Post-Translational Modifications in Determining the Modulatory Effects of Myosuppressin on the Ligatured Cardiac Ganglion of the American Lobster, *Homarus americanus*

Emily Oleisky

INTRODUCTION

Within any organism, there is a need for behavioral flexibility, so that when unforeseen environmental pressures arise, the nervous system output can help the organism adapt and survive (Wright, T. F. et al., 2010). While the mechanisms of the rhythmic patterns of nervous signaling are generally well understood, the details of how these systems react when altered is still being studied.

Central pattern generators (CPGs) are neural networks that produce rhythmic electrical patterns that regulate behavior without the need for sensory input, such as breathing, chewing, and walking (Delcomyn, F., 1980). While vertebrate CPGs are part of extensive and complex systems, invertebrate CPGs provide simple model systems based on similar mechanisms and are useful for study. Unlike the heart of vertebrates, which contracts regularly without an external stimulus, the neurogenic crustacean heart is part of a nervous system and requires neural signals for contraction (Goy, M. F., 2005). The American lobster heart is a model organ for understanding the extrinsic control of CPGs, as the cardiac ganglion provides a physical platform to study the neurophysiological mechanisms through which CPGs function (Cooke, I. M., 2002).

The cardiac neuromuscular system of *Homarus americanus* is a simple system centered around a CPG: the cardiac ganglion (CG). The crustacean cardiac ganglion is a neural network of nine cells: five anterior motor neurons, and four posterior pacemaker interneurons. These neurons exhibit spontaneous bursting activity and contribute to the muscle contractions that

define the beating of the heart (Cooke, I. M., 2002). Cross-neuronal communication between these nine neurons is an essential characteristic in defining the rhythmic and patterned output of the of the CG. Previous data have shown that the motor and pacemaker neurons are coupled, and burst in-phase (Cooke, I. M., 2002).

Communication between neurons is an essential characteristic of CPG systems. While many chemical signaling molecules have been classified to have modulatory effects on neuronal output, neuropeptides are a particularly expansive class of neurohormones that are known to affect change on nervous systems. Peptides, short sequences of amino acids linked by amide bonds, are often encoded within genomes as precursor proteins, or prepro-hormones, that include a signal sequence at the amino (N)-terminus. After transcription and translation, within the neurosecretory pathway, various forms of enzymatic cleavage and post-translational modifications (PTMs) result in neuropeptide modification, including carboxy (C)-terminus amidation, cyclization of N-terminal glutamine/glutamic acid residues, disulfide bridging between cysteines, and sulfation of tyrosines (Christie, et al., 2010). Variance in PTMs has proved to affect the relative bioactivity of the neuropeptide itself, and the change that it exhibits within the neuronal system.

Myosuppressin (pQDLDHVFLRFamide), a member of the larger FMRFamide-like peptide family, is a well characterized peptide endogenous to the American lobster known to be delivered locally from other neuroendocrine organs to exert a hormonal effect (Stevens et al., 2009). Mass spectrometric studies have shown that the full isoform of myosuppressin is not only present in *H. americanus,* but conserved across 32 species in seven decapod crustacean infraorders (Stemmler, et al., 2007). This conservation prompted further research involving the

cardiac neuromuscular system, in which the effects of myosuppressin were characterized across various preparations of the American lobster heart, including whole heart and isolated ganglion preparations. Myosuppressin elicited a dose-dependent decrease in frequency and increase in amplitude of heart contractions in whole heart preparations, and a dose-dependent decrease in cycle frequency in the isolated CG (Stevens et al., 2009).

A novel neuropeptide, QDLDHVFLRFamide, was detected in the nervous system and the neuroendocrine periphery, and later classified as a member of the myosuppressin subfamily of the larger FMRFamide peptide family due to its structural similarity to myosuppressin, despite cyclization of N-terminal glutamine (Ma, et al., 2008). The idea of various isoforms of myosuppressin raised the question of importance of PTMs as they relate to neurohormonal modulation, and prompted the basis for comparison across various modified isoforms of myosuppressin.

