdysteroid levels surge in response to prothoracicotropic hormone release from the brain (insects) or a drop in MIH release from the eyestalk X-organ/sinus gland complex (crustaceans). The animal immediately stops feeding and undergoes apolysis, whereby the cuticle detaches from underlying epidermal cells. Apolysis involves not only the integument surrounding the animal but also the linings of the respiratory system, foregut, and hindgut. Synthesis of new cuticle ensues, and when complete, the molt is terminated on ecdysis of the old cuticle. A recent review compares the neuroendocrine control of ecdysis in insects and crustaceans (Ayali 2009).

Ecdysis in insects results from performance of stereotypic behaviors initiated and scheduled by a complex peptide signaling cascade. Successive events include tracheal inflation, pre-ecdysis behaviors to loosen the old cuticle, ecdysis behaviors for shedding it, and postecdysis behaviors designed to expand,

harden, and darken the new cuticle (Park et al. 1999, 2002; Zitnan et al. 1999; reviewed in Truman 2005; Zitnan et al. 2007). Early events in the signaling cascade depend on release of ecdysis-triggering hormones (ETHs) from endocrine Inka cells, which respond to rising and falling levels of ecdysteroids in different ways. While elevation of ecdysteroids at the beginning of the molt induces expression of the ETH gene and loading of Inka cells with peptide secretory product, competence to release these peptides requires declining ecdysteroid levels and a subsequent round of gene expression late in the molt (Zitnan et al. 1999; Kingan and Adams 2000). Rising ecdysteroid levels also induce synthesis of ETH receptors in central nervous system (CNS) neurons. The stage is then set for ETH release and termination of the molt by ecdysis. Lack of ETH brought about through either gene excision (Park et al. 2002) or interference with its release (K. H. Cho and M. E. Adams, in preparation) produces lethal ecdysis deficiencies.

Scheduling of ecdysis behaviors involves selective activation of ETH receptor neurons in the CNS. Two subtypes of ETHRs (ETHR-A, ETHR-B) are expressed. Identification of neurons that express ETHR-A has led to new insights about ecdysis control in moths and flies. ETHR-A is expressed in numerous “peptidergic ensembles,” or groups of central neurons implicated in behavior initiation. These include neurons that release eclosion hormone (EH), FMRFamide, kinins, CRF-like diuretic hormones, crustacean cardioactive peptide (CCAP), myoinhibitory peptide (MIP), and bursicon (Kim et al. 2006a, 2006b). In *Manduca sexta*, central L_{3,4} neurons expressing ETHR-A co-release kinins and CRF-like diuretic hormones. Application of this peptide cocktail to the isolated CNS elicits fictive pre-ecdysis-like motor patterns, implicating the L_{3,4} neurons in recruitment of this behavior. Another key ETHR-A ensemble, the IN704 neurons, co-releases CCAP and MIP. Co-application of CCAP and MIP induces fictive ecdysis motor patterns, suggesting that IN704 neurons initiate ecdysis behavior. It is also known that IN704 neurons mobilize cGMP before ecdysis in response to eclosion hormone release (Ewer et al. 1997). Hence, ETH appears to regulate IN704 neurons and ecdysis initiation both directly and indirectly through EH neurons. These experiments implicate ETH in the release of peptide cotransmitters for recruitment of central pattern generators involved in pre-ecdysis and ecdysis behaviors.

Hypothesized roles for ETHR neurons in ecdysis scheduling were tested by monitoring intracellular calcium mobilization during ETH-induced behaviors using transgenic *Drosophila* (Kim et al. 2006b). Peptide promoters were used to drive cell-specific expression of the fluorescent calcium reporter GCaMP in selected ETHR-A peptidergic ensembles. We found that calcium is mobilized sequentially, first in FMRFamide neurons, followed by EH neurons, CCAP/MIP neurons, and finally CCAP/MIP/bursicon neurons. Sequential recruitment of ETHR-A neurons suggests that they are involved in activation of successive behavioral steps during ecdysis. Further hypothesis testing regarding the roles of ETHR-A neurons in ecdysis recruitment now involves cell-specific expression of genes that

may allow for selective activation of candidate ETHR-A neurons to ascertain whether anticipated behaviors are elicited.

In summary, ecdysis behavioral sequences in moths (*M. sexta* and *Bombyx mori*) and flies (*Drosophila melanogaster*) provide favorable models for understanding how ecdysteroids and peptides regulate assembly, activation, and scheduling of neural substrates for behavior.

