Peripheral modulation of cardiac contractions in the American lobster, *Homarus americanus*, by the peptide myosuppressin is mediated by effects on the cardiac muscle itself

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Abstract

A substantial factor for behavioral flexibility is modulation — largely via neuropeptides — which occurs at multiple sites including neurons, muscles, and the neuromuscular junction (NMJ). Complex modulation distributed across multiple sites provides an interesting question: does modulation at multiple locations lead to greater dynamics than one receptor site alone? The cardiac neuromuscular system of the American lobster (*Homarus americanus*), driven by a central pattern generator called the cardiac ganglion (CG), is a model system for peptide modulation. The peptide myosuppressin (pQDLDHVFLRFamide) has been shown in the whole heart to decrease contraction frequency, largely due to its effects on the CG, as well as increase contraction amplitude by acting on periphery of the neuromuscular system, either at the cardiac muscle, the NMJ, or both. This set of experiments addresses the location(s) at which myosuppressin exerts its effects at the periphery. To elucidate myosuppressin’s effects on the cardiac muscle, the CG was removed, and muscle contractions were stimulated with L-glutamate while superfusing myosuppressin. Myosuppressin increased glutamate-evoked contraction amplitude in the isolated muscle, suggesting that myosuppressin exerts its peripheral effects directly on the cardiac muscle. To examine effects on the NMJ, excitatory junction potentials were evoked by stimulating of the motor nerve and intracellularly recording a single muscle fiber both in control saline and in the presence of myosuppressin. Myosuppressin did not modulate the amplitude of EJPs suggesting myosuppressin acts at the muscle and not at the NMJ, to cause an increase in contraction amplitude.
Introduction

All nervous systems are modulated by molecules such as amines, amino acids, and peptides. Receptors for these modulators are expressed on various locations including neurons, muscles, and the neuromuscular junction (NMJ); thus, circulating hormones and modulators can not only influence the voltage characteristics of those receptor locations, but also have consequences on an entire circuit that are difficult to predict. It is interesting to consider how modulation at more than one location occurs, and if modulation at multiple sites leads to greater dynamics than one receptor site alone.

1.1 Central Pattern Generators (CPGs)

Central pattern generators (CPGs) are neural networks that, when activated, generate repetitive rhythmic behaviors and patterns of electrical activity in the absence of rhythmic central or peripheral inputs (Marder & Bucher, 2001; Dickinson, 2006). These complex circuits are present in both vertebrates and invertebrates and control behaviors such as swimming, walking, and breathing (Marder & Bucher, 2001). Although rhythmic input is not necessary for CPG functioning, many CPGs are modulated by rhythmic sensory feedback and non-rhythmic modulatory input (Marder, 2011). These network of neurons, the motor efferents, and the innervated muscle comprise the CPG-effector system with neurons in a motor CPG innervating muscles and driving contractions. CPGs continue to be studied because of their ability to produce fictive activity in vitro, allowing insight into neural circuit functioning and dynamics – particularly when the connectivity of the network is known.

1.2 Neuromodulation of CPGs

Flexibility in CPG networks can arise from neurohormones and neurotransmitters such as amines and neuropeptides that are able to modulate CPGs by altering action potential firing
patterns, thereby providing organisms with behavioral flexibility (Brezina et al., 2010). This framework arises from the fact that in the animal, a CPG is likely never in a non-modulated or “control” state. Experimentally, we can isolate nervous systems to their base components and add modulators back into the system and observe their effects. However, there is some disconnect between how the nervous system acts in a dish with applied modulators, compared to how it typically functions in an animal (always modulated). This modulation is important as most species require flexibility in CPG output to be able to adequately respond to environmental and sensory cues (Dickinson et al., 2008, Marder, 2012). Neuromodulators have effects on both CPG neurons and on peripheral targets; for example, altering muscle contractions by way of acting on the NMJ or directly on the muscle itself (the components of the periphery) (Erxleben et al., 1995; Dickinson et al., 2015a). The peptide of interest in this study, myosuppressin, is one of several peptides that modulates CPG-effector systems in a dynamic way.

Neuropeptides, primarily short covalently bound chains of amino acids, are the largest class of neuromodulators and are necessary for communication within neuronal systems (Dickinson et al., 2016a; Briggman & Kristan, 2008; Russo, 2017). Peptidergic signaling is present even in the simplest of organisms and is important across species (Varoqueaux et al., 2018). Signaling peptides are commonly encoded within genomes as prepro-hormones or precursor proteins that have a signal sequence at the amino (N)-terminus (Oleisky et al., 2020). Furthermore, peptides are often modified after transcription and translation through enzymatic cleavage, folding, and post-translational modifications (PTMs). These modifications change peptide structure, making them biologically active and adding stabilization for cellular
interaction, which can alter the peptide's ability to modulate neuronal output (Christie et al., 2010).

Understanding how modulation comes about (i.e., the sites and sources) is useful as predictions are possible to determine the location at which a neuromodulator exerts its effects (such as on neurons, muscles, and/or the NMJ). Modulation of peripheral targets can affect presynaptic, postsynaptic responses, and/or muscle contractions. Presynaptically, modulators have receptors on the presynaptic membrane of the motor nerve terminal. In the case of the NMJ, postsynaptic modulation of calcium release from internal stores is important in determining contraction characteristics (e.g., increasing calcium results in a larger contraction). Additionally, there are receptors present at the postsynaptic region of muscle and modulators have the capacity to alter muscle membrane resistance and receptor availability and affinity (Wu & Cooper, 2012; Levitan & Kaczmarek, 2015).

1.3 The lobster heart CPG-effector system

The American lobster (Homarus americanus) heart is an ideal system to use when investigating neuromodulation due to its simplicity and well-characterized anatomy and physiology. Whereas a myogenic human heart can contract independently of neural input, contractions of the neurogenic lobster heart are generated by a neuronal circuit (Cooke, 2002). So long as physiological saline is present, the heart produces fictive activity in vitro – this activity provides a behaviorally salient correlate with no exogenous input/application needed for these circuits to function. Heart contractions are driven by a CPG called the cardiac ganglion (CG) (Cooke, 2002) (Figure 1). The CG is a Y-shaped ganglion in the dorsal wall of the heart and consists of 9 cells: five anterior motor neurons (large cells or LCs) and four posterior premotor neurons (small cells or SCs) that are chemically and electrically coupled
(i.e., connected by electrotonic synapses) to generate synchronous, rhythmic, and monophasic bursting activity (Cooke, 2002) (Figure 1).

Figure 1. Schematic of cardiac ganglion (CG) and diagram of synaptic connectivity of CG neurons. Left, Schematic of the CG with the location of motor neurons (red) and premotor neurons (blue). Right, Diagram of synaptic connectivity of large (LC) and small cells (SC) denoting the electrical (resistor symbol) and chemical (filled triangle) coupling of all the motor and premotor neurons. All to all electrical coupling within cell type.
Specifically, the small cells (pacemaker cells) generate frequent driver potentials that produce bursts of action potentials; they synapse onto the large cells (motor cells), which synchronously fire bursts of action potentials that cause rhythmic heart contractions (Hartline, 1967; Marder & Calabrese, 1996) (Figure 2). Mechanistically, the driver potentials are sustained calcium-based depolarizations which initiate bursts of sodium-based action potentials in the spike initiating zone. The axons of these neurons extend to innervate the cardiac muscle (Yazawa et al., 1999). Therefore, the synaptic connections between the pacemaker neurons and motor neurons that innervate muscle fibers produce a monophasic rhythmic output that drives the heartbeat (Figure 2).

