Localization and expression of molt-inhibiting hormone and nitric oxide synthase in the central nervous system of the green shore crab, *Carcinus maenas*, and the blackback land crab, *Gecarcinus lateralis*

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ABSTRACT

In decapod crustaceans, molting is controlled by the pulsatile release of molt-inhibiting hormone (MIH) from neurosecretory cells in the X-organ/sinus gland (XO/SG) complex in the eyestalk ganglia (ESG). A drop in MIH release triggers molting by activating the molting gland or Y-organ (YO). Post-transcriptional mechanisms ultimately control MIH levels in the hemolymph. Neurotransmitter-mediated electrical activity controls Ca^{2+} -dependent vesicular release of MIH from the SG axon terminals, which may be modulated by nitric oxide (NO). In green shore crab, *Carcinus maenas*, nitric oxide synthase (NOS) protein and NO are present in the SG. Moreover, *C. maenas* are refractory to eyestalk ablation (ESA), suggesting other regions of the nervous system secrete sufficient amounts of MIH to prevent molting. By contrast, ESA induces molting in the blackback land crab, *Gecarcinus lateralis*. Double-label immunofluorescence microscopy and quantitative polymerase chain reaction were used to localize and quantify MIH and NOS proteins and transcripts, respectively, in the ESG, brain, and thoracic ganglion (TG) of *C. maenas* and *G. lateralis*. In ESG, MIH- and NOS-immunopositive cells were closely associated in the SG of both species; confocal microscopy showed that NOS was localized in cells adjacent to MIH-positive axon terminals. In brain, MIH-positive cells were located in a small number of cells in the olfactory lobe; no NOS immunofluorescence was detected. In TG, MIH and NOS were localized in cell clusters between the segmental nerves. In *G. lateralis*, *Gl-MIH* and *Gl-crustacean hyperglycemic hormone (CHH)* mRNA levels were $\sim 10^5$ -fold higher in ESG than in brain or TG of intermolt animals, indicating that the ESG is the primary source of these neuropeptides. *Gl-NOS* and *Gl-elongation factor (EF2)* mRNA levels were also higher in the ESG. Molt stage had little or no effect on *CHH*, *NOS*, *NOS-interacting protein (NOS-IP)*, membrane *Guanylyl Cyclase-II (GC-II)*, and NO-independent *GC-III* expression in the ESG of

both species. By contrast, *MIH* and *NO receptor GC-I beta subunit (GC-I β)* transcripts were increased during premolt and postmolt stages in *G. lateralis*, but not in *C. maenas*. MIH immunopositive cells in the brain and TG may be a secondary source of MIH; the release of MIH from these sources may contribute to the difference between the two species in response to ESA. The MIH-immunopositive cells in the TG may be the source of an MIH-like factor that mediates molt inhibition by limb bud autotomy. The association of MIH- and NOS-labeled cells in the ESG and TG suggests that NO may modulate MIH release. A model is proposed in which NO-dependent activation of GC-I inhibits Ca²⁺-dependent fusion of MIH vesicles with the nerve terminal membrane; the resulting decrease in MIH activates the YO and the animal enters premolt.

1. INTRODUCTION

Growth in decapod crustaceans is characterized by the shedding of the exoskeleton in a process known as molting or ecdysis. Immediately after ecdysis, the new exoskeleton expands to increase the interior space for tissue growth. Steroid molting hormones (ecdysteroids) are synthesized by a pair of Y-organs (YO) and coordinate the physiological processes required for successful ecdysis (for reviews see Chang and Mykles, 2011; Lachaise et al., 1993; Skinner, 1985). Ecdysteroid synthesis is negatively regulated by molt-inhibiting hormone (MIH), a neuropeptide produced in the X-organ/sinus gland (XO/SG) complex of the eyestalk ganglia (ESG) (for reviews see Hopkins, 2012; Skinner, 1985; Webster, 2015b). Molting is initiated by a reduction in MIH secretion by the XO/SG complex. The decrease in MIH in the hemolymph activates the YO, which increases ecdysteroidogenesis and drives the transition from intermolt to premolt stages (for reviews see Chang and Mykles, 2011; Covi et al., 2012; Mykles, 2011).

Neurotransmitter-mediated electrical activity controls the release of neuropeptides from the XO/SG complex. Synaptic vesicles transport MIH, crustacean hyperglycemic hormone (CHH), and other neuropeptides from cell bodies in the XO and brain along axonal tracts that terminate in the SG, where neuropeptides are released into the hemolymph (for reviews see Bliss and Welsh, 1952; García and Aréchiga, 1998; Hopkins, 2012; Skinner, 1985). Within the SG, the axons are highly branched and are supported by a matrix of astrocyte-like glial cells (Dirksen, 1992; reviewed by García and Aréchiga, 1998). Neuropeptide secretion from SG axon terminals requires Ca^{2+} influx through voltage-gated Ca^{2+} channels; a negative feedback loop inhibits secretion after protracted channel activation (Cooke, 1985; Cooke and Sullivan, 1982; Keller et al., 1994; Meyers and Cooke, 1997; Richmond et al., 1996).

NO modulates synaptic transmission in the central nervous system (CNS) of decapod crustaceans. Presynaptic depression at neuromuscular junctions is mediated by NO (Aonuma et al., 2000). Two classes of interneurons of the terminal abdominal ganglion respond differently to NO; stimulation of NO/cGMP signaling enhances synaptic transmission in Class 1 interneurons, while NO/cGMP signaling depresses transmission in Class 2 interneurons (Aonuma, 2002; Aonuma and Newland, 2001, 2002). NO donors inhibit synaptic transmission of interneurons in the olfactory lobes of the brain (Johansson and Mellon, 1998). NO may also modulate neuropeptide release from the XO neurons. NOS protein is localized in the SG of crayfish (*Procambarus clarkii*) and green crab (*Carcinus maenas*) and NO is produced in the SG of *C. maenas* (Lee et al., 2000; Pitts and Mykles, 2015). The distribution of NO-FL fluorescence in the SG suggests that NOS may be localized in the glial cells (Pitts and Mykles, 2015). As the half-life of NO is seconds in aqueous solutions (for reviews see Garthwaite, 2016; Moncada and Bolanos, 2006), the close proximity of NO production, as indicated by NO-FL fluorescence, to the SG axon terminals supports the hypothesis that NO regulates neurosecretion (Pitts and Mykles, 2015). An NO-binding protein in the SG releases NO over an extended period, thus potentially prolonging the effects of NO on the neurosecretory axons (Pitts and Mykles, 2015).

