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Patsy S. Dickinson
Bowdoin College

Andrew Calkins
Bowdoin College

Jake S. Stevens
Bowdoin College

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Related neuropeptides use different balances of unitary mechanisms to modulate the cardiac neuromuscular system in the American lobster, *Homarus americanus*

Patsy S. Dickinson, Andrew Calkins, and Jake S. Stevens
Department of Biology and Neuroscience Program, Bowdoin College, Brunswick, Maine

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Dickinson PS, Calkins A, Stevens JS. Related neuropeptides use different balances of unitary mechanisms to modulate the cardiac neuromuscular system in the American lobster, *Homarus americanus. J Neurophysiol* 113: 856–870, 2015. First published November 12, 2014; doi:10.1152/jn.00585.2014.—To produce flexible outputs, neural networks controlling rhythmic motor behaviors can be modulated at multiple levels, including the pattern generator itself, sensory feedback, and the response of the muscle to a given pattern of motor output. We examined the role of two related neuropeptides, GYSDRNYLRFamide (GYS) and SGRNFLRFamide (SGRN), in modulating the neurogenic lobster heartbeat, which is controlled by the cardiac ganglion (CG). When perfused though an isolated whole heart at low concentrations, both peptides elicited increases in contraction amplitude and frequency. At higher concentrations, both peptides continued to elicit increases in contraction amplitude, but GYS caused a decrease in contraction frequency, while SGRN did not alter frequency. To determine the sites at which these peptides induce their effects, we examined the effects of the peptides on the periphery and on the isolated CG. When we removed the CG and stimulated the motor nerve with constant bursts of stimuli, both GYS and SGRN increased contraction amplitude, indicating that each peptide modulates the muscle or the neuromuscular junction. When applied to the isolated CG, neither peptide altered burst frequency at low peptide concentrations; at higher concentrations, SGRN decreased burst frequency, whereas GYS continued to have no effect on frequency. Together, these data suggest that the two peptides elicit some of their effects using different mechanisms; in particular, given the known feedback pathways within this system, the importance of the negative (nitric oxide) relative to the positive (stretch) feedback pathways may differ in the presence of the two peptides.

cardiac ganglion; FMRFamide-like peptide; feedback

ADAPTIVE RHYTHMIC BEHAVIOR is determined not only by the output of the nervous system, but also by the interactions of the nervous system with the periphery. Output from the nervous system is transformed into movement at the level of the muscles by way of the neuromuscular transform (Brezina et al. 2000a, 2000b; Brezina and Weiss 2000; Williams et al. 2013); the resulting movements determine the level and nature of feedback from the periphery, which can in turn affect the output of the nervous system. Thus the generation of rhythmic movements is determined by the integration of activity, inputs, and interactions of multiple components of the multilayered neuromuscular system: the central pattern generator (CPG) and motor neurons, the neuromuscular junctions (NMJs) and muscles, and feedback systems.

Address for reprint requests and other correspondence: P. S. Dickinson, 6500 College Station, Brunswick, ME 04011 (e-mail: pdickins@bowdoin.edu).

Moreover, the components that make up each of these layers are subject to modulation by both locally released and circulating modulators. Consequently, motor outputs are flexible, enabling organisms to produce behaviors that are appropriate as internal or external conditions change. The mechanisms by which CPGs are modulated, both by circulating neurohormones and by the outputs of projection neurons, have been studied for many years (reviewed in Brezina 2010; Goulding 2009; Guertin 2009; Guertin and Steuer 2009; Hooper and DiCaprio 2004; Marder 1991, 2000; Marder and Bucher 2001, 2007; Marder et al. 2005; Pearson 2000; Selverston 2010; Selverston and Ayers 2006; Simmers et al. 1995; Stein 2009). Neuromodulators can alter intrinsic membrane properties of pattern generating neurons and can modulate the synaptic interactions between them. Similarly, they can alter the NMJ or the contractile properties of muscles (e.g., Beilin and Pasztor 1989; Bishop et al. 1984, 1987; Brezina et al. 2000b; Erxleben et al. 1995; Jorge-Rivera and Marder 1996, 1997). Moreover, the stretch receptors that make up many feedback systems are subject to neuromodulation (e.g., Billimoria et al. 2006; Birmingham et al. 2003; Pasztor and Bush 1987, 1989; Pasztor and Golas 1993), and sensory feedback can in return modulate, particularly on a cycle to cycle basis, the output of CPGs (reviewed in Buschges et al. 2008; Rossignol et al. 2006). What is less clear is how these multiple sites are modulated in concert to produce a predictable and appropriate pattern of movements.

The crustacean cardiac neuromuscular system, including that of the American lobster, *Homarus americanus*, has several characteristics that lend themselves to studies of the modulation of rhythmic output at multiple sites. Rhythmic contractions of the neurogenic heart are driven by regular bursting activity in the CPG that comprises the cardiac ganglion (CG) (Anderson and Cooke 1971; Cooke 2002; Cooke and Hartline 1975; Hartline 1967; Hartline and Cooke 1969). The CG receives feedback via two known pathways. First, the neurons within the CG have stretch-sensitive dendrites (Alexandrowicz 1932; Garcia-Crescioni et al. 2010; Sakurai and Wilkens 2003). Second, the production of nitric oxide (NO) by cardiac muscle decreases cycle frequency in the *Homarus* heart (Mahadevan et al. 2004). Additionally, the heart in decapod crustaceans is modulated by a large number of neuropeptides as well as amines (see Cooke 2002; Cruz-Bermudez and Marder 2007; Dickinson et al. 2007; Fort et al. 2004, 2007a, 2007b; Mercier et al. 2003; Mercier and Russenes 1992; Stevens et al. 2009).

Because the CG is located within the lumen of the heart, and most cardiac modulators are delivered to the heart hormonally, the CG and cardiac muscles are nearly always exposed to modulators in concert. Modulators thus exert their effects simultaneously at multiple levels of this pattern generator-effector system, as has been shown for myosuppressin in the lobster (Stevens et al. 2009) and for dopamine, crustacean cardioactive peptide, and several FMRFamide-like peptides (FLPs) in the crab *Callinectes sapidus* (Fort et al. 2004, 2007a, 2007b).

H. americanus contains at least 84 neuropeptides belonging to 17 peptide families (Ma et al. 2008). One of the larger families, the FLPs, contains 19 peptides that share a similar LRFamide ending (Ma et al. 2008). Among these are several YLRF peptides, including GYSDRNYLRFamide (GYS), and several FLRF peptides, including SGRNFLRFamide (SGRN). Both GYS and SGRN have been identified in the brain, ventral nerve cord, and stomatogastric ganglion of *H. americanus*; SGRN has also been identified in the pericardial organs (Ma et al. 2008). A number of different members of this peptide family have been shown to modulate the activity of the crustacean cardiac neuromuscular system. Nearly all of these peptides elicit increases in both contraction frequency and amplitude at physiological concentrations (Fort et al. 2007a). Of the FLPs that have been examined, only myosuppressin (pQDLDHVFLRFamide) has been shown to consistently elicit mixed effects, causing an increase in contraction amplitude but a decrease in frequency (Stevens et al. 2009). One question that thus arises is whether all of these peptides, particularly members of the same family, exert unique modulatory effects on a given neuromuscular system, such as the cardiac system; answers to this question may help us to understand why there are so many modulators acting on a single system.

If related peptides exert similar overall effects, we can then ask whether different modulators use the same or different mechanisms to alter the outcome in the same way. Previous modeling work has shown that similar patterns of neural activity can be generated by many different parameter sets (Goldman et al. 2001; Marder and Taylor 2011; Prinz et al. 2004; Taylor et al. 2009). These results agree with experimental observations; variability across animals of the same species has been observed for many circuit components, including the strength of intrinsic ionic currents (Schulz et al. 2006; Swensen and Bean 2005), the strength of synaptic interactions (Goaillard et al. 2009; Roffman et al. 2012), the voltage-dependence of ionic currents (Amendola et al. 2012), and the number and identity of cells comprising a circuit (Daur et al. 2012). We can now ask whether similar global patterns of modulation are likewise supported by multiple mechanisms on a systems level.

To answer these questions, we have examined the modulatory effects of two FLPs, GYS and SGRN, on the cardiac neuromuscular system. Because both GYS and SGRN are members of this family, we sought to determine whether they, like most other members of this peptide family, enhance cardiac activity. Additionally, we asked whether these closely related peptides used similar mechanisms to cause their effects, as well as whether they elicit effects at the same sites within the cardiac neuromuscular system.

MATERIALS AND METHODS

Male and female *Homarus americanus*, weighing 400–600 g, were purchased from local, Maine seafood markets and kept in tanks of recirculating sea water at 10–12°C on a 12:12-h light-dark cycle. Lobsters were fed weekly with chopped squid or shrimp.

Heart Preparations

To enable us to examine and compare the effects of the peptides on different parts of the cardiac neuromuscular system, we used several different types of preparations, all of which have been described in detail in Stevens et al. (2009). Briefly, lobsters were packed in ice for 20–30 min before dissection. For all of the experiments described here, the heart was removed from the lobster and immediately placed into cold physiological saline (composition in $mmol^{-1}$: 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20.00 $MgSO_4$, 3.91 Na_2SO_4, 11.45 Trizma base and 4.82 maleic acid; pH 7.45; Stevens et al. 2009).

Whole Heart Recordings

The neurogenic heart of *H. americanus* is controlled by a pattern generator located in the CG, which also receives feedback from the cardiac muscle. We therefore recorded the force of contraction, as well as the activity of the CG, in semi-intact whole hearts, in which all feedback and feedforward pathways were intact. For these experiments, the heart was left attached to a section of the dorsal thoracic carapace, which was removed from the animal and pinned in a Sylgard 170-lined dish (KR Anderson, Santa Clara, CA); the posterior artery was cannulated with polyethylene tubing and perfused at a flow rate of ~2.5 ml/min. The temperature of the inflow saline was maintained at 9–11°C using an in-line Peltier temperature regulator (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instrument, Hamden, CT). A second perfusion line directed cooled saline over the top of the heart to help maintain temperature.