Adipokinetic Hormones and Control of Energy Metabolism in Insects

A major function of adipokinetic/hypertrehalosemic/hyperprolinemic hormones (AKHs) is to mobilize readily available substrates into the hemolymph from stores in the fat body during periods of high energy demand. This may be locomotory activity in the form of flight or swimming, for example (Gäde et al. 2007). After the hormone is released, it binds to a GPCR and signals through adenylate cyclase or phospholipase C to activate a glycogen phosphorylase or a triacylglycerol lipase, respectively (Gäde and Auerswald 2003). Fat oxidation mainly fuels the contraction of flight muscles in a stink bug (*Nezara viridula*) and a spittle bug (*Locris arithmetica*). Moreover, low doses of the endogenous AKH (in the form of a synthetic peptide) injected into the appropriate donor species results in the mobilization of lipids from the fat body into the hemolymph, which suggests that locomotory energy metabolism is based on fat oxidation.

AKH in insects and red pigment-concentrating hormone (RPCH) in crustaceans are either octa-, nona-, or decapeptides with blocked N- and C-termini (Gäde 2004). A deduced sequence for an RPCH from the cladoceran *Daphnia* (Dappu-RPCH) is rather similar in structure and function to insect AKHs and has no chromatophorotropic activity in shrimps but adipokinetic activity in an insect (Marco and Gäde 2008). A decapod RPCH (Panbo-RPCH) is present in certain pentatomid hemipteran insects (Gäde 2009). To date, more than 40 different AKH structures are known from insects (Gäde 2009). It appears that a number of insects at the base of the evolutionary lineage, Ephemeroptera and certain Odononata, contain the peptide Anaim-AKH (Gäde and Marco 2005; Gäde 2009). At the other side of the spectrum in highly evolved orders, the peptide Aedae-AKH has been isolated in an alderfly (Megaloptera), that is, *Sialis lutaria*, and fully characterized by mass spectrometry (Gäde et al. 2009). This AKH is also presumed present (on the basis of genomic data) in the yellow fever mosquito, *Aedes aegypti*. The propeptide of this AKH has been cloned (Kaufmann et al. 2008).

Peptidergic Control of the Circulatory System of the Stick Insect

Circulatory systems help move nutrients, gases, wastes, and indeed, hormones to and from cells of the body such that their homeostasis can be maintained. The four components of the vertebrate circulatory system are (1) the heart, which is the main propulsive organ; (2) the arterial system, which delivers blood to the tissues; (3) the capillaries, which allow for transfer

of items between the blood and the cells; and (4) the venous system, which is responsible for returning blood to the heart. This intricate grouping of blood vessels allows vertebrate circulation to be highly controlled so that blood is routed and rerouted to specific tissues as demand is increased or decreased. Arthropods generally have an open circulatory system, with the hemolymph (blood) directly bathing the tissues. During circulation in insects, hemolymph typically enters the abdominally located heart (aided by contraction of alary muscles) through incurrent ostia (valves), then travels anteriorly through the aorta (which lies in the thorax), and then enters the head capsule (in some insects, reversal of this flow can occur). Hemolymph then leaves the head capsule and moves through the body cavity, allowing exchange of nutrients and wastes at tissues and the circulation of hormones, before entering the heart again. This “simple” circulatory system is essentially similar to that found in some crustaceans; however, other crustaceans have evolved quite complex distribution systems that use arteries to deliver oxygenated hemolymph to specific tissues, while deoxygenated hemolymph is collected through a complex system of sinuses that deliver the hemolymph to the gills. Following oxygenation, this hemolymph passes from the gills through the branchiocardiac canals, which open into the pericardium (McMahon 2001).