Recent research in the Dickinson Lab at Bowdoin College examined the effects of two PTMs of myosuppressin, the cyclization of the (N)-terminal glutamine and the (C)-terminal amidation, across three peptide isoforms. In isolated whole heart preparations, both the full form of myosuppressin, with both PTMs, and myosuppressin lacking cyclized glutamine elicited a decrease in contraction frequency and brief decrease in contraction amplitude followed by a steep increase (10^{-6}M tested). On the other hand, the non-amidated form of myosuppressin again caused a decrease in contraction frequency, but only a decrease in contraction amplitude (10^{-6}M tested; Stanhope, M.E., 2016). These findings, in addition to bioinformatic analyses that predicted three unique myosuppressin receptors in the CG, suggest differential capabilities of the myosuppressin isoforms in eliciting quantifiable change to peptide application.

In this study, the effects of the three isoforms of myosuppressin (pQDLDHVFLRFamide, QDLDHVFLRFamide, pQDLDHVFLRF) were characterized on intact and ligatured preparations of the CG in an attempt to quantify the effect of PTMs on a focused area of the cardiac neuromuscular system. Not only did the preparation of the intact CG provide data that informed the effects of these myosuppressin isoforms when the motor and pacemaker neurons of the CG were coupled, but the ligatured ganglion preparation provided unique insight into the differential response of the large and small cells.

METHODS

Animal husbandry

Hard and soft shell, male and female American lobsters (*Homarus americanus*) were purchased from local seafood suppliers (Bath & Brunswick, ME). Lobsters were housed in aquaria with circulating natural seawater between 10 - 12^{o}C. The tanks were kept at Bowdoin College with 12 hour light-dark cycles. Lobsters were fed a diet of chopped shrimp.

Synthesis of myosuppressin isoforms

Myosuppressin isoforms were custom synthesized by GenScript (Piscataway, NJ). Due to the relatively low aqueous solubility of myosuppessin, the lyophilized isoforms were first dissolved in 15% DMSO, and then diluted with deionized water to create 10^{-3}M stock solutions. Stock solutions were aliquoted into small quantities to reduce continual thawing and refreezing, and stored at -25^{o}C until needed for application. 10^{-6}M solutions were made immediately preceding application through a dilution of the peptide stock solution with physiological saline (composition in mmol^{-1}: 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20.00 $MgSO_4$, 3.91 Na_2SO_4, 11.45 Trizma base and 4.82, maleic acid; pH: 7.45).

Physiology

Lobsters were put on ice for a minimum of 30 minutes to be anesthetized prior to dissection. A gross dissection of the lobster involved removal of the tail, legs, and claws, prior to a dorsal dissection of the heart from the cephalothoracic carapace. Isolated hearts were pinned in a small Sylgard 184 (KR Anderson, Santa Clara, CA)-lined dish with cold (4ºC) physiological saline. A superficial incision was made along the ventral midline of the heart and the ambient cardiac tissue was pinned back to expose the cardiac ganglion. The motor nerves of the CG were cut as far from the central branching point as possible to ensure the intactness of the most distal anterior motor neurons and portion of the posterior artery was left intact not only to preserve the distal posterior pacemaker neurons, but to be used for pinning. Once removed, the isolated CG was pinned in a fresh Sylgard-lined dish with cold physiological saline.

Placement of the Ligature and Vaseline Wells

One unraveled fiber from 0.1mm 6-0 Suture Silk was used tie a slack knot around the anterior trunk of the CG as a ligature. While the placement of the ligature varied by individual, the ligature was consistently placed anterior to cell 4 (Figure 1) and the small cell spike initiation zones, in order to solely silence the axonal communication of the large cells past the ligature.

Three vaseline wells were placed on the ganglion, in order to record the electrophysiological output of the motor and pacemaker neurons. The wells located anterior to cells 1 and 2 (Figure 1) recorded only large cell bursting, as the projections of the small cells do not extend past these anterior motor neuron cell bodies. The well located on the trunk of the CG, immediately anterior to cell 5 (Figure 1), recorded both large and small cell bursting in the intact ganglion, but only small cell bursting in the ligatured ganglion.