To measure contraction force, the anterior arteries were attached, using 6/0 suture silk, to a Grass FT03 force-displacement transducer (Astro-med, West Warwick, RI) at an angle of ~30° from horizontal. To maintain an appropriate level of stretch, the thread was pulled to a baseline force of 2 g. The signal was amplified with an ETH-250 Bridge/Bio Amplifier and a model 410 Brownlee Precision Instrumentation amplifier. Data were recorded on a computer using a CED 1401 digitizer and Spike2 V6 or 7 (Cambridge Electronic Design, Cambridge, UK).

To record from the CG while simultaneously recording force, a small slit was made on the ventral side of the heart. A suction electrode was inserted and used to gently suction one of the two anterolateral motor nerves. Neuronal activity was amplified with a 1700 A-M Systems Differential AC Amplifier (Sequim, WA) and a Brownlee Precision amplifier.

Isolated Cardiac Ganglion

To record effects of the peptides on the CG itself, the heart was opened along its ventral surface, and the ganglion was manually dissected from the isolated heart, taking care to keep all 9 neurons and a length of each of the motor nerves intact. The ganglion was pinned to a Sylgard-lined dish filled with physiological saline. A petroleum jelly well was used to isolate a small portion of a motor nerve; pin electrodes were placed in the wells to record the activity of the motor nerve, with electrodes placed outside of the well serving as grounds. The signal was amplified and recorded using the same equipment used to record the ganglion in the semi-intact preparation. During recordings, the ganglion was constantly superfused with 10°C saline at a flow rate of 5.0 ml/min.

Stimulated Heart

To record the responses of the periphery (muscle/NMJ) to peptides, we used a preparation in which the CG was removed, but the motor nerves remained intact. This allowed us to eliminate effects mediated by changes in CG output. For these experiments, the heart was prepared as described above. However, once the slit was made in the heart, the region of the ganglion containing the 9 neurons was cut and removed from the heart. One of the two anterolateral nerves that extend into the heart muscle was pulled into a suction electrode. The motor nerve was stimulated through the suction electrode, using stimulation patterns generated in Spike2. Specifically, repeated bursts of stimulations (0.5 ms, 60 Hz; 200 ms burst duration) were administered to the heart at a frequency of 1 burst per second, with 45 s of rest between each group of 15 bursts. This pattern of stimulation enabled us to maintain stimulations for several hours without decrement of contraction amplitude in most preparations. The group of 15 bursts was sufficient to enable the heart, which shows considerable facilitation within bursts (Anderson and Cooke 1971), as well as burst-to-burst increases in contraction amplitude with repeated stimulations (Stevens et al. 2009), to reach a steady-state amplitude of contraction.

Peptides

GYS and SGRN were synthesized by GenScript (Piscataway, NJ) and were dissolved in deionized water to make 10^{-3} M stock solutions. The peptides were stored at −20°C to prevent degradation. Peptides were diluted in physiological saline to the appropriate concentration just before use.

Before any peptides were applied to the heart preparations, the preparations were allowed to stabilize for approximately 1 h. Peptides were applied through the perfusion system for 6 min, by which time the effects had stabilized. The preparation was allowed to recover in control saline for 1 h before another peptide application.

Data Analysis

Data were analyzed in Spike2, using custom scripts to analyze contraction parameters. To analyze bursting parameters, scripts from the Bucher laboratory, freely available at http://stg.rutgers.edu/Resources.html, were used. The data were further analyzed using Microsoft Excel, where we normalized the data for variable starting values by calculating the percent change between the control and peptide parameters for both contraction and bursting parameters. The control values used were those recorded during the 200 s immediately before peptide application, and the experimental value used was the average of the parameters measured during the 200 s of peak peptide effect. Contraction amplitude was measured as the change in force from baseline to the peak of contraction. Contraction durations were measured at half-amplitude. Contraction and burst frequency were calculated as the 1/period, where the contraction period was measured as the time between the peaks of adjacent contractions, and burst period was measured from the start of one burst to the start of the next burst. Duty cycle was calculated as burst duration/burst period. The data were graphed in Prism5 (Graphpad, La Jolla, CA), and significant changes from baseline were determined using one-sample, two-tailed *t*-tests with a significance threshold of 0.05; these statistics are presented in the figure legends. Threshold was considered to be the lowest concentration at which the peptide elicited significant effects. However, the actual threshold could have been anywhere between that value and the next lower concentration. Comparisons between GYS and SGRN at single peptide concentrations were made using two-tailed *t*-tests; comparisons between multiple concentrations were made using ANOVA followed by Tukey's multiple-comparisons test.

RESULTS

To determine the global effects of GYS and SGRN, as well as the sites at which they exerted effects, we examined the effects of these peptides in whole hearts, in isolated CGs, and in preparations in which we elicited controlled patterns of motor stimulation.

Effects of GYS and SGRN in Whole Heart Preparations Differed at Low and High Peptide Concentrations

When applied at concentrations ranging from 10^{-11} to 10^{-8} M, both GYS and SGRN altered multiple aspects of cardiac contractions (Figs. 1 and 2). As can be seen in the recording in Fig. 1, both contraction amplitude and heartbeat frequency increased when the heart was perfused with peptide. Both parameters returned to control values within 5–15 min after the return to control saline.

Contraction amplitude. The most striking effect of both peptides was a dose-dependent increase in contraction amplitude (Fig. 2, *A* and *B*). Threshold for this effect appeared to be slightly lower for GYS, which elicited significant increases (16.7 ± 5.1%) even at a concentration as low as 10^{-11} M, suggesting that the threshold is below 10^{-11} M. Threshold in SGRN appeared to be between 10^{-10} and 10^{-11} M. On the whole, however, the effects of the two peptides on contraction amplitude were similar, with increases in amplitude of less than 30% at 10^{-10} and 10^{-11} M, increasing to over 100% at 10^{-8} M (SGRN: 165 ± 14.7%; GYS: 132.1 ± 12.1%; Fig. 2, *A* and *B*).

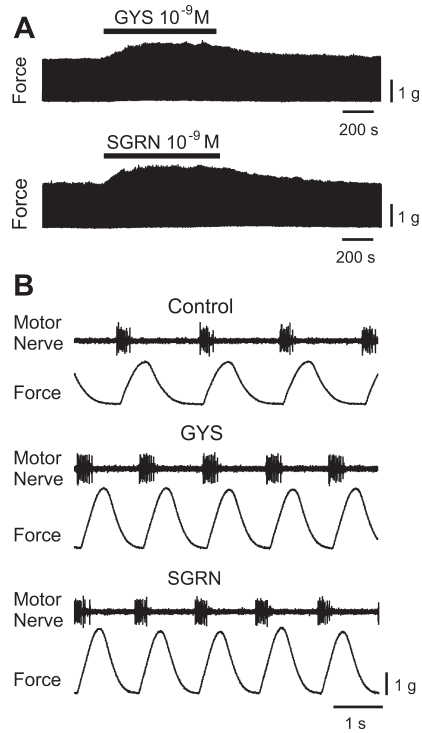


Fig. 1. When superfused at concentrations of 10^{-9} M, both GYSDRNYLRFamide (GYS) and SGRNFLRFamide (SGRN) elicited increases in contraction amplitude and frequency, which washed out when the preparation was returned to control saline. Muscle force was recorded with a force-displacement transducer, while electrical activity on the anterolateral motor nerve was recorded using a suction electrode inserted through a small slit in the heart. *A*: slow time-base recordings show the global time course of the increase in force. *B*: expanded recordings show motor neuron bursts and individual heartbeats.