Importantly, within the CPG-effector system are two closed, intrinsic feedback pathways modulating the bursting pattern of the CG and stabilizing the heart's activity: the positive feedback pathway from stretch-sensitive dendrites and the negative nitric oxide (NO) feedback pathway (Mahadevan et al. 2004; Dickinson et al., 2015a; Dickinson et al., 2016b). Muscle stretch activates the stretch pathway via mechanosensitive dendrites that provide

Figure 2. Schematic of Lobster heart with example traces of muscle contraction and extracellular CG recordings. Top trace (blue) denotes an example of a muscle contraction recording from the entire heart via force transducer. Bottom (green) provides an example of an extracellular CG recording from large cells. Note the CG bursts reflect a 1:1 ratio with heart contractions.
feedback to the cardiac ganglion, increasing burst frequency of the CG (Alexandrowitz, 1932; Maynard & Maynard, 1960; Chin-Purcell, 2014; Qu, 2017). Specifically, the communication between the dendrites of the motor and pacemaker neurons and the muscle fibers activates this pathway (the stretch of CG dendrites signals changes in heart volume) (Alexandrowitz, 1932; Hartline, 1979; Yazawa et al., 1999; Cooke, 2002). NO feedback inhibits and slows the heartbeat by decreasing contraction frequency (Mahadevan et al. 2004). Mechanistically, increased calcium concentration from enhanced muscle contraction induces the release of NO, which in turn acts on the CG (Mahadevan et al. 2004). The two feedback pathways, in addition to modulation by neuropeptides with the CG, control the lobster heartbeat.
1.3.1 **Physiology of the cardiac striated muscle in decapods**

The *H. americanus* cardiac muscle consists of striated myocardial cells (striated muscle) that are non-propagating fibers (Sherman and Burrage, 1979, Millar et al., 2005). Like many other invertebrates, the crustacean striated muscles show graded responses to neural input (Hooper & Weaver, 2000; Millar et al., 2005). Contractions are controlled by the amount of calcium in the muscle, and modulation of calcium levels affects muscle activity (Berridge et al., 2000; Endo, 2009; Williams et al., 2013). The sarcoplasmic reticulum sequesters calcium in the muscle fiber. As fibers become depolarized by CPG-generated action potentials the

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**Figure 3. Lobster heart modulation via two intrinsic feedback pathways: Stretch and Nitric oxide.** Stretch is believed to be the positive feedback pathway and increases burst frequency. Nitric oxide negative feedback pathway decreases burst frequency.
sarcoplasmic reticulum releases calcium (Endo, 2009). Neuromodulators are known to effect muscle activity via modulation of calcium dynamics (Levitan and Kaczmarek, 2015).

Hartline (1967) showed that the two postero-lateral nerves (PLNs) of the CG each have three axon branches. These stem from the three most anterior cells in the ganglion, and all three cells have axons directed caudally down the trunk of the ganglion. On the trunk, each axon branches in a symmetrical manner sending one process out the right PLN and one out the left. The PLN’s have been shown to be identical (Anderson & Cooke, 1971).

1.3.2 Physiology of the Neuromuscular Junction (NMJ)

The crustacean NMJ – the chemical synapse between the motor neuron and the muscle fiber – is a model system to study the properties of synaptic transmission and integration/facilitation (Worden, 1997; Worden, 1998; Johnson et al., 2013; Titlow & Cooper, 2018). Most vertebrate skeletal muscles fire action potentials that cause contraction. However, most invertebrate muscle contraction is a graded function of depolarization with the muscles being multiply innervated. In crustaceans, neurotransmitter release at the NMJ results in a depolarization of the muscle membrane potential, known as an excitatory junction potential (EJP). This depolarization of the muscle fibers causes the muscle contraction, which is proportional in size to the amount of depolarization (Titlow & Cooper, 2018). Specifically, the EJPs in the lobster cardiac system are driven by the neurotransmitter glutamate released from the LCs, which quickly increases sodium currents, causing a rapid depolarization that is followed by a slower decay in the synaptic potential (Jan & Jan, 1976; Wu & Cooper, 2012; Titlow & Cooper, 2018).

Amplitude of EJPs can vary in crustaceans and, as muscles are non-spiking, the amplitude of the contraction is a direct function of the depolarization/membrane potential.
(Atwood et al., 1994; Atwood et al., 1995; Titlow & Cooper, 2018). Thus, EJP amplitude is an important factor to consider as a larger EJP generally corresponds to a larger muscle contraction in crustaceans (Titlow & Cooper, 2018). Amplitude is influenced by elements including the density of receptors on the postsynaptic membrane, the resistance of the membrane, the amount of transmitter released from the presynaptic cell, the presence of circulating modulators, and the type of muscle response (Atwood et al., 1994; Atwood et al., 1995; Titlow & Cooper, 2018). An increase in the probability of transmitter release is largely due to three factors: duration of depolarization, presynaptic membrane voltage, and the concentration and proportion of extracellular calcium (Cooke & Lipkin, 1972).

Furthermore, facilitation is believed to occur because of a buildup of calcium at release sites from repeated action potentials; this build up is thought to be unable to decrease to resting levels fast enough before the next stimulus (Katz, B, 1968; Titlow & Cooper, 2018). Facilitation generally acts on timescales shorter than 1 second (generally in the hundreds of milliseconds) (Zucker & Regehr, 2002). Evidence for this theory was shown by Katz and Miledi (1968) where a focal extracellular pipette was used to administer Ca\(^{2+}\) ions to neuromuscular junctions in a Ca\(^{2+}\) free medium. They showed that a conditional impulse did not release transmitter in this absence of Ca\(^{2+}\). Furthermore, it has been shown that potentiation depends on the presence of external Ca\(^{2+}\) during conditioning stimulation (Rosenthal, 1969; Weinreich, 1971; Erulkar & Rahamimoff, 1978). Additionally, there is evidence that continuous stimulation at frequencies that are greater than or equal to 5 Hz for between 20-30 minutes causes a two to five-fold increase in amplitude of EJPs: this facilitation is seemingly a result of sodium accumulation in the nerve terminals (Sherman & Atwood, 1971).
Miniature EJPs (mEJPs) are the result of spontaneously occurring unitary release from the presynaptic terminal (Cooke & Lipkin, 1972; Katz & Thesleff, 1957; Segal et al., 1985). mEJPs have mechanistically similar counterparts in other tissues including miniature end-plate potentials (MEPPs in vertebrate muscles, or miniature postsynaptic potentials (mPSPs) (neuron- to- neuron synapses)) (Katz & Thesleff, 1957; Segal et al., 1985; Jack et al., 1991). ‘End-plates’ describing the post-synaptic location of a vertebrate muscle fiber in contact with motor nerve endings (Cooke & Lipkin, 1972). The initial observation of miniature end plate potentials helped reveal the mechanism of chemical transmission (Fatt and Katz, 1952). Early work with mEJPs related the amount of mEJPs to transmitter release in response to nerve stimulation and presented evidence for an increase in probability in quantal release during nerve stimulation (Del Castillo and Katz, 1954a-b).