Two-pronged stainless steel electrodes were used to record the electrical difference across the Vaseline wells, and quantify the bursting of the CG. The electrode signal was amplified with a 1700 A-M Systems Differential AC Amplifier (Sequim, WA, USA) and a model 440 Brownlee Precision Instrumentation amplifier, and then sent through a CED Micro 1401 digitizer and recorded using Spike2.17 software.

Perfusion and Recording

Since maximal cardiac output in the American lobster heart has been shown to occur around 10°C (Worden et al., 2006), the Sylgard dish containing the CG was perfused with physiological saline held at a temperature between 9°C and 13°C using an in-line Peltier temperature regulator (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT) and monitored using a temperature probe (Warner Instruments, Hamden, CT). The saline was perfused at a rate of 5ml/m over the cardiac ganglion, and consistent perfusion was achieved using a Rabbit peristaltic pump (Gilson, Middleton, WI).

Fifty milliliters of each isoform of myosuppressin was applied at a concentration of 10^{-6}M was applied to the isolated, intact CG to allow for a baseline recording of the peptide's effects on the coupled neurons. The order of isoform application on the intact CG was randomized by individual to ensure the response seen to peptide modulation was unique to the individual isoform. Preparations were perfused with plain saline following peptide application was approximately 45 minutes, to allow the previous peptide to completely wash out before another peptide application.

After perfusion of all three isoforms of myosuppressin and a final saline wash, the ligature was tightened. Utmost care was taken when tightening the ligature with forceps, so as

not to disturb the vaseline wells on either side of the ligature, or irreversibly damage the trunk of the ganglion. The taught ligature silenced both chemical and electrical communication between the large and small cells, allowing electrophysiological output to be recorded and quantified for each neuronal cell type independently. Upon tightening the ligature, the ganglion output was initially extremely variable, and further saline perfusion was continued until consistent, stable, and patterned output returned.

Once strong post-ligature patterning was achieved, all three isoforms of myosuppressin in a randomized order were perfused over the decoupled CG, interspersed by saline washes.

Data Analysis

Isolated CG recordings were analyzed in Spike2.17 using scripts written by Dirk Bucher (Whitney Laboratory for Marine Bioscience: www.whitney.ufl.edu/BucherLab/), analysis tools found in Spike2, and scripts previously written by the Dickinson Lab.

These scripts quantified burst characteristics, such as number of spikes per burst, burst duration, cycle frequency and duty cycle as a function of time. The data were analyzed using a compilation of macro programs written for Microsoft Excel and Prism 7 across each isoform on the intact and ligatured CG.

RESULTS

The three differentially modified isoforms of myosuppressin (pQDLDHVFLRFamide, QDLDHVFLRFamide, pQDLDHVFLRF) were prefused through intact and ligatured CG preparations of individual lobsters (n = 6) at a concentration of 10^{-6}M. Application of the full isoform of myosuppressin (Full; pQDLDHVFLRFamide) resulted in an increase in burst

duration across the small and large cells in both the ligatured and intact ganglion (Figure 2). This result supported previous findings in the Dickinson Lab (Stevens, et al., 2009).