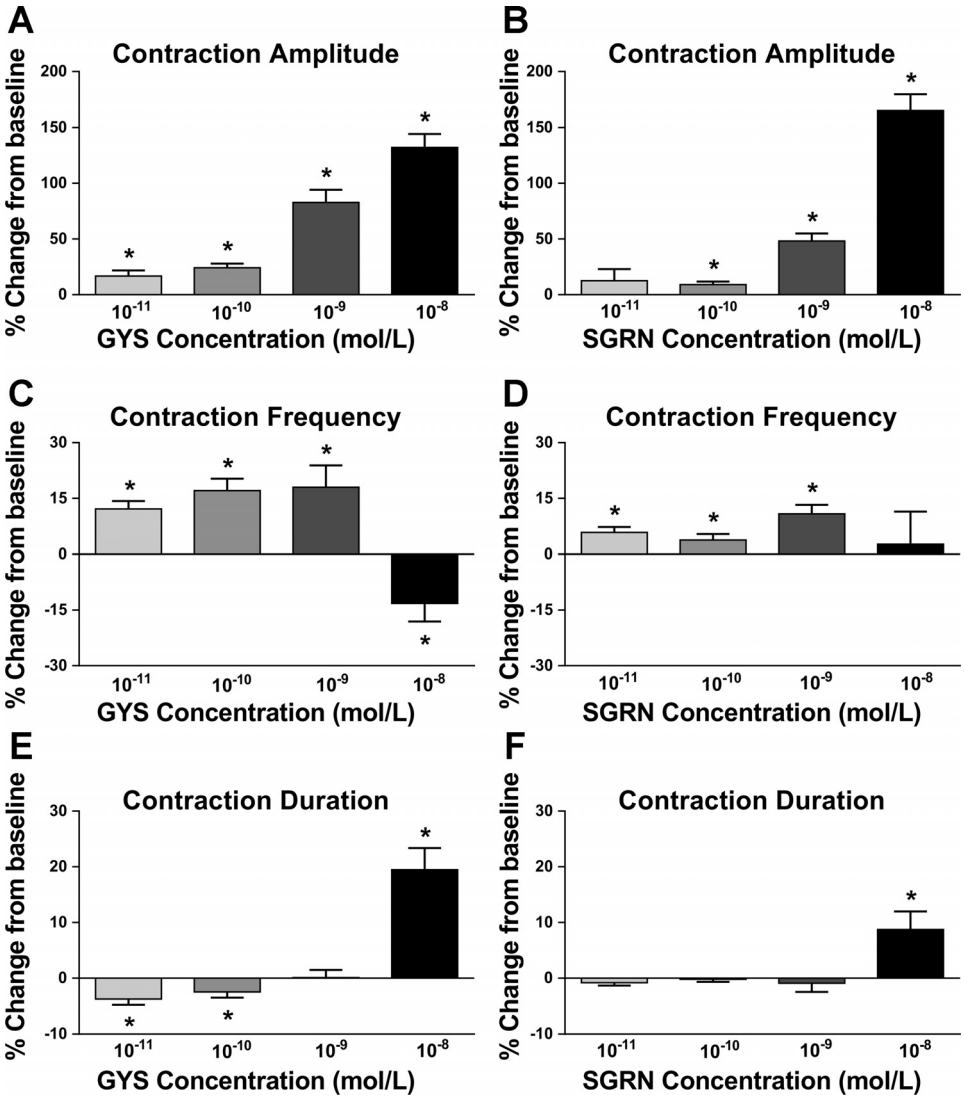


Fig. 2. Both GYS (*A*, *C*, and *E*) and SGRN (*B*, *D*, and *F*) altered contraction parameters when perfused through whole heart preparations. Thresholds, indicated by changes significantly greater than 0, for changes in at least some contraction parameters, were as low as 10^{-11} M in both peptides. *A* and *B*: contraction amplitude increased with increasing peptide concentration for both peptides. *C* and *D*: contraction frequency showed small (<20%) but significant increases in both peptides at concentrations below 10^{-8} M; at 10^{-8} M, however, frequency decreased significantly in GYS and was unaltered by perfusion with SGRN. *E* and *F*: contraction duration was essentially unchanged at low peptide concentrations, showing only a slight (<10%) decrease in GYS at 10^{-10} and 10^{-11} M. In contrast, when peptides were perfused through the heart at 10^{-8} M, contraction duration increased somewhat. *Values significantly different from 0; one-sample *t*-test; GYS 10^{-11} $n = 23$; GYS 10^{-10} $n = 21$; GYS 10^{-9} $n = 11$; GYS 10^{-8} $n = 14$; SGRN 10^{-11} $n = 10$; SGRN 10^{-10} $n = 13$; SGRN 10^{-9} $n = 14$; SGRN 10^{-8} $n = 9$.

Contraction frequency. Like contraction amplitude, contraction frequency increased in response to both peptides at low concentrations, with significant increases at concentrations as low as 10^{-11} M for both peptides. However, frequency increases at these concentrations (10^{-11} through 10^{-9} M) were relatively modest, with increases of only 18.0 ± 5.9% in GYS and 10.8 ± 2.4% in SGRN at 10^{-9} M.

Although the effects of the two peptides on amplitude were similar at all concentrations, the effects of the two peptides on frequency diverged at 10^{-8} M. In contrast to its effects at lower concentrations, 10^{-8} M GYS elicited a significant decrease in contraction frequency, with an average change of −13.2 ± 4.9% (Fig. 2*C*). The effects of SGRN at this concentration also differed from those recorded at lower concentrations. However, the effects of 10^{-8} M SGRN were quite variable, ranging from a −33% to 42% change; this is evident in the larger error bars at 10^{-8} M (Fig. 2*D*). Consequently, averaged over preparations, SGRN at 10^{-8} M did not significantly alter contraction frequency. This divergence of effects of the two peptides at higher concentrations led us to ask whether the same mechanisms were responsible for any of the effects of these two related peptides, and at what point the mechanisms diverged.

Contraction duration. Contraction duration was relatively unchanged by perfusion of either peptide at concentrations ranging from 10^{-11} to 10^{-9} M (Fig. 2, *E* and *F*). Statistically, there was a significant decrease in duration at both 10^{-11} and 10^{-10} M in GYS, but not in SGRN; however, these decreases were extremely small (less than 5%). In contrast to the lower concentrations, both peptides elicited modest increases in contraction duration when perfused at 10^{-8} M; GYS caused duration to increase by 19.5 ± 3.9%, while SGRN elicited an increase of 8.7 ± 3.2%.

To examine the mechanisms that might underlie the similarities and the differences in the response of the heart to these related peptides, we examined their effects both on the motor pattern generator that drives contractions and on the NMJ/cardiac muscle. Because the lobster heart is neurogenic, contraction is driven by output from the CG; thus one might predict that the effects of the peptides on the isolated CG, together with any effects on the NMJ/muscle, would directly determine contraction parameters. However, because the crustacean CG receives feedback from the heart muscle via both NO (Mahadevan et al. 2004) and stretch pathways (Alexandrowicz 1932; Garcia-Crescioni et al. 2010; Sakurai and Wilkens 2003), the effects of modulators on the CG output can

differ significantly when the ganglion is isolated, with feedback eliminated, vs. when it is in situ within the heart, with feedback pathways intact (e.g., Fort et al. 2004, 2007a, 2007b; Stevens et al. 2009). We thus examined the effects of SGRN and GYS on bursting parameters, particularly burst duration, cycle frequency and burst duty cycle, in the CG in both situations.

GYS and SGRN Exert Differential Effects on the Motor Pattern Generator of the Isolated Cardiac Ganglion

Burst cycle frequency in the isolated CG. Although both peptides elicited small increases in frequency at low peptide concentrations in the whole heart, neither SGRN nor GYS had any effect on cycle frequency of the isolated CG when applied at the lower concentrations (10^{-8} M or less), indicating that threshold in the isolated CG is much higher than in the whole heart. Surprisingly, even at concentrations of 10^{-8} and 10^{-7} M, there was no significant effect of GYS on cycle frequency across the group of preparations, although there was considerable variability in the responses of individual ganglia, as seen by the sizes of the error bars (Fig. 3*A*). Only when SGRN was applied at a concentrations of 10^{-7} M was cycle frequency significantly altered (Fig. 3*B*). Equally surprising, although SGRN elicited increases in contraction frequency in the whole heart, it had the opposite effect in the isolated CG: burst frequency decreased significantly at 10^{-7} M ($-27.4 \pm 6.6\%$; Fig. 3*B*).

Burst duration in the isolated CG. In contrast to their relatively small effects on the duration of whole heart contractions, both FLPs caused an increase in the duration of motor nerve bursts. These increases had a lower threshold in GYS (10^{-9} M) than in SGRN (10^{-8} M). At a concentration of 10^{-8} M, which was at or above threshold for both peptides, the effects were also more pronounced in GYS than in SGRN [Fig. 3, *C* and *D*; GYS $296.3 \pm 33.1\%$; SGRN $87.9 \pm 18.7\%$; $P < 0.0001$, *t*-test, degrees of freedom (df) = 21]. At higher concentrations (10^{-7} M), burst duration continued to increase in SGRN (to $209.1 \pm 42.7\%$; 10^{-8} vs. 10^{-7}, $P < 0.05$ Tukey's test, df = 25), but not in GYS ($251.3 \pm 35.2\%$; 10^{-8} vs. 10^{-7}, $P > 0.05$).

These differences in the responses of both cycle frequency and burst duration to the two peptides led to substantial differences in the effects of the peptides on burst duty cycle (Fig. 3, *E* and *F*). Thus, in spite of the fact that cycle frequency did not increase in the presence of GYS, the duty cycle of the