The long-accepted view is that MIH synthesis and secretion is restricted to the ESG (for reviews see Hopkins, 2012; Skinner, 1985; Webster, 2015b). However, recent studies report that MIH mRNA and peptide are present in other regions of the CNS, such as the brain, optic nerve, ventral nerve cord, and thoracic ganglion (TG) (Abuhagr et al., 2014; Chan et al., 1998; Chang et al., 1999; Lu et al., 2001; Stewart et al., 2013; Sun, 1995; Tiu and Chan, 2007; Zhu et al., 2011). Extra-eyestalk sources of MIH provide a plausible explanation for why, in some species, the removal of the eyestalks does not induce molting. Adult *C. maenas* are refractory to eyestalk

ablation (ESA) and MIH mRNA is detected in the brain and TG (Abuhagr et al., 2014). By contrast, ESA induces molting in the blackback land crab, *Gecarcinus lateralis* (Abuhagr et al., 2016; Covi et al., 2010). In the TG of *Portunus pelagicus*, MIH is localized to cell clusters that are adjacent to the sternal artery, suggesting that MIH synthesized in the TG is directly released into the hemolymph (Stewart et al., 2013). Physiological studies also indicate that limb autotomy factor - proecdysis (LAF_{pro}) is an MIH-like peptide in secondary (2°) limb regenerates (Yu et al., 2002). LAF_{pro} is thought to mediate the suspension of premolt processes by limb bud autotomy (for reviews see Chang and Mykles, 2011; Mykles, 2001). Taken together, there is growing evidence that auxiliary sources of MIH and MIH-like factors can have important roles in molt regulation.

The aim of this study was to compare the localization of MIH and NOS proteins and the mRNA levels of MIH, NOS, and NO/cGMP pathway genes in the CNS of two decapod species, *C. maenas* and *G. lateralis*, that respond differently to ESA. Double-label immunofluorescence microscopy was used to localize MIH and NOS proteins in the same sections of the ESG, brain, and TG. A novel NOS interacting protein (Gl-NOS-IP), identified from a *G. lateralis* YO transcriptome (Das et al., 2016), was characterized. Quantitative PCR (qPCR) was used to quantify the transcript levels of *Gl-MIH*, *Gl-CHH*, *Gl-NOS*, and *Gl-elongation factor 2 (EF2)* in the ESG, brain, and TG of intermolt animals. The effects of molt stage on *Gl-MIH*, *Gl-CHH*, *Gl-NOS*, *Gl-NOS-IP*, NO receptor guanylyl cyclase (GC) beta subunit (*Gl-GC-Iβ*), membrane receptor GC (*Gl-GC-II*), and soluble NO-independent GC (*Gl-GC-III*) expression in the ESG were also quantified by qPCR.

2. MATERIALS AND METHODS

2.1. Animals

Adult male *G. lateralis* were collected in the Dominican Republic, shipped to Denver, CO, and maintained at ~27 °C in ~85% relative humidity on a 12 h:12 h light:dark cycle (Covi et al., 2010). Molting was induced by multiple leg autotomy (MLA); progress through premolt was monitored by the growth of limb regenerates (R index = regenerate length x 100/carapace width (Covi et al., 2010; Yu et al., 2002). Intermolt animals had an R index of 8-12.9; early premolt (stage D₀), R = 13-15.9; mid premolt (stage D₁), R = 16-18.9; and late premolt (stage D₂), R = 19-22 (Covi et al., 2010).

Adult male *C. maenas* were collected from Bodega Harbor, Bodega Bay, CA and maintained in the flowing sea water system at the University of California Davis Bodega Marine Laboratory at ambient temperature (12-15 °C).

Hemolymph ecdysteroid titer was quantified using a competitive enzyme-linked immunoassay (Abuhagr et al., 2014; Kingan, 1989).

2.2. Double-label immunofluorescence microscopy

A polyclonal antibody was raised in rabbits against a 14-mer peptide sequence near the N-terminus of the MIH mature protein (residues #7 to #20; GenBank accession DQ473354) by Pacific Immunology Corp. (Ramona, CA, USA). The antibody reacted with a ~7-kDa protein in Western blots of SG extracts from *C. maenas* and *G. lateralis* (data not shown). ESG, TG, and brain (supraesophageal ganglion) from *G. lateralis* and *C. maenas* were fixed in 4% paraformaldehyde for ~24 h at room temperature (RT), dehydrated in an ethanol and xylene series, and embedded in paraffin. Sections (12 µm) were affixed to OptiPlus™ Positive-Charged

Microscope Barrier Slides (BioGenex, Fremont, CA, USA) by heating overnight at ~35 °C, deparaffinized in xylenes, and rehydrated in an ethanol series. Slides were placed in pre-boiled 0.01 M sodium citrate buffer (pH 6) and heated in a pressure cooker for 3 min. Sections were blocked in 2% goat serum at 4 °C for 1 h and incubated overnight at 4 °C in the first primary antibody (1:100 anti-*G. lateralis* MIH peptide), washed in Tris-buffered saline plus 0.05% Tween-20 (TBST; 5 mM Tris-HCl, pH 7.4, and 150 mM NaCl), and then incubated 1 h at RT in the first secondary antibody (1:100 Alexa Fluor488 conjugated to the F(ab')₂ fragment; ThermoFisher Scientific, Grand Island, NY, USA). This was followed by overnight incubation at 4 °C in the second primary (1:50 anti-universal NOS; ThermoFisher Scientific), washed in TBST, and incubated 1 h at RT in the second secondary antibody [1:5000 Alexa Fluor555; ThermoFisher Scientific; Lewis Carl et al., 1993). Slides were mounted with Vectasheild 4'6'-diamindino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingam, CA, USA) and sealed with nail polish. Every third section was stained with hematoxylin and eosin Y (H&E) to image tissue structure.

H&E images at 5x and 40x were captured with a Leica CTR 5500 microscope equipped with a Leica DFC 450 camera. Low (10x) and high (40x) fluorescent images were captured with a Zeiss Axiovert 200M microscope equipped with a Hamamatsu ORCA-ER cooled charge-coupled device camera (Olympus America, Inc. Melville, NY, USA) at 350 nm excitation, 460 nm emission for DAPI (500 ms); 570 nm excitation, 590 nm emission for Rhodamine Red (Alexa555, 1500 ms); and 494 nm excitation, 518 nm emission for FITC (Alexa488, 500 ms).

2.3. Quantification of gene expression in *G. lateralis*

RNA was isolated from ESG, brain, and TG using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacture's protocol (for detailed protocol see Pitts and Mykles, 2015). cDNA was synthesized with Transcriptor Reverse Transcriptase (Roche Diagnostics, Indianapolis, IN, USA) and an oligo-dT primer (50 µmol).