In a single preparation, there can be different classes of mEJPs defined by distinct shape and amplitude, which suggests they are being released from different terminals (but likely because of different receptors). mEJPs have been noted to be highly variable in their occurrence and lack consistency (Fatt & Katz, 1951; Katz & Thesleff, 1957). In particular, the mean frequency has been shown to be unstable and progressively changes with the intervals between mEJPs varying, with long periods of time with no bursts (Fatt & Katz, 1951). mEJPs occasionally produce short high-frequency bursts that have been attributed to extraneous stimulation or brief interaction between contributing units (likely because very little Ca\textsuperscript{2+} is needed for a single mEJP) (Fatt & Katz, 1951).

1.3.3 Modulation of the cardiac neuromuscular system

Neuropeptides both extrinsically and intrinsically modulate the cardiac neuromuscular system (Harris-Warrick & Marder, 1991; Katz, 1995; Dickinson et al., 2015b). The lobster has
an open circulatory system: hemolymph moves into the heart from the pericardial space through the ostia that are both dorsally and ventrally located on the heart and is pumped out via the anterior and posterior arteries (Maynard & Maynard, 1960) (Figure 4). Extrinsic modulators can be released locally or hormonally; the hormonally released modulators are synthesized outside the cardiac neuromuscular system, then released into the hemolymph to travel through the circulatory system (Cooke, 2002). The locally released extrinsic modulators are also synthesized outside of the cardiac neuromuscular system in locations with nerves to the cardiac ganglion and are released at nerve terminals in the CG or receptors in the periphery (Chen et al., 2007; Dickinson et al., 2015b). Intrinsic modulators are synthesized, released, and directly act on the CG.

**Figure 4.** Schematic of the entire lobster heart showing the CG (orange) and the ostia labeled. Heart oriented posterior to anterior from left to right with the ventral side up. Adapted from MacMillan (2013).
1.4 Myosuppressin

Myosuppressin (pQDLDHVFLRFamide) is a highly conserved (present in neural tissues of 32 species in seven decapod crustacean infraorders; Stemmler et al., 2007) and well-studied neuropeptide that is endogenous to *H. americanus* and has known effects on the heart (Stemmler et al., 2007; Stevens et al., 2009; Oleisky et al., 2020; Oleisky et al., 2022). Named for its inhibitory effects on insect muscle tissue, crustacean myosuppressin was identified via mass-spectrometry in lobster commissural ganglia (Nässel, 2002; Stemmler et al., 2007). Myosuppressin is part of the FMRFamide-like peptide (FLP) family and characterized by its C terminal motif- HVFLRFamide (Stevens et al., 2009). In many arthropods myosuppressin has inhibitory effects, including in *Drosophila melanogaster* where myosuppressin acts to decrease *in vivo* heart rate in a dose-dependent manner, and in the crayfish heart where some isoforms decrease contraction rate (Mercier & Russenes 1992; Nichols 2003; Tanaka, 2016).

Stevens et al. (2009) provided evidence that myosuppressin acts at multiple sites within the cardiac neuromuscular system of *H. americanus* and can result in muscular excitation as well as decreases in heart frequency. Myosuppressin’s effects were observed across four types of preparations: the intact animal, the heart *in vitro*, the isolated CG, and a stimulated heart muscle preparation. In the whole heart, myosuppressin causes a rapid decrease in contraction frequency and amplitude followed by a large increase in amplitude (Stevens et al., 2009) (Figure 5). In the isolated CG, myosuppressin (10^{-7} M) increases the duration of action potential bursts and decreases their cycle frequency by hyperpolarizing the membrane potential of cell bodies of the neurons in the pattern generator (Stevens et al., 2009) (Figure 6). Thus, these changes in the CG explain the decrease in contraction frequency seen in the recording of the whole heart with myosuppressin but do not explain the changes in contraction amplitude. In a preparation with spontaneous motor input eliminated and with electrical stimulation of the
motor nerve end to evoke transmitter release and thus a contraction, myosuppressin acted to increase the amplitude of cardiac contractions (Stevens et al., 2009) (Figure 7). This suggests that myosuppressin acts at the periphery, (i.e., the NMJ or muscle), as well as in the CG itself, but it is unclear whether myosuppressin acts on the muscle, the NMJ, or both that results in an increased contraction amplitude observed in the whole heart.

Prepronosuppressin mRNA transcripts are found in CG tissue, indicating it is synthesized and released locally (Oleisky et al., 2020). Additionally, there is evidence that there is no FMRFa-like immunoreactivity, and since myosuppressin is a FMRFamide-like peptide, it is probably not observed in the CG (Stevens et al., 2009). So, modulation of the cardiac neuromuscular system via FMRFa-like peptides (including myosuppressin), is hormonal (Stevens et al., 2009).

Five putative myosuppressin receptors (MSR I – V) have been identified from a homology search of the lobster transcriptome from brain and eyestalk ganglia, and cardiac ganglion-specific transcriptomes (Oleisky et al., 2020; 2022). All five HaMSRs were identified in the cardiac muscle, but among the eight replicates examined, this expression was not consistent, which could suggest conditional regulation of transcription or limited transcript abundance (Figure 8) (Oleisky et al., 2022).

In this study, I investigated where myosuppressin acts at the periphery of the neuromuscular system, to cause an increase in contraction amplitude. Because there are myosuppressin receptors on the neurons and the muscles, we examined the response after removing the nervous system. I found that myosuppressin increased the amplitude of glutamate-evoked contractions in the isolated muscle but did not increase the amplitude of excitatory junction potentials (at the NMJ). This suggests that myosuppressin acts directly on
the cardiac muscle and not at the NMJ, to cause the observed increase in amplitude in the whole heart.
Figure 5. In the whole heart, myosuppressin causes a decrease in contraction frequency and amplitude followed by a large increase in amplitude. Adapted from Stevens et al. (2009). Upper trace denotes myosuppressin’s (10^-6) complete effects on the whole heart. Bottom trace shows a faster timescale of myosuppressin’s (10^-6) initial effects from the section indicated by black bars on the top trace.
Figure 6. In the isolated CG, myosuppressin hyperpolarizes the membrane potential of cell bodies, increases burst duration, and decreases cycle frequency. Adapted from Stevens et al. (2009). Ant Lat nerve: anterolateral nerve.
Figure 7. In a preparation with the motor cells removed and consistent electrical input to elicit contractions, myosuppressin increases the amplitude of stimulated contractions. Bottom overlay shows control traces in green and traces with myosuppressin (10^{-7}M) in green. Adapted from Stevens et al. (2009).