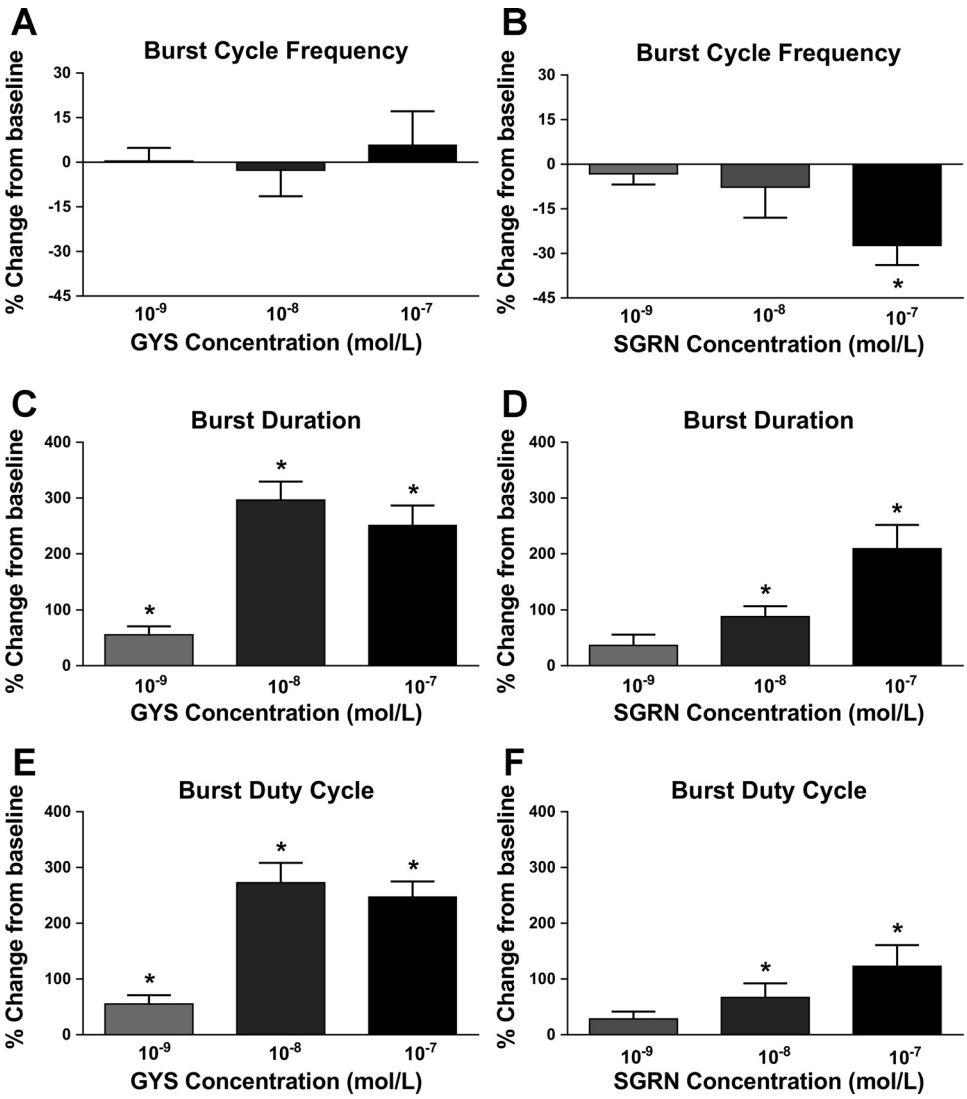


Fig. 3. When superfused over the isolated cardiac ganglion (CG), GYS and SGRN exerted different modulatory effects on the motor neuronal bursting pattern. *A* and *B*: cycle frequency was unchanged by superfusion with GYS (*A*), but showed dose-dependent decreases in response to SGRN (*B*). *C* and *D*: both peptides elicited increases in motor neuron burst duration. *C*: these increases were significantly greater than 0 at concentrations ranging from 10^{-9} to 10^{-7} M in GYS; this effect appeared to saturate at 10^{-8} M, showing no further increase when the concentration was raised from 10^{-8} to 10^{-7} M. *D*: in SGRN, threshold was 10^{-8} M, and this effect did not appear to saturate over the concentrations tested. *E* and *F*: burst duty cycle increased in both peptides. *E*: since cycle frequency did not change significantly in GYS, the pattern of increased in duty cycle paralleled that of the increases in burst duration. *F*: in SGRN, increases in duty cycle were significantly greater than 0 at both 10^{-8} and 10^{-7} M, but these increases were modest (<50%) since the increase in burst duration was accompanied by an increase in cycle period. *Values significantly different from 0; one-sample *t*-test; GYS 10^{-9} $n = 9$; GYS 10^{-8} $n = 13$; GYS 10^{-7} $n = 9$; SGRN 10^{-9} $n = 9$; SGRN 10^{-8} $n = 10$; SGRN 10^{-7} $n = 10$.

isolated CG increased at all concentrations, with large increases (greater than 200%) at the two higher concentrations (10^{-9} M: 55.1 ± 15.8%; 10^{-8} M: 272.7 ± 35.7%; 10^{-7} M: 247.4 ± 27.5%). In the presence of SGRN at concentrations greater than 10^{-9} M, burst duration increased, while cycle frequency decreased; however, because both of these effects were modest, particularly at 10^{-8} M, the increases in burst duty cycle (66.8 ± 25.4%) at that concentration were considerably smaller than those recorded in GYS (GYS vs. SGRN at 10^{-8} M, $P < 0.001$, *t*-test, df = 21). Similarly, when SGRN was applied at a concentration of 10^{-7} M, duty cycle increased (by 122.7 ± 37.96%); this increase was again significantly smaller than that recorded in GYS (GYS vs. SGRN at 10^{-7} M, $P = 0.02$, *t*-test, df = 21).

GYS and SGRN Exert Similar Effects on the CG In Situ at Low Concentrations, but Different Effects at High Concentrations

A major difference between the state of the motor pattern generator of the CG in the whole heart and that in the isolated ganglion is the presence of feedback to the ganglion. In the whole heart, the CG receives feedback from stretch receptors as well as from NO. To examine the effects of the peptides on the CG with feedback intact, we recorded the motor output using a suction electrode inserted through a small opening in the ventral surface of the heart.

Burst cycle frequency. Since burst cycle frequency directly determines contraction frequency, effects of the peptides on burst frequency were identical to those observed for whole heart contraction frequency. Thus frequency increased slightly at low (10^{-9} through 10^{-11} M) concentrations of both peptides, but effects diverged at 10^{-8} M (Fig. 2, *C* and *D*). At this concentration, GYS caused a decrease in frequency, while SGRN did not, on average, alter cycle frequency.

Burst duration and duty cycle. Burst duration did not change significantly in either peptide at very low peptide concentrations (10^{-10} and 10^{-11} M; Fig. 4, *A* and *B*). However, duty cycle increased somewhat (~20%) in the presence of GYS, a consequence of the increased cycle frequency (Fig. 2, *C* and *D*) coupled with a nonsignificant increase in burst duration (Fig. 4*C*). Duty cycle in SGRN remained unchanged at these peptide concentrations (Fig. 4*D*).

These differences between the two peptides' effects on burst duration and duty cycle were augmented at higher concentrations. At 10^{-9} M, burst duration increased by 31.6% in GYS, but did not change in SGRN (GYS vs. SGRN, $P < 0.0001$, df = 22). Since both peptides elicited similar small (~10%) increases in cycle frequency at this concentration, duty cycle likewise increased, with a larger increase in GYS than in SGRN (GYS: 52.8 ± 4.9%; SGRN: 14.9 ± 2.5%; $P < 0.0001$, df = 22; Fig. 4, *C* and *D*).

These changes in frequency and duty cycle might be expected to alter contraction amplitude because of the nonlinear relations of the neuromuscular transform (Williams et al. 2013). However, most of the changes in burst parameters at these low peptide concentrations, although statistically significant across preparations, are nonetheless quite small, so the changes in contraction amplitude due solely to changes in burst parameters might also be expected to be minimal at low peptide concentrations.

At 10^{-8} M peptide, burst duration increased substantially in both peptides (Fig. 4, *A* and *B*), but the increase was over twice as large in GYS (137.2 ± 18.0%) as in SGRN (64.5 ± 16.9%; GYS vs. SGRN, $P = 0.01$, df = 21). Accompanying that increase in burst duration was a decrease in cycle frequency in GYS, but not in SGRN. However, because the decrease in cycle frequency was relatively small compared with the increase in burst duration, duty cycle in GYS increased substantially (99.4 ± 14.0%). Duty cycle likewise in-

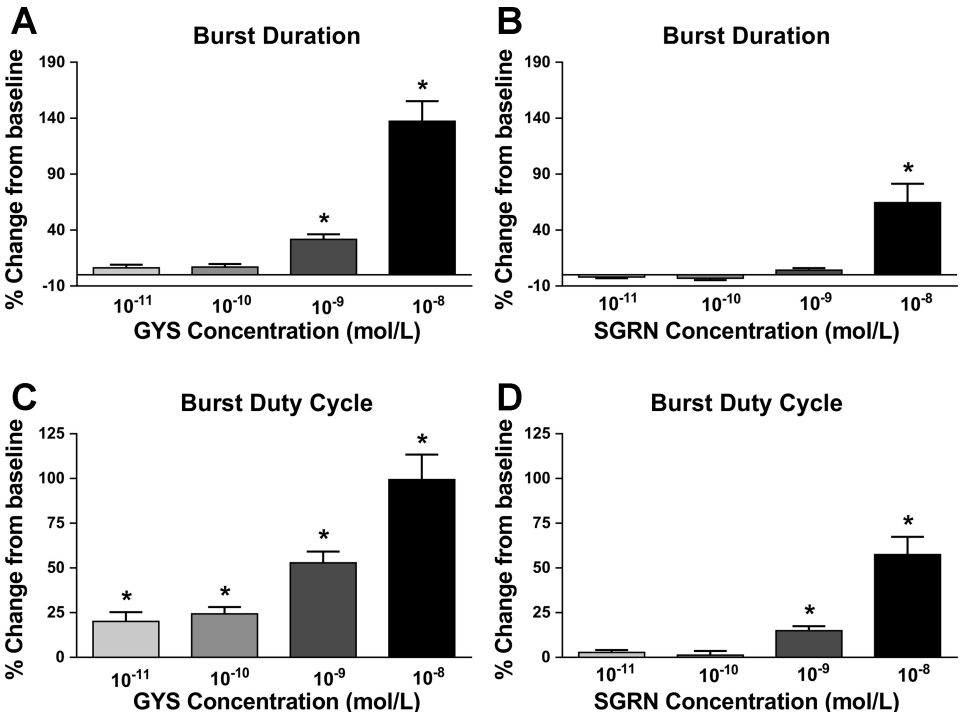


Fig. 4. The overall patterns of changes in burst duration and duty cycle recorded when the CG was still embedded in the heart, and therefore subject to feedback, were similar in the two peptides, although thresholds and the extent of the changes differed. *A* and *B*: burst duration did not change in either peptide at concentrations of 10^{-10} or 10^{-11} M, but it increased somewhat at 10^{-9} M, and substantially (>100%) at 10^{-8}M GYS (*A*). *B*: there was no change in burst duration in SGRN except at the highest concentration tested, 10^{-8} M. *C* and *D*: burst duty cycle increased in both peptides, but threshold differed. *C*: duty cycle increased in a dose-dependent manner in GYS, with significant changes at all concentrations tested. *D*: in SGRN, duty cycle, like burst duration, did not change at 10^{-11} or 10^{-10} M. Although burst duration did not increase in 10^{-9} M SGRN, duty cycle nonetheless increased because contraction frequency increased. At 10^{-8} M, the increase in burst duration coupled with no significant change in cycle frequency resulted in an increase in duty cycle. *Values significantly different from 0; one-sample *t*-test; GYS 10^{-11} $n = 23$; GYS 10^{-10} $n = 21$; GYS 10^{-9} $n = 11$; GYS 10^{-8} $n = 14$; SGRN 10^{-11} $n = 10$; SGRN 10^{-10} $n = 13$; SGRN 10^{-9} $n = 14$; SGRN 10^{-8} $n = 9$.

creased, although to a smaller extent, in response to perfusion of the heart with 10^{-8} M SGRN (57.4 ± 9.9%; GYS vs. SGRN, $P = 0.04$, df = 21).