A Light Cycler 480 thermal cycler (Roche) was used to quantify levels of *Gl-EF2* (GenBank accession AY552550), *Gl-GC-Iβ* (DQ355434), *Gl-GC-II* (DQ355437), *Gl-GC-III* (DQ355438), *Gl-NOS* (AY552549), *Gl-NOS-IP* (KU214505; c141319_g1_i1; Das et al., 2016), *Gl-MIH* (DQ473354), *Gl-CHH* (DQ473354), *Cm-EF2* (GU808334), *Cm-MIH* (X75995), *Cm-CHH* (X17596), *Cm-NOS* (GQ862349), *Cm-GC-Iβ* (JQ911525), *Cm-GC-II* (JQ911527), and *Cm-GC-III* (JQ911526). For all genes except *Gl-NOS*, *Gl-MIH*, and *Cm-NOS*, SYBR green was used to quantify mRNA in both species. Reactions (10 µL) consisted of 5 µL 2x SYBR Green I Master mix (Roche), 0.5 µL each of 10 mM forward and reverse primers (Table 1 or see Lee et al., 2007b for *Gl-GC-Iβ*, *Gl-GC-II* and *Gl-GC-III* primer sequences; Lee et al., 2007a for *Gl-CHH* primer sequences; McDonald et al., 2011 for *Cm-EF2* primer sequences; and Abuhagr et al., 2014 for *Cm-GC-II* and *Cm-GC-III* primer sequences), 3 µL nuclease-free water, and 1 µL cDNA or PCR product standard. *Gl-NOS*, *Cm-NOS*, and *Gl-MIH* were quantified using universal Probe #17 (NOS) and #13 (MIH) (Roche). Reactions (10 µL) for *Gl-NOS*, *Gl-MIH*, and *Cm-NOS* contained 5 µL LightCycler 480 Probes Master (Roche) 0.2 µL each of 20 mM forward and reverse primers (Table 1), 0.2 µL universal Probe #17 or #13, 1.9 µL nuclease free water, and 2.5 µL of cDNA or PCR product. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min. For each gene, a series of dsDNA standards were produced by 10-fold serial

dilutions and transcript concentrations calculated with the LightCycler 480 software (Roche, version 1.5). Transcript copy numbers per μg total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the molecular weight of the dsDNA products.

2.4. Data analysis and software

Statistical analysis was performed using JMP version 12.0 (SAS Institute, Inc., Cary, NC, USA) and graphs were generated with SigmaPlot version 12.0 (Systat Software Inc., San Jose CA, USA). All qPCR data was log transformed to reduce variances from the mean. A Brown-Forsythe test ($p < 0.05$) confirmed the variances among log-transformed data were equivalent. Means for transcript abundance were compared using an analysis of variance (ANOVA) and *post-hoc* multiple comparisons were made using Tukey-Kramer HSD tests. A paired t-test was used to compare the means for transcript abundance between intact and other molt stages. Data are presented as mean $\pm$ 1 S.E. and the level of significance was set at $\alpha = 0.05$. Fold changes indicated in the text were calculated based on transcript copy number for each gene.

3. RESULTS

3.1 Immunofluorescent localization of MIH and NOS in the CNS

The nomenclature from Stewart et al. (2013), Krieger et al. (2012), and Sandeman et al. (2015) was used for the identification of anatomical regions and cellular features of the *C. maenas* and *G. lateralis* eyestalk ganglia, brain, and thoracic ganglion. The only difference is that we used a separate numbering of the cell clusters for each organ; Stewart et al. (2013) used a continuous numbering system.

The ESG is organized into the medulla terminalis (MT), medulla interna (MI), and the medulla externa (ME) neuropils (Fig. 1). The XO consists of a cluster of cell bodies located in the MT. Nerve fibers from the XO terminate in the sinus gland (SG) located lateral to the MI/ME junction (Fig. 1). The SG has strong eosin Y staining due to high concentrations of neuropeptides and the presence of few nuclei. Both MIH and NOS were localized in the SG of *G. lateralis* (Fig. 2A) and *C. maenas*, (Fig. 2B). The MIH (green) and NOS (red) fluorescence overlapped in some areas, producing a yellow color in the merged images. This indicated that MIH and NOS were localized in close proximity in the 12- μ m sections. Confocal microscopy showed that MIH was present in the axons and NOS was present cells adjacent to the MIH-immunopositive cells in the SG of *G. lateralis* (Fig. 3). NOS immunofluorescence was diffuse, indicating that NOS was distributed throughout the cytoplasm of these cells. Control sections incubated with either pre-immune serum (MIH) or buffered saline without primary antibody (NOS) showed no immunofluorescence (data not shown).

The TG controls the movement of the appendages. It is a bilaterally symmetrical structure fused with the subesophageal ganglion at the anterior end and the abdominal ganglia (AG) at the posterior end (Fig. 4). There are five pairs of segmental nerves (sn1-5) that innervate the claws and walking legs (Fig. 4). Five cell clusters are positioned along the midline (Fig. 4, clusters #1 to #5 numbered from the anterior to posterior). Cell cluster #1 in the subesophageal ganglion contains a pair of giant neurons (Fig. 4, inset; Matsumoto, 1954). Seven cell clusters are positioned laterally between the segmental nerves (Fig. 4, clusters #6 to 12). Posteriorly, there is a pair of cell clusters located between sn5 and the AG (Fig. 4, cluster #13) and a pair of cell clusters lateral to the AG and abdominal nerve (Fig. 4, cluster #14).

MIH and NOS immunopositive cells were identified in the lateral cell clusters located between the segmental nerves (clusters #6 -#12) and cell cluster #1. MIH and NOS was localized adjacent to the giant neurons in both species (Fig. 5; arrows). Figure 5A shows the images of cluster #12, located between sn4 and sn5, in *G. lateralis* and Figure 5B shows images of cluster #11, located between sn3 and sn4, in *C. maenas*. There were fewer MIH immunopositive cells than NOS-immunopositive cells in the TG of both species (Fig. 5). There was close association of the MIH and NOS immunofluorescence in some of the cells (Fig. 5, arrowheads); other cells were labeled with either the MIH (green) or NOS (red) antibodies (Fig. 5).

The brain or supraesophageal ganglion is divided into the protocerebrum, deutocerebrum, and tritocerebrum, from anterior to posterior; the brain architecture of *C. maenas* is described in more detail by Krieger et al. (2012). A pair of large olfactory lobes are positioned laterally (Fig. 6, OL). The protocerebrum projects by way of the optic nerve (optn) to the ESG to innervate the medulla terminalis (Fig. 6; Sandeman, 1982). The protocerebrum is divided by the central body (CB) into the anterior-medial protocerebral neuropils (AMPN) and the posterior-medial protocerebral neuropils (PMPN) (Fig. 6). The deutocerebrum innervates the first pair of antennae and contains the median antenna I neuropil (MAN), OL, and the accessory lobes (AL) (Fig. 6). The tritocerebrum innervates the second pair of antennae and contains the antenna II neuropils (Fig. 6, AnN). There are nine cell clusters in the brain (Fig. 6). Cell cluster #1, which is located along the midline anterior to the AMPN, contains a diverse cell population that includes giant neurons (Stewart et al., 2013). A pair of cell clusters (#2 and #6) is associated with the OL; a pair of cell clusters (#3 and #4) is located in the tritocerebrum; and a pair of cell clusters (#5) is located posterior to the AL (Fig. 6). MIH immunofluorescence was restricted to a few cells in the

OL in both species (Fig. 7). None of the cells in the cell clusters were MIH immunopositive. NOS immunofluorescence was not detected in the brain regions of either species (Fig. 7).

