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Interaction of stretch feedback and beat regularity in response to AMGSEFLamide in the heart of *Homarus americanus*

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An Honors Project for the Program of Neuroscience

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Abstract

Central pattern generators (CPGs) are neural circuits whose component neurons possess intrinsic properties and synaptic connections that allow them to generate rhythmic motor outputs in the absence of sensory feedback and descending inputs. The cardiac ganglion (CG) is a nine-cell CPG located in the American lobster, *Homarus americanus*. Its motor neurons synapse directly onto the myocardium to induce contraction of the heart and generate beating patterns. Stretch of the myocardium feeds back to the CG through mechano-sensitive dendrites. This feedback is generally excitatory and is thought to play a role in maintaining regularity in the beating pattern of the heart. The novel peptide AMGSEFLamide has been observed to induce irregular beating patterns when applied at high concentrations. This study investigated the interaction between stretch-related feedback and AMGSEFLamide modulation in generating irregular beating patterns in the whole heart of *Homarus americanus*. It was hypothesized that greater longitudinal stretch of the heart would result in greater regularity in the instantaneous beat frequency, based on previous findings that stretch-sensitive dendrites play a role in the regulation of the heartbeat. Furthermore, it was predicted that the elimination of stretch feedback via deafferentation of the heart would augment the irregularity induced by AMGSEFLamide.

Data showed significantly increased irregularity in beating, as well as higher order beat cycles in response to 10^{-6} M AMGSEFLamide application. While this effect was pronounced in some preparations, others showed little or no irregularity in their response to the neuromodulator. Longitudinal stretch did not reliably alter baseline variability in frequency, nor did it influence the modulatory effect of AMGSEFLamide. Deafferentation did not significantly alter baseline irregularity. Deafferented preparations did, however, exhibit a trend of responding to

AMGSEFLamide with a greater percent increase in the coefficient of variation of frequency compared to when afferents were intact.

Further study will utilize transverse and biaxial loading to better activate stretch feedback pathways. Additionally, the efficacy of other irregularity-inducing modulators in the study of heartbeat regulation should be considered.

Introduction

Central Pattern Generators

Rhythmic motor outputs are generated and controlled by central pattern generators (CPGs). These neural circuits create patterns in the absence of input from sensory systems or other descending sources (Marder et al. 2001). This intrinsic rhythmicity allows for essential physiological functions to continuously cycle semi-independently from environmental cues. Some of the first substantial evidence of CPG control of rhythmic movement was in the observation of fictive motor patterns, which are seen when a neural circuit is removed from the rest of the body and continues to fire in a discernable pattern. This pattern would presumably generate rhythmic motor outputs if the circuit were still connected to the tissue it was dissected from (Wallén and Williams, 1984; Pearson, 2000). Thus, the motor pattern is "fictive" since the neural circuit is generating stimulatory bursts but has no muscle to stimulate. Importantly, the generation of fictive motor patterns confirm the idea that CPGs can intrinsically produce patterns in the absence of central or higher order neural inputs.

While CPGs can, by definition, function when removed from sensory or higher order inputs, some CPGs do require some sort of stimulation to initiate their patterns of activity. For example, the isolated brain stem and spinal cord of the rat can generate rhythmic outputs, but only upon the application of modulators such as serotonin or other excitatory signaling molecules, including NMDA (N-methyl-D-aspartate; Cazalets et al. 1992; Alford et al. 2003). This demonstrates that the patterns produced by CPGs are *semi*-independent from outside influence, meaning that while they can cycle in the absence of input, the nature of the pattern can be initiated and modulated by a multitude of signals and factors. Even basic functions like heartbeat, breathing, and digestion are controlled by CPG networks that change their motor

activity patterns across physiological and environmental contexts (Zhang et al. 2009). For example, neurogenic hearts are controlled by ganglia that act as CPGs to generate rhythmic, yet flexible, beating patterns. The flexibility of a motor system controlled by a CPG is dependent, in part, upon neuromodulators. Modulation can occur through a multitude of mechanisms. Generally, modulators exert their impact by either the changing of membrane properties in a CPG neuron or neurons (through the opening of ion channels, activating G-protein coupled receptors, etc.) or the changing of synaptic strength between cells within and downstream of the CPG (Dickinson 2006). Uncovering the mechanisms and consequences of CPG modulation is essential to understanding the enormous flexibility in the outputs produced by rhythmic motor systems.

The American lobster, Homarus americanus

The crustacean nervous system has long served as a model for the study of CPG networks (Clarac and Pearlstein, 2007). Both the intrinsic neuronal properties that allow for continuous cycling, and the extrinsic modulation of these cycles are (somewhat) easily studied in these neural circuits that are simple in connectivity, yet diverse in the range of outputs they can produce. In many crustacean systems, CPGs responsible for distinct, and easily measured, motor outputs such as digestion, locomotion, and the beating of the heart consist of networks as small as double-digit, and sometimes even single digit, numbers of neurons (Simmers et al. 1995). These neurons can have a range of identities, from motor neurons to interneurons that communicate, often bidirectionally, to intrinsically generate patterns and manifest motor outputs entrained to these patterns. The scale of these networks has allowed for identification of the

individual neurons within them. This is the case in both the cardiac and stomatogastric CPG networks in the lobster.

Homarus americanus, the American lobster, has been extensively studied for the simple CPGs in its cardiac and stomatogastric nervous systems (Stein 2009; Calabrese et al. 2016). Endemic to the Atlantic coast of North America, *H. americanus* is especially abundant along the coast of Maine. Much of the research around *H. americanus* has attempted to unravel the relationship between its CPG networks and its neurosecretory systems. The major site of neuroendocrine control is in the eyestalks of *Homarus*. This was initially discovered through experiments that removed the eyestalks of crayfish, and then transplanted then, to determine the hormonal relevance of the tissue (Brown and Cunningham, 1939). This led to the discovery of the sinus glands in *Homarus*, which are made up of projections from the eyestalk ganglia to regions of circulating hemolymph. These glands, along with additional structures throughout the body, including the pericardial and commissural organs, release neurohormones into the open circulatory system of *Homarus,* where they act on a multitude of targets to influence physiological functions like molting and blood-glucose control (Hopkins 2012). One signaling molecule that utilizes this mechanism in crustaceans is dopamine, which is projected to and released from the pericardial organs into circulation (Fort et. al, 2004). Targets of these neurohormones include the CPGs of the cardiac and stomatogastric systems, which are modulated through this endocrine delivery mechanism.

The Cardiac Ganglion

The heart of *Homarus* is neurogenic, meaning its beating is controlled by a central pattern generator. The cardiac ganglion (CG) is embedded in the dorsal wall of the myocardium, the

musculature of the heart, and stimulates contraction of the muscle. The CG consists of 9 neurons in total; 4 excitatory small cells, also called pacemaker cells, which synapse onto 5 large motor neurons, which in turn innervate and stimulate the heart muscle itself. The CG is a Y-shaped structure, with the base at the posterior end of the heart, and the branches extending in the anterior direction. The 4 small cells are found at the base of the Y, posterior to the posterolateral nerve branch. Motor cell bodies are found more anteriorly along the trunk of the Y, and make up either side anterior to the anterolateral nerve branch as well. Studies that have transected the *Homarus* CG at various points in its anatomy suggest that the small cells are capable of maintaining a bursting pattern independent of input from the large cells. Data from the same study suggested that the large cells require input from the small cells to generate a bursting pattern (Mayeri 1973), though recent unpublished data from the Dickinson Lab suggest that large cells can produce bursting patterns when physically separated from the small cells via a ligature.

The cells of the CG are tightly electrically coupled, meaning that charge can passively diffuse between them through gap junctions (Watanabe and Bullock, 1959). This coupling is essential to the rhythmicity of the motor outputs from the large cells (Tazaki 1972). The CG in *Homarus* fires in distinct bursts. These bursts begin with spikes in the small pacemaker cells, followed by rapid spikes from both the small and large cells. Shortly after this point the spike frequency of the large cells tapers off and eventually stops (Hartline 1979). The final phase of the burst is variable across individuals, with both the large and small cells exhibiting variable patterns (Williams et. al, 2013). The full bursting pattern repeats after an intermittent period of no firing. In the absence of input, the integrity of this cycle remains robust, with parameters like spike frequency, burst duration, and burst period holding relatively constant. These parameters are subject to change, however, when the CG is modulated chemically or otherwise.

Endogenous Modulatory and Sensory Feedback Systems

Amines and neuropeptides are classes of molecules that can modulate the lobster CG. These signaling molecules can act as neurohormones when released into the hemolymph from the pericardial glands, eyestalk ganglia, and other endocrine structures throughout the body (Beltz and Kravitz 1986). This neuroendocrine mechanism of signaling bathes the entire ganglion in the modulator, rather than acting at a single synapse, like the classical neurotransmitter model of signaling. This means that these amines and peptides can presumably exert their modulatory effects at receptors along the entirety of the CG, rather than just at specific synapses. Given this mechanism of action, the CG can be isolated for recording purposes and bathed in neuromodulators with the expectation that they will induce a similar neuronal effect as they would *in vivo*.

Despite the benefits of the isolated CG preparation, it is important to note that removal of the ganglion from the organism eliminates several endogenous feedback systems. In fact, in *Callinectes*, the isolated CG has been found to exhibit a significantly greater burst frequency and burst duration than recordings from CGs still embedded in the myocardium (Fort et. al, 2004). The heart provides feedback to the CG primarily through myocardial stretch and nitric oxide signaling. Nitric oxide is synthesized in the lobster myocardium during contraction via nitric oxide synthase. Nitric oxide synthase is necessary for this production, since pharmacological blockage of the enzyme eliminates NO synthesis (Casares et al. 2006). NO behaves as a gaseous neuromodulator that exerts its effect on the CG directly. Studies that demonstrate the generation of cGMP, a common second messenger, in the isolated CG in response to an exogenous NO donor provide evidence in support of this feedback mechanism (Scholz et al. 2002). While the exact cellular mechanism of NO neuromodulation is unclear, it has been shown to act on both the

whole heart and the isolated CG when administered exogenously. In a whole heart preparation, NO reduced heart beat amplitude and frequency, and in an isolated CG preparation, it decreased burst frequency (Mahadevan et al. 2004). This generally inhibitory effect of NO in *Homarus* is consistent with its actions in many other organisms. Since NO seems to be generated in the heart muscle, the presence of the myocardium is important to better understand how modulation occurs in the context of the entire cardiac system.

Conversely, stretch of the myocardium has an excitatory effect on the CG. Research has identified mechano-sensitive dendrites on CG neurons, which could provide the mechanism through which stretch excites the heart. The stretch sensitivity of these dendrites could be due in part to piezo ion channels, which contribute to mechanically-activated cation currents in multiple vertebrate models (Coste et al, 2010). Unpublished transcriptomic data in the Dickinson Lab has indicated the presence of piezo in the CG. While severing these dendrites has no influence on the isolated CG, doing so in a semi-intact heart reduces contraction amplitude (García-Crescioni et al. 2010). This suggests that by stretching during contraction, the myocardium activates these mechanosensitive dendrites and influences the CG in an excitatory manner.

While its effect on the contraction of the myocardium is clearly excitatory, stretch applied directly to the CG has been found to induce a dose-dependent hyperpolarization, followed by a reduction in burst frequency, in CG neurons in another crustacean, *Ligia*. Importantly, this hyperpolarization eventually contributes to post-inhibitory rebound that increases burst frequency above baseline upon relaxation of the applied stretch (Sakurai and Wilkens 2003). These stretch-dependent changes in membrane potential seem to be more complex in *Homarus*, where neither injection of single hyperpolarizing nor depolarizing currents were sufficient to mimic the complex and multi-phasic response of the CG to stretch (Qu, 2017). This complex

response has been characterized via intracellular recordings from single CG motor neurons, which exhibit a delay in bursting upon increased stretch, an increase in burst frequency upon holding of an increased stretch value, and an increase in burst duration upon relaxation from that stretch (Qu, 2017). The stretch feedback pathway is further complicated by the variability of the motor response observed with respect to frequency. One study in the Dickinson Lab found that in response to longitudinal stretch the frequency of the heartbeat increased in 87% of preparations (Chin-Purcell, 2014), while another study in the same lab found that frequency only increased in approximately 50% of lobsters, with others exhibiting either decreases or no change in contraction frequency upon longitudinal stretch (Dickinson, 2014). Thus, while stretch feedback is clearly excitatory in the way it consistently increases contraction amplitude, it is less clear how it influences contraction frequency, as well as how the changes it causes in CG burst parameters translate into these motor responses.