In addition to the main circulatory system incorporating the “dorsal vessel,” insects have accessory pulsatile organs or auxiliary hearts that help with circulation in long appendages, such as the legs, antennae, and wings (reviewed in Pass 2000). Visceral muscle contraction may also participate in circulation; for example, the ovaries of locusts and midgut of *Rhodnius prolixus* are considered accessory pulsatile organs in that their movement allows for hemolymph to be moved throughout the body cavity (Maddrell 1964; Orchard and Lange 1994). Some insects also possess excurrent ostia, which are present on the heart or aorta and allow hemolymph to pass out of the dorsal vessel directly into the sinus around the alimentary canal, the perivisceral cavity (Nutting 1951). It has recently been shown in the Vietnamese stick insect, *Baculum extradentatum*, that these excurrent ostia contain muscle fibers (Ejaz and Lange 2008). The presence of accessory pulsatile organs, along with excurrent ostia, allows the circulatory system to be more efficient and flexible in its flow throughout the body cavity, with the potential of directing hemolymph to tissues in need, reminiscent of that suggested for some crustaceans (McGaw et al. 1994, 1995). This notion of flexibility would also extend to the distribution of hormones. In addition, the hormones themselves might be capable of altering heart rate, accelerating their own circulation, and modifying the excurrent ostia or accessory pulsatile organs and thereby controlling their own microcirculation. What evidence might there be supporting such notions? There are many examples of neuropeptides acting as cardioacceleratory agents on the heart or on an accessory pulsatile organ in arthropods (reviewed in McGaw et al. 1995; McMahon 2001; Nässel 2002). The heart and pulsatile organs may be the final target of the neuropeptide. However, cardioacceleration would inevitably lead to the circulation of the neuropeptides themselves through-

out the body to distant target sites; similarly, there are many examples of these cardioacceleratory peptides having physiological effects on tissues other than the heart in insects (Nässel 2002). Furthermore, on examining the heart/aorta in more detail, one finds a differential distribution of neuropeptides throughout the various parts in insects (Table 2). Thus, immunoreactive processes positive for proctolin, CCAP, and FMRFamide-related peptides (FaRPs) are present within the segmental nerves that project to the dorsal vessel and in processes projecting over the heart, alary muscles, and incurrent ostia (Ejaz and Lange 2008; Lange et al. 2008). Proctolin and FaRPs, but not CCAP-like immunoreactive processes, are also associated with the excurrent ostia (Ejaz and Lange 2008; Lange et al. 2008). This distribution might imply that proctolin, CCAP, and FaRPs might influence contraction of the alary muscles, heart, and incurrent ostia and that proctolin and FaRPs might control the openings of the excurrent ostia. Furthermore, FaRP-like immunoreactivity, but not immunoreactivity associated with proctolin or CCAP, is associated with the lateral cardiac nerve and the lateral cardiac neurons lying along the nerve (Ejaz and Lange 2008; Lange et al. 2008). Thus, FaRPs may play an additional local control in the contraction properties of the heart.

The excurrent ostia in *B. extradentatum* and in the African migratory locust, *Locusta migratoria*, are made up of a mass of cells that produces openings between the heart lumen and the perivisceral cavity (Lange and da Silva 2007). Muscle fibers have been shown to be associated with the excurrent ostia using phalloidin staining (Lange and da Silva 2007; Ejaz and Lange 2008), and electron microscope sections illustrate the presence of nerve processes and, indeed, neuromuscular junctions (R. da Silva and A. B. Lange, unpublished data). These morphological data, along with the immunohistochemical staining of proctolin and FaRP-like processes on the excurrent ostia, provide strong evidence that these excurrent ostia might be neurally controlled but also under modulation by neuropeptides. Opening and closing of these excurrent ostia could potentially result in microcirculatory changes of hemolymph flow.

Physiological assays reveal that CCAP, proctolin, and some FaRPs are cardioacceleratory, increasing heartbeat frequency in a dose-dependent manner (Ejaz and Lange 2008; Lange et al. 2008). One member of the myosuppressin subfamily of FaRPs,

Table 2: Differential distribution of neuropeptides (revealed by immunohistochemistry) within the innervation to the dorsal vessel of *Baculum extradentatum*

	Proctolin	CCAP	FaRPs
Heart	Yes	Yes	Yes
Alary muscles	Yes	Yes	Yes
Incurrent ostia	Yes	Yes	Yes
Excurrent ostia	Yes	No	Yes
Lateral cardiac neurons	No	No	Yes

Note. CCAP, crustacean cardioactive peptide; FaRPs, FMRFamide-related peptides.

SchistoFLRFamide, decreases heartbeat frequency (Lange et al. 2008). Similarly, in crustaceans, these same families of peptides have been shown to control aspects of heartbeat frequency, stroke volume, and distribution of hemolymph (McGaw et al. 1994, 1995). Heartbeat frequency might translate into an increase in circulation volume, and so recently, we established an assay whereby we could monitor the flow of hemolymph along the dorsal vessel and out through the anterior end of the aorta in insects. Using dye, we have observed flow through the incurrent ostia into the heart and then anteriorly along the dorsal vessel. Dye also leaves the dorsal vessel through the excurrent ostia. Preliminary results reveal that the flow of fluid through the dorsal vessel is indeed increased by CCAP and proctolin, although at very high beat rate, this flow is actually decreased (R. da Silva and A. B. Lange, unpublished data). We are using this assay to define the changes in flow of fluid under differing manipulations, in particular under the influence of neuropeptides, and we are also examining whether the flow through the excurrent ostia is modified.