3.2. Expression of neuropeptides and NO/cGMP signaling genes in CNS tissues

The mRNA levels of *Gl-MIH*, *Gl-CHH*, *Gl-NOS*, and *Gl-EF2* in brain, TG, and ESG from intermolt *G. lateralis* were quantified by qPCR. The transcript levels of all four genes were highest in the ESG and lowest in the TG, but the magnitude differences varied among the four genes (Fig. 8). As expected, expression of both neuropeptides was higher in the ESG compared to the brain and TG. The *Gl-MIH* mRNA level in the ESG was 1.5×10^5 -fold greater than that in the brain and 7.8×10^5 -fold greater than that in the TG (Fig. 8). The *Gl-CHH* mRNA level in the ESG was 7×10^5 -fold greater than that in the brain and 8.5×10^5 -fold greater than that in the TG. Expression of both neuropeptides was also significantly higher in the brain compared to the TG (MIH, 5-fold; CHH, 12.2-fold). *Gl-NOS* mRNA level was 11-fold higher in the ESG than that in the brain and 133-fold higher than that in the TG. *Gl-NOS* was 12-fold greater in the brain compared to the TG (Fig. 8). *Gl-EF2* expression was highest in the ESG (4.8×10^2 -fold greater than the brain, 3.3×10^3 -fold greater than the TG). The *Gl-EF2* level in the brain was 7-fold higher than that in the TG.

There were significant effects of molt stage on gene expression in the ESG from *G. lateralis*. The hemolymph ecdysteroid titers were 6 pg/ μ L for intermolt (stage C₄) animals, 26 pg/ μ L for early premolt (stage D₀; R index ~14), 70 pg/ μ L for mid premolt (stage D₁; R index ~17), 104 pg/ μ L for late premolt (stage D₂; R index ~21), and 2 pg/ μ L for 10 days postmolt (stage B). The mRNA levels of *Gl-MIH*, *Gl-GC-IB*, and *Gl-EF2* were significantly greater at premolt and postmolt stages than those at intermolt (Fig. 9A). *Gl-MIH* mRNA level was

significantly higher at early premolt (by 49-fold), mid premolt (by 172-fold), and post molt (by 44-fold) compared to intermolt animals. *Gl-MIH* mRNA level at mid premolt was significantly higher (by ~17-fold) than the levels at the other molt stages. *Gl-GC-I β* and *Gl-EF2* mRNA levels at the premolt and post stages were ~180-fold and ~800-fold, respectively, higher than the levels at intermolt (Fig. 9A). Other genes displayed molt stage-specific increases in expression. *Gl-CHH* and *Gl-GC-II* mRNA levels were ~70-fold and ~150-fold higher, respectively, at mid premolt than those at intermolt (Fig. 9A). The *Gl-GC-III* mRNA level was increased 3.6×10^3 -fold at postmolt compared to intermolt animals. A contig encoding an NOS interacting protein (Gl-NOS-IP) was obtained from an analysis of the *G. lateralis* YO transcriptome (Das et al., 2016) (Supplementary Fig. 1). There was no significant effect of molt stage on either *Gl-NOS-IP* or *Gl-NOS* mRNA levels (Fig. 9A).

Molt stage had little effect on gene expression in the ESG of *C. maenas*. Only animals at intermolt, early premolt (Stage D₀), and postmolt (stages B, C₁₋₃) stages were available at the time of tissue collection. Animals at mid and late premolt were not available. The hemolymph ecdysteroid titers were 7 pg/ μ L for intermolt animals, 43 pg/ μ L for early premolt, and 2 pg/ μ L for postmolt. mRNA levels of *Cm-CHH*, *Cm-NOS*, *GC-I β* , and *Cm-GC-III* were not affected by molt stage (Fig. 9B). *Cm-MIH* mRNA level in postmolt ESG was 30-fold higher than that at intermolt. *Cm-GC-II* and *Cm-EF2* mRNA levels were 15-fold and 10-fold, respectively, lower than those at intermolt (Fig. 9B).

4. DISCUSSION

MIH is the primary regulator of molting in decapod crustaceans (for reviews see Chang and Mykles, 2011; Chung et al., 2010). The prevailing view is that MIH is restricted to the ESG,

while CHH is expressed in the ESG and other tissues. The tissue distributions are consistent with the known functions of MIH and CHH: MIH regulates molting, whereas CHH controls a variety of physiological processes (for reviews see Chung et al., 2010; Lee et al., 2014; Webster, 2015a). However, data are accumulating that challenge the thinking that MIH solely functions in molt control and is not expressed in extra-eyestalk tissues. MIH stimulates vitellogenesis in adult *C. sapidus* and may be involved in *C. sapidus* embryonic development (Chung et al., 2010; Zmora et al., 2009). In *G. lateralis*, the ESG expressed high levels of MIH and CHH, but MIH and CHH mRNAs were also detected in the brain and TG at lower levels (Fig. 8). Using Northern blots or end-point RT-PCR, MIH mRNA was detected in the ESG and other regions of the nervous system, such as the optic nerve, ventral nerve cord, and thoracic/abdominal ganglion of *Cancer pagurus* (Lu et al., 2001); the brain and TG of *S. paramamosain* (Huang et al., 2015), *Portunus trituberculatus* (Zhu et al., 2011), and *C. maenas* (Abuhagr et al., 2014); the brain of *Charybdis feriatius* (Chan et al., 1998) and *Penaeus vannamei* (Sun, 1995); and the TG of *Metapenaeus ensis* (Tiu and Chan, 2007). Interestingly, there are reports of MIH protein in the tegumental glands of *Penaeus monodon* (Namvongsakool et al., 2015) and MIH mRNA in the ovary, testis, posterior spermatic duct, and bristle in the ejaculatory duct of *P. trituberculatus* (Zhu et al., 2011).

As RT-PCR is highly sensitive, a question is whether the mRNA in extra-eyestalk tissues is translated to produce MIH protein in sufficient amounts to have a physiological function. It is possible that detection of MIH in extra-eyestalk tissues, particularly in non-nervous tissues (Zhu et al., 2011), resulted from contamination. The mRNA levels of MIH and CHH in the brain and TG are four to five orders of magnitude lower than those in the ESG (Fig. 8), suggesting that the neuropeptides are expressed in a small number of cells. Western blot analysis detected MIH

protein in extracts of the ESG, brain, and TG of *P. pelagicus* (Stewart et al., 2013). MIH immunolabeled cells and neuron tracts were identified in the brain, esophageal ganglion, ventral nerve cord, and TG of *P. pelagicus* (Stewart et al., 2013). MIH immunopositive cells were identified in the brain and TG of *C. maenas* and *G. lateralis* (Figs. 5 and 7). In *P. monodon*, tegumental glands could be a significant extra-ESG source of MIH (Namvongsakool et al., 2015). These data indicate that the MIH mRNA in the brain and TG is being translated into protein and the MIH protein levels are high enough to be detected in soluble extracts by Western blotting.