Active and Passive Forces in the Cardiac System

To effectively manipulate the stretch-state of the heart, it is essential to understand the active and passive forces at play in its beating. The passive force refers to the tension in the heart muscle during diastole, the relaxation phase of the beat when the heart fills. The active force is the difference in tension between the passive force, and that felt at the peak of the beat, during systole. These parameters are related to stretch through the Frank-Starling law, which states that the stroke volume of the heart is directly proportional to volume of blood in the heart. This is based in the direct relationship between the length of cardiomyocytes, and the force of the contraction they generate (Delicce and Makaryus, 2020). This does not describe the activity of skeletal muscle, which typically follows a length-tension curve where a peak tension is met,

followed by a decrease in tension with increasing length (Burkholder and Lieber, 2001). Cardiac muscle, however, tends to increase in contraction force consistently with increasing length along its operational range. While extensively studied in vertebrates, this phenomenon is less established in crustaceans.

Recent research has investigated the length-tension relationship in the lobster cardiac system. The length-tension curve characterized in the lobster follows the Frank-Starling model, with increased tension in response to increased muscle length (Maguire, 2019). Lengthening of the heart longitudinally, transversely, and biaxially (in both directions simultaneously) yielded increases in both passive and active forces. Furthermore, the influence on the passive force was anisotropic in that the passive force was more drastically changed by longitudinal stretch than transverse stretch (Dickinson et. al, 2016). This finding is important because the heart fills in three dimensions, and thus expands and contracts along both axes. Furthermore, it lays the foundation for interactions between modulators and stretch feedback, both of which can influence passive and active forces simultaneously.

The Whole Heart Preparation

Due to the feedback that the myocardium provides to the CG in the form of NO production and stretch, it is important to study modulation of the cardiac system in the context of both the CG and the heart muscle. The two systems are constantly feeding back to one another *in vivo,* and so preparations that include this interaction will more closely replicate the physiology of the intact organism. The whole heart preparation of *Homarus* consists of the entire myocardium, removed from the body, with the intact CG still embedded in the musculature of the dorsal wall. The posterior artery can be cannulated so that saline can be perfused, alongside

any neuromodulators under investigation. This preparation records the beats of the heart by tying off the anterior arteries and connecting them to a force transducer with suture silk (Dickinson et al., 2016). This *ex vivo* preparation does not directly record the electrical activity of the CG, but it does allow researchers to observe the motor output that the CG generates in the context of both feedback systems and neuromodulators. The semi-intact working heart preparation was developed to resolve this issue, as it allows for the simultaneous recording of both neural impulses from the CG, and physical contractions from the heart muscle (Fort et. al, 2004).

Variability in Responses of the Lobster Cardiac System to Neuromodulators

Certain neuromodulators have consistent robust effects on the whole heart of *Homarus*. Myosuppressin, an endogenous FMRFamide-like peptide, reliably reduces contraction frequency and increases contraction amplitude in whole heart preparations. Consistent with these data, injection *in vivo* causes reductions in beat frequency, while perfusion on the isolated CG elicits a decrease in burst frequency (Stevens et al., 2009). Similarly, the neuropeptide proctolin induces increases in contraction amplitude in the whole heart (Wilkens et al., 2004). While the effects of certain modulators seem to be fairly consistent across individuals, and in some cases across many species of crustacea, there are modulators that produce a high level of variation in the responses they elicit.

Inter-animal variability is a relevant factor when it comes to the effect of certain modulators (Hamood and Marder, 2015). The effect of C-type allatostatin has been found to demonstrate a great deal of inter-animal variability. The peptide decreases contraction frequency consistently. In some preparations, however, it increases contraction amplitude, while it elicits a decrease in others. The study that found this was able to pinpoint the variation to the effect of C-

type allatostatin to the CG, since preparations that experienced the same effect on the whole heart experienced the same effect on the CG, while preparations that saw different effects on the whole heart were also differentially modulated at the CG (Wiwatpanit et al., 2012). This shows how not all CGs in *Homarus* respond equally to modulation, and how this difference can have dramatic consequences to the motor outputs generated across individuals.

The activity of the cardiac system is also dependent upon environmental factors such as temperature. Across a range of ecologically relevant temperatures, the *ex vivo* heart of *Homarus* tends to increase in both contraction amplitude and contraction frequency with increases in temperature (Worden et al., 2006). One recent study performed noninvasive recordings of cardiac activity over the course of temperature ramps in *Cancer borealis*. Heart rate increased with temperature until a "crash" temperature was reached, meaning a temperature at which the heartbeat became irregular and then halted until cooling (Kushinski et al., 2019). These findings make temperature a relevant factor to consider when assessing how chemical signals may modulate the activity of the heart.

Understanding which factors may be contributing to the "baseline" state of the cardiac system is important because the activity state of CPG systems can influence the effect that modulators have on that system. This state dependence of CPG modulation has been well documented in the crustacean stomatogastric nervous system. For example, the neuropeptide proctolin tends to impart an effect of greater magnitude on stomatogastric nervous systems with lower baseline activity levels than on systems with higher baseline activity in *Cancer borealis* (Nusbaum and Marder, 1989). Even though this state dependence has not been extensively studied in the *Homarus* cardiac system, it should be considered when evaluating the potential

effect of neuromodulators, considering the large inter-animal variability that is seen in baseline cardiac output.

Irregularity in the Lobster Heartbeat

Many modulators tend to elicit a well-defined and reproducible change in motor output parameters; however, it is possible for motor systems to respond with irregularity to certain modulators. Multiple peptides have been found to induce irregular heartbeat patterns in crustacean whole heart preparations. Application of certain FMRFamide-like peptides to the whole heart of *Callinectes sapidus* has been observed to induce different "modes" of regularity in some but not all preparations (Fort et al. 2007). These modes include regular, irregular, and higher order cycle responses. A regular response to the peptide refers to the typical gradual change in contraction parameters, where an irregular response denotes a response that varies in amplitude and frequency between individual beats. Higher order cycles consist of a series of contractions that are irregular from beat to beat, but that repeat in a discernable pattern consisting of multiple beats. It was found that the whole heart exhibited all three modes in response to FMRFamide-like peptides, specifically TNRNFLRFamide, SDRNFLRFamide, and GYNRSFLRFamide, particularly at high concentrations. Higher order patterns tended to contain 2-10 beats per cycle. Additionally, the whole heart often underwent sudden mode switches that tended to be more common upon addition or removal of the peptide (Fort et al. 2007).

Similar irregularity has been observed in the *C. sapidus* whole heart in response to crustacean cardioactive peptide (CCAP). This peptide was found to exert effects on both the CG and the myocardium itself. This could be concluded because CCAP increased burst frequency and burst duration in the isolated CG, while exerting the exact opposite effect on CGs still

embedded in the myocardium. The same reversal was observed in the effect of FMRFamide-like peptides. This dual action has been proposed as a possible explanation for the irregularity and higher order cycles observed, since the peptide may be exerting differential modulatory effects on the distinct tissues of the system (Fort et al. 2007). Mathematical models of these complex dynamical outputs in biological systems have attempted to distinguish whether outputs of this nature are in fact chaotic, or if they display some underlying periodic variation. The output of the crustacean CG has been used to study this phenomenon, as the duty cycle of CG bursting appears to exhibit a nonlinear relationship from cycle to cycle. One theory proposes that a perturbation that influences both of these nonlinearly related parameters (the duty cycle of each respective burst cycle) could potentially underlie periodic variations that have been observed, such as higher order beating cycles (Hokkanen 2000). If interaction with both the myocardium and the CG independently is a unifying characteristic of these irregularity-inducing modulators, then perhaps they are interacting with one of the major feedback systems that conveys information from the myocardium to the CG: the stretch feedback system.

Stretch feedback, as previously discussed, has been well established in the literature as an excitatory input to the CG. What is less established, however, is how myocardial stretch may inform how the CG, and consequently the myocardium, reacts to inputs from other sources. Experiments in which the CG was deafferented in a semi-intact heart preparation in *Homarus* indicate that stretch feedback may be an important factor in the maintenance of regularity in the heartbeat (Chin-Purcell, 2014). CGs were deafferented by severing their mechano-sensitive dendrites, which effectively eliminated the ability of the CG to integrate stretch information from the myocardium. The result of blocking stretch feedback in this way was increased variability of heart rate, as measured by the variation in instantaneous frequency of heart beats (Chin-Purcell,

2014). This is consistent with studies of sensory feedback stabilization of motor patterns in other models. For example, the locust flight central pattern generator receives sensory feedback regarding the state of its effector muscles from the tegula, which is a proprioceptor that communicates downward movement of the wing. Stimulation of the tegula lead to the reduction and regulation of the frequency of the motor output (Ausborn et. al, 2007). If stretch feedback is in fact working to regulate variability in the motor output of the heart, then it should be considered when assessing the generation of seemingly irregular heartbeats.

GSEFLamides

Neuropeptides are a large class of signaling proteins that can act as both neurohormones and neurotransmitters in the nervous system. They follow the same general transcription to translation mechanism of protein synthesis, with key attributes specific to the neuropeptide class. Neuropeptides tend to be small proteins, often ranging from 4 to 100 amino acids in length. A signal sequence brings the mRNA to the rough endoplasmic reticulum (ER), where it interacts with a ribosome so that the peptide is synthesized within the lumen of the ER, rather than in the cytoplasm. After the signal sequence is cleaved, the prepropeptide is packaged into a vesicle and trafficked to the golgi apparatus, where it is cleaved at dibasic cleavage sites in order to release the active peptide sequence or sequences (multiple sequences can arise from a single prepropeptide). After post-translational modification, folded, active neuropeptides are condensed into dense-cored vesicles. These vesicles are typically transported to terminal buttons, where their exocytosis and release are controlled by depolarization-activated inward calcium currents (Levitan and Kaczmarek, 1991).

The GSEFLamides are a family of novel neuropeptides that were only recently identified in *Homarus americanus*. Members of this peptide family share the common carboxyl end sequence -GSEFLamide. Through transcriptome mining techniques, the GSEFLamides were predicted from eyestalk ganglia tissue samples (Christie et al., 2017). Two transcripts were uncovered as encoding for N-terminal partial preprohormones, while a single transcript was identified as a C-terminal partial protein. Either of the N-terminal transcripts products could potentially be combined with the C-terminal product to form a full preprohormone. From these transcripts, 6 isoforms of the GSEFLamide family were identified: IGSEFLamide, MGSEFLamide, AMGSEFLamide, ALGSEFLamide, VMGSEFLamide, and AVGSEFLamide. Since the identification of their transcripts in the eyestalks of *Homarus*, the GSEFLamides have been synthesized *in vitro* for assessment of their potential modulatory action in the nervous system.

Extracts from the brain of *Homarus americanus* have identified endogenous production of all six isoforms of the GSEFLamide family. AMGSEFLamide, the most abundant of the GSEFLamide isoforms in the *H. americanus* brain, has been shown to exert neuromodulatory effects on both the cardiac and stomatogastric ganglia. In an isolated CG preparation, AMGSEFLamide causes a decrease in burst frequency, while eliciting increases in burst duration and duty cycle (Dickinson et al., 2019). Unpublished data from the Dickinson Lab reports that in a whole heart preparation, AMGSEFLamide can elicit pronounced irregular beating patterns. This makes the novel peptide AMGSEFLamide an excellent candidate to investigate the role of stretch in modulating regularity in the heart beat of *Homarus*.

Present Study

In this study, the neuromodulatory effect of the novel peptide AMGSEFLamide was investigated with respect to longitudinal stretch of the myocardium as well as the state of stretch feedback in a whole heart preparation in *Homarus americanus*. Specifically, the incidence of irregularity was assessed, with the hypothesis that increased longitudinal stretch would work to stabilize the beating of the heart and effectively reduce the irregularity in beat frequency that AMGSEFLamide typically generates. Additionally, the response of the heart to the same modulatory perturbation was assessed after the elimination of stretch feedback through deafferentation. It was predicted that the removal of regulatory stretch feedback would amplify the irregular response induced by AMGSEFLamide. Findings of this study could further our understanding of how distinct intrinsic and extrinsic modulatory systems interact to generate complex motor outputs.

Methods

Animals

Homarus americanus were obtained from local seafood markets in Brunswick, Maine. They were kept for up to 3 weeks in recirculating seawater aquaria. Water was kept at 10-12ºC and the animals were maintained on a 12 hour:12 hour light:dark cycle. Feeding consisted of providing chopped squid or shrimp on a weekly basis (Dickinson et al., 2016).