Figure 8. PCR-based confirmation of HaMSR expression in cardiac muscle. Adapted from Oleisky et al. (2022). Arrowheads indicate products of expected sizes. All products were sequence validated.
Methods

2.1 Animals

Adult (~500g) *H. americanus* were purchased from local seafood retailers in Brunswick, Maine, USA. Individuals were housed in re-circulating natural seawater aquaria and were maintained at 10-12°C on a 12-hr/12-hr light/dark cycle. Lobsters included females and males and all molt stages were used. Their diet consisted of chopped shrimp or squid once a week. Experimental and animal care procedures were performed following protocols approved by Bowdoin College.

2.2 Physiological Experiments

2.2.1 Experimental Setup and Lobster Dissection

In preparation for physiological experiments, individual lobsters were anesthetized by packing in ice for ~30 min prior to isolation of the heart. The heart is positioned anterior to the tail and is dorsally situated. The heart was removed from the body of the animal as well as the cephalothoracic carapace (with the hypodermis attached) via manual microdissection in chilled (8–10°C) physiological lobster saline (composition in mM: 479.12 NaCl, 12.74 KCl, 13.67 CaCl2, 20.00 MgSO4, 3.91 Na2SO4, 11.45 Trizma base, and 4.82 maleic acid; pH: 7.45 (room temperature pH); as described in Dickinson et al. 2018). The heart was opened along the ventral axis to expose the intact cardiac ganglion and cardiac muscles under a dissecting microscope.

Throughout experiments the temperature of physiological saline was maintained between 10-12°C via a Peltier temperature regulator (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA) and monitored with a temperature probe (Warner Instruments, Hamden, CT, USA). Physiological saline was
superfused across the heart with a Rabbit peristaltic pump (Gilson, Middleton, WI, USA) at a flow rate of ~5ml/min.

2.2.2 Myosuppressin Synthesis and Preparation

Myosuppressin (pQDLDHVFLRFamide; MW = 1272.46 g/mol) was custom synthesized by GenScript Corporation (Piscataway, NJ, USA) and bath applied using the peristaltic pump. Myosuppressin has relatively low aqueous solubility and was dissolved in DMSO (15%) and then diluted in deionized water to make $10^{-3}$ M stock solutions (Stevens et al., 2009). The $10^{-3}$ M myosuppressin stock solution was stored in small aliquots at -20°C and diluted in room temperature lobster saline to desired concentrations immediately before experimental use.

2.2.3 The Cardiac Muscle

To examine whether myosuppressin exerts its effects directly on the muscle itself, cardiac muscle contractions were measured using either of the two lateral muscles (Figure 9) inside the lobster heart. The CG was removed to eliminate spontaneous neural input (spontaneous contractions). Removal of the CG both eliminated intrinsic stimulation of the muscle fibers from stimulus and bypassed the NMJ to independently assess muscle activity in response to myosuppressin. To measure muscle contractions, a SI-H optical force transducer (WPI Inc., Sarasota, FL) and a 1700 A-M Systems Differential AC Amplifier (Sequim, WA, USA) were used with methods adapted from Maguire (2019). After a healthy bundle of muscle fibers was located, hooks were glued perpendicular to each end of the bundle using GluTure topical tissue adhesive (Zoetis Inc., Kalamazoo, MI). Healthy bundles were identified by visualizing contractions of the muscle fibers with the nervous system still in the heart (before removal of the CG). Before glue was applied, the fiber bundle was briefly dried to ensure that
there was not any physiological saline in the region, ensuring the glue would adequately stick to the hooks. During this process the preparation would be out of saline for less than 30 seconds, which caused no known harm to the heart (Xuan, 2017). Hooks were used to stabilize the muscle movement and were attached to manipulators to allow for positioning on muscles. The optical force transducer was placed on top of the muscle between the hooks. The force transducer was used to measure the muscle contractions and calibrated to measure this force of contraction in grams (Micro 1401; CED, Milton, Cambridge, UK).

Figure 9. Annotated ventral view image of the lobster heart from Maguire (2019). Location of the lateral (longitudinal) muscles denoted and outlined. CR transverse muscle stands for “Circle’s region” named after Circle Xuan, an Honor’s Student in 2017.
After isolating the cardiac muscle, a muscle contraction was stimulated via L-glutamate, the neurotransmitter used by the motor neurons. L-glutamate was administered via an Aladdin Single-Syringe pump (WPI, Sarasota, FL) and microelectrode (AM systems, Sequim, WA) that was connected to an electrode positioned to focally apply glutamate ($5.5 \times 10^{-4}$ M) onto one of the lateral muscles with the following parameters: 0.02ml was applied every 250s at a rate of 0.85 $\mu$L/min (i.e., as a 1.41s application). A control experiment with lobster saline focally applied onto the muscle was done to validate that the mechanical force of the application was not causing the contraction (data not shown). Concentration was determined via a set of experiments observing sensitization/desensitization with different glutamate concentrations ($10^{-4} – 10^{-3}$). Physiological saline was constantly perfused across the heart until the glutamate-evoked muscle contractions were stable in size. These glutamate-evoked contractions were used as a comparison regarding the amplitude of contraction with myosuppressin superfused. Once stable in size, bath application of myosuppressin ($10^{-6}$ M) was continuously administered over the heart for 30 minutes with periodic glutamate application with the same parameters as in control saline (.85ul/min, 0.02ml every 250s with a 1.41s). After applying myosuppressin, the preparation was washed with lobster saline to ensure contractions returned to baseline while glutamate was periodically administered (same shape/size as glutamate-evoked contractions before the myosuppressin application) (Figure 10). Only preparations in which the contractions returned to baseline – indicating a healthy heart muscle/undamaged muscle – were analyzed (10/11).
Figure 10. **Annotated image of muscle contraction experimental set up.** After eliminating input from the large cells (the CG was removed before the start of the experiment), focal L-glutamate ($5 \times 10^{-4}$M) was applied to evoke contractions. Lateral muscle contractions were recorded via optical force transducer (calibrated to start at 0g) on the surface of a single muscle bundle. Experimental design adapted from Maguire (2019) and Jordan (2020). Image courtesy of Matt Maguire.

2.2.4 The Neuromuscular Junction (NMJ)

As with the cardiac muscle protocol, the CG was removed to eliminate spontaneous EJPs. Methods to elicit and record EJPs were adapted from Jordan (2020). One posterior lateral nerve ending was stimulated with a suction electrode (hand cut and fire polished) using a pulse stimulator (model 2100 Isolated Pulse Stimulator, A-M System, Sequim, WA). This elicited a contraction in the muscle fiber(s) that it innervates. An intracellular electrode was inserted into one of those contracting fibers; the resting membrane potentials of the muscle fibers were between -30 and -70 mV.
To record activity at the NMJ, EJPs were recorded via microelectrode filled with a solution that mimics the muscle (squid cytoplasmic fill: 20 mM NaCl, 15 mM Na2SO4, 10 mM Hepes, 400 mM potassium gluconate, 10 mM MgCl2) (Hooper et al., 2015). Electrodes had a resistance within the range of 12MΩ -22MΩ. The electrode was inserted into a single muscle fiber on either of the lateral muscles of the heart with intracellular activity monitored via an AxoClamp 2B amplifier (Axon Instruments, Molecular Device, San Jose, CA) (Figure 11, Figure 12).