In the brain, MIH immunolabeling is mostly localized in nerve tracts; only a small number of neuron cell bodies are MIH immunopositive (Fig. 7 and Stewart et al., 2013). Stewart et al. (2013) hypothesized that the nerve tracts convey MIH from the ESG via the ventral nerve cord through the esophageal ganglion and circumesophageal connectives to the TG. However, Bliss and Welsh (1952) proposed that the nerve tracts convey MIH in the opposite direction, from neurosecretory cells in the TG to the ESG. The presence of MIH-immunopositive neurons in the TG antero-medial cell clusters support retrograde transport of MIH to the ESG (Fig. 5 and Stewart et al., 2013). The localization of MIH immunopositive cell bodies in brain varied among *P. pelagicus*, *C. maenas*, and *G. lateralis*. In *P. pelagicus*, MIH is localized in cells in cell cluster #17 (same as cell cluster #3 using our numbering scheme in Fig. 6; Stewart et al., 2013). In *C. maenas* and *G. lateralis*, the cells in this and the other cell clusters were MIH negative. The only MIH-immunopositive cells were outside the clusters and associated with the olfactory lobe (Fig. 7). The function of these cells is enigmatic. Clusters of neurosecretory cells are located in the medial and lateral regions of the brain of *G. lateralis* and *C. maenas* (for review see Bliss and Welsh, 1952); the locations of the neurosecretory cells correspond to the cell clusters identified

histologically (Fig. 6; Stewart et al., 2013). The cells may contribute MIH to the SG. Bliss and Welsh (1952) reported nerve fibers leading from neurosecretory cells in the brain via the optic nerve to the SG. Nerve tracts in the ESG, brain, ventral nerve cord, esophageal ganglion, and circumesophageal connectives contain MIH-positive material, providing strong evidence that MIH is being transported between parts of the CNS (Stewart et al., 2013). Unfortunately, we were not able to confirm the observations on *P. pelagicus*, as the double immunofluorescence technique did not label the nerve tracts in the brains of *G. lateralis* and *C. maenas* (Fig. 7). The origin and transport direction of MIH in the CNS nerve tracts remain an area for further investigation.

In *G. lateralis*, autotomy of at least five walking legs induces a precocious molt, mediated by a putative limb autotomy factor - anecdysis (LAF_{an}) produced by the primary (1°) LBs, to regain mobility (see reviews by Chang and Mykles, 2011 and Skinner, 1985). LAF_{an} remains uncharacterized. A second factor, designated LAF_{pro} , is an inhibitory factor released from secondary (2°) LBs to suspend premolt processes ~3 weeks, allowing time for regrowth of a new regenerate (see reviews by Chang and Mykles, 2011 and Skinner, 1985; Yu et al., 2002). LAF_{pro} is an MIH-like peptide that appears to inhibit YO ecdysteroidogenesis, as LB autotomy lowers hemolymph ecdysteroid titers (Yu et al., 2002). The site of LAF_{pro} synthesis is unknown. It may be synthesized in the 2° LBs, but neurosecretory cells have not been identified. The MIH-immunopositive neurons in cell clusters associated with the segmental nerves may be the source of LAF_{pro} , assuming the antibody recognizes LAF_{pro} . Double-label immunofluorescence microscopy detected MIH in cell bodies, but did not detect MIH in neuron processes (Fig. 5). However, Stewart et al. (2013) identified neuron tracts in the segmental nerve of *P. pelagicus* that exhibited strong MIH labeling. This suggests that MIH is synthesized in the TG cell bodies

and is transported to the autotomy plane, where it is released by the 2° LBs and inhibits YO ecdysteroid secretion. The injection of extracts equivalent of one or two 2° LB inhibits 1° LB growth in premolt *G. lateralis* for 2 days, which indicates that there is sufficient LAF_{pro} in the extracts to elicit a systemic effect (Yu et al., 2002). The proposed role of neurosecretory cells in the TG with molt-inhibitory activity is not without precedent. CHH neurosecretory cells are located at the base of the second root of the third thoracic ganglion and in the subesophageal ganglion of American lobster, *Homarus americanus* (Chang et al., 1999). The antibody used to identify the CHH neurosecretory cells does not distinguish between the CHH-A and CHH-B forms (Chang et al., 1998). CHH-A, also designated MIH, has both molt-inhibitory and hyperglycemic activities, whereas CHH-B only has hyperglycemic activity (Chang et al., 1990). ES-ablated juvenile *H. americanus* molt more frequently and still show the cyclical changes in hemolymph ecdysteroid titers (Chang and Bruce, 1980). ES-ablated adult *H. americanus* had measurable levels of CHH in the hemolymph after 1 year and demonstrated emersion-induced increases in hemolymph CHH levels (Chang et al., 1998). These data indicate that extra-ESG sources of MIH and MIH-like proteins have physiological functions and can even serve an auxiliary function in the absence of the ESG. This may explain why adult *C. maenas* are refractory to ES ablation, although ESA has no effect on *Cm-MIH* expression in the brain and TG (Abuhagr et al., 2014).

The regulation of MIH expression in the XO/SG complex is not fully understood. There is no consistent pattern of *MIH* mRNA levels in the ESG over the molt cycle among decapods. In the green mud crab, *Scylla paramamosain*, *Sp-MIH* mRNA level is lower during premolt, suggesting that transcriptional regulation contributes to the drop in MIH secretion, although the premolt substage was not specified (Huang et al., 2015). In other species, the patterns of *MIH*

mRNA levels are not consistent with transcription playing a major role in initiating premolt. *MIH* mRNA levels either remain relatively constant, increase during premolt, or are high stage D₁ and then decline at stages D₂ and D₃₋₄ (Chung and Webster, 2003; Lee et al., 1998; Namvongsakool et al., 2015; Ohira et al., 1997; Techa and Chung, 2015). In *G. lateralis*, *Gl-MIH* mRNA level is elevated throughout premolt (Fig. 9A). In *C. maenas*, there is no decrease in *Cm-MIH* mRNA level in early premolt (Fig. 9B and Chung and Webster, 2003). Moreover, the *MIH* mRNA levels in the ESG are not correlated with *MIH* protein concentrations in the hemolymph (Chung and Webster, 2003, 2005; Techa and Chung, 2015). Molt stage has little or no effect on *CHH* expression in *G. lateralis* and *C. maenas* (Fig. 9 and Chung and Webster, 2003). Taken together, there is little evidence indicating that down-regulation of *MIH* transcription triggers the transition from intermolt to premolt. However, transcriptional control of the *MIH* gene may play a role in the repression of the YO during postmolt. *MIH* mRNA levels are elevated in the ESG of postmolt *C. maenas* (Fig. 9B), *Callinectes sapidus* (Lee et al., 1998), *Penaeus japonicus* (Ohira et al., 1997), and *S. paramamosain* (Huang et al., 2015), and remain elevated in postmolt *G. lateralis* (Fig. 9A).