Dissection

Lobsters were anesthetized by immersing them in ice for 30 minutes prior to dissection. The ventral nerve cord was severed and the tail removed from the thorax, along with legs and claws. The heart, which adheres to the dorsal wall of the thorax, was removed along with a window of the carapace. Care was taken not to stretch the posterior artery and to cut the artery as ventrally as possible, so that it could be easily cannulated. The preparation was then submerged in cold 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) and pinned in sylgard with the heart ventral-side up.

Whole Heart Preparation

The perfusion system was arranged so that saline flowed over the posterior end of the heart. Outflow tubing was placed at the anterior end, so that fresh saline was being pulled across the heart. Room temperature saline was passed through an in-line peltier temperature regulator (CLV100 bipolar temperature controller and SCV 20 solution heater/cooler; Warner Instruments, Hamden, CT, USA) so that it would be perfused at a controlled 10ºC.

The ventral-most posterior artery of the heart was cannulated with a single inflow polyethylene tube so that saline could be perfused directly into the lumen of the heart at approximately 2.5 ml/minute. The artery was considered sufficiently cannulated once the valve was broken. The artery was tied tightly to the inflow tubing with 6-0 suture silk. Suture silk (6-0) was also used to tie the 5 anterior arteries together. The other end of the silk was tied to a force transducer (see below) so that the force of the heart's beats could be amplified by an ETH 50 bridge amplifier, Model 44 Brownlee Precision Instrumentation. The signal was then recorded with a Cambridge Electronic Design (CED) Micro 1401 digitizer (CED, Cambridge, UK) (Dickinson et al., 2016). Spike7 was the data acquisition software used.

Manual Stretch Experiment

In the manual stretch experiments, the suture silk connected to the anterior arteries was secured to a Grass FT03 force-displacement transducer (Astro-Med, West Warwick, RI) attached to a manipulator. Preparations were subject to 3 different stretch levels, 1.5, 2.5, and 3.5 grams of passive force, in a randomized order. Once manipulated to the desired passive force, the preparation was allowed 1 hour to equilibrate with normal saline perfusing all the while. After equilibration, 10^{-7} M AMGSEFLamide in lobster saline (synthesized by GenScript, Piscataway, USA) was perfused for 10 minutes, followed by a 45-minute saline wash. After the wash, AMGSEFLamide at 10^{-6} M was perfused for 10 minutes followed by another wash. These concentrations of AMGSEFLamide were chosen after preliminary experimentation indicated that they most reliably induced heartbeat irregularity compared to more dilute concentrations. Then, the force transducer was manipulated to bring the passive force on the heart to the next desired

value. The heart was washed and allowed to equilibrate at this new force for an additional 30 minutes. The same peptide perfusion protocol was followed for each force value.

AMGSEFLamide Response Duration Experiment

A similar protocol to the manual stretch experiment was followed for an experiment aimed at assessing both the short- and long-term effects of AMGSEFLamide on the whole heart. In this experiment, the heart was equilibrated to a stretch value of 1.5, 2.5, or 3.5g force for an hour. Following this equilibration, AMGSEFLamide at 10^{-6} M was perfused for 40 minutes. This allowed for the assessment of transient and lasting effects of the peptide on the heart beating pattern. The same wash out and force adjustment protocol described above was used so that the concentrated peptide was perfused for 40 minutes at each of the 3 stretch values.

Aurora Conditioning Experiments

The Aurora conditioning experiment was designed to eliminate the effects of stretch relaxation observed over the course of the manual stretch experiments. For these experiments, the suture silk tied to the anterior arteries of the whole heart preparation was secured to an Aurora Scientific Dual Mode Lever System (Model 300C, Aurora Scientific, Aurora, ON, Canada). Adjustments were made so that the heart was pulled up at a roughly 30^{o} angle from the horizontal, and the lever arm of the Aurora made a roughly 90^{o} angle with the suture silk (Dickinson et al., 2016). The preparation was then allowed to equilibrate at the desired passive force, which was again randomized between 1.5, 2.5, and 3.5 grams. This force was manually acquired through the manipulator that the Aurora was mounted on. After the equilibration period, the preparation was subject to 3 consecutive stretch ramps. Stretch ramps consisted of stretching

of the heart from the baseline passive stretch value, to a length 2.5mm greater than this value, and back down to baseline. Stretch was altered at a constant rate and ramps lasted 120 seconds in total (Chin-Purcell, 2014). Force was simultaneous applied and recorded through the Aurora system during stretch ramps. After the termination of a ramp, preparations often fell below their original baseline passive force value. Because of this, force was manually returned to baseline before the initiation of the subsequent ramp 2 minutes after the termination of the preceding ramp. After stretch conditioning, the protocol for the response phase experiment was followed, with three stretch ramps completed each time the heart was equilibrated to a new force value.

Deafferentation Experiments

After Aurora conditioning experiments, preparations were subject to deafferentation, so that the influence of the mechano-sensitive afferents on the modulation via AMGSEFLamide could be assessed. A small incision was made posteriorly along the midline of the ventral wall of the heart muscle. This incision was extended anteriorly until the trunk and anterolateral branch of the cardiac ganglion (CG) was visible. Care was taken to minimize extension of the incision in the anterior direction, so as to reduce the severing of any motor efferents in the ventral wall. Glass hooks were then used to expand the opening transversely, to maximize visualization of the CG. 0.01% Janus Green dye was perfused for 40 seconds and promptly washed out to stain neural tissue in the preparation. Sensory afferents were identified according to a diagram provided by Chin-Purcell (2014; Figure 2), though notable deviation from this schematic exists in the placement and number of afferents across individuals. Full deafferentation was attempted in the region of the CG between the posterolateral nerves and immediately anterior to the anterolateral nerves. Complete deafferentation was not possible without compromising the motor

output of the heart. After deafferentation, the heart was allowed to equilibrate for 45 minutes before applications of the peptide.

Data Analysis

Higher order cycles were defined as consisting of at least 2 irregular successive beats, the pattern of which repeated at least 3 times in full before any sort of mode switch. Images of waveforms were generated in CorelDraw (Corel Corp., Ottawa, ON). Contraction amplitude and instantaneous beat frequency data were acquired from Spike 2 software (CED, Cambridge, UK) by creating cursors that identified successive beat peaks, along with the relaxation troughs between them. As double-beating was common in response to the peptide, it was necessary to firmly define what constituted distinct beats. A double beating pattern was defined as two distinct beats if the smaller amplitude beat was at least 25% the amplitude of the higher amplitude beat and a downswing of at least 25% of the preceding beat's amplitude was completed prior to the upswing of the second beat. Analysis of contraction amplitude and instantaneous frequency values over the course of peptide applications and washes was performed in Microsoft Excel and Prism8. Irregularity was quantified by calculating the coefficient of variation in instantaneous frequency, which was then compared across different concentrations of AMGSEFLamide application, as well as different passive force values, with calculated values of percent change from baseline coefficient of variation.

All values of coefficient of variation that were either combined or directly compared to one another were generated from the same number of heartbeats, since the coefficient of variation is dependent upon the sample size. Each AMGSEFLamide application period was split into three sections of equal number of beats for analysis. The size of these sections was based on

the peptide application period within that particular analysis that contained the least number of heartbeats. For example, if two 10-minute peptide applications were being compared, one containing 60 beats and one containing 100, both would be sectioned into three 20 beat groups. While these groups would span the entire application period for the application with less beats (60), the application with more beats (100) would be sectioned into the first 20 beats, the second 20 beats, and the final 20 beats (beginning from the end of the application and moving backwards) with beats 41-80 excluded. This ensured that both the initial and final responses to the peptide could be observed, with the "middle" response given by section 2. Baseline values were taken immediately prior to the introduction of the peptide. When percent change from baseline was calculated, baseline coefficient of variation followed the restrictions of a single application section, meaning that in this example, all baseline measurements would be generated from the 20 beats immediately preceding AMGSEFLamide application. When just the absolute coefficient of variation was compared, each measurement was based on exactly 100 beats.

Results

AMGSEFLamide induced irregularity in the lobster heartbeat

Perfusion of AMGSEFLamide at a concentration of 10^{-6} M caused the frequency of the heartbeat to become less regular. Comparison of the beat frequency at baseline to that immediately after the first application of the peptide in each preparation revealed a significant increase in the mean coefficient of variation of the frequency from 0.10 to 0.24 (paired t-test, p=0.0094; Figure 3D). Similarly, when applications were separated by passive stretch value, AMGSEFLamide elicited a statistically significant increase in the coefficient of variation of frequency at 1.5 (p=0.0404; Figure 3A), 2.5 (p=0.0285; Figure 3B), and 3.5 grams passive force (p=0.007; Figure 3C). This indicates that the instantaneous frequency from beat to beat varied more widely after application of the peptide compared to the baseline variation in frequency. In terms of the appearance of the beating pattern, this change manifested in a more irregular, and sometimes even arrhythmic waveform.

Of the preparations that responded to AMGSEFLamide with irregularity in instantaneous frequency, many exhibited higher order patterns of irregularity (n = 8). These cycles, defined as consisting of at least 2 irregular successive beats the pattern of which repeated at least 3 times in full, varied in number of beats per cycle and number of cycle repetitions (Figure 4). Higher order cycles were often initiated and terminated with sudden mode switches from either regular or irregular states (Figure 4D).

Application of AMGSEFLamide for 40 minutes demonstrated that the irregularity observed in beat frequency is a transient effect that gradually recovers over extended exposure. While the instantaneous beat frequency was highly irregular during the first 400 seconds of AMGSEFLamide application, frequency stabilized after this point. Small bouts of irregularity

were observed after this initial stabilization, though they became less frequent and further spaced from one another with time (Figure 5A). These bouts of irregularity were near identical in composition and appeared to be higher order cycles with increasing durations of regular bouts between them. Of the two response phase experiments performed, one preparation showed irregularity in instantaneous beat frequency that declined with time, while the other showed no irregularity upon application of AMGSEFLamide (Figure 5B).

Modulation via AMGSEFLamide exhibited notable inter-animal variability

Response to AMGSEFLamide showed a notable degree of inter-animal variability. All preparations (n = 13) responded in some way to AMGSEFLamide. Preparations did not, however, consistently respond with irregularity in beat frequency and amplitude. Irregularity was primarily quantified by instantaneous beat frequency. While the majority preparations (10 out of 13) exhibited some degree of sporadic change in instantaneous frequency upon application of AMGSEFLamide (Figure 6A), others (3 out of 13) showed only a gradual, continuous increase in frequency with the peptide (Figure 6B). Importantly, preparations often could not be classified as consistently irregular or consistently regular in their response. The degree of irregularity of the response existed on a spectrum, with some preparations even responding with regularity to one application, and then irregularity with the next.

The magnitude of change in the coefficient of variation of frequency was also widely variable across preparations. While some hearts responded with modest increases in irregularity from baseline on the order of magnitude of 1-9%, others exhibited increases of over 2000%. Some hearts even responded to AMGSEFLamide with modest reductions in coefficient of variation of frequency, suggesting an opposing and potentially stabilizing effect of the peptide on

the heartbeat in certain individuals. Importantly, all of this variation occurred in response to application of the peptide at the same concentration of 10^{-6} M. A large degree of inter-animal variability was observed in the baseline coefficient of variation across preparations as well, with values ranging from 0.02 to 0.42 (Figure 7A). A linear regression analysis showed no correlation between the baseline coefficient of variation and the magnitude of the percent change from baseline upon application of 10^{-6} M AMGSEFLamide (R^2=0.1426; Figure 7B).

Longitudinal stretch of the heart did not influence irregularity in response to AMGSEFLamide

The longitudinal stretch of the heart was manipulated to one of three passive force values within a physiologically relevant range. After conditioning stretch ramps, hearts were held at a given passive force to assess the impact of that longitudinal stretch state on the variability of the motor output. Longitudinal stretch alone did not significantly alter the regularity of the heartbeat frequency (One-way ANOVA, p=0.3168; Figure 8). No clear trend could be observed relating the variability of the frequency to the passive force the heart was conditioned to. This held true both for baseline values of hearts at their "original" passive force value (prior to any applications of AMGSEFLamide), and for baseline values at stretches the heart was brought to after previous experimentation at different passive force values.