The posterior lateral nerve (PLN) ending was stimulated with three to four electrical pulses at a frequency of 2.5Hz to evoke three to four EJPs (Grass S88 dual output square pulse stimulator; AM systems, Sequim, WA). The stimuli were delivered every hundred seconds and each stimulus pulse had a duration of 0.5 ms (5 x 10⁻⁴ second). Parameters were adapted from Jordan (2020). A range of voltages (2V-7V) were used as each nerve/preparation required a different threshold voltage to evoke an EJP. Once EJPs were being elicited consistently, myosuppressin was superfused over the preparation for twenty minutes (Stevens et al., 2009). This was followed by a 40–50-minute saline wash to ensure the myosuppressin was completely removed from the preparation and activity could return to baseline. mEJPs occurred spontaneously in the preparations (Figure 13).

Analogue signals were recorded on Spike (2v7 and 2v9 software; CED, Milton, Cambridge, UK) and digitized at 10kHz using a Micro 1401 (CED, Milton, Cambridge, UK).
Figure 11. Annotated image of NMJ (EJP) experimental set up. EJPs were elicited with 0.5ms pulses delivered via suction electrode on the CG nerve ending and recorded with an intracellular electrode inserted into a cell within a single contracting muscle fiber.

2.3- Data Analysis

2.3.1 The Cardiac Muscle

The glutamate-evoked contractions were measured using a custom script in Spike2. The script measured five points (the stimulation artifact, the minimum value of the contraction, the maximum value, and both half-widths) to find the peak of the contraction, the baseline tension, and the duration. To compare the amplitude and baseline changes of the contractions in physiological saline and in myosuppressin, the four contractions in saline were compared to four contractions in the myosuppressin at peak effect (within subjects) ($N=10$).
2.3.2 The Neuromuscular Junction

To measure the amplitudes of the stimulated EJPs (EJP 1, EJP 2, EJP 3) the “peak finder” function and the horizontal cursors function in Spike2 were used. Due to facilitation, each EJP was analyzed individually as three separate groups, EJP 1, 2, and 3, in the control saline, myosuppressin application, and the wash. If four EJPs were recorded, the first three EJPs were used (in case there were any effects of facilitation). For each preparation, the amplitudes of the trains of EJPs prior to myosuppressin superfusion (control) were averaged to keep a consistent number of events across experiments. If there were more than seven EJPs in a given preparation, the last seven EJPs were averaged in each condition. mEJPs were measured by class type via the horizontal cursors. All mEJPs were equal to or less than 10% of the stimulated EJP.

2.3.3 Statistical Analysis of Cardiac Muscle and NMJ experiments

Prism was used for statistical analysis and graphing (v9.0 GraphPad Software, Inc., San Diego, California). A paired t-test was used to compare between saline and myosuppressin conditions, using a cutoff of $p \leq 0.05$. A Kolmogorov-Smirnov (KS) test was used for the mEJPs to assess the distribution of the amplitude. A gaussian function curve was applied to the histogram of mEJPs. mEJPs were normalized, i.e., the frequency histogram presented as % of total, as the frequency was inconsistent between preparations.

Results

3.1 Myosuppressin significantly increases the glutamate-evoked contraction amplitude in the isolated cardiac muscle

The presence of myosuppressin receptor (MSR) mRNA in the cardiac muscle tissue (as well as the ganglion) suggests that these receptors are present at the cardiac muscle, where
myosuppressin binding may result in the increased contraction amplitude observed in the whole heart recordings (Oleisky et al. 2020; 2022) (Figure 8). To assess this at the level of the muscle, 10^{-6} M myosuppressin was superfused over the cardiac muscle with the CG removed, and muscle contractions were recorded in response to glutamate focal application.

Myosuppressin increased glutamate-evoked contraction amplitude in the isolated muscle, demonstrating that myosuppressin exerts peripheral effects directly on the cardiac muscle (Paired t-test, \( p=0.0068, N=10 \)) (Figure 14, Figure 15). To visually compare the amplitude of recorded contractions in myosuppressin and those in saline, example contraction traces were overlayed and aligned to contraction onset (Figure 14).

In addition to the changes observed in contraction amplitude, in some preparations the baseline tension were variably affected. Baseline tension remained constant (Figure 14), increased, or decreased from the initial set point of 0g in different preparations (change in both directions). The range of change also varied dramatically from 36.7\% (increase from 0g) to -32.07\% (decrease from 0g) (Figure 16). To ensure the observed change in amplitude was a function of myosuppressin, and not a function of the changing baseline, baseline tension was analyzed. There was no difference in mean baseline tension as a function of myosuppressin superfusion (Paired t-test, \( p=0.2285, N=10 \)) (Figure 16).

Other aspects of evoked contractions were considered, including duration of contraction. However, there was a large amount of variability between preparations and no significant change in contraction duration was observed in control saline and myosuppressin (Paired t-test, \( p=0.1339, N=10 \)) (Figure 17, Figure 18). The range of percent change of duration of contraction ranged dramatically from a 705\% increase in duration of the contraction to -52.9\%, a decrease in duration of contraction in myosuppressin as compared to control saline
To compare the duration of contractions visually, a single glutamate-evoked contraction in myosuppressin and a single contraction in saline were normalized (Figure 17). Thus, myosuppressin (10⁻⁶ M) primarily contributed to an increase in contraction amplitude of glutamate-evoked contractions with non-significant changes in baseline tension and the duration of contraction (Figures 14-18).

3.2 Myosuppressin does not exert its effects at the NMJ

3.2.1 Myosuppressin has no significant effect on stimulated EJPs

With MSR expression in the CG and cardiac muscle tissue, it is possible that there are receptors at the NMJ. However, it is not clear where the receptors are localized as there is currently not a working antibody. Therefore, myosuppressin binding at the NMJ could contribute to the increased contraction amplitude observed in the whole heart recordings (Figure 5)– and would be seen as an increased EJP amplitude. EJPs were recorded from a single muscle fiber in control saline and myosuppressin (10⁻⁷ M). At the NMJ, myosuppressin did not alter the amplitude of EJPs, suggesting that myosuppressin does not act to increase muscle contraction amplitude by affecting EJP dynamics (Figure 19, Figure 20). To visualize the difference (or lack thereof) in amplitude between EJPs in saline and myosuppressin (10⁻⁷ M), example traces from one set of stimulated EJPs were overlayed (Figure 19). EJPs were analyzed separately rather than a set of three (EJP 1, EJP 2, EJP 3) to account for possible effects of facilitation. None of the three EJPs changed in amplitude from control saline to myosuppressin (10⁻⁷ M) (Figure 19) (Paired t-test; p>0.05; N= 10).