Interestingly, based on the neuromuscular transform (Williams et al. 2013), one would predict that increases in cycle frequency should lead to lower contraction amplitudes, if duty cycle remained constant. In contrast, except in extreme cases, increases in duty cycle should lead to increases in contraction amplitude. Thus, in GYS, we would predict that both factors should promote increased contraction amplitude; in SGRN, these two changes should oppose one another, so that we might expect to record a smaller increase in contraction amplitude in SGRN than in GYS. In contrast to this prediction, the changes in contraction amplitude resulting from perfusion with 10^{-8} M GYS and 10^{-8} M SGRN were not significantly different (*t*-test, $P = 0.1$, df = 21), and the nonsignificant changes trended in the other direction (Fig. 2, *A* and *B*).

Both Peptides Increase Contraction Amplitude at Peripheral Sites

One explanation for the differences in responses to the peptides in the whole heart compared with the isolated CG might be differences in their effects on the NMJ and/or cardiac muscle. We thus examined the effects of each peptide at the periphery by stimulating one of the anterolateral motor nerves, while recording contraction amplitude. This allowed us to eliminate differential effects of the peptides on the ganglionic output, so that we could directly compare the responses of the peripheral neuromuscular components to the peptides. Although the whole heart can continue to function in isolation for many hours, our laboratory has previously found (Stevens et al. 2009) that the preparation is much less resilient when the motor nerves are stimulated. Thus we stimulated the motor nerve in groups of 15 bursts at 1 burst per sec, with 45 s between each set of bursts. This also enabled us to examine the effects of the peptides on facilitation at the level of the NMJ/muscle. In this paradigm, the initial contraction was quite small; the heart showed extensive facilitation over the next several bursts, but had virtually always reached steady state by around the 10th burst (Fig. 5). Thus we measured the amplitudes of the first contraction in each series to determine the effects on the periphery in the absence of interburst effects, such as facilitation or depression from one burst to the next; we measured the average of the last two contractions to examine the effects of the peptides on steady-state contractions.

Qualitatively, the effects of these two peptides were very similar (compare Fig. 5, *A* and *B*): both peptides elicited increases in contraction amplitude at steady state (i.e., the last 2 contractions in a series of 15) when applied at concentrations of 10^{-10} M and greater; contraction amplitude increased with increasing peptide concentrations in both peptides (Fig. 6, *A* and *B*; Tukey's test, $P < 0.01$ for all comparisons). Moreover, the percent changes recorded in the two peptides were remarkably similar at all concentrations (10^{-10} M: GYS 10.5 ± 3.3%; SGRN 11.5 ± 2%; 10^{-9} M: GYS 30.6 ± 6.6%; SGRN 32.9 ± 7.4%; 10^{-8} M: GYS 135.5 ± 18.2; SGRN 118.3 ± 13.4%). When we compared the effects of the two peptides on the first contraction in a group of 15, rather than on the steady-state response, we found that the overall trend was maintained, but the extent of the changes was larger at all concentrations above threshold ($P < 0.05$ for all comparisons of *contraction 1* vs. *contraction 15*), particularly in GYS (Fig. 6, *C* and *D*). For example, the amplitude of the first contraction in a series increased by 102.4 ± 26.1% in 10^{-9} M GYS (vs. 30.6 ± 6.6 for the 15th contraction, $P = 0.02$) and 390.5 ± 112.7% in 10^{-8} M GYS (vs. 135.5 ± 18.2 for the 15th contraction, $P = 0.04$). Consequently, there was somewhat less facilitation (from *contraction 1* to *contraction 15*) in the presence of the peptides. Specifically, facilitation decreased significantly at concentrations of 10^{-9} M and higher in both peptides (paired *t*-tests; $P < 0.05$; Fig. 7, *A* and *B*). Additionally, we noted that, as the amplitude of the first contraction in a series increased in the peptides, the extent of the facilitation from the first to the second contraction decreased, with significant decreases at concentrations of 10^{-10} M and higher in GYS ($P < 0.05$) and 10^{-9} M and higher in SGRN ($P < 0.05$) (Fig. 7, *C* and *D*). In contrast, the extent to which contraction continued to facilitate between the second and fifteenth contractions, which was relatively small even in control conditions, did not change in the presence of the peptides (Fig. 7, *E* and *F*). Taken together, these data strongly suggest that effects of the two peptides on

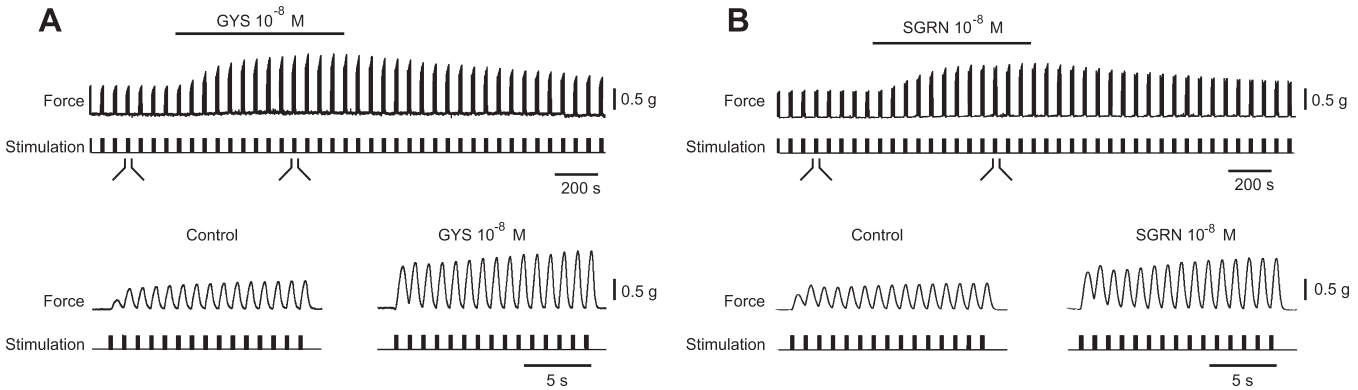


Fig. 5. Perfusion with either GYS (*A*) or SGRN (*B*) at 10^{-8} M elicited large increases in the amplitude of contractions that resulted from controlled and consistent motor nerve stimulation; amplitude gradually returned to control values when the peptide was washed off. The CG was removed from the heart, and an anterolateral nerves was stimulated with 200-ms-long bursts of pulses (each 0.5 ms in duration) repeated at 1 Hz. To prevent damage from repeated stimulation, bursts were delivered in bouts of 15 bursts, followed by 45 s of recovery time. The cardiac muscle showed significant facilitation within each group of 15 bursts (*bottom*), and amplitude increased until it stabilized for the last few contractions. Both the initial contraction in a group and the final, facilitated contraction were larger in peptide than in control saline.

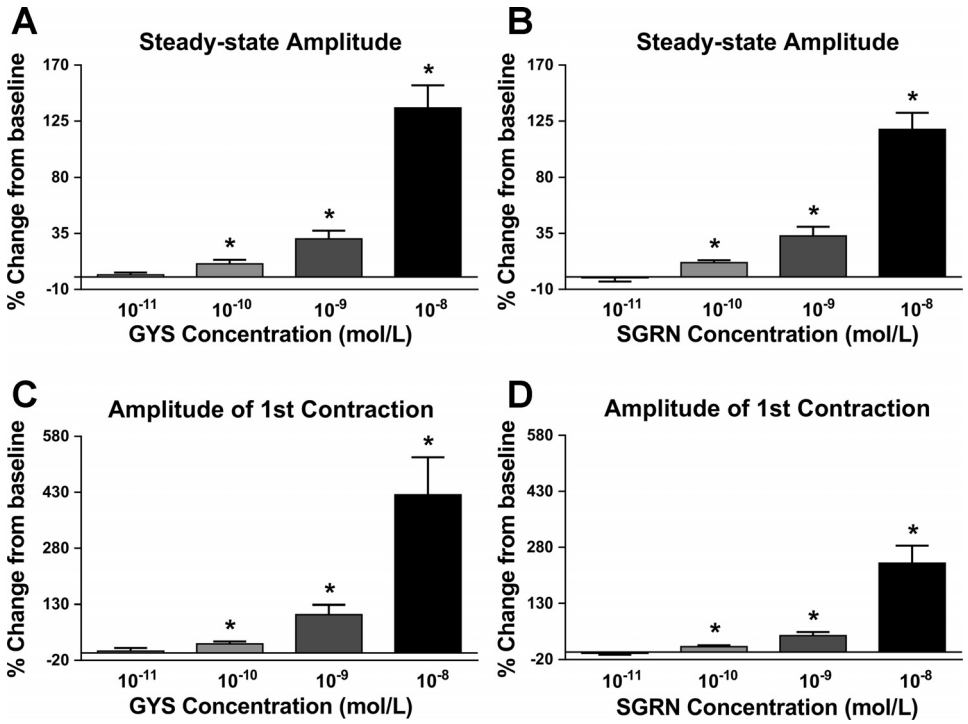


Fig. 6. Both GYS (*A* and *C*) and SGRN (*B* and *D*) elicited dose-dependent changes in contraction amplitude when applied to stimulated nerve-muscle preparations in which motor neuronal input was held constant. Bursts of stimuli 200 ms in length were delivered to the motor nerve at a frequency of 1 Hz in bouts of 15 bursts separated by 45 s of recovery time. *A* and *B*: graphed are the averages of the last two contractions in each bout of 15 bursts; at this point, the heart had reached a steady-state amplitude. The effects of the two peptides on this preparation were very similar, with thresholds of $\sim 10^{-10}$ M, and increases to over 100% when the peptides were perfused at 10^{-8} M. *C* and *D*: both peptides elicited increases in contraction amplitude in response to the first train in each bout. Increases were significant at concentrations of 10^{-10} M in both peptides, and increased strongly when peptide concentration was increased from 10^{-9} M to 10^{-8} M ($P < 0.0001$, Tukey multiple comparisons). *Values significantly different from 0; one-sample *t*-test; GYS 10^{-11} $n = 18$; GYS 10^{-10} $n = 18$; GYS 10^{-9} $n = 17$; GYS 10^{-8} $n = 13$; SGRN 10^{-11} $n = 10$; SGRN 10^{-10} $n = 12$; SGRN 10^{-9} $n = 21$; SGRN 10^{-8} $n = 11$. First contraction sample sizes GYS 10^{-11} $n = 13$; GYS 10^{-10} $n = 11$; GYS 10^{-9} $n = 9$; GYS 10^{-8} $n = 7$; SGRN 10^{-11} $n = 9$; SGRN 10^{-10} $n = 11$; SGRN 10^{-9} $n = 18$; SGRN 10^{-8} $n = 8$.

the periphery are major contributors to the increased contraction amplitude elicited by both GYS and SGRN.