Longitudinal stretch did not influence the degree of irregularity with which hearts responded to AMGSEFLamide (One-way ANOVA, p=0.6153). Preparations held at both 1.5 and 3.5 grams passive force seemed to respond with greater increases in coefficient of variation of frequency than those held at 2.5 grams, though these trends were not statistically significant. There was no temporal trend in the magnitude of the response. Preparations held at 1.5 grams passive force had an initial average percent increase of 305%, compared to a peak percent

increase of 485%, while preparations held at 3.5 grams passive force had an initial average increase of 344%, compared to a peak average of 240% (Figure 9).

Deafferentation potentially altered the response to AMGSEFLamide modulation

Deafferentation did not consistently increase or decrease the coefficient of variation of beat frequency. The deafferentation procedure did, however, tend to marginally alter the coefficient of variation, with percent increases or decreases on the order of magnitude of 0 to 100% for 4 of the 5 preparations. A single preparation increased in coefficient of variation of frequency by over 3000% after deafferentation but this was deemed an outlier. While not statistically significant, the mean baseline coefficient of variation for deafferented preparations was 0.26, while that for the intact was 0.04 (Wilcoxon test, $p>0.99$; Figure 10).

While deafferentation alone did not directly influence the regularity of the heartbeat, the procedure may have altered the degree to which hearts responded with variability in frequency to modulation by AMGSEFLamide. Both the initial and middle responses demonstrated trends of increased percent change in variability of frequency in deafferented preparations compared to the responses observed in those same preparations prior to deafferentation. In the middle of the response, the coefficient of variation of intact preparations increased by 28% on average, while that of deafferented preparations increased by 937%. While not statistically significant (Wilcoxon test, $p=0.3125$), these trends point towards an augmentation of the heart's response to AMGSEFLamide post-deafferentation (Figure 11A, B). A linear regression of the baseline coefficient of variation after deafferentation as a function of the percent change in coefficient of variation in response to 10^{-6} M AMGSEFLamide (after removal of the outlier preparation) suggests a potential correlation between the two ($R^2=0.7454$, $p=0.1367$; Figure 11C).

Analysis of individuals revealed that certain preparations that did not respond to AMGSEFLamide modulation with any irregularity while intact, responded to the peptide with irregularity after deafferentation. An intact preparation with stable beat frequency at baseline (Figure 12A1) responded to AMGSEFLamide with an increase in frequency, but no notable changes in the variability of frequency (Figure 12A2, A3). After deafferentation, baseline frequency was still stable (Figure 12B1), but AMGSEFLamide perfusion induced immediate and pronounced irregularity in frequency (Figure 12B2, B3).

One preparation exhibited the exact opposite effect. The heart responded with irregularity in frequency to AMGSEFLamide while intact. Once deafferented, however, the coefficient of variation of frequency was reduced upon perfusion of AMGSEFLamide (Figure 13). This suggests that modulation via AMGSEFLamide may have stabilized the frequency of this deafferented heart (Figure 13B, C). Of all the deafferented hearts studied, this heart did exhibit the greatest coefficient of variation of 1.11 at post-deafferentation baseline (Figure 11).

Discussion

AMGSEFLamide promotes irregularity in the lobster heart beating pattern

Perfusion of the novel neuropeptide AMGSEFLamide was found to induce irregularity in the beating patterns of the intact *ex vivo* heart of *Homarus americanus*. This irregularity was often qualitatively visible in heartbeat traces. In this study, regularity of the heart was quantified using the instantaneous frequency of the heartbeat, which is the inverse of the beat period, or the time elapsed between the peak of one beat to peak of the subsequent beat. The coefficient of variation of the frequency was calculated to determine the degree to which the instantaneous frequency varied within a given number of heartbeats. Thus, low coefficients of variation reflected a more stable instantaneous frequency and were considered to describe more "regular" beating patterns. Conversely, high coefficients of variation reflected large variation in frequency and therefore indicated more "irregular" patterns. It is important to note that baseline coefficient of variation of frequency values were variable across preparations (typically ranging between 0 and 0.3). Because of this, percent change from baseline of the coefficient of variation was often calculated when assessing the degree to which perturbations promoted irregularity.

Initial experiments perfused AMGSEFLamide at concentrations of 10^{-7} and 10^{-6} M, both of which consistently modulated the cardiac motor pattern in some fashion. AMGSEFLamide at 10^{-6} M more consistently elicited the patterns of irregularity we were interested in evaluating, so this concentration was used exclusively in subsequent experiments and analysis. When applied at 10^{-6} M, AMGSEFLamide induced a statistically significant increase in the coefficient of variation of frequency from baseline in AMG-naïve hearts (paired t-test, $p=0.0094$). This analysis includes the small number of preparations that did not respond to the peptide with increased irregularity (Figure 3D). Given this, it can be concluded that AMGSEFLamide

generally tends to promote irregular beating patterns at high concentrations, despite inter-animal variability in responsiveness to the peptide (Figures 6, 7). This inter-animal variability is not uncommon in crustacean physiology, with different individuals responding to the same modulators with changes in electrophysiological and motor parameters of different magnitudes and sometimes in different directions (Hamood and Marder, 2015; Wiwatpanit et al., 2012). The general finding that AMGSEFLamide modulates the motor output of the *ex vivo* heart supports research that describes its ability to modulate the CG pattern (Dickinson et. al, 2019). How AMGSEFLamide's modulation of specific CG burst parameters translates into the motor patterns observed is not yet clear.

Modulation via AMGSEFLamide is characterized by higher order cycles of beating

The effect of AMGSEFLamide cannot be characterized as a distinct shift to a complete and sustained chaotic beating pattern. Rather, irregularity was observed on a spectrum, and often varied within the period of a single peptide application. Complex beating patterns can be simplified into three general categories: regular, irregular, and higher order cycle (Fort et. al, 2007). Of 13 preparations, 8 responded to modulation by AMGSEFLamide with some period of higher order cycling. These patterns varied widely in the number of beats per cycle, and in the number of cycles repeated (Figure 4). Furthermore, cycles were initiated and terminated with sudden mode switches to regularity or irregularity, as previously described (Fort et. al, 2007).

Extended AMGSEFLamide application experiments revealed what we will refer to as the extended higher order cycle. The extended higher order cycle denotes long-form cycles of primarily regular beats book-ended by identical, or near identical, spurts of irregularity. In the extended application experiments, the extended higher order cycle was observed toward the end

of the 40-minute AMGSEFLamide application, while the heart was recovering to its baseline variability in frequency. The periods of regularity within the cycle can be seen extending as the time between the irregular bouts grows longer (Figure 5). A similar extended higher order cycle pattern was occasionally observed during the washout period following a 10-minute AMGSEFLamide application. While these patterns do not meet the criteria of a higher order cycle as described by Fort, since the cycle periods seem to be increasing with each repetition, they could represent an important transition state as the modulatory impact of AMGSEFLamide gives way to some potential stabilizing force that returns the heart to its natural rhythm and regularity.

The physiological significance of higher order cycles is still unclear. Some theories posit that they may demonstrate the complex and dynamic interaction of the distinct effects that single modulators can exert across multiple sites centrally and peripherally. Put more simply, the overall effect that a modulator is observed to exert on a motor pattern results from the integration of the individual effects that it exerts on different locations in the CPG-effector system. Crustacean cardio-active peptide (CCAP) has been determined to act on multiple sites in the CG and the periphery of the *Callinectes* cardiac CPG-effector system and has been shown to sometimes elicit higher order cycles in the beating pattern (Fort et. al, 2007). The same has been observed with FMRFamide-like peptides in *Callinectes* (Fort et. al, 2007). This theory of integration is supported by this CCAP study, as it was found that the effect of the peptide on the CG bursting pattern was opposite in the isolated CG to what was seen in the semi-intact preparation (where the CG is still embedded in its peripheral musculature). Beyond the direct effects of a modulator, the parameters that are altered by the modulator can in turn influence one another as well as sensory inputs that feedback into the system, further complicating predictions

of the overall output and contributing to the variability and potentially even chaotic nature of that output (Hokkanen 2000). If the generation of higher order cycles by AMGSEFLamide is in fact evidence of these complex dynamic interactions, then further study should investigate the peptide's modulatory ability at the periphery, as its central action has already been established (Dickinson et. al, 2019). Utilizing higher-order-cycle-inducing peptides like CCAP, FMRFamide-like peptide, and AMGSEFLamide could lead to better understandings of how modulation at multiple sites contributes to the generation of complex, variable outputs in seemingly simple systems.

Longitudinal stretch does not influence how the heartbeat is modulated by AMGSEFLamide

We found no statistically significant effect of uniaxial longitudinal stretch on the variability of the heartbeat frequency, as measured by the coefficient of variation. No clear effect was observed from manipulation of the passive force on the heart alone (Figure 8). While increasing the passive force via longitudinal stretch was sufficient to increase contraction amplitude in crabs and lobsters (García-Crescioni et. al, 2010; Dickinson et. al, 2016), this cannot necessarily be conflated with activation of the stretch-feedback pathway, since the increase could be due solely to the length-tension relationship of the cardiac muscle (Maquire 2019). Furthermore, deafferentation studies as they are currently executed cannot confirm whether increased contraction amplitude with stretch is due to direct feedback to the CG, since the deafferentation procedure requires the cutting of the heart's ventral muscle tissue, which affects contraction amplitude and thus confounds the effect of deafferentation. Given this, contraction frequency is the parameter that can most effectively assess the mechanisms by which stretch feedback alters the CG bursting pattern. In this study, manipulating passive force by

increasing longitudinal stretch was not sufficient to activate the stretch-regulation pathway described in recent deafferentation studies (Chin-Purcell, 2014). This finding is, however, consistent with a past study that found that longitudinal stretch did not significantly alter the coefficient of variation of the burst duration of an individual CG motor neuron (Qu, 2017). These discrepancies could be related to the variability in the direction and magnitude of the frequency response to stretch that has been documented in *Homarus* (Dickinson, 2014).

It is possible that simple longitudinal stretch does not disrupt the system to the degree that deafferentation does, making it so that it does not yield the same observable difference in regularity. Additionally, longitudinal stretch may have been insufficient because it does not accurately represent the way the heart stretches as it fills and empties *in vivo*. The heart expands in three dimensions as it fills, stretching longitudinally and circumferentially. Furthermore, the active and passive forces of the heartbeat are anisotropic in that stretch differentially influences them depending on the axis through which it is applied (Dickinson et. al, 2016). It is possible that the activation of the mechano-sensitive dendrites in the heart is anisotropic as well, and that they respond more reliably to stretches that mirror that of the *in vivo* heart filling. With this in mind, further study could experiment with transverse and biaxial stretches of the heart and determine whether they are sufficient to support the stretch-regulation hypothesis. Previous studies have also manipulated the perfusion pressure on the whole heart preparation to observe stretch-dependent changes in the amplitude of contractions, which could be a useful tool for mimicking the stretch experienced during diastole *in vivo* (Kuramoto and Ebara, 1984).

Consistent with the lack of effect of stretch on baseline coefficient of variation, the longitudinal stretch of the heart as measured by the passive force did not significantly influence the degree to which preparations responded with irregularity to 10^{-6} M AMGSEFLamide.

Preparations held at 1.5 and 3.5 grams passive force seem to exhibit a trend of greater irregularity in response to AMGSEFLamide compared to those held at 2.5 grams, however this effect is not significant in either the initial or the peak response phase (Figure 9). If this effect were significant, it would still be inconsistent with the predictions proposed by this study. If longitudinal stretch were activating the stretch-regulation pathway we hypothesized, we would in theory observe the smallest increases in coefficient of variation in preparations held at 3.5 grams, followed by those at 2.5 grams, and the largest increases in those at 1.5 grams passive force. Thus, if further study were to prove these trends significant, it would not support a linear model of increased longitudinal stretch yielding increased heartbeat regularity.

While not statistically significant in either case, it is worth noting that preparations held at 2.5 grams passive force had the greatest baseline coefficient of variation of frequency (Figure 8), as well as the smallest increase in coefficient of variation up application of AMGSEFLamide (Figure 9). One potential interpretation of this trend observed at 2.5 grams passive force is that AMGSEFLamide modulates the cardiac motor pattern in a state-dependent manner. State dependence refers to the variable magnitude of effects a neuromodulator can induce depending on the existing activity level or "state" of the network being acted upon (Nusbaum and Marder, 1989). Preparations held at 2.5 grams passive force showed a greater average coefficient of variation of frequency at baseline, 0.21, compared to those held at 1.5 and 3.5 grams passive force, 0.04 and 0.09, respectively (Figure 8). If preparations held at 2.5 grams passive force tended to have greater baseline irregularity, regardless of whether or not that irregularity was related to the passive force, then the ability of AMGSEFLamide to modulate them could have been effectively capped by the preexisting state of the system. The concept of an irregularity

state dependence is consistent with some observations in deafferentation experiments and will be discussed further.