In conclusion, although defined as an open circulatory system, the circulation of hemolymph in insects might be finely controlled at the level of alary muscles, heart, incurrent ostia, and excurrent ostia, using a variety of neuropeptide inputs. Also, quite apart from the possibility that neuropeptides control microcirculation of hemolymph per se is the notion that they are controlling their own microcirculation to their own distant target sites.

Neuroendocrine Control of Fluid Transport in a Blood-Gorging Insect

Numerous studies have shown that osmotic and ionic regulation in arthropods is controlled by the neuroendocrine system (Coast et al. 2002; Chung and Webster 2006). In crustaceans, a wide variety of neuroendocrine tissues has been implicated in salt and water balance. The eyestalk appears to be the most likely source of a true osmoregulatory hormone, with CHH being a strong candidate (Spanings-Pierrot et al. 2000; Chung and Webster 2006). More complete information is available in insects, where the excretory system is composed of the Malpighian tubules and hindgut, which are controlled by diuretic hormones (DHs) and antidiuretic hormones (ADHs; Coast et al. 2002). Water availability in insects is dependent on such things as developmental stage, locomotory activity, and nutritional state, and so water is regulated, with the major site of regulation being the excretory system. Thus, DHs and ADHs are used to control hemolymph volume and composition while allowing nitrogenous wastes, toxic substances, and excess water and salts to be voided. An extreme example of this is shown by *Rhodnius prolixus*, which uses multiple neuroactive chemicals to coordinate diuresis following a blood meal (reviewed in Orchard 2006, 2009). *Rhodnius prolixus* is an obligatory blood feeder, capable of taking blood meals that are 10–12 times their initial body mass. *Rhodnius prolixus* then enters a phase whereby the nutrient component of the blood meal is concentrated within the anterior midgut (AMG) and excess H₂O and

NaCl are eliminated (Fig. 2). Thus, H₂O and NaCl are absorbed across the epithelium of the AMG into the hemolymph, and H₂O, NaCl, and KCl are secreted across the epithelium of the upper Malpighian tubules (MTs) from the hemolymph into the lumen. This urine is then modified by reabsorption of KCl into the hemolymph across the epithelium of the lower MTs. The modified urine, hypo-osmotic to the hemolymph and rich in NaCl, is emptied into the hindgut, where it is periodically expelled without further modification. Diuresis in *R. prolixus* is very rapid, with ion transport stimulated 1,000-fold across the upper MTs and the insect excreting a volume of fluid equivalent to 10 times the hemolymph volume within 3 h of feeding (for details, see Maddrell 1976, 1991).

Salt and H₂O homeostasis must be tightly regulated in the face of such a large blood meal and the massive movement of fluid, and one can imagine that there must be a precise match between fluid transport across the AMG and the MTs. How, then, are these tissues coordinated such that they are biased toward a new physiological state that preserves volume as well as ionic and osmotic balance of the hemolymph? The answer lies in the coordinated actions of DHs represented by serotonin and neuropeptides and ADHs released into the hemolymph and acting in concert on AMG and MTs (reviewed in Orchard 2009).

Serotonin is released into the hemolymph from dorsal unpaired median neurons that lie within the mesothoracic ganglionic mass (MTGM) and that produce neurohemal sites on abdominal nerves. The peak titer of serotonin (115 nM) is reached within 5 min of the start of gorging (Lange et al. 1989) and is capable of stimulating absorption of H₂O and NaCl from the lumen of the AMG and secretion of H₂O, NaCl, and KCl by the upper MTs (Farmer et al. 1981). At the same time, serotonin acts on the lower MTs to stimulate reabsorption of KCl (Table 3; reviewed in Orchard 2006, 2009). By the time gorging is completed, the serotonin titer has dropped to 40 nM and may not be of sufficient concentration to act on the AMG or upper MTs (but may still be of sufficient titer to stimulate the lower MTs). Serotonin appears to act through cAMP in AMG and MTs (Orchard 2009). Diuresis, however, persists for 3 h following gorging, and this diuresis can be attributed to the release of at least one peptidergic DH, a CRF-related peptide, Rhopr-DH (Te Brugge et al. 1999; V. A. Te Brugge, personal communication). This peptidergic DH is synthesized and released from posterior lateral neurosecretory cells of the MTGM, which have neurohemal sites on abdominal nerves. Rhopr-DH elevates cAMP content of AMG and upper MTs and stimulates absorption across the AMG and secretion by the upper MTs (V. A. Te Brugge, personal communication; Table 3). Rhopr-DH does not stimulate reabsorption of KCl from the lower MTs (Donini et al. 2008), which can still be stimulated by the remaining titer of serotonin.