DISCUSSION

The lobster cardiac neuromuscular system is deceptively complex: a central pattern generator (the CG) generates a single-phase rhythmic output, which is translated into contraction of the single heart chamber via the neuromuscular transform, a nonlinear transformation that encompasses properties of both the NMJ and the cardiac muscle. Additionally, however, the CG receives feedback from NO generated by cardiac muscle, which slows the CPG frequency (Mahadevan et al. 2004), and from stretch-sensitive processes of the CG neurons, the effects of which have not yet been directly recorded in *Homarus*, but which are thought to increase cycle frequency upon stretch (Cooke 2002).

By comparing the modulatory effects of two members of the same peptide family on both the overall functional output of the complete neuromuscular system and on many of the individual components that make up the system, we are able to address two major questions. First, we asked whether different members of the same peptide family use similar mechanisms to exert their effects, particularly when those effects are qualitatively similar. We examined two FLPs, GYS and SGRN, both of which elicit increases in contraction amplitude and frequency at low concentrations. At higher concentrations, although they both remain excitatory in most respects, the effects of the peptides diverge. Second, because we can examine the effects of the FLPs on the ganglion and the periphery, we asked whether these effects, together with the presumed effects of the feedback, are sufficient to explain changes in the frequency, amplitude and duration of contractions when the peptides are applied simultaneously to all components of the neuromuscular system.

Factors Determining Contraction Frequency

The crustacean heart is neurogenic, which implies that contraction frequency is determined directly by the cycle frequency of bursting in the motor neurons of the CG. In simultaneous recordings of contractions and CG output, this was always the case, indicating that the heart is indeed strictly neurogenic. Even in the presence of these excitatory FLPs, cardiac muscle is a strict follower of neuronal input. This contrasts with certain muscles in other crustaceans, for example, the shrimp *Palaemon serratus*, in which some muscles in the foregut can become myogenic in the present of other FLPs (Meyrand and Marder 1991).

However, cardiac muscle itself can strongly influence the output of the CG via feedback mechanisms, as has been previously suggested in studies of the effects of NO on the lobster heart (Mahadevan et al. 2004) and in examinations of the effects of stretch on isopod and crab hearts (Garcia-Crescioni et al. 2010; Sakurai and Wilkens 2003). In our study, these effects are evident when one compares the effects of the

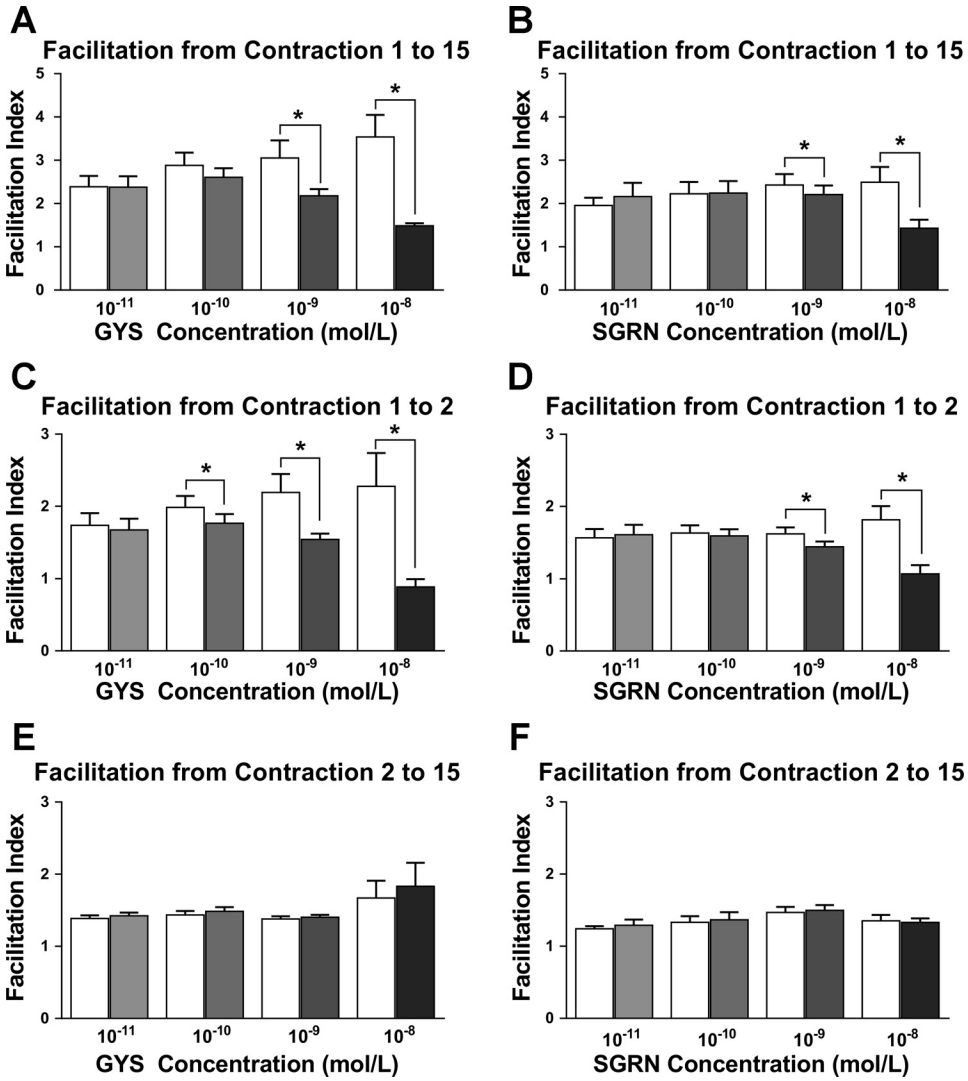


Fig. 7. GYS and SGRN both altered facilitation of contraction amplitude recorded in response to repeated stimulation of the motor nerve (200-ms bursts delivered to the motor nerve at a frequency of 1 Hz in bouts of 15 bursts separated by 45 s of recovery time). Facilitation index was calculated as (amplitude of the later contraction)/(amplitude of the earlier contraction); thus an index or 1.0 indicates no facilitation. For each preparation, the facilitation index for the relevant contractions in control saline is depicted in the open bars; the facilitation index from the same preparations in peptide is shown in gray bars. *A* and *B*: facilitation over the course of the entire set of stimuli, i.e., from the first to the fifteenth contraction, was unaffected by low concentrations of either peptide, but decreased in the presence of both GYS (*A*) and SGRN (*B*) when the peptides were perfused through the heart at concentrations of 10^{-9} or 10^{-8} M. *C* and *D*: most of the decrease in facilitation in the presence of peptide occurred between the first and second contractions, with significant decreases in facilitation in the presence of GYS (*C*) at concentrations of 10^{-10} M and higher, and in the presence of SGRN (*D*) at concentrations of 10^{-9} and 10^{-8} M. *E* and *F*: to determine the extent to which GYS (*E*) and SGRN (*F*) affected facilitation after the second contraction, we calculated a facilitation index from the second to the fifteenth contraction. Neither peptide altered this facilitation index at any concentration. Open bars: control saline; gray bars, saline with peptide. Paired *t*-tests were used to compare each set of facilitation indices to their matched controls. *Significant differences; $P < 0.05$. GYS 10^{-11} $n = 13$; GYS 10^{-10} $n = 13$; GYS 10^{-9} $n = 8$; GYS 10^{-8} $n = 5$; SGRN 10^{-11} $n = 9$; SGRN 10^{-10} $n = 11$; SGRN 10^{-9} $n = 17$; SGRN 10^{-8} $n = 7$. Note that, although the facilitation index appears to increase in control saline in *A* and *B*, these differences between controls were not significant (ANOVA, $P > 0.3$).

two peptides on the output of the isolated CG to their effects on the in situ CG. These data suggest that the feedback systems play a critical role in determining the overall motor output of the integrated system.

For both of the FLPs examined here, threshold was considerably lower for effects exerted at the periphery (i.e., in whole heart and stimulated preparations) than for effects on the CG itself. The peripheral threshold of around 10^{-10} M is comparable to or lower than the concentrations of most peptides that have been measured in hemolymph. For example, measurements of FLPs, using a radioimmunoassay based on a FMRFamide antibody in lobster, suggested concentrations on the order of 10^{-11} to 10^{-10} M (Kobierski et al. 1987). However, the authors point out that the antibody used in this assay targets FMRFamide, while the native peptides in lobster are extended FLRFamides, and likely do not bind as effectively, suggesting that these values underestimate the actual peptide concentrations. In the snail *Helix aspersa*, a radioimmunoassay using a more targeted antibody suggested that concentrations of a FMRFamide-related peptide ranged between 3 and 57×10^{-9} M (Price et al. 1985), while measurements in locusts suggest concentrations of $\sim$5–8 $\times$ 10^{-9} M (Robb and Evans 1990), and concentrations in the blood-sucking bug *Rhodnius* ranged from about 10^{-9} M to 2×10^{-8} M (Elia et al. 1993).

Measurements of allatostatins in insects suggest that these peptides are in the nanomolar range (Bendena et al. 1997). Hemolymph concentrations as high as 3–4 $\times$ 10^{-8} M have been reported for other peptides in insects and shrimp (e.g., ecdysis triggering hormone and vitellogenin inhibiting hormone) (Fastner et al. 2007; Kang et al. 2014; Zitnan et al. 1999). The lower end of reported concentrations in these species is on the order of 10^{-10} M (Fastner et al. 2007; Kang et al. 2014; Zitnan et al. 1999). The factors that control the release of both GYS and SGRN are unknown, but it seems likely that their concentrations in the heart could span a similar range. Thus concentrations would sometimes be at the high end of our tested concentrations, with effects on both the periphery and the CG; under other physiological conditions, they might be at the lower concentrations tested, where they exert effects only on the periphery.