This state-dependent hypothesis, and assessment of the interaction of longitudinal stretch and AMGSEFLamide in general, require further data collection for a more robust statistical analysis. Ideally, experiments would be performed in which AMGSEFLamide is applied while the heart simultaneously undergoes gradual stretch ramps (Dickinson et. al, 2016). This would be an important next step, since this study assessed the effect of a longitudinal stretch that the heart had been allowed to equilibrate to. Given the characterization of the stretch response in the CG as multi-phasic (Qu, 2017), the induction of irregularity in the heart via external modulation should be studied in relation to simultaneous changes in the heart's stretch state. This experiment, however, would likely not work with AMGSEFLamide, given the transient nature of the irregularity it induces (Figure 5). Extended application experiments should evaluate whether the irregularity induced by CCAP and FMRFamide-like peptides is lasting, to determine if these neuromodulators could be better candidates for this future research.

Deafferentation points to a role of stretch feedback in regulating modulation

Deafferentation did not significantly change baseline irregularity, as measured by the coefficient of variation in frequency. It is important to note that variability in frequency is not the end-all-be-all in quantifying irregularity, though it is the metric used in a previous study describing the change in beat regularity that results from deafferentation (Chin-Purcell, 2014). Our findings, however, are not necessarily completely inconsistent with those of Chin-Purcell, who found that deafferentation significantly increased the coefficient of variation of frequency in the whole heart (n=16). While not statistically significant, the mean coefficient of variation of

deafferented preparations, 0.26, was notably higher than that of intact preparations, 0.04, though it is important to note that an outlier with a sizably larger baseline coefficient of variation post-deafferentation is included in these averages (Figure 10). With an n value of 5, a larger sample size would give a more representative picture of how deafferentation is or is not altering this heartbeat parameter.

The deafferentation procedure itself is likely a source of error, as complete deafferentation was not possible in the whole heart preparation. Deafferentation was considered successful if all afferents between the posterolateral and anterolateral branches of the CG were severed (Figure 2). More posterior afferents were spared given their proximity to the small cells of the CG and to the posterior artery, where the heart was cannulated. Thus, even in "successful" deafferentations, stretch feedback was presumably still present to some degree. Given the inter-animal variability in the placement of the afferent projections along the CG, it is conceivable that certain hearts had a greater proportion of afferents in the deafferentation region compared to others (Alexandrowicz 1932). Given this, two hearts that were considered equally "deafferented" by the definition used in this study could have had different proportions of their total afferents ligated. While this is likely a minor source of error, the fact that preparations were not necessarily equivalently deafferented is nonetheless noteworthy.

While the n values for this portion of the study are not sufficient to yield a well-powered statistical analysis, trends were observed that suggest a possible interaction between the state of stretch feedback and the response to AMGSEFLamide. Specifically, deafferented preparations experienced a greater average increase in coefficient of variation of frequency in both their initial (979%) and peak (937%) responses to AMGSEFLamide compared to intact preparations (243% and 28%, respectively; Figure 11). Each preparation was investigated in its intact state and then

deafferented and assessed again. This means that the inherent responsivity of each individual to AMGSEFLamide was accounted for in the comparison. Furthermore, we used percent change from baseline values, with both the intact and deafferented states having their own respective baselines, so any changes in regularity that may have resulted from deafferentation alone were considered as well. These trends suggest that removal, or reduction, of stretch feedback via deafferentation augments the destabilizing modulatory effect of AMGSEFLamide. From this, it can be extrapolated that in the intact heart stretch feedback works to counteract external, irregularity-inducing modulation by AMGSEFLamide.

The concept of a stretch-feedback-dependent modulatory ability of AMGSEFLamide is further supported by observations made in individual preparations subject to deafferentation. Of the 5 successfully deafferented preparations, 2 did not respond to AMGSEFLamide with irregularity while intact, but then responded to the peptide with irregularity after deafferentation. Examples of recordings from the preparation with the more pronounced shift to irregularity are shown (Figure 12). The baseline frequency, while increasing slightly upon deafferentation, was not notably more variable after deafferentation. In fact, the average baseline coefficient of variation while intact was 0.08, compared to the average baseline after deafferentation of 0.03, which indicates that the beating pattern was slightly more stable after deafferentation. Despite the lack of an increase in irregularity with deafferentation, the preparation responded to AMGSEFLamide with a substantial 1566% increase in coefficient of variation; in contrast, when intact, it responded with a 58% decrease in that parameter. This suggests that by removing stretch feedback, we augmented the ability of AMGSEFLamide to induce irregularity in the beating pattern of the heart. Again, this points to the interaction of stretch feedback and

AMGSEFLamide modulation in the production of the overall output in the intact CPG-effector system.

Conversely, the exact opposite effect was observed in 1 of the 5 deafferented preparations. The reversal of function across these two preparations could suggest a state dependence, where the action of AMGSEFLamide is dependent upon the existing state of regularity in the heartbeat. The preparation, after exhibiting irregularity in response to AMGSEFLamide with an 100% increase in coefficient of variation while intact, was stabilized by the peptide with a 71% decrease after deafferentation (Figure 13). While it is difficult to reconcile this finding with what was observed in the preparation discussed in the previous paragraph, this could be an example of a state-dependent action of AMGSEFLamide, given that the baseline coefficient of variation of the preparation in question after deafferentation was 1.14. This is far greater than the post-deafferentation baseline values of any of the other 4 preparations studied, in which mean coefficient of variation was only 0.04. So, the baseline state of this outlier preparation was substantially more irregular than that of the other deafferents. Thus, the existing variability in frequency may have been approaching an irregularity ceiling. As a result, AMGSEFLamide stabilized the beat frequency by reducing the variability. This is supported by the preliminary correlation between the baseline deafferent coefficient of variation and the magnitude of the irregularity response of the deafferented heart to AMGSEFLamide (Figure 11C). Similar activity suggesting a state dependent action of modulators has been previously described in crustaceans. In *Cancer borealis,* CCAP applied to the isolated stomatogastric ganglion (STG) frequently produced a decrease in the frequency of the pyloric rhythm in preparations that exhibited a relatively fast pyloric rhythm at baseline. Conversely, CCAP produced increases in the frequency of the pyloric rhythm in preparations in which the rhythm

was slow at baseline (Weimann et. al, 1997). In the context of this experiment, the state dependent theory requires substantially more investigation to confirm; however, it does have the potential to explain the extreme differences in responses that were observed in the individual deafferented hearts.

Conclusions

The novel neuropeptide AMGSEFLamide was found to significantly dysregulate the heartbeat of *Homarus americanus*, making it a useful tool for further study of the interaction between internal feedback and extrinsic modulation with respect to the regularity of cardiac output. Longitudinal stretch did not significantly alter the regularity of the heartbeat, nor did it seem to influence the irregularity with which hearts responded to modulation by AMGSEFLamide. Further study should assess the ability of transverse and biaxial stretches of the heart to influence these parameters. Finally, preliminary evidence from deafferentation studies suggest that the removal, or reduction, of stretch feedback could enhance the irregularity-inducing capacity of AMGSEFLamide. Further study is necessary to assess the significance of this trend, and the overall relationship between sensory feedback and external modulation in central pattern generator systems.

Figures

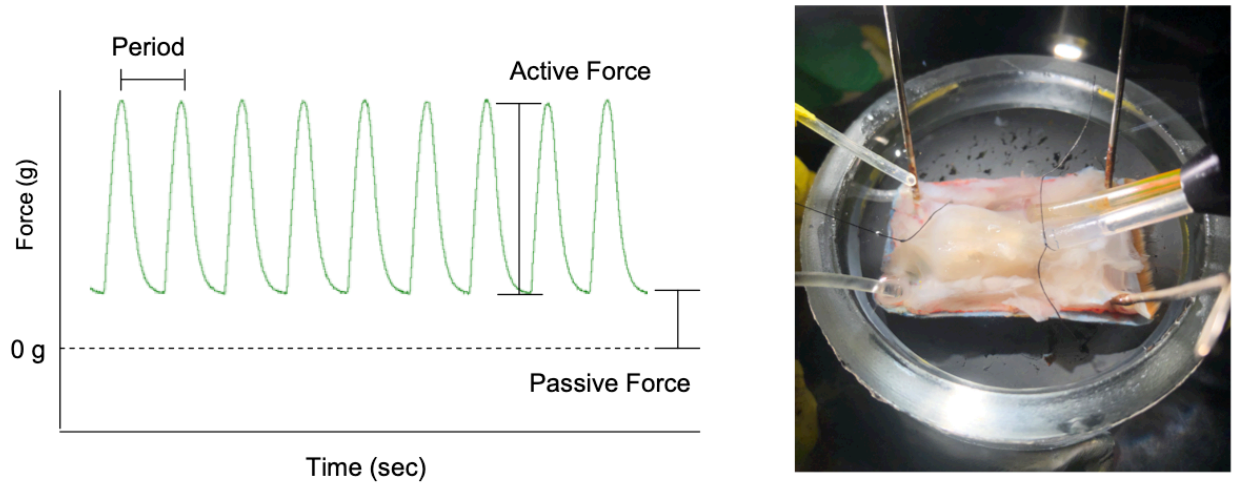

Figure 1. Images of the intact whole heart preparation and the force vs. time trace generated from it. Contractions of the heart muscle are measured by the suture silk that ties off the 5 anterior arteries on one end, and is secured to a force transducer on the other. The trace shows a typical heartbeat waveform and denotes the period (the time from peak to peak), passive force (the tension in the heart when at its most relaxed), and active force (the difference in force from the passive value to the peak of a beat).

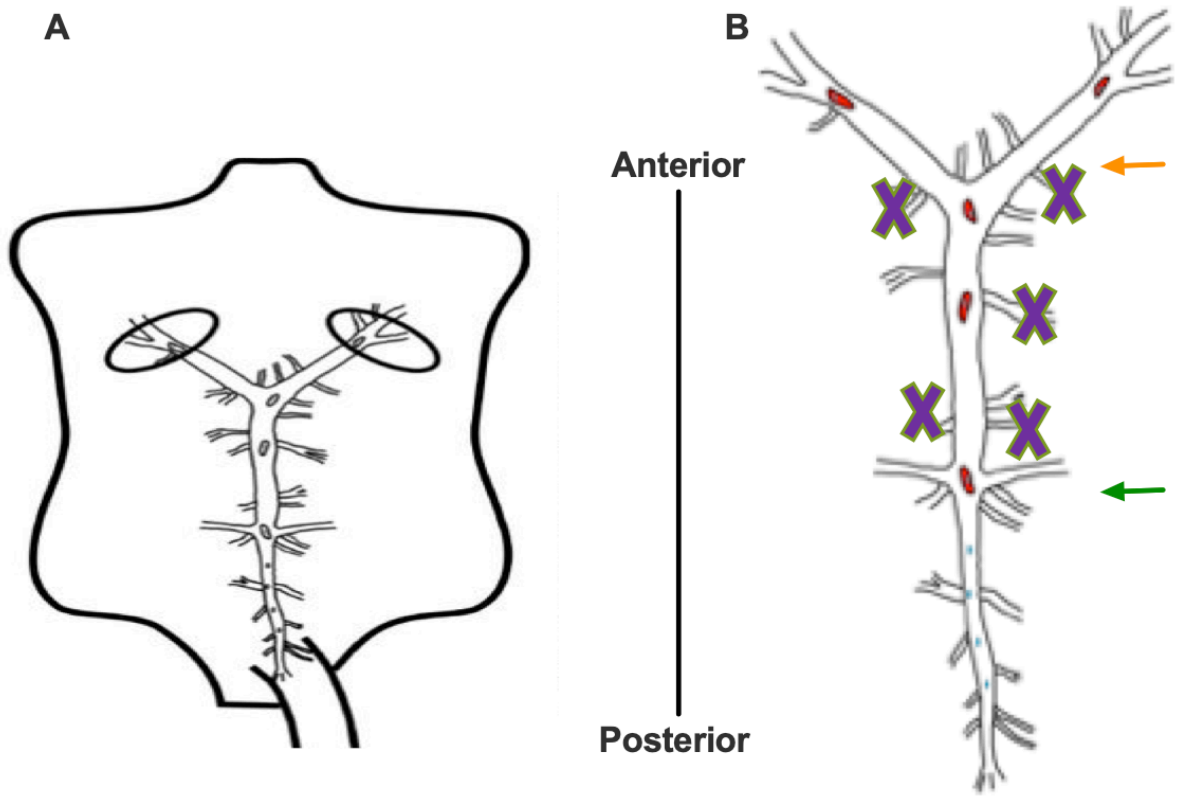

Figure 2. The placement of the cardiac ganglion (CG), the 9-cell central pattern generator within the neurogenic heart of *Homarus americanus* (A). Four small driver cells are located posteriorly (blue) while 5 large motor neurons extend anteriorly (red). X's mark the likely sites of mechano-sensitive afferents that were ligated during the deafferentation procedure (B). Only afferents between the posterolateral (green arrow) and anterolateral (orange arrow) nerve branches of the CG were targeted.