3.2.2 Myosuppressin’s impact on spontaneous mEJPs is not fully understood

In some EJP preparations, mEJPs were observed (Figure 13). The mEJPs occurred spontaneously and analysis of mEJPs did not yield definitive results (responses were extremely
variable). Of preparations with mEJPs, only two produced responses reliable enough to be analyzed. Thus, with this small of a sample size, these data are extremely preliminary. In one preparation, there was one observed class of mEJPs (Figure 21) and in another preparation, three distinct classes were observed (Figure 22a-c). Classes of the second preparation were grouped by amplitude, duration, and waveform shape. There was an observed overall change in the distribution of amplitude (KS test; $p<0.0001; p=0.0482$) (with myosuppressin mEJPs smaller than control mEJPs) (Figure 21, 22a) in two cases. However, it is important to note that one preparation (Figure 21) does not include a wash which makes those results inconclusive as this could just be significant decrease in amplitude over time as seen in another preparation (Figure 22b). Furthermore, in another class (Figure 22c) the distribution of mEJP in amplitude was significant different between control and myosuppressin ($10^{-7}$ M) (KS test; $p=0.0393$) but was also significantly different for control and wash (KS test; $p=0.0014$), indicating a lack of change from the myosuppressin itself. Finally, the sample size for one class was extremely small ($N=1$; sampled mEJPs in each condition: saline, 8; myosuppressin, 7; saline wash, 15) making the distribution less reliable (Figure 22a). These results suggest that myosuppressin acts directly on the cardiac muscle to increase contraction amplitude and not at the NMJ.
Figure 12. **Spontaneous EJPs vs Stimulated EJPs.** Top, spontaneous EJPs were recorded in a heart with the CG intact in a contracting muscle fiber. Bottom, example trace of stimulated EJPs with the CG removed.
Figure 13. Comparison of spontaneous miniature EJPs in control lobster saline (dark blue) to min EJPs in myosuppressin (10^{-7} M) (light blue). Myosuppressin does not appear to change the amplitude of the spontaneous mEJPs.
Figure 14. Example glutamate evoked contractions in physiological saline and myosuppressin (10^{-6} M). **Top,** myosuppressin (10^{-6} M) (orange) significantly increased glutamate-evoked contraction amplitude compared to control in lobster saline (saline, yellow). **Bottom,** effects of myosuppressin (10^{-6} M, orange), were reversed when the preparation was washed with control saline (yellow).
Figure 15. Myosuppressin significantly increases the glutamate-evoked contraction amplitude in the isolated cardiac muscle. **Left**, Comparison of mean amplitude (grams) for control contractions in lobster saline (0.019g ± 0.01g) and in myosuppressin 10^{-6} M (0.028g ± 0.02g). There is a significant mean difference between conditions (Paired t-test, \( p=0.0068, N=10 \)). **Right**, Percent change of the amplitude of contraction from control to myosuppressin 10^{-6} M ranged from 19.8% to 165%. Error bars represent SD.
Figure 16. Myosuppressin did not significantly alter baseline tension in cardiac muscles. **Left,** Comparison of mean baseline level for control contractions in lobster saline (0.025g ± 0.05g) and in myosuppressin $10^{-6}$ M (0.024g ± 0.05g). Force transducer calibrated to start at 0g, tension measured to that baseline. There is no significant change in mean baseline level (Paired $t$-test, $p=0.5900$, $N=10$). **Right,** Percent change of the baseline tension from control to myosuppressin $10^{-6}$ M ranged from -32% to 36%. Error bars represent SD.
Figure 17. Normalized curves demonstrating myosuppressin ($10^{-6}$ M) largely did not act to change the duration of glutamate-evoked contractions. Yellow trace, control saline; orange, myosuppressin ($10^{-6}$ M).
Figure 18. Myosuppressin did not significantly duration of glutamate-evoked contraction in the isolated muscle. Left, Comparison of mean duration (s) for control contractions in lobster saline (21.6s ± 23.4s) and in myosuppressin 10⁻⁶ M (28.14s ± 26.8s). There was no significant mean difference between conditions (Paired t-test, $p=0.1339$, N=10). Right, Percent change of the duration of contraction from control to myosuppressin 10⁻⁶ M ranged from -52.9% to 705%. Error bars represent SD.
Figure 19. Comparison of stimulated EJPs in control lobster saline (light green) to stimulated EJPs in myosuppressin ($10^{-7}$ M) (dark green). Bottom trace is an overlay of the two above traces. Myosuppressin does not appear to change the amplitude of EJPs.
Figure 20. Myosuppressin does not significantly increase the amplitude of EJPs. The mean amplitude of the first three EJPs in a train of EJPs was measured in control saline (light green) EJP 1 (6.31mV ± 4.9mV), EJP 2 (5.56mV ± 4.0mV), EJP 3 (5.90mV ± 4.1mV) and during the application of myosuppressin (10⁻⁷ M) (dark green; EJP 1 (5.78mV ± 4.5mV), EJP 2 (4.86 ± 3.6mV), EJP 3 (5.28mV ± 3.6mV); (mean ± SD). There was no significant difference in the amplitude of EJPs between control and myosuppressin (Paired t-test; p>0.05; N= 10). Data combined with experiments completed by Audrey Jordan.
Figure 21. Distribution of changes in mEJP in amplitude in response to myosuppressin (10^{-7} M). The amplitude of mEJPs was measured in control saline (red; 1.47 ± 0.0) and myosuppressin (10^{-7} M) (myo) (blue; 1.38 ± 0.0). Myosuppressin application resulted in an altered overall distribution of amplitude (KS test; p<0.0001). No wash was recorded for this preparation. A gaussian distribution curve was applied for each condition: myosuppressin (10^{-7} M) (R^2=0.886) and control saline (R^2=0.942) (N=1; sampled mEJPs in each condition: control, 251; myo, 231). ****p<0.0005.
Figure 22. Distribution of changes in mEJP in amplitude in response to myosuppressin (10⁻⁷ M). A,B,C represent three classes of EJPs from a single preparation. A, The amplitude of mEJPs was measured in control saline (red; 0.332 ± 0.0133) and myosuppressin (10⁻⁷ M) (myo) (blue; 0.279 ± 0.146) and wash (orange; 0.322 ± 0.009). Myosuppressin application did significantly altered distribution of amplitude considering control and myosuppressin (10⁻⁷ M) (KS test; p=0.0482), did not for myosuppressin (10⁻⁷ M) and wash (KS test; p=0.294), nor for control and wash (KS test; p=0.496). A gaussian distribution curve was applied for each condition: control saline (R²=0.7847), myosuppressin (10⁻⁷ M) (R²=0.332), and wash (R²=0.430). (N=1; sampled mEJPs in each condition: control, 8; myo, 7; saline wash, 15) B, The amplitude of mEJPs was measured in control saline (red; 0.844 ± 0.0228) and myosuppressin (10⁻⁷ M) (blue; 0.812 ± 0.0135) and wash (orange; 0.786 ± 0.0121). Myosuppressin application did significantly altered distribution of amplitude considering control and myosuppressin (10⁻⁷ M) (KS test; p=0.0393), did not for myosuppressin (10⁻⁷ M) and wash (KS test; p=0.294), and was significantly different for control and wash (KS test; p=0.0014). A gaussian distribution curve was applied for each condition: control saline (R²=0.980), myosuppressin (10⁻⁷ M) (R²=0.972), and wash (R²=0.943) (N=1; sampled mEJPs in each condition: control, 12; myo, 53; saline wash, 33). C, The amplitude of mEJPs was measured in control saline (red; 1.61 ± 0.03) and myosuppressin (10⁻⁷ M) (blue; 1.32 ± 0.01) and wash (orange; 1.22 ± 0.0157). Myosuppressin application did not significantly alter distribution of amplitude considering control and myosuppressin (10⁻⁷ M) (KS test; p=0.9934), did for myosuppressin (10⁻⁷ M) and wash (KS test; p<0.0001), and was significantly different for control and wash (KS test; p<0.0001). A gaussian distribution curve was applied for each condition: control saline (R²=0.5855), myosuppressin (10⁻⁷ M) (R²=0.962), and wash (R²=0.834) (N=1; sampled mEJPs in each condition control, 22; myo, 88; saline wash, 194). *p<0.05, **p<0.005, ****p<0.0005.
Discussion