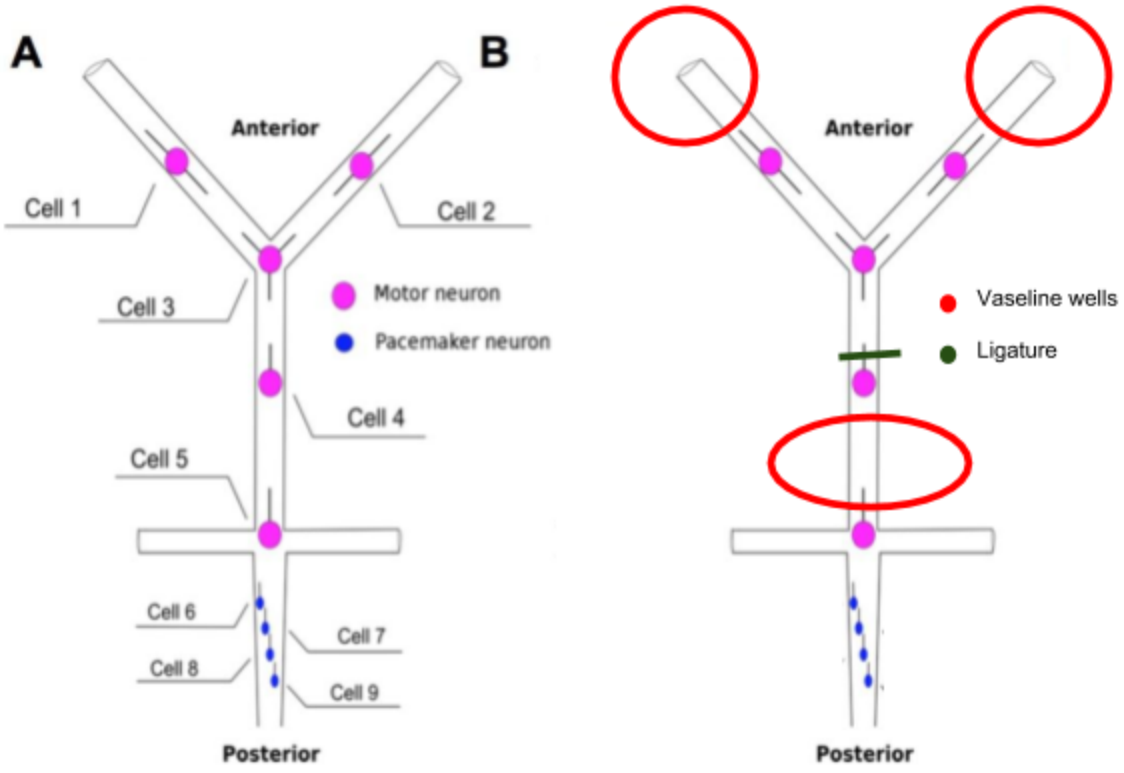
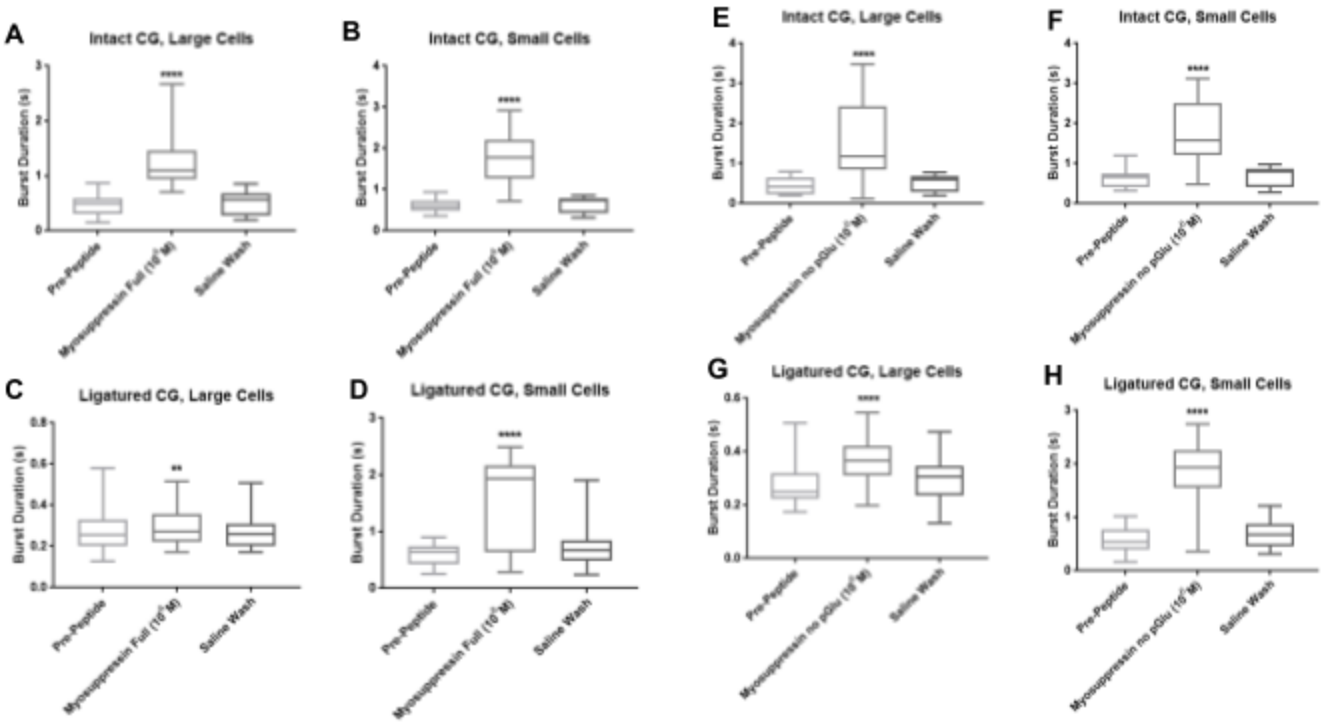