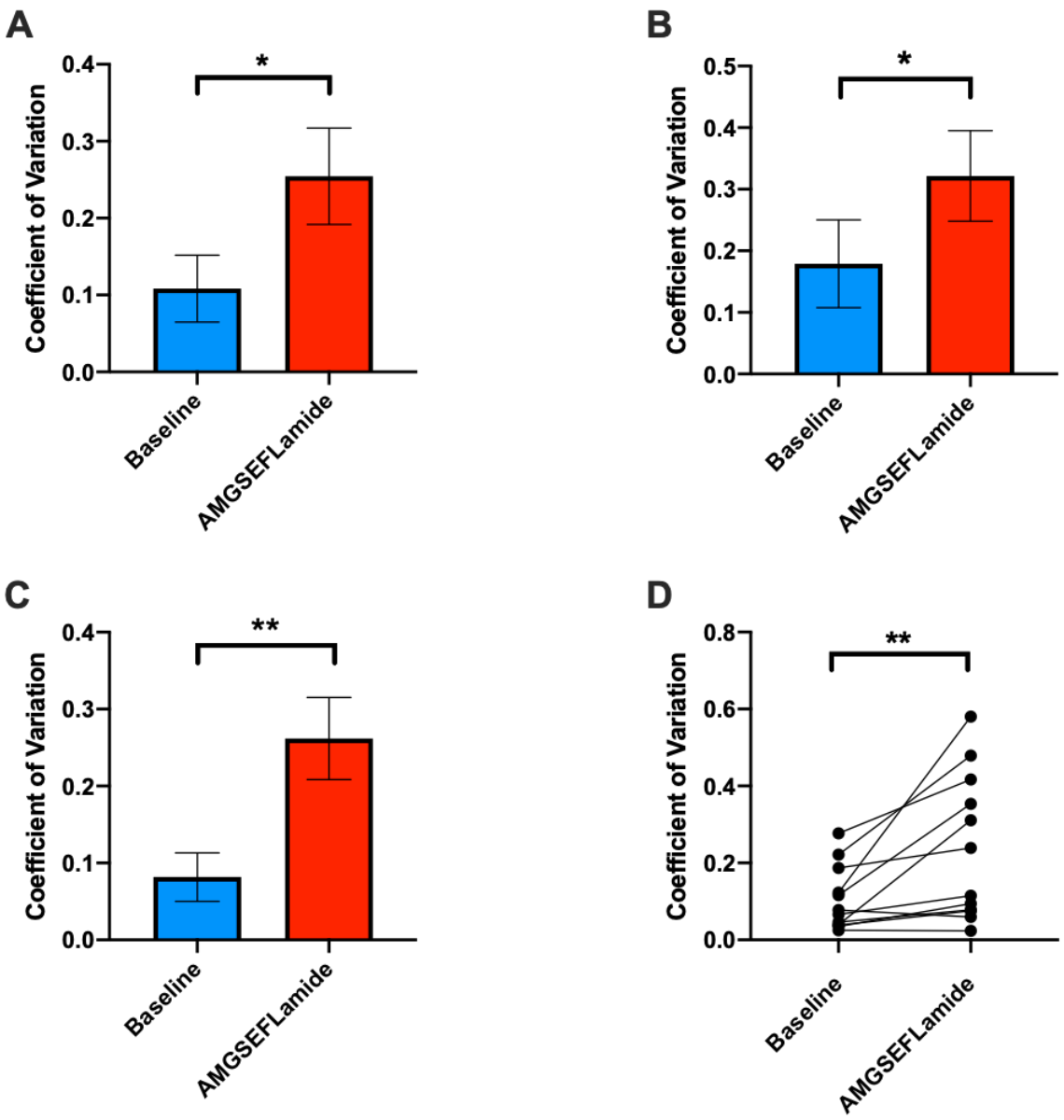

Figure 3. AMGSEFLamide induced irregularity in the frequency of the heartbeat. The coefficient of variation of the instantaneouos beat frequency was calculated from 100 beats for preparations at baseline and immediately upon application of 10^{-6} M AMGSEFLamide. The coefficent of variation of freqeuncy was significantly greater in the presence of AMGSEFLamide compared to baseline for preparations at 1.5 grams (n=9, paired t-test, p=0.0404; A), 2.5 grams (n=10, paired t-test, p=0.0285; B), and 3.5 grams passive force (n=11, paired t-test, p=0.007; C) indicating an increase in the irregularity of the heartbeat. The change in coefficient of variation from baseline was positive for the majority of individual preparations, and was statistically significant upon first application to AMG-naive preparations (n=11, paired t-test, p=0.0094; D).

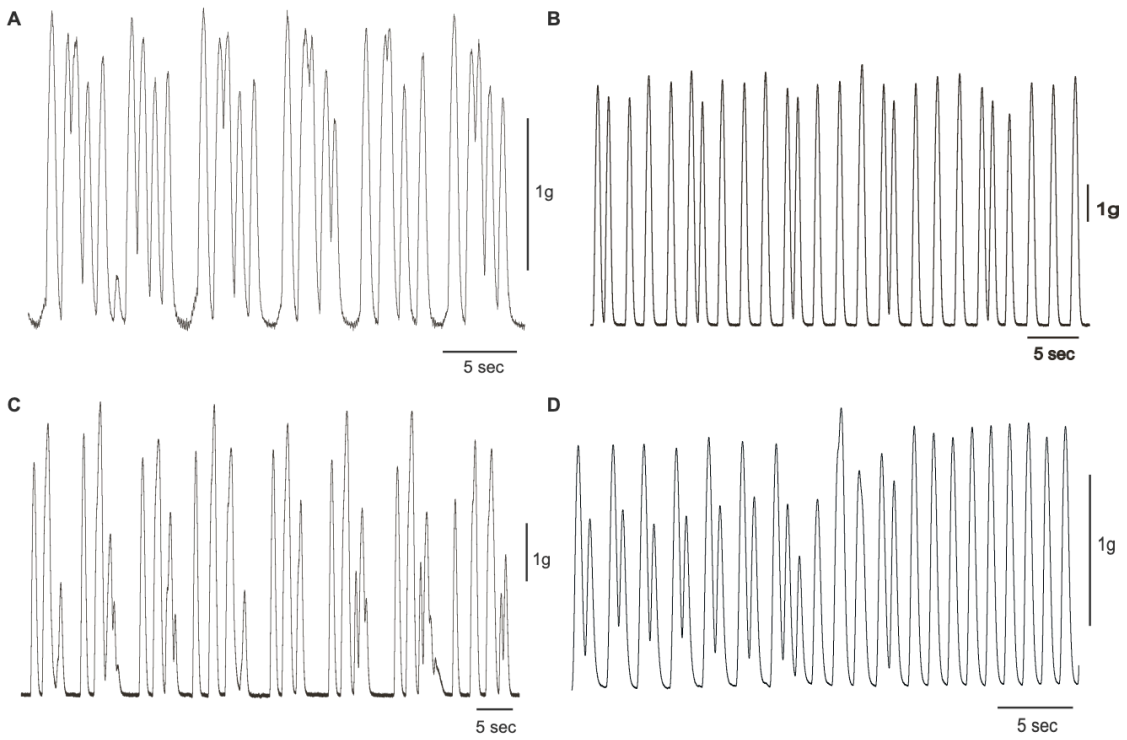

Figure 4. Higher order patterns emerge in response to AMGSEFLamide at multiple concentrations and at multiple passive force values. Higher order cycles were defined as consisting of at least 2 irregular successive beats, the pattern of which repeated at least 3 times in full before any sort of mode switch. (A) 10^{-6} molar concentration of AMGSEFLamide at 2.5 grams passive force, (B) 10^{-6} molar concentration at 3.5 grams, (C) 10^{-6} molar at 2.5 grams, (D) 10^{-6} molar at 2.5 grams. Higher order cycles were observed at 10^{-7} molar concentration, but tended to be less pronounced. Patterns vary in number of beats per cycle, ranging from 3 (C) to 6 (D). (D) demonstrates a mode switch from a higher order cycle to a regular pattern.

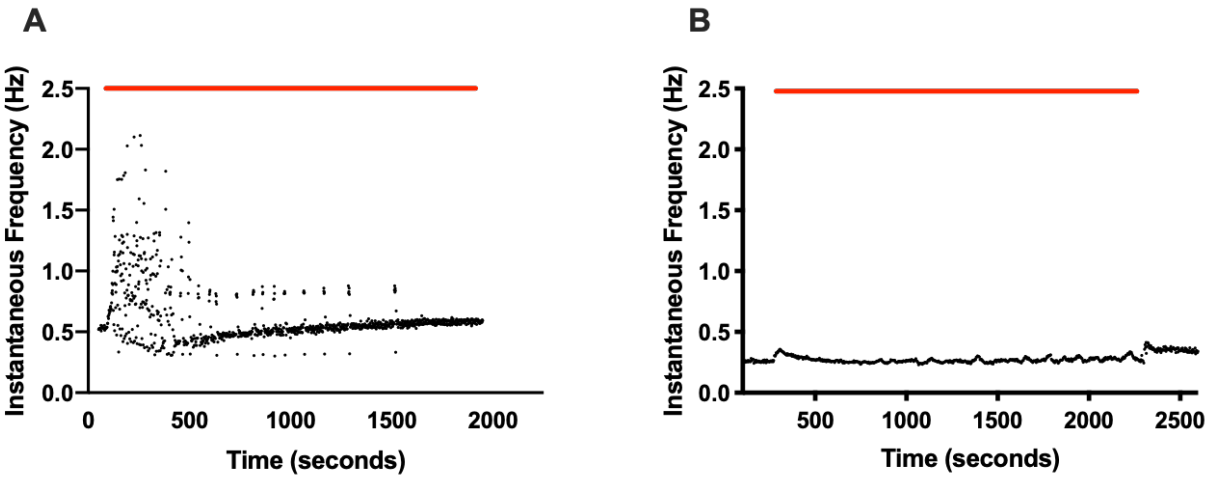

Figure 5. The irregularity induced by AMGSEFLamide is a transient effect (**A**). The red line indicates the roughly 40-minute period during which 10^{-6} molar AMGSEFLamide was applied. After approximately 400 seconds of sustained irregularity, regularity in instantaneous frequency gradually recovered (**A**). A separate preparation did not respond to sustained application of AMGSEFLamide with any irregularity (**B**).