4.1 Myosuppressin exerts effects at the periphery

This work aimed to elucidate the peripheral site at which myosuppressin acts to cause an increase in amplitude of cardiac contractions, as seen in the whole heart, in the *H. americanus*. The observed complex changes in cardiac physiology in response to myosuppressin – an increase in amplitude and a decrease in frequency – suggested that modulation occurs at more than one site. This provides an interesting opportunity to examine how complex modulation leads to greater dynamics than at any one site alone. Modulation can be dynamic, with unique effects on a single neuron that are dependent on factors including concentration of modulators and modulators having different timescales of action at different locations. When extrapolated to a whole network, it is likely that for invertebrate responses (where responses are graded), we would see non-linear correlations with a large pool of possible outcomes that are difficult to predict (Jorge-Rivera & Marder, 1996; Jorge-Rivera et al., 1998; Swenson & Marder, 2000; Swenson & Marder, 2001). Interestingly, these data provide evidence that myosuppressin acts directly at the cardiac muscle but not at the NMJ to increase the amplitude of contractions.

The lack of observed modulation of EJP amplitude in myosuppressin is a surprising result, considering that most work shows modulators that enhance contraction amplitude also enhanced EJP amplitude (Jorge-Rivera & Marder, 1996, Jorge-Rivera et al., 1998, Wilkens et al., 2005). EJPs of the gastric mill 4 (gm4) and gastric mill 6 (gm6) muscle of the crab, *Cancer borealis*, were shown to increase in amplitude in response to TNRNFLRFamide, serotonin, proctolin, and dopamine, and decrease in amplitude slightly in response to Allatostatin C. The changes in EJP amplitude were smaller than the observed contraction but did so in the same direction (for all modulators). Jorge-Rivera & Marder (1996), provided evidence that nerve
evoked contractions and EJPs in *Cancer borealis* both increased in amplitude in response to the peptide TNRNFLRFamide. However, the change in EJP amplitude was modest compared to the percent increase of contraction (% increase, EJP amplitude <20%; contraction, 120%) (Jorge-Rivera & Marder, 1996).

In addition to the contraction amplitude, it is interesting to consider the duration of contraction. These data presented suggest that myosuppressin does not significantly change the duration of contraction; however, there was a great deal of variability in responses to myosuppressin (a -52.9% to 705% change). In aplasia, SCP<sub>A</sub>, SCP<sub>B</sub> (SCP; small cardioactive peptide family), and serotonin increase the amplitude and relaxation rate of the accessory radula closer (ARC) muscle contractions (Weiss et al., 1978; Weiss et al., 1992). The ARC muscles are responsible for the ‘closer muscle’ of aplasia which is part of the feeding system. It is hypothesized that this modulation occurs to maintain the appropriate feeding behavior (Weiss et al., 1978; Weiss et al., 1992; Fox & Lloyd, 1997; Lum et al., 2005). Interestingly, in the lobster heart, myosuppressin does not increase the relaxation rate (decrease the duration), and it is possible that because the frequency of contractions were much slower (contractions were elicited every 100s), there was no physiologically relevant reason from them to return – essentially there was time for the muscle to take longer to relax.

It is unclear why the duration of contractions does not appear to change. Factors including sex, year (completed across two summers), and size of the lobster had no correlation with the duration of contraction (data not shown). There is a great deal of variation; however, all contractions that started with a duration above 20s showed a significant increase in duration of contraction for that group. However, this increase is still small (data not shown) and because there is such a small increase, this could not be physiologically relevant. Additionally, it is
unclear what determined contraction duration to begin with. Thus, it is difficult to predict what could be causing a change in duration. For example, any changes could be purely experimental: a preparation with an increased baseline tension could have moved the heart closer to the electrode administering glutamate, causing the glutamate-evoked contractions to change shape.

At this point, it is not clear the myosuppressin is the sole cause of any duration changes and due to the variability of response, more preparations would need to be examined to state this conclusively. Stevens et al. (2009) showed that both in the whole heart and the controlled nerve stimulated heart, myosuppressin caused an increase in duration of contraction. The increase was much smaller in the stimulated heart, with most of the increase in contraction duration attributed to the nervous system (Figure 23.).

![Figure 23. Overlayed single contractions with bath application of myosuppressin (10^{-7}) and control saline for heart contractions evoked via a nerve stimulation (A) and for an intact heart (spontaneous contractions) (B). Myosuppressin increased the duration of contraction in both preparations, but to a smaller extent in the stimulated heart. Adapted from Stevens et al (2009).](image)

It would be useful in the future to conduct the same experiments with other peptides such as calcitonin-like diuretic hormone (CLDH), which is known to increase the frequency and
amplitude of contraction and decreases contraction duration in the whole heart of the lobster and see if modulation occurs at the muscle (Christie et al., 2010).

### 4.2 Myosuppressin could exert different effects through a range of signaling pathways

One explanation for myosuppressin acting at the cardiac muscle but not the NMJ (via EJPs) to increase contraction amplitude, is that the distribution of myosuppressin receptors in the tissue leads to differing responses. Oleisky et al. (2020) provided evidence that myosuppressin, when applied to the motor neurons and pacemaker interneurons separately, produced unique responses. The proposed hypothesis for the distinct responses was possibly that the known myosuppressin receptors (I-V) exert different effects as well as have a varied distribution.

More specifically, Oleisky et al. (2022) showed that the nonamidated myosuppressin (pQDLDHVFLRFG) only affected the nervous system but did not affect the muscle. This indicates that receptors present in the muscle could be different (and/or acting differently) than those in the cardiac ganglion. Specifically, suggesting that there is at least one receptor that acts at the cardiac ganglion and not at the muscle. Interestingly, all five known myosuppressin receptors are seen to be present in the muscle (Oleisky et al. 2022) (Figure 8.). However, it is possible that one of the receptors in the muscle could be expressed at such low levels that it is not contributing to the muscle response. There is no qPCR done to conform this, but visually inspecting the expression of receptors in PCR it is possible that receptor III could be in the muscle but not contribute to the response, as the PCR band is extremely faint (Oleisky et al. 2020). Furthermore, receptor III is expressed in all three motor cells and one pacemaking cell in the CG (Oleisky et al. 2020). Additionally, MSR V has a strong PCR band and is not seen to be expressed at the CG, suggesting MSR V could be strongly mediating the response at the
muscle. It is however important to note that of the 8 lobsters used, 6/8 expressed MSR V (Oleisky et al. 2022) (Figure 8.).