The release of *MIH* from axon terminals in the SG is controlled by synaptic inputs from ESG neurons. Serotonin (5-hydroxytryptamine or 5-HT) and γ -aminobutyric acid mediate synaptic transmission between neurons in the medulla terminalis and the XO neurons (see review by García and Aréchiga, 1998). Depolarization of the XO neurons stimulates a Ca²⁺-dependent exocytosis of neurosecretory peptide (Keller et al., 1994; Richmond et al., 1996). Injection of 5-HT in *C. maenas* increases hemolymph glucose level and decreases hemolymph ecdysteroid level, which supports the stimulation of *CHH* and *MIH* release, respectively, by 5-HT (Robert et al., 2016). Data from this and other studies suggest that the control of *MIH* secretion involves

nitric oxide. NOS protein is localized to the SGs of *C. maenas*, *G. lateralis*, and *P. clarkii* (Figs. 2, 3; Lee et al., 2000; Pitts and Mykles, 2015). Moreover, a Cu-FL compound, which reacts with NO to produce a highly fluorescent NO-FL product, showed that the SG is a major site of NO production in the ESG (Pitts and Mykles, 2015). Images of the NO-FL fluorescence (Pitts and Mykles, 2015) and the MIH/NOS immunofluorescence labeling (Fig. 3) indicate that the NOS and NO are located in cells distinct from the MIH-labeled axons. Our interpretation of the images is that NO production is restricted to the glial cells. The close association of NO production in supporting cells with the axon termini suggests that NO modulates MIH secretion. In the SG of *C. maenas*, NO is sequestered by an NO-binding protein, which is proposed to modulate neuropeptide secretion over a prolonged period (Pitts and Mykles, 2015). NO modulates synaptic transmission in neuromuscular junctions and abdominal ganglion and brain interneurons in decapod nervous systems (Aonuma, 2002; Aonuma et al., 2000; Aonuma and Newland, 2001, 2002; Johansson and Mellon, 1998). In the TG, MIH and NOS are closely localized, suggesting that NO also regulates MIH release from the TG neurosecretory cells.

NOS immunoreactivity is not detected in the brain of either species. This is surprising given that NOS mRNA is detected in the brain of *C. maenas*, *G. lateralis*, and *Marsupenaeus japonicus* (Fig. 8; Abuhagr et al., 2014; Inada et al., 2010). NOS protein, either detected by NADPH diaphorase histochemistry or with NOS immunocytochemistry, is localized in various regions of the brain, including the olfactory lobe, of *P. clarkii*, *H. americanus*, and *Hemigrapsus sanguineus* (Benton et al., 2007; Dyuizen et al., 2012; Johansson and Mellon, 1998; Scholz and Truman, 2000; Scholz et al., 1998; Song et al., 2009). The uNOS antibody is directed to a highly conserved sequence in NOS proteins from a wide spectrum of invertebrate and vertebrate species. In *Drosophila*, truncated NOS isoforms generated by alternative splicing may lack the

conserved sequence and therefore not react with the uNOS antibody (Regulski and Tully, 1995; Stasiv et al., 2001). A truncated NOS isoform in *G. lateralis* was reported by (Kim et al., 2004), but was not characterized further. A formaldehyde-sensitive NOS activity is expressed in insect brain and thoracic ganglion (Ott and Elphick, 2002). These data raise the possibility that the brain expresses an alternative NOS isoform that is not recognized by the uNOS antibody.

NO may modulate neurosecretion by cGMP-dependent signaling. The NOS expressed in decapod crustacean tissues is a Ca^{2+} /calmodulin (CaM)-dependent form homologous to insect NOS and mammalian eNOS and nNOS (Kim et al., 2004). The reductase and oxidase domains in the NOS catalyze the production L-citrulline and NO from L-arginine and O_2 (see review by Daff, 2010). An ortholog of mammalian NOS interacting protein (NOS-IP) was identified, for the first time, in a *G. lateralis* YO transcriptome (Supplementary Fig. 1). NOS-IP blocks NOS activation in mammals (Su, 2014) and may have a similar function in decapod crustaceans. NO binds and activates NO-dependent guanylyl cyclase (GC-I), which catalyzes the conversion of GTP to cGMP. It is a heterodimer composed of alpha and beta (catalytic) subunits. Crustacean tissues also express a membrane receptor GC (GC-II), which appears to be the receptor for CHH, and a soluble NO-insensitive GC (GC-III) that is only found in invertebrates (Chang and Mykles, 2011; Lee et al., 2007c; Morton, 2004). Increased cGMP activates cGMP-dependent protein kinase (PKG), which has been identified in the *G. lateralis* YO transcriptome (Das et al., 2016).

The effects of molt stage on the expression of the NO/cGMP signaling pathway genes were quantified in the ESG of *C. maenas* and *G. lateralis*. *G. lateralis* were induced to molt by multiple leg autotomy (MLA) and ESG was harvested from intermolt, premolt (early or stage D₀; mid or stage D₁; and late or stage D₂), and 10 days postmolt animals. As *C. maenas* are refractory to molt induction by MLA or ESA (Abuhagr et al., 2014), ESG was harvested from

animals undergoing spontaneous molts. Unfortunately, animals in mid and late premolt stages were not available at the time of collection; ESG was harvested from intermolt (stage C₄), early premolt (stage D₀), and postmolt (stage B, C₁₋₃) animals. *Gl-NOS-IP* was expressed at very low levels, which were not affected by molt stage ($<10^2$ copy #/ μ g RNA; Fig. 9A), suggesting that *Gl-NOS-IP* mRNA level does not regulate NOS activity in the ESG. *Gl-NOS* and *Gl-GC-I β* were expressed at high levels in intermolt animals and *Gl-GC-I β* mRNA level was significantly higher at all premolt and postmolt stages (Fig. 9A). *Gl-GC-II* and *Gl-GC-III*, which served as reference GCs not part of NO/cGMP signaling pathway, were expressed at lower levels than *Gl-GC-I β* at all molt stages (Fig. 9A). Molt stage had little or no effect on *Gl-GC-II* and *Gl-GC-III* mRNA levels, except for a small, but significant, increase in mid premolt for *Gl-GC-II* and a large increase in postmolt for *Gl-GC-III*. Interestingly, there was a large increase in *Gl-EF2* mRNA level that paralleled the increases in *Gl-MIH* mRNA level in all premolt and postmolt stages (Fig. 9A). As EF2 is required for translation, the combined high mRNA levels of *Gl-EF2* and *Gl-MIH* may result in increased synthesis of Gl-MIH in the ESG during premolt and postmolt. Assuming that the increase in *Gl-GC-I β* mRNA occurs in the XO/SG complex, the up regulation of *Gl-GC-I β* could prolong the effects of NO by sustaining higher cGMP concentrations. The effect may be enhanced further in postmolt by the up regulation of *Gl-GC-III*. GC-III mediates the action of eclosion hormone during pupal ecdysis in *Manduca sexta* (Morton and Simpson, 2002) and may play a similar role in the ESG. By contrast, molt stage had little or no effect on the mRNA levels of *Cm-NOS*, *Cm-GC-I β* , *Cm-GC-II*, *Cm-GC-III*, and *Cm-EF2* in the *C. maenas* (Fig. 9B), suggesting that the activities of these proteins are not transcriptionally regulated in the ESG in this species.