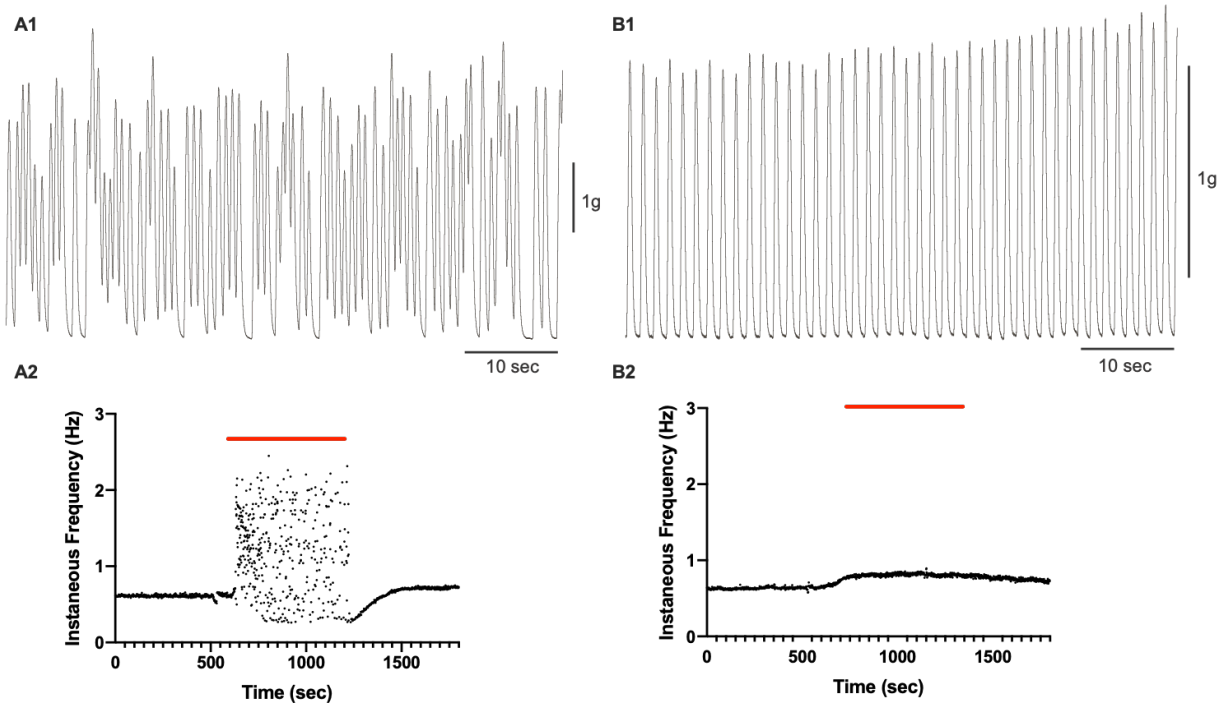

Figure 6. Response to AMGSEFLamide shows notable inter-animal variability. Both preparations were subject to 10^{-6} molar AMGSEFLamide at 3.5 grams passive force for 600 seconds. Red lines indicate the period during which the peptide was perfused through the preparation. Letters indicate data from the same preparation. Preparation A shows immediate and sustained irregularity in instantaneous beat frequency upon peptide application that is rapidly recovered from upon saline wash (A2). Preparation B shows a rapid, yet continuous increase in frequency, with minimal variability from beat to beat (B2). Waveform images (A1, B1) show 60 seconds of heartbeats exactly 60 seconds after introduction of AMGSEFLamide.

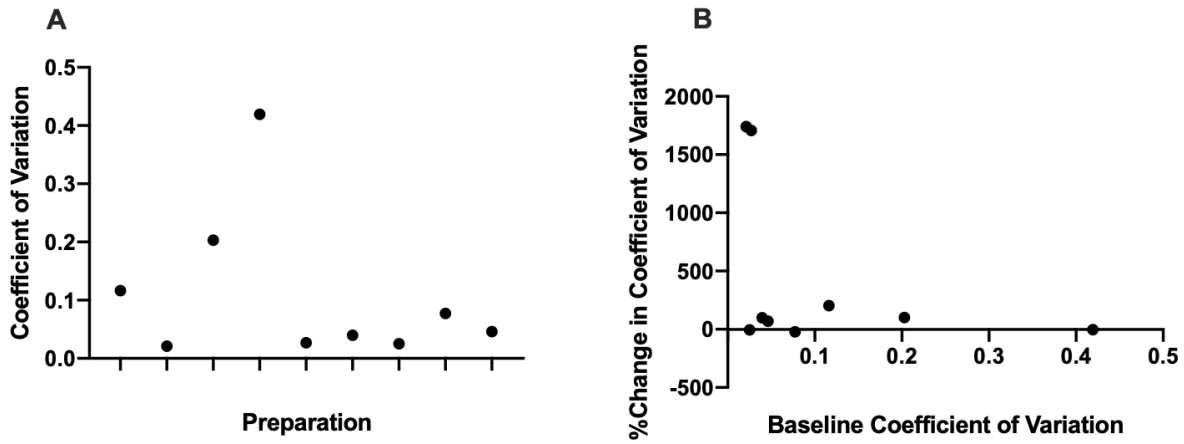

Figure 7. A large degree of inter-animal variability was observed in the initial coefficient of variation of beat frequency prior to application of 10^{-6} M AMGSEFLamide. Baseline values were calculated from 100 beats from preparations (n=9) during the equilibration with which they were held at a passive force of 1.5 grams (A). Percent change in coefficient of variation was calculated for the same preparations with the same 100 beats of baseline compared to the first 100 beats following perfusion of 10^{-6} M AMGSEFLamide. A scatter plot of percent change versus baseline coefficient of variation shows no clear correlation between the two (R^2=0.1426; B).

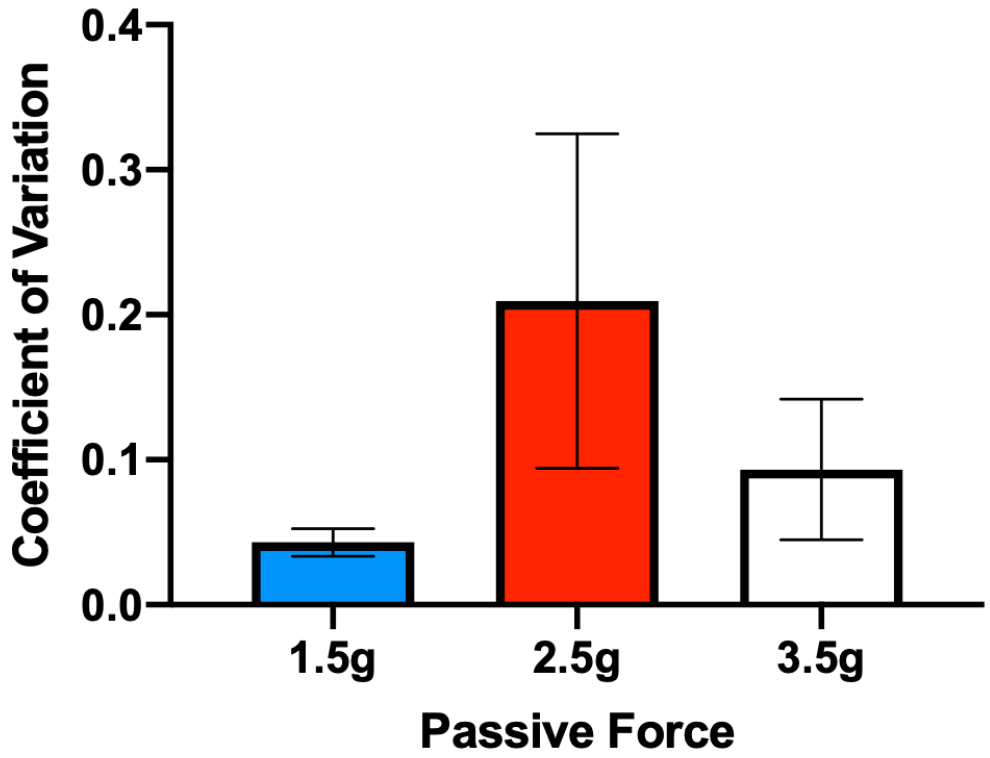

Figure 8. Longitudinal stretch alone did not significantly alter the coefficient of variation of heartbeat frequency (Ordinary one-way ANOVA, p=0.3168). Coefficient of variation values were calculated from 100 beats from preparations (n=8) after completing the stretch ramp procedure for each of the 3 passive force values. Longitudinal stretch was manipulated by holding the passive force at 1.5, 2.5, or 3.5 grams.

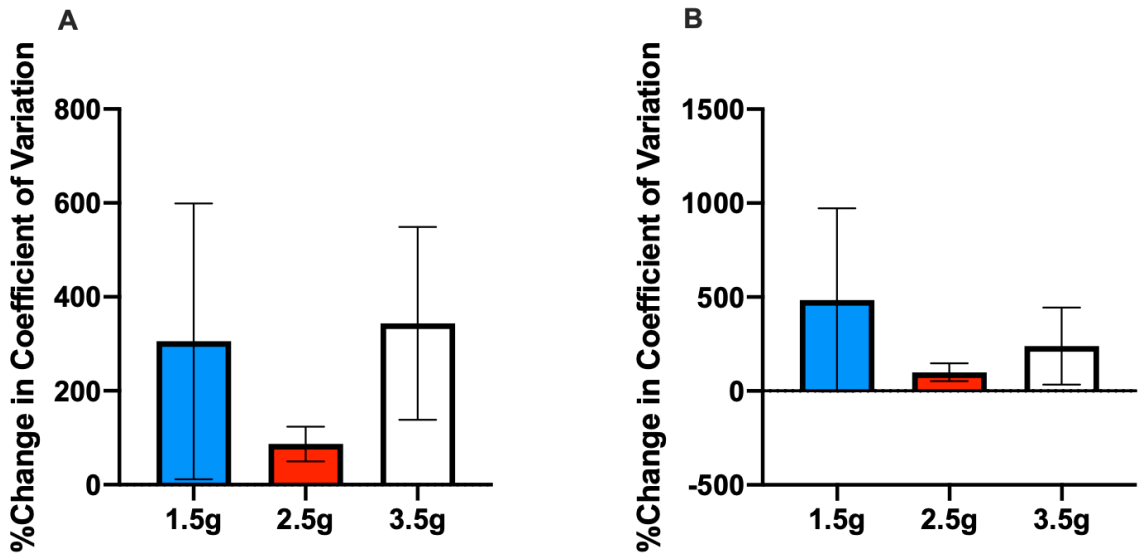

Figure 9. Longitudinal stretch alone did not significantly influence the degree to which preparations responded to AMGSEFLamide with irregularity, as measured by the percent change in the coefficient of variation of the frequency (n=8). The initial response (A) refers to the first 71 beats detected within the 600 second AMGSEFLamide perfusion period (Ordinary one-way ANOVA, p=0.6153). The peak response (B) refers the second 71 beats in the application period. Longitudinal stretch was manipulated by holding preparations at 1.5, 2.5, or 3.5 grams passive force (Ordinary one-way ANOVA, p=0.6363).

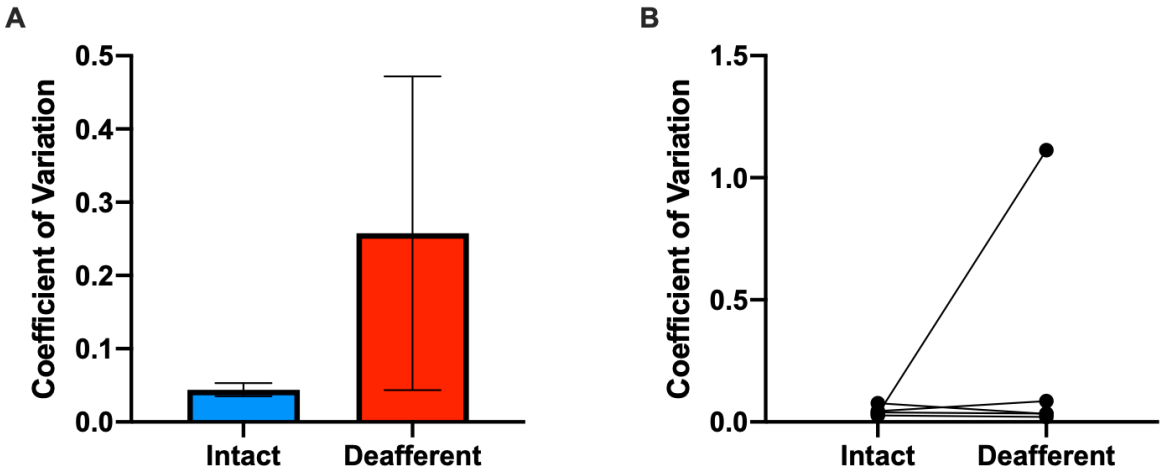

Figure 10. Deafferentation did not significantly alter the coefficient of variation of freqeuncy (n=5, Wilcoxon test, p>0.99; A). A single outlier experieced a large increase in coefficient of variation after deafferentation, however, the remaining 4 preparations experienced only marginal increases or decreases in coefficient of variation after deafferentation (B).