4.3 Preliminary evidence suggests myosuppressin does not change the amplitude of miniature EJPs

The amplitude of an EJP is a result of a combination of the number of vesicles and the amplitude of response to each vesicle (Cooke & Lipkin, 1972; Jack et al., 1991; Korn & Faber, 1991). Because presynaptic neurons spontaneously release individual vesicles, those result in a small response, known as mEJPs. As there was no change in the EJP amplitude in control saline compared to EJPs in myosuppressin, we hypothesized that the mEJPs would likely also not significantly change in response to myosuppressin. More specifically, a change in mEJP amplitude reflects a change occurring at the NMJ (postsynaptically) as the response to a single vesicle is getting bigger. This could be indicative of more transmitter (more vesicles) or a bigger response to each vesicle (Cooke & Lipkin, 1972). If the mEJPs were decreasing in size this would indicate that each vesicle caused a smaller postsynaptic response. Because an EJP is determined by the number of mEJPs and related vesicles, and because the amplitude of stimulated EJPs are constant, if mEJPs were decreasing in size, there would have to be a proportional increase in the number of vesicles released. While this is not impossible, this author is aware of no such cases that have been documented. As mentioned previously, changes in EJP amplitude is important to consider as, in crustaceans, amplitude is a direct function of the depolarization (muscles are non-spiking) (Atwood et al., 1994; Atwood et al., 1995; Titlow & Cooper, 2018). This in contrast to vertebrate muscles where the size of PSPs does correlate with muscle contraction as to firing is an “all or none” event.
mEJPs were not consistently present in each preparation. Thus, only a small same size was obtained ($N=2$). mEJP analysis indicated a lack of change of mEJP amplitude from myosuppressin ($10^{-7}$). A preliminary pass of the data suggests that myosuppressin might significantly decrease in mEJP amplitude; however, this is likely not the case. First, the clear trend seen in the first preparation (Figure 21) lacks the wash. This makes the results inconclusive as it is possible that the mEJPs were continually decreasing in amplitude throughout the preparation. This is seen in the second EJP class in the second preparation (Figure 22b). Although there is a significant change in amplitude from the myosuppressin application to the wash, this is also the case when comparing the distribution of the control and the wash, indicating this gradual decrease (Figure 22b). Furthermore, the trend observed in first class in the second preparation (Figure 22a), although significant, is a product of an extremely low sample size ($N=1$; sampled mEJPs in each condition control, 8, myosuppressin, 7; wash, 15). Similarly in the third class in the second example (Figure 22c), the distribution is significantly different in control compared to myosuppressin and the control compared to the wash. However, the myosuppressin condition and the wash distributions are not different from each other, suggesting a lack of change. Thus, the results suggest a lack of change with the application, or if anything a slight decrease in amplitude of spontaneous mEJPs in myosuppressin. Taken together, these data suggest that myosuppressin does not act on the NMJ via mEJPs or EJPs to cause an increase in contraction amplitude as observed in the whole heart.

4.4 Proctolin, which modulates the muscle through L-type calcium channels, provides a possible mechanism for myosuppressin’s effects on the cardiac muscle

A neuromodulator that has been well studied at the periphery – at both the NMJ and the muscle – is proctolin (Wilkens et al., 2005). In another arthropod, *Drosophila melanogaster*,

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proctolin did not appear to modulate the amplitude of excitatory junction potentials but did increase the amplitude of nerve-evoked contractions in a dose-dependent manner (Ormerod et al., 2016). It additionally induced sustained muscle contractions in preparations with the CNS removed and no other stimuli applied (Ormerod et al., 2016). In the marine isopod, *Idotea baltica*, proctolin acts to increase muscle membrane resistance at the postsynaptic membrane, which leads to the increased amplitude of EJPs (Erxleben et al., 1995).

Wilkens et al. (2005) determined that proctolin exerts its effects on the periphery at both the NMJ and muscle to increase cardiac contraction amplitude. Furthermore, they showed that the extent of the lobster’s cardiac muscle contraction was proportional to calcium increase, suggesting that proctolin does affect calcium dynamics.

Mechanistically, proctolin was shown to affect calcium release from the L-type calcium channels in the sarcoplasmic reticulum (SR). When an L-type calcium blocker (ryanodine) was applied, contractions were suppressed even when proctolin was applied, indicating that sequestered calcium (from the SR) is essential to cause enhanced contractions (Wilkens et al., 2005). Caffeine elicits release from the SR and when applied in the presence of proctolin, the observed contractions were faster and stronger (compared to contractions with no caffeine) (Wilkens et al., 2005). Understanding the calcium dynamics in conjunction with myosuppressin would be an interesting next step in this work; it is very possible that myosuppressin could be acting in the same manner as proctolin and by inducing increased release of calcium from internal stores.

In future experiments it would be interesting to measure the size of the PLN that the EJP experiments stimulate from. This would allow correlations to be made between the size of
the nerve and evoked EJP size and allow analysis to see if there are any patterns seen in response to myosuppressin application.

**4.5 Conclusions**

Taken together, these data suggest that myosuppressin acts on the isolated cardiac muscle, not at the NMJ via EJPs (including mEJPs) to increase the contraction amplitude, as observed in the whole heart (Figure 24). Myosuppressin provides a unique example of a modulator where the global effects of modulation are understood, as well as how and where it acts. This includes on both types of neurons (Oleisky et al., 2020; 2022) as well as the periphery—with particular evidence for direct action on the muscle fibers (shown here). These data provide an additional layer of understanding that myosuppressin acts directly on the cardiac muscle, possibly via calcium dynamics. The one aspect of this system where myosuppressin’s effects have not yet been characterized, is on the feedback system. This is currently being investigated by a student in the Dickinson lab with particular emphasis on the stretch feedback pathway.
Figure 24. Myosuppressin acts at multiple sites including the cardiac ganglion and at the periphery. Future work requires investigation of the mechanism of action myosuppressin takes to increase the contraction amplitude at the muscle, which is hypothesized to be via calcium dynamics. Additionally, myosuppressin’s impact on the feedback pathways has not been fully characterized.
References


Cellular and molecular life sciences: CMLS 67: 4135-4169


during stretch in the heart of the lobster Homarus americanus are anisotropic and are altered by neuromodulators. J. Exp. Biol., 219:1187-1202.


Mahadevan, A., Lappe, J., Rhyne, R.T., Cruz-Bermudez, N.D., Marder, E, and Goy, M.F.

Marder E., and Calabrese R.L. Principles of rhythmic motor pattern generation.
Physiological reviews 76: 687-717, 1996.


Calcium sensitivity of neurotransmitter release differs at phasic and tonic synapses. *Journal of Neuroscience, 25*(12), 3113-3125.


