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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 ~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-Iβ*) 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 Ca^{2+}-dependent fusion of MIH vesicles with the nerve terminal membrane; the resulting decrease in MIH activates the YO and the animal enters premolt.

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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).

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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 (Dircksen, 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 cylase (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 × 100 / carapace width (Covi et al., 2010; Yu et al., 2002). Intermolt animals had an R index of 8–12.9; early premolt (stage D_0), $R = 13$–15.9; mid premolt (stage D_1), $R = 16$–18.9; and late premolt (stage D_2), $R = 19$–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 5× and 40× were captured with a Leica CTR 5500 microscope equipped with a Leica DFC 450 camera. Low (10×) and high (40×) fluorescent images were captured with a Zeiss Axiovert 200 M microscope equipped with a Hammatsu 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).

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