Figure 10 presents a hypothetical model for the modulation of neuropeptide release by NO in the SG. It is analogous to the inhibition of catecholamine secretion in the mammalian adrenal gland through the activation of NO/cGMP-dependent signaling (Olson et al., 2012; Schwarz et al., 1998). A two-cell model is proposed, based on the double-label immunolabeling of MIH and NOS images (Figs. 2, 3), the localization of NO production as determined by NO-FL fluorescence images (Pitts and Mykles, 2015), and electrophysiological studies of SG neurosecretory axons (Cooke, 1985; Cooke and Sullivan, 1982; Keller et al., 1994; Meyers and Cooke, 1997; Richmond et al., 1996). In the axon terminal, Ca^{2+} influx via voltage-gated Ca^{2+} channels triggers vesicle fusion with the membrane and neuropeptide secretion into the hemolymph. NO produced in the supporting glial cells, triggered by Ca^{2+} stimulation of NOS, diffuses into the axon terminal and activates GC-I. Increased cGMP in the cytoplasm activates PKG, which phosphorylates and inactivates the Ca^{2+} channel. The lower cytoplasmic Ca^{2+} inhibits secretion. The inhibition of the Ca^{2+} channel is prolonged by the release of NO from an NO-binding protein (Pitts and Mykles, 2015) and/or up regulation of *Gl-GC-I β* (Fig. 9A). Suppression of MIH secretion would be especially crucial in premolt, as a lower MIH hemolymph level drives the transition from intermolt to premolt, as well as sustains premolt processes (for review see Chang and Mykles, 2011). The up regulation of both *Gl-GC-I β* and *Gl-GC-III* in the ESG of postmolt animals suggests the inhibition of neurosecretion extends into the postmolt stage. As the YO is in a repressed state in postmolt crabs (for review see Chang and Mykles, 2011), MIH may not be needed to inhibit ecdysteroidogenesis until the YO transitions back to the MIH-sensitive basal state by the intermolt stage.

In summary, MIH level in the hemolymph is largely determined by the frequency of MIH release from the axon terminals in the SG (for reviews see Chang and Mykles, 2011; Chung et

al., 2010). MIH mRNA level in the ESG is not correlated with MIH protein level in the hemolymph over the molt cycle, indicating that transcriptional regulation does not drive hormonal control of molting. MIH and NOS are expressed in the ESG, brain, and TG of *C. maenas* and *G. lateralis* and other decapod species. Here we show for the first time a close association of MIH- and NOS-expressing cells in the SG and the TG using double-label immunofluorescence microscopy, suggesting that NO inhibits MIH secretion via a cGMP-dependent signaling pathway (Fig. 10). Further studies quantifying MIH release from the SG in the presence of NOS and GC-I antagonists, NO donors, and NO scavengers will provide stronger evidence to establish the exact relationship between NO and neurosecretion of peptides from the SG. Alternative sources of MIH and MIH-like factors in the brain and TG may explain why adult *C. maenas* do not respond to ESA and why LBA suspends premolt processes in *G. lateralis*. The MIH-immunopositive cells associated with the TG segmental nerves may be the source of LAF_{pro} in 2° LB extracts. There is evidence that MIH has a role in embryogenesis and reproduction (for review see Chung et al., 2010), which challenges the view that MIH functions solely in molt regulation.

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List of Abbreviations

abn: abdominal nerve

AL: accessory lobe

AMPN: anterior-medial protocerebral neuropil

AnM – antenna I neuropil

CB: central body

cGMP: 3'5'-guanosine monophosphate

CHH: crustacean hyperglycemic hormone

CNS: central nervous system

EF2: elongation factor 2

ELISA: enzyme-linked immunosorbent assay

ESG: eyestalk ganglia

GC-II: membrane receptor class II guanylyl cyclase

GC-III: soluble class III guanylyl cyclase

GC-I β : soluble class I guanylyl cyclase beta subunit

GN: giant neuron

Hec: hemocoele

H&E: hematoxylin and eosin Y

MAN: median antenna I neuropil

ME: medulla externa

MI: medulla interna

MIH: molt inhibiting hormone

MLA: multiple leg autotomy

MT: medulla terminalis

NADPH: nicotinamide adenine dinucleotide phosphate

NO: nitric oxide

NOS: nitric oxide synthase

NOS-IP: nitric oxide synthase inhibitory protein

OGTN: olfactory globular tract neuropil

OL: olfactory lobe

optn: optic nerve tract

PMPN: posterior-medial protocerebral neuropil

qPCR: quantitative polymerase chain reaction

SG: sinus gland

sn: segmental nerve

SN: small neuron

SNAP: soluble NSF attachment protein

SNARE: soluble NSF attachment protein receptor

TG: thoracic ganglion

XO: X-organ

YO: Y-organ

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Table 1. qPCR primer Sequences for NOS, NOS-IP, and MIH.

Primer	Sequence (5' – 3')	Product size (bp)
Gl-NOS F1	CATCACCCACCCTGAGTACA	60
Gl-NOS R1	GGGAGGCCATACCATTGAA	
Gl-NOS-IP F1	GCAGCCTCTCCCTTCACCC	178
Gl-NOS-IP R1	GCCTCACCCAACCTCCTCCAAC	
Gl-MIH F1	AACATTGACTTCCTGTGGTGTG	71
Gl-MIH R1	ACGAGCTCACACCGATACGT	
Cm-NOS F1	ATGGGTCCAAGGTTCTACTCC	82
Cm-NOS R1	ACTGTACAATAGCCACCGTCA	
Cm-MIH F2	CGAGCAGCGGCGAGAGTTATC	275
Cm-MIH R2	GATGAAGGAGGGAGAGACGAC	
Cm-CHH F2	GTGACGATTGTTACAACCTCTACAG	166
Cm-CHH R2	GTTTACTTCTTCCTGCCAACCATC	
Cm-GC-I β F1	CTACCTCCCCGAGACTGACTTAATG	372
Cm-GC-I β R1	GAGCAGTAGTCGGTGAAGCCC	

Figure Legends

Fig. 1. Histological organization of the eyestalk ganglia of *C. maenas*. A longitudinal section of the ESG was stained with hemotoxylin and eosin Y. The ESG is organized into the medulla terminalis (MT), medulla interna (MI), medulla externa (ME) neuropils. The X-organ (XO) is located peripheral to the MT and sends axons dorsally to the sinus gland (SG) located at the ME/MI junction.