Interestingly, serotonin is colocalized with a calcitonin-related DH, Rhopr-DH₃₁, and Rhopr-DH₃₁ is colocalized with a kinin-like DH, Rhopr-K (Te Brugge et al. 2001, 2005, 2008). However, neither Rhopr-DH₃₁ nor Rhopr-K can stimulate absorption across the AMG (V. A. Te Brugge, personal com-

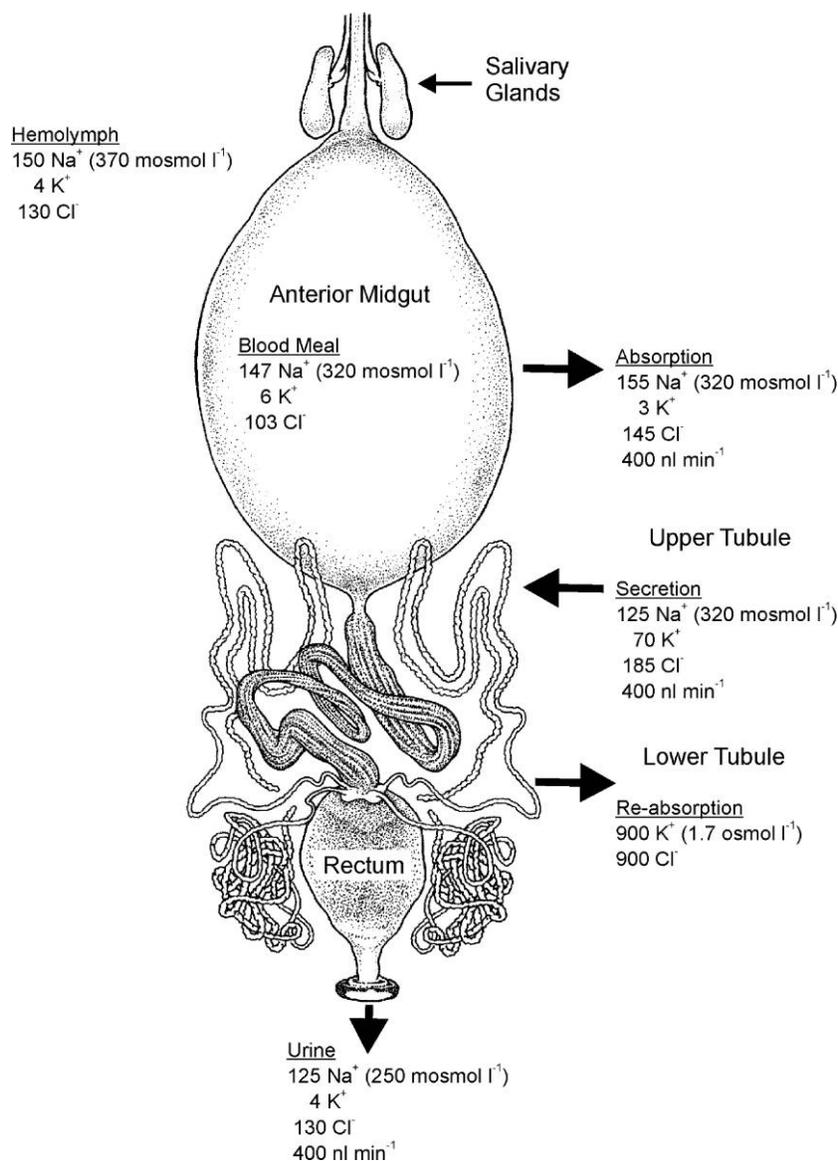


Figure 2. Osmotic and ionic concentrations and fluid transport in fifth instar *Rhodnius prolixus* in response to a blood meal. Based on Maddrell (1976). Drawing by Zach McLaughlin.

munication), and neither is very active at stimulating secretion by the upper MTs. All of the colocalized factors, however, act as myoactive agents by increasing the frequency of contractions of muscles of the AMG and the hindgut (V. A. Te Brugge, personal communication) and so would appear to be involved in a range of physiological processes associated with feeding.