Figure 1. Organization of the cardiac ganglion. A) Five large motor neurons are located in the anterior portion of the cardiac ganglion, and are electrically and chemically coupled to the four small pacemaker, or pre-motor neurons located in the posterior trunk of the cardiac ganglion. B) Placement of the Vaseline wells and ligature on the isolated cardiac ganglion. Adapted from Mortimer, A. L. (2012).


A
Intact CG, Large Cells
B
Intact CG, Small Cells
C
Ligatured CG, Large Cells
D
Ligatured CG, Small Cells
E
Intact CG, Large Cells
F
Intact CG, Small Cells
G
Ligatured CG, Large Cells
H
Ligatured CG, Small Cells
Burst Duration (s)
****
**
Pre-Peptide
Myosuppressin Full (10⁻⁶ M)
Myosuppressin no pGlu (10⁻⁶ M)
Saline Wash

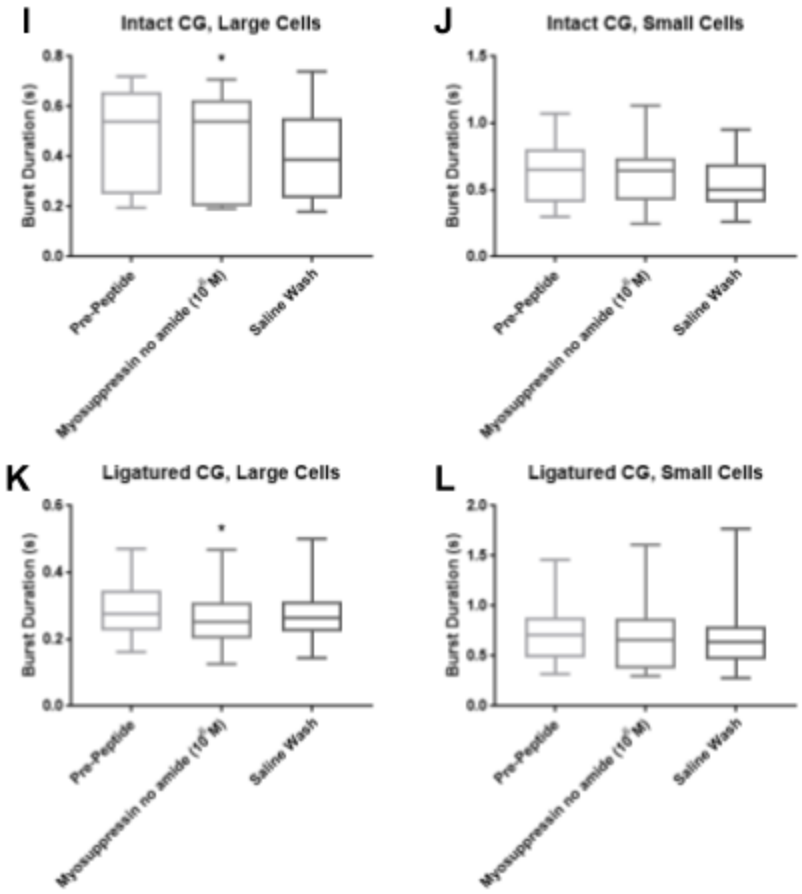


Figure 2. The average burst duration for ten bursts per preparation (n = 6) sampled prior to peptide application (pre-peptide), at the end of myosuppressin application (Myosuppressin Full/no pGlu/no amide 10^{-6}M), and at the end of the saline wash (saline wash). Paired t-tests demonstrated a
statistically significant increase in burst duration across the large and small cells of the intact and ligatured CG when myosupressin full (A-D) and myosupressin no pGlu (E-H) were applied.

LITERATURE CITED

Christie, A. E., Stemmler, E. A., & Dickinson, P. S. (2010). Crustacean neuropeptides. *Cellular and Molecular Life Sciences, 67*(24), 4135-4169.

Christie, A. E., Stevens, J. S., Bowers, M. R., Chapline, M. C., Jensen, D. A., Schegg, K. M., . . . Dickinson, P. S. (2010). Identification of a calcitonin-like diuretic hormone that functions as an intrinsic modulator of the American lobster, *Homarus americanus*, cardiac neuromuscular system. *The Journal of Experimental Biology, 213*(1), 118-127.

Cooke, I. M. (2002). Reliable, responsive pacemaking and pattern generation with minimal cell numbers: The crustacean cardiac ganglion. *Biological Bulletin, 202*(2), 108-136.

Delcomyn, F. (1980). Even "simple" systems are more complex than we thick. *Behavioral and Brain Sciences, 3*(4), 544.