Fig. 2. Double-label MIH and NOS immunofluorescence in the eyestalk ganglia of *G. lateralis* (A) and *C. maenas* (B). Immunofluorescence images at low (10x) and high (40x) magnification shows MIH (green) and NOS (red) localized in the SG. The yellow color in the overlay images of the NOS and MIH fluorescence indicate the close proximity of the two proteins. Cell nuclei are stained with DAPI (blue).

Fig. 3. Confocal images of MIH and NOS localization in the sinus gland of *G. lateralis*.

Representative confocal images at 40x from a 20-slice z-stack show that MIH immunofluorescence (green) is restricted to axon terminals and NOS Immunofluorescence (red) is restricted to adjacent cells. The position number of each image in the stack is indicated. The full stack can be viewed as a video in the online version of this manuscript (Supplementary Fig. 2). Cell nuclei are stained with DAPI (blue).

Fig. 4. Histological organization of the thoracic ganglion of *C. maenas*. A montage image of a horizontal section of the TG was stained with hemotoxylin and eosin Y. In brachyuran crabs, the TG incorporates the subesophageal ganglion with a prominent cell cluster (#1) of giant neurons

(inset; 40x objective), small neurons, and glial cells and the abdominal ganglion (AG) with the abdominal nerve (abn) projecting posteriorly. Five paired segmental nerves (sn1-5) project to the thoracic appendages. Cell clusters #1-5 are positioned along the midline axis. Paired medial cell clusters #6-8 and paired lateral cell clusters #9-13 are positioned between the segmental nerves. Two cell clusters (#14) are lateral to the abdominal nerve. Hec, hemocoele.

Fig. 5. Double immunofluorescence of MIH and NOS in the TG of *G. lateralis* (A) and *C. maenas* (B). Immunofluorescence images at low (10x) and high (40x) magnification shows MIH (green) and NOS (red) staining at the periphery of giant neurosecretory cells (GN; arrows) between segmental nerves (sn) 4 and 5. As in the ESG, the overlay shows NOS and MIH in close proximity (arrowheads). Cell nuclei are stained with DAPI (blue).

Fig. 6. Histological organization of the brain of *C. maenas*. A horizontal section of the brain was stained with hemotoxylin and eosin Y. The brain is divided into the protocerebrum, deutocerebrum, and tritocerebrum. The protocerebrum contains the anterior-medial cells (cluster #1), a pair of anterior-medial protocerebral neuropils (AMPN), and a pair of posterior-medial protocerebral neuropils (PMPN); the AMPN and PMPN are separated by a central body (CB). The two optic nerves (optn) project from the AMPN and PMPN to the eyestalk ganglia. The deutocerebrum contains a pair of dorsal lateral cell clusters (#2), a pair of olfactory lobe cell clusters (#6), the median antenna neuropil (MAN), a pair of olfactory lobes (OL), and a pair of olfactory globular tract neuropils (OGTN). The tritocerebrum contains a pair of posterior medial cell clusters (#3), a pair of ventral lateral cell clusters (#4), a pair of posterior lateral cell clusters (#5), a pair of accessory lobes (AL), and a pair of antenna II neuropils (AnN).

Fig. 7. Double-label MIH and NOS immunofluorescence in the brain of *G. lateralis* (A) and *C. maenas* (B). Immunofluorescence of the olfactory lobe (OL) region in the deuterocephalon; images captured at low (10x) and high (40x) magnifications. MIH (green) was localized in a small number of small neurons (arrowheads); no MIH-positive cells were detected in other brain regions. No NOS immunofluorescence was detected in the OL or in other brain regions. Cell nuclei are stained with DAPI (blue).

Fig. 8. Expression of *Gl-MIH*, *Gl-CHH*, *Gl-NOS*, and *Gl-EF2* in CNS tissues from intermolt *G. lateralis*. mRNA levels were quantified in eyestalk ganglia (ESG), brain (Br), and thoracic ganglion (TG) from intermolt animals using qPCR. Data are presented as mean $\pm$ 1 S.E. (ESG, n = 12; TG, n = 20; brain, n = 18). Brackets indicate significant differences between the means at $P < 0.05$.

Fig. 9. Effects of molting on expression of neuropeptides and NO/cGMP signaling pathway components in the ESG of *G. lateralis* (A) and *C. maenas* (B). mRNA levels were quantified by qPCR. (A) ESG were harvested from intermolt, early premolt, mid premolt, late premolt, and postmolt animals. Data presented as mean $\pm$ 1 S.E. (intermolt, n = 9-12; early premolt, n = 32-37; mid premolt, n = 29-32; late premolt, n = 50-54; and postmolt, n = 12-13). Stages with means that were significantly different from intermolt are indicated with an asterisk (*). Means of early premolt, mid premolt, late premolt, and postmolt stages that were significantly different are indicated with a shared letter. Only significant differences with magnitudes of 3-fold or greater are labeled. (B) ESG were harvested from intermolt, early premolt, and postmolt animals;

animals at mid and late premolt were not available at the time of collection from the Bodega Harbor population. Data presented as mean $\pm$ 1 S.E. (intermolt, n = 18; early premolt, n = 12; and postmolt, n = 21). Stages with means that were significantly different from intermolt are indicated with an asterisk (*). Means of early premolt and postmolt stages that were significantly different are indicated with a shared letter. Only significant differences with magnitudes of 3-fold or greater are labeled.

Fig. 10. Proposed two-cell model for NO/cGMP-dependent regulation of MIH release in the sinus gland. In glial cells, Ca^{2+} influx through a voltage-gated Ca^{2+} channel activates a Ca^{2+} /calmodulin-dependent nitric oxide synthase (NOS); NO diffuses across the glial cell membrane into the axon terminals of molt-inhibiting hormone (MIH) neurosecretory cells. NO activates guanylyl cyclase I (GC-I), which converts GTP to cGMP. cGMP-dependent protein kinase G (PKG) blocks activation of a voltage-gated Ca^{2+} channel, resulting in inhibition of secretory vesicle fusion with the axon membrane and reduced neuropeptide release to the hemolymph.

Figure 2

Figure 5

Figure 7

A. *Gecarcinus lateralis*

B. *Carcinus maenas*