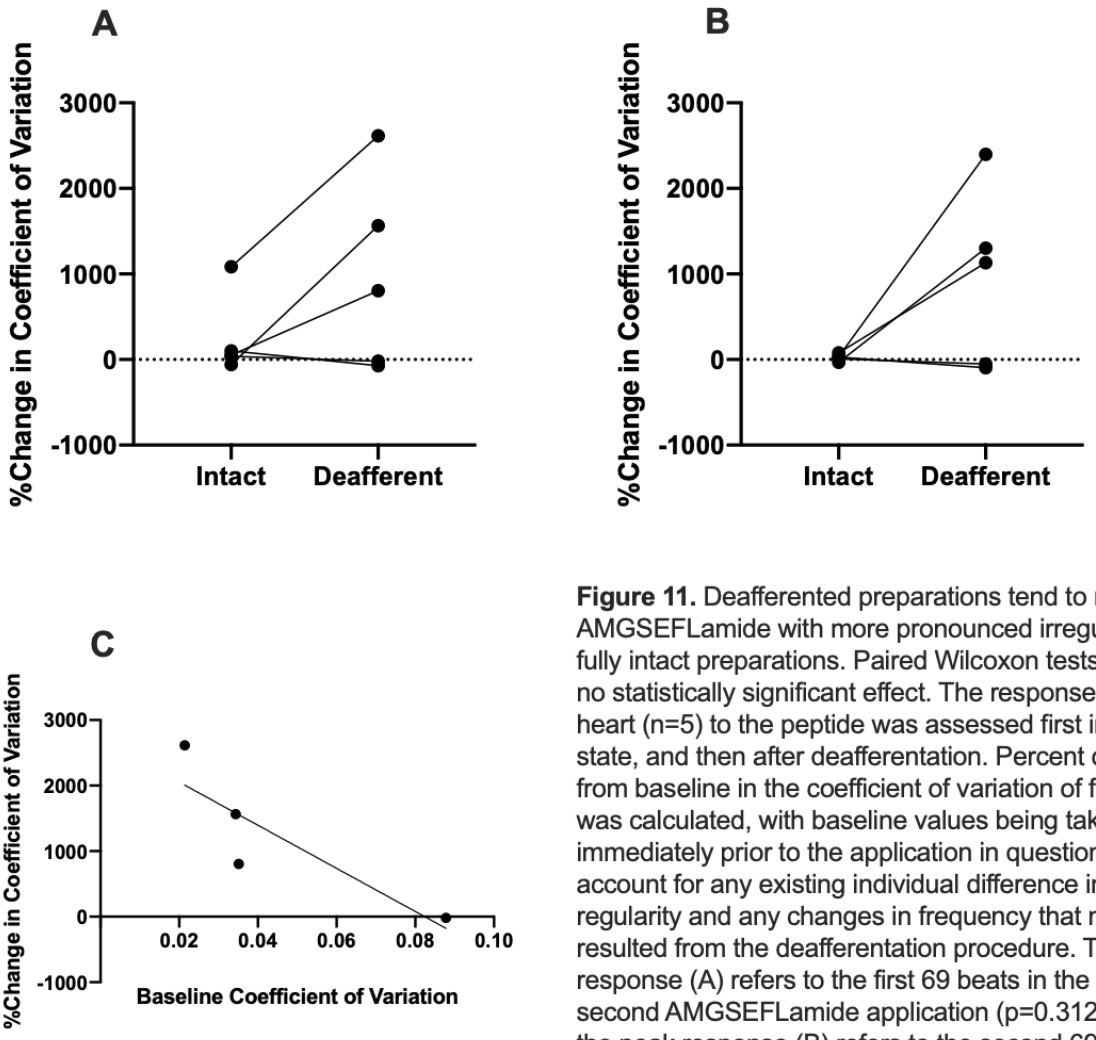

Figure 11. Deafferented preparations tend to respond to AMGSEFLamide with more pronounced irregularity than fully intact preparations. Paired Wilcoxon tests revealed no statistically significant effect. The response of each heart (n=5) to the peptide was assessed first in its intact state, and then after deafferentation. Percent change from baseline in the coefficient of variation of frequency was calculated, with baseline values being taken immediately prior to the application in question, so as to account for any existing individual difference in regularity and any changes in frequency that may have resulted from the deafferentation procedure. The initial response (A) refers to the first 69 beats in the 600 second AMGSEFLamide application (p=0.3125), and the peak response (B) refers to the second 69 beats in the application period (p=0.3125). A preliminary linear regression analysis suggests a potential correlation between the baseline coefficient of variation in deafferent preparations and the percent change in coefficient of variation upon application of 10^{-6} M AMGSEFLamide (R^2=0.7454, p=0.1367).

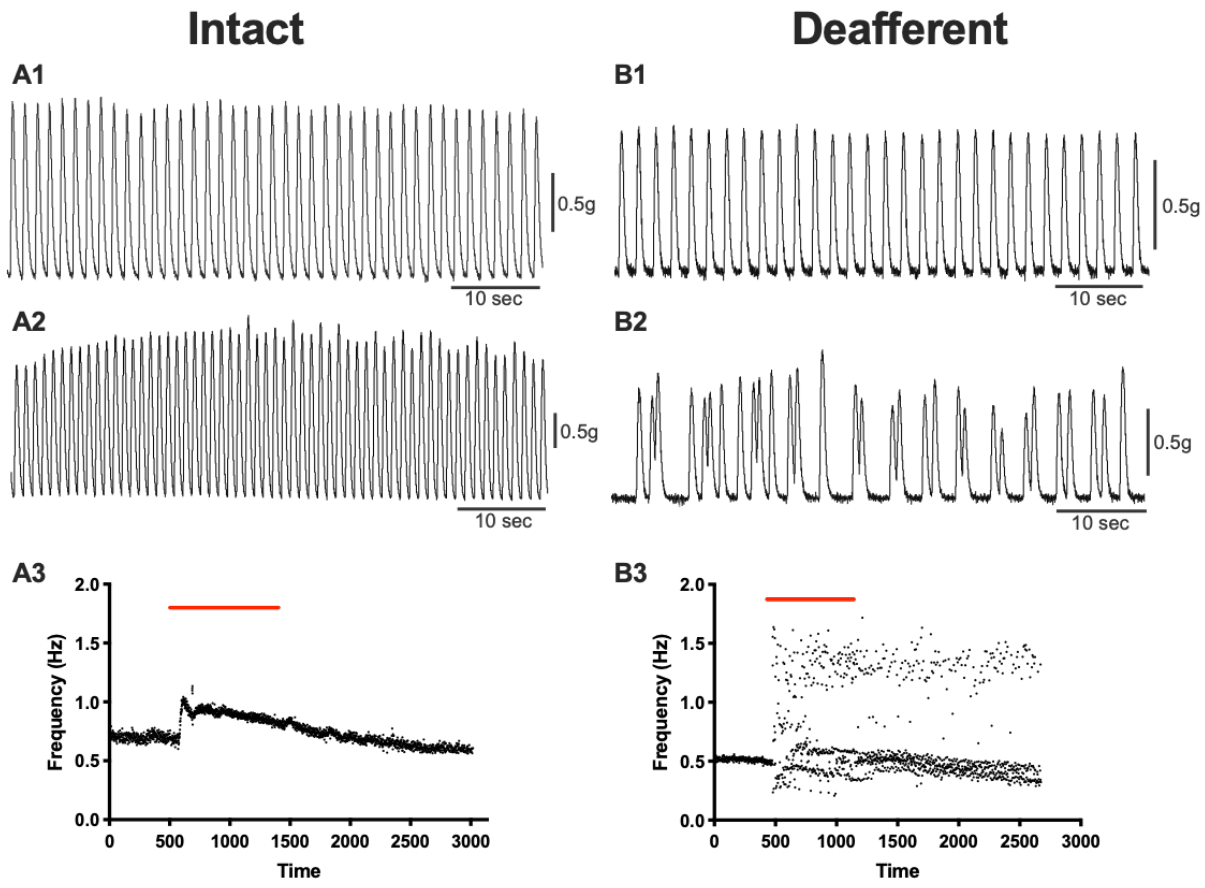

Figure 12. A single preparation did not respond with any irregularity upon application of 10^{-6} M AMGSEFLamide while fully intact, however, then responded to the peptide with irregularity after deafferentation. The intact preparation (A) shows only an increase in frequency from baseline (A1) in the first 60 seconds of AMGSEFLamide perfusion (A2). The time of peptide perfusion is denoted by the red bar in the frequency vs. time graph for the intact preparation (A3). Once deafferented (B), the preparation went from a stable baseline frequency (B1) to pronounced irregularity in the first 60 seconds of AMGSEFLamide application (B2) and for the duration of the perfusion (B3).

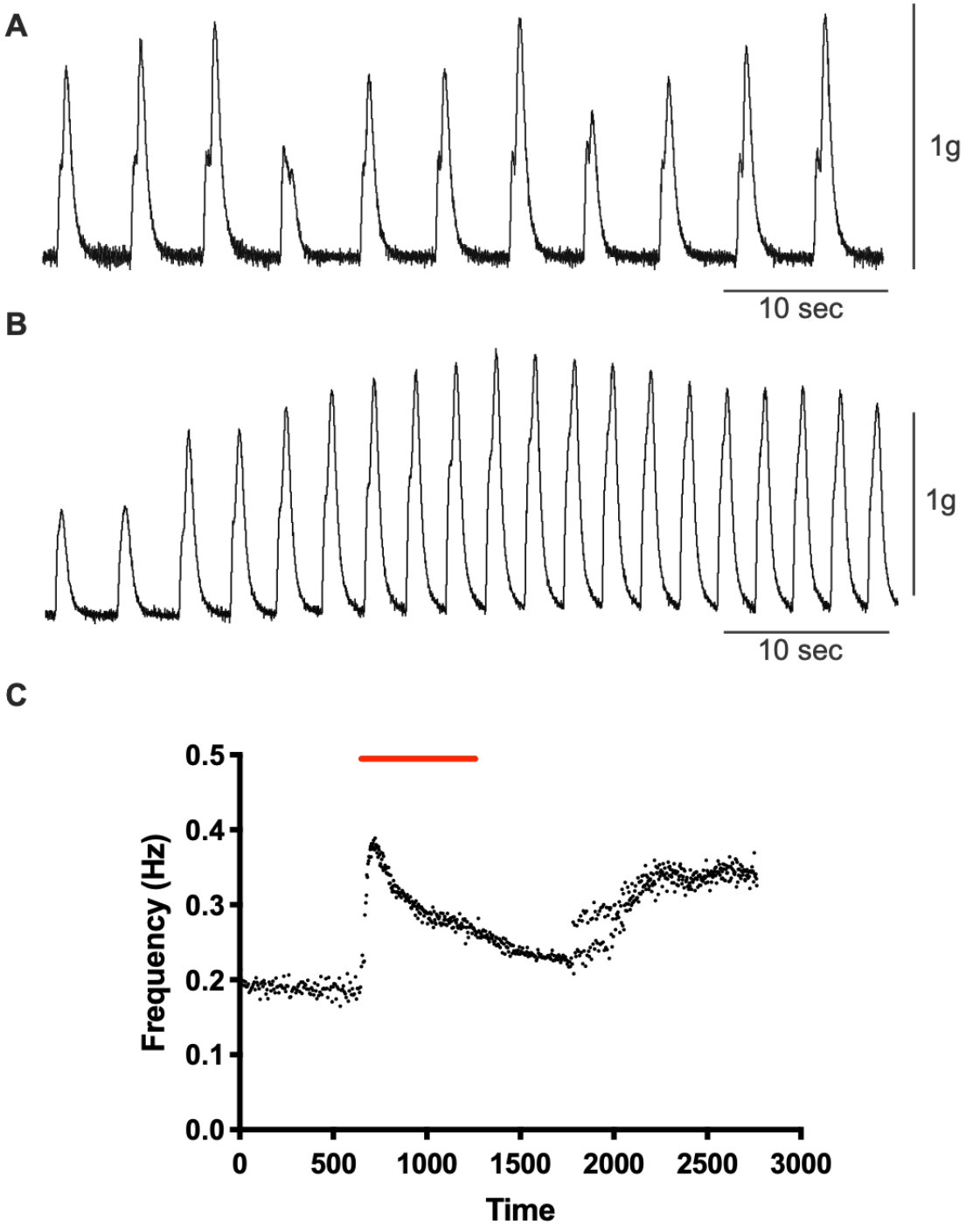

Figure 13. A single preparation that responded to AMGSEFLamide with irregularity when intact, experienced a stabilizing effect upon 10^{-6} molar AMGSEFLamide application after deafferentation. The preparation exhibited the highest baseline coefficient of variation of frequency after deafferentation of all the deafferent hearts. Irregularity in the baseline waveform (A) immediately gives way to regularity in the initial 60 seconds of AMGSEFLamide perfusion (B). The time of AMGSEFLamide perfusion is denoted in the frequency vs. time graph by the red bar (C).

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