Two different peptides that modulate the heart in another crustacean, the crab *Callinectes sapidus*, likely display similar differences in thresholds at the periphery and on the CG. Although the threshold for peripheral actions was not directly determined, the effects of the native FLP CalFLP on the whole heart in *Callinectes* resembled those recorded here for GYS and SGRN, with thresholds around 10^{-10} M, and effects on the isolated CG only at higher concentrations (Fort et al. 2007a). Additionally, Fort et al. (2007b) found that crustacean cardioactive peptide perfused through the semi-intact crab heart caused increases in contraction amplitude at concentrations that did not elicit changes in motor neuron output (burst frequency or duration), again suggesting a purely peripheral effect. Interestingly, thresholds for myosuppressin, a FLP that elicits increases in amplitude but decreases in frequency in the lobster heart (Stevens et al. 2009), appear to be similar in the whole heart and in the isolated CG, with the threshold for effects on frequency lower than that for effects on amplitude. Nonetheless, myosuppressin strongly increased contraction amplitude in response to controlled stimulation, indicating that this peptide also exerts its effects at the muscle/NMJ as well as on the CG.

In the present study, very low peptide concentrations (i.e., at or below 10^{-9} M) did not alter cycle frequency in the isolated CG, but did elicit small, but significant, increases in frequency in the whole heart. Interestingly, although GYS caused a small increase in burst duration in the isolated CG, there was no increase when the ganglion was in situ within the heart, so this does not contribute to the effects of GYS at these low concentrations in the whole heart. At these same concentrations, both peptides likewise caused increases in contraction amplitude in response to controlled motor neuronal input (i.e., in stimulated preparations). As diagrammed in Fig. 8, *A* and *B*, these data suggest that the effects of low concentrations of both peptides on frequency are largely mediated indirectly through feedback from the muscles. Because the peptides increase contraction amplitude directly at the level of the muscle/NMJ, we can postulate that increased contraction amplitude causes an increase in stretch, which then feeds back to the CG via stretch-sensitive dendrites, exciting the neurons of the pattern generator and thereby increasing burst frequency. A similar mechanism was postulated to explain the effects of FLPs in the crab, *C. sapidus* (Fort et al. 2007a). Subsequent studies of the stretch-sensitive dendrites in *Callinectes* suggested that increased contraction amplitude might instead slow the rhythm: eliminating stretch feedback by deafferentation or deefferentation actually caused cycle frequency to increase, suggesting that the feedback from larger contractions might normally slow the rhythm. Nonetheless, tonic passive stretch generally caused an increase in cycle frequency in these same experiments (Garcia-Crescioni et al. 2010), suggesting that the excitatory effect of stretch predominates. Interestingly, recordings from CG neurons in the isopod, *Ligia pallasii*, the only crustacean in which the response to stretch has been recorded intracellularly, indicate that stretch causes a hyperpolarization of the pattern-generating neurons in the CG, resulting in a decrease in cycle frequency in response to tonic stretch. However, because these neurons show considerable postinhibitory rebound, bursts after short stretches, which mimic those that would result from heart contractions, are not only stronger but are also phase-advanced in this species (Sakurai and Wilkens 2003). The effects of stretch on contraction parameters in the lobster have thus far been less thoroughly studied. However, recent data suggest that contraction frequency and amplitude in the *Homarus* heart usually increase in response to tonic stretch (Dickinson et al. 2014; Harmon et al. 2014). The extent to which contraction must increase to activate NO synthase and the negative NO feedback pathway is not known. However, it is plausible that the increased stretch from stronger contractions in the presence of the peptides at low concentrations might be insufficient to active the NO pathway, but sufficient to activate the stretch pathway, thereby indirectly resulting in an increased CG frequency at low peptide concentrations (Fig. 8, *A* and *B*). At these concentrations, both the effects of the two peptides and the mechanisms that underlie them appear to be very similar.

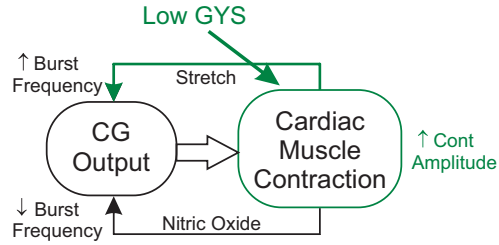
As peptide concentrations were increased to levels that also affected the CG, the effects became more complex and multifaceted (see Fig. 8, *C* and *D*). Thus, at concentrations of 10^{-8} to 10^{-7} M, both peptides not only elicited large (>100%) changes in contraction amplitude at the periphery, but they also exerted direct effects on the CG. In contrast to their effects on the NMJ/muscle, the two peptides differed in their effects on the CG (Fig. 8, *C* and *D*). Notably, however, neither peptide increased cycle frequency: GYS did not alter cycle frequency, while SRGN caused a significant (~35%) decrease in frequency when applied to the isolated CG. To explain the peptide effects on the whole heart, we must consider the primary effects of each peptide on both the periphery and the CG, as well as the roles of feedback in the cardiac neuromuscular system as a whole.

High concentrations of GYS did not alter the frequency of motor neuron bursts in the isolated CG, but caused a decrease in contraction frequency in the whole heart (Fig. 8*C*). Thus the effects on the CG itself cannot directly cause the observed decrease in contraction frequency, indicating that the decrease must result from the interactions of the CG with feedback from the cardiac muscle. Given the generally positive and negative effects of stretch and NO feedback, respectively, this in turn suggests that the dominant feedback in the presence of GYS is negative feedback, presumably exerted by NO (see Fig. 8*C*).

In contrast, SGRN caused a significant decrease in cycle frequency in the isolated CG. However, in the presence of high

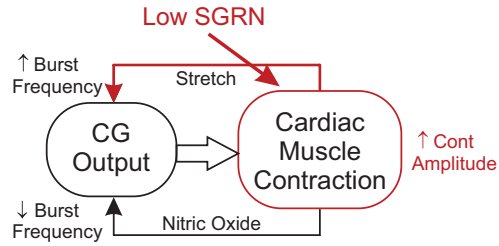
A

	Low GYS		
	Whole Heart	Isolated CG	Stim Muscle
Cycle Frequency	+	0	
Burst Duration	0	+	
Contraction Amplitude	+		+



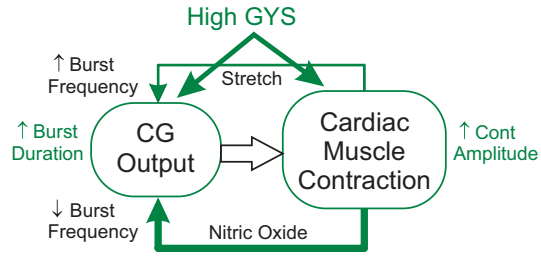
B

	Low SGRN		
	Whole Heart	Isolated CG	Stim Muscle
Cycle Frequency	+	0	
Burst Duration	0	0	
Contraction Amplitude	+		+



C

	High GYS		
	Whole Heart	Isolated CG	Stim Muscle
Cycle Frequency	-	0	
Burst Duration	+	+	
Contraction Amplitude	+		+



D

	High SGRN		
	Whole Heart	Isolated CG	Stim Muscle
Cycle Frequency	0	-	
Burst Duration	+	+	
Contraction Amplitude	+		+

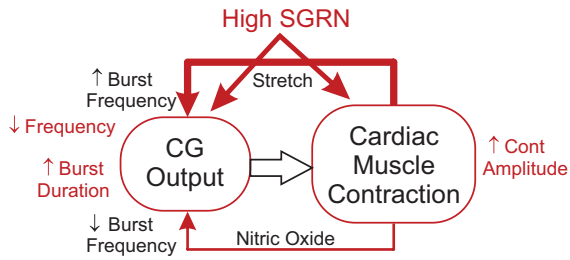


Fig. 8. Diagrammatic depiction of the cardiac neuromuscular system and its control and modulation by GYS and SGRN at high and low concentrations. Output from the CG acts on the cardiac muscle (open arrow) to elicit heart contractions. These contractions in turn are thought to control the generation of nitric oxide, which feeds back to the ganglion, inhibiting it to cause a decrease in cycle frequency (*bottom* arrow). Contractions also stretch the stretch-sensitive dendrites of the CG neurons; this is thought to result in positive feedback, and an increase in cycle frequency (*top* arrow). The effects of the peptides are shown in green (SGRN) and red (GYS) in each panel, with thicker arrows indicating effects that are predicted to be stronger. Tables list the observed effects of the peptides on the whole heart, the isolated CG, and the stimulated muscle preparation. *A*: in the presence of low (10^{-10} to 10^{-9} M) GYS, contraction amplitude increases peripherally; burst duration increases in the isolated CG, but not in the whole heart. Thus the effects on frequency appear to be mediated by an increase in the stretch feedback resulting from the peripheral enhancement of contraction amplitude. *B*: similar to *A*, in the presence of low (10^{-10} to 10^{-9} M) SGRN, contraction amplitude increases peripherally. Thus the effects on frequency appear to be mediated by an increase in the stretch feedback resulting from the peripheral enhancement of contraction amplitude. *C*: higher concentrations of GYS (10^{-8} M) elicit not only an increase in peripheral contraction amplitude, but also increases in burst duration within the CG itself, sufficient to result in increased burst duration in the whole heart. However, although there is no direct effect on cycle frequency in the CG, cycle frequency in the whole heart decreases, suggesting that the impact of the nitric oxide feedback pathway predominates (thick green arrow). *D*: higher concentrations of SGRN (10^{-8} M) elicit not only an increase in peripheral contraction amplitude, but, like GYS, increases in burst duration within the CG itself, which in turn leads to increased burst duration in the whole heart. In spite of the increased burst duration and the decreased cycle frequency in the isolated CG, cycle frequency remains unchanged in the whole heart, suggesting that the impact of the stretch feedback pathway predominates (thick red arrow). Because burst duration increases while frequency remains constant, duty cycle increases, which may also contribute to the increases in contraction amplitude. +, Increase; −, decrease; 0, no significant change.

concentrations of SGRN, whole heart contraction frequency did not change (Fig. 8*D*). If negative NO feedback were dominant, as we predict for GYS feedback, then the heartbeat should decrease even more than in GYS. Instead, we postulate that, to overcome the decreased frequency that the effects of SGRN on the CG alone should elicit, positive feedback, presumably mediated by stretch in response to the larger contraction amplitude, dominates in the presence of SGRN.