Goy, M. F. (2005). Nitric oxide: An inhibitory retrograde modulator in the crustacean heart. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 142*(2), 151-163.

Ma, M., Chen, R., Sousa, G. L., Bors, E. K., Kwiatkowski, M., Goiney, C. C., . . . Li, L. (2008). Mass spectral characterization of peptide transmitters/hormones in the nervous system and neuroendocrine organs of the American lobster *Homarus americanus*. *General and Comparative Endocrinology, 156*(2), 395-409.

Mortimer, A. L. (2012). *Localization of Homam CLDH and its Effects on the Cardiac Central Pattern Generator in Homarus Americanus.* (Bachelor's Degree), Bowdoin College, Brunswick, ME.

Stanhope, M.E. (2016). Investigating the bioactivity of differentially modified isoforms of the neuromodulator, myosuppressin, on the cardiac neuromuscular system of the American Lobster, *Homarus americanus.* (Independent Study), Bowdoin College, Brunswick, ME.

Stemmler, E. A., Cashman, C. R., Messinger, D. I., Gardner, N. P., Dickinson, P. S., & Christie, A. E. (2007). High-mass-resolution direct-tissue MALDI-FTMS reveals broad conservation of three neuropeptides (APSGFLGMRamide, GYRKPPFNGSIFamide and pQDLDHVFLRFamide) across members of seven decapod crustacean infraorders. *Peptides, 28*(11), 2104-2115.

Stevens, J.S., Cashman, C. R., Smith, C.M., Beale, K.M., Towle, D.W., Christie, A.E., Dickinson, P.S. (2009). “The peptide hormone pQDLDHVFLRFamide (crustacean myosuppressin) modeulates the Homarus americanus cardiac neuromuscular system at multiple sites. *Journal of Experimental Biology.* 212(24):3961-3976.

Worden, Clark C.M., & Conaway M. (2006). Temperature dependence of cardiac performance in the lobster Homarus americanus. *The Journal of Experimental Biology 209,* 1024 - 1034.

Wright, T. F., Eberhard, J., Hobson, E., Avery, M. L., & Russello, M. (2010). Behavioral flexibility and species invasions: the adaptive flexibility hypothesis. *Ethology Ecology & Evolution, 22*(4), 393-404.