Diuresis may be terminated in a number of ways. The obvious one is that the DHs cease being released and their hemolymph titer drops below threshold levels. Alternatively, or in addition, an ADH might be released that stops absorption from the AMG and secretion by the MTs. There is now growing evidence that *R. prolixus* does indeed use an ADH. Thus, one of the peptides from the CAPA gene, Rhopr-CAPA-2 (a CAP₂₆-related peptide), appears to be released from ventral-paired median neurosecretory cells of the MTGM that have neuro-

hemal sites on abdominal nerves (Paluzzi and Orchard 2006; Paluzzi et al. 2009). Rhopr-CAPA-2 inhibits serotonin and Rhopr-DH-stimulated absorption across the AMG and secretion by the MTs (Paluzzi et al. 2009; J.-P. Paluzzi, personal communication; Table 3). Thus, Rhopr-CAPA-2 might well be an ADH in *R. prolixus*, rapidly inhibiting postprandial diuresis. The mode of action of Rhopr-CAPA-2 is unclear but might well involve interplay between levels of cGMP and cAMP (J.-P. Paluzzi and J. Ianowski, personal communication).

Conclusion

Growth, energy metabolism, fluid transport, and hemolymph circulation require the coordinated and precisely timed release of multiple neuropeptides that allow for flexibility in messaging.

Table 3: Involvement of diuretic and antidiuretic hormones following blood feeding in *Rhodnius prolixus*

	Anterior Midgut	Upper MTs	Lower MTs
Serotonin	Increases cAMP and stimulates absorption	Increases cAMP and stimulates secretion	Increases cAMP and stimulates reabsorption
Rhopr-DH	Increases cAMP and stimulates absorption	Increases cAMP and stimulates secretion	No effect on reabsorption
Rhopr-CAPA-2	Inhibits absorption in stimulated preparations	Inhibits secretion in stimulated preparations	Not determined

Note. MTs, Malpighian tubules; Rhopr-DH, *Rhodnius prolixus* CRF-related diuretic hormone; Rhopr-CAPA-2, *R. prolixus* CAP_{2b}-related peptide.

Since many arthropod neuropeptides bind to GPCRs and a receptor binds a specific ligand, neuropeptide diversity can be estimated from genomic analysis. For example, the completed genomes of three insect species reveal a large number of neuropeptide GPCRs: 48 in flour beetle (*Tribolium castaneum*), 45 in fruit fly (*Drosophila melanogaster*), and 35 in honeybee (*Apis mellifera*; Hauser et al. 2006, 2008). The neuropeptide ligands for many of these receptors have been identified (Hauser et al. 2006, 2008; Nässel and Homberg 2006; Li et al. 2008). An analysis of expressed sequence tags reveals the presence of related neuropeptides in crustaceans (Christie et al. 2008; Gard et al. 2009). In decapod crustaceans, MIH and CHH inhibit molting in response to environmental conditions or stress by activating signaling pathways that increase cAMP and cGMP. The CHH receptor appears to be a membrane GC, but the MIH receptor remains unidentified. A neuropeptide cascade, triggered by the release of ETH by Inka cells, coordinates the stereotyped movements during exuviation in insects (Zitnan et al. 2007). Short neuropeptides that signal through GPCRs and increase intracellular levels of cAMP, Ca²⁺, and IP₃ activate phosphorylases and lipases and are, hence, responsible for mobilizing the circulating metabolites trehalose, diacylglycerols, and proline that are needed for intense locomotory activity, such as flight. Neuropeptides, acting through GPCRs, control circulation by affecting the organs that move and distribute the hemolymph (e.g., heart, alary muscles, and ostia); CCAP, proctolin, and some FaRPs are cardioacceleratory, whereas some FaRPs (e.g., myosuppressin) are inhibitory. The diuretic process in *Rhodnius prolixus* is another illustration of how multiple neurohormones, colocalized in discrete neurosecretory cells, enable the animal to cope with osmotic challenges associated with gorging on massive blood meals. Thus, the diversity of neuropeptides, often in concert with biogenic amines and ecdysteroids, enables arthropods to fine-tune the timing and physiological responsiveness of tissues under differing challenges.

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