Taken together, these data suggest that these FLPs likely modulate feedback in addition to their effects on the CG and the periphery. Moreover, they suggest that the related peptides modulate the two feedback pathways differentially. This in turn suggests that the peptides, although structurally similar, likely act on different receptors. To the best of our knowledge, virtually nothing is currently known about FLRFamide receptors in crustaceans. However, studies on several other systems suggest the plausibility of related peptides activating different receptors. In the locust, for example, it appears that different receptors may respond to the FLRFamide peptides (particularly myosuppressins) in abdominal muscles than in muscles of the oviduct (Lange and Cheung 1999). In both *Caenorhabditis elegans* and *Drosophila melanogaster*, single neuropeptides, including those in the FMRFamide family, have been shown to activate two or more receptors; conversely, the same receptors are often activated by more than one related peptide, making for complex signaling possibilities (Johnson et al. 2003; Klose et al. 2010; Li 2005; Maynard et al. 2013). Among the most intriguing of these studies is that by Klose et al. (2010), demonstrating that two receptors are required for enhancement at the NMJ in a larval *Drosophila* muscle.

As transcriptomic and genomic information for the lobster becomes available, it will be interesting to determine the numbers, specificity, and distributions of receptors for FLPs in the cardiac neuromuscular system.

Factors Determining Contraction Amplitude

Contraction amplitude in the cardiac neuromuscular system is determined by the characteristics of the motor neuronal bursts and the nonlinear neuromuscular transform (Brezina et al. 2000a; Brezina and Weiss 2000), which describes contraction amplitude as a function of burst frequency and duty cycle (Williams et al. 2013). Because the lobster heart shows burst-to-burst increases in amplitude, contraction amplitude used in plots of the neuromuscular transform is based on the final steady-state values, which presumably reflect the contraction amplitude that would be recorded in a heart during ongoing activity. Changes in either the shape or the scaling of the neuromuscular surface will result in changes in contraction amplitude, as will changes in the burst parameters. As we have demonstrated, both peptides lead to an increase in contraction amplitude in response to consistent patterns of stimulation, resulting in changes in the neuromuscular transform. However, it is not yet clear whether the shape of the surface changes or if the surface is simply scaled. The mechanisms that underlie the increased contraction amplitude in response to defined stimulation are likewise not clear. Possibilities include effects within the muscle itself as well as effects at the NMJ. The fact that the amplitude of the first contraction within a bout is increased by the peptides suggests the possibility that intraburst facilitation is enhanced; however, changes within the muscle or global increases in transmitter release could likewise account for this increase.

In the lobster heart, maximum contraction amplitude tends to occur at relatively low frequency combined with a modest duty cycle (see Fig. 9). If we overlay the general changes elicited by GYS and SGRN in the whole heart on a map of the cardiac neuromuscular transform, we see contradictory effects for both peptides at the lower concentrations. At low peptide concentrations (10^{-10} to 10^{-9} M), both peptides elicit increases in both cycle frequency and duty cycle. Increases in cycle frequency lead, on average, to decreases in contraction amplitude, while the increase in duty cycle should on average increase contraction amplitude (Fig. 9*A*). The changes in duty cycle were somewhat larger for GYS than for SGRN at both concentrations; the changes in frequency were larger in GYS than in SGRN at 10^{-10} M, but effects of the two peptides did not differ significantly from one another at 10^{-9} M. However, because the changes in duty cycle and frequency should cause opposing effects in both peptides, it is not evident that the changes in burst parameters alone should necessarily lead to an increase in amplitude when either peptide is perfused at this concentration. This suggests the possibility that most of the increase in contraction amplitude at low peptide concentrations results from effects in the periphery.

At higher concentrations (e.g., 10^{-8} M), both peptides still elicit increases in duty cycle, which is predicted by the neuromuscular transform to result in increased contraction amplitude (Fig. 9*B*), with GYS eliciting a somewhat larger increase in duty cycle than SGRN. However, at this concentration, the effects on cycle frequency differ between the peptides: GYS elicits a decrease in cycle frequency, which should result in an increased contraction, while SGRN does not alter frequency. Because the changes in both cycle frequency and duty cycle should result in increases in contraction amplitude in GYS, but only duty cycle is altered by SGRN at this concentration, and, because the increase in duty cycle is larger in GYS than in SGRN, the neuromuscular transform alone would predict that 10^{-8} M GYS would induce larger increases in contraction amplitude than would 10^{-8} M SGRN. That is not, however, the case, since the increases in contraction amplitude do not differ significantly between the two peptides. It is possible that the difference is due to differences in the changes that the peptides

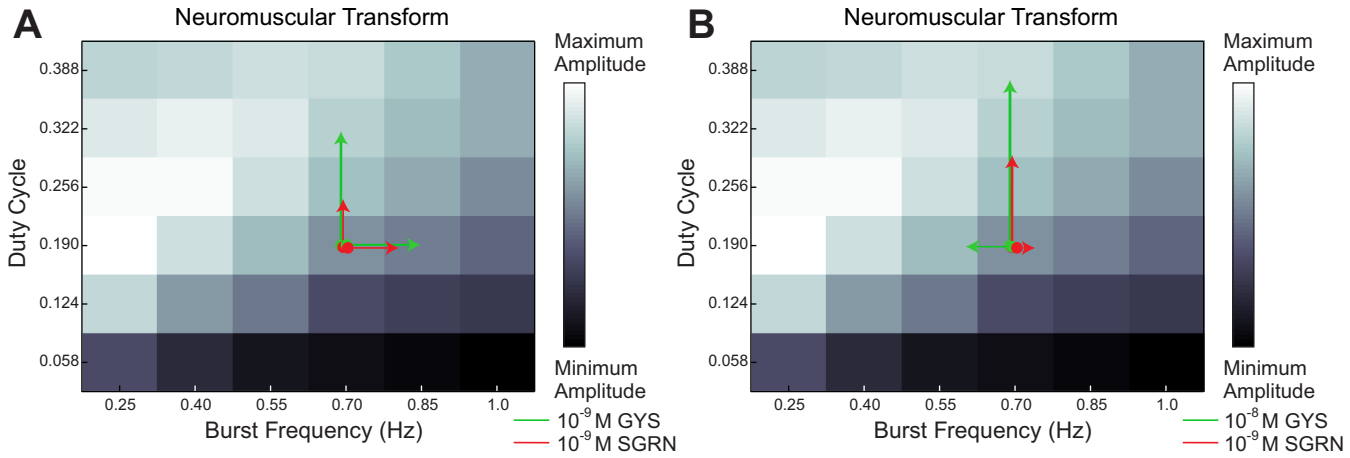


Fig. 9. Heat-map depiction of the neuromuscular transform for the heart of *H. americanus*, showing the general changes that are predicted in both duty cycle and cycle frequency in the two peptides, GYS (green) and SGRN (red). The neuromuscular transform heat-map illustrates the normalized contraction amplitude that resulted from stimulating the motor nerve over a range of cycle frequency and duty cycle pairs. Lighter colors represent larger contractions. The average starting values for activity in the whole heart are represented by circles, which are slightly offset so that they are visible. The average changes in each parameter are represented by the arrows. *A*: perfusion of either peptide at 10^{-9} M resulted in increases in both duty cycle and burst frequency. The increase in duty cycle would be predicted to cause an increase in contraction amplitude, while the increase in cycle frequency would be predicted to cause a decrease in amplitude. *B*: when the peptides were perfused at 10^{-8} M, GYS elicited an increase in duty cycle, but a decrease in frequency; SGRN elicited an increase in duty cycle, but did not significantly alter frequency. Globally, these changes are predicted to result in increased contraction amplitude, with a larger increase in GYS than in SGRN. [Neuromuscular transform heat-map modified from Williams et al. 2013 with permission.]

elicit at the muscle or NMJ, which would be reflected in changes in the shape or scaling of the neuromuscular transform.

Redundant Peptide Modulators?

Why there are so many neuropeptides, many of which are related members of single peptide families, in a given species, is a question that remains unanswered. Based on studies in *Aplysia*, *C. elegans*, and *Drosophila*, a number of hypotheses have been suggested. First, it is possible that such peptides are to a large extent redundant and are the result of gene duplication, which in turn has relaxed constraints on the evolution of these peptides (Brezina et al. 1995). Such duplication would ultimately provide a substrate for further evolution if selective pressure changed. Such may be the case for the myomodulins in *Aplysia*, in which the related peptides appear to have identical defects on one current, although they have divergent effects on another (Brezina et al. 1995). Similarly, at the *Drosophila* NMJ, seven FLPs encoded by the *D. melanogaster FMRFamide* gene appear to be functionally redundant (Hewes et al. 1998). The FLPs encoded by the *flp-18* gene in *C. elegans* likewise appear to be almost completely redundant, having similar effects and similar potencies on two receptors (Kubiak et al. 2008). However, there are several genes in *C. elegans* that encode FLPs, and evidence suggests that the selectivity for different receptors may vary between other FLPs (Mertens et al. 2006). Overall, data from *C. elegans* suggest that signaling by related peptides may be very complex, with multiple peptides each capable of activating multiple receptors to cause a wide range of effects (Li 2005). This would simultaneously provide redundancy and complexity of potential behavioral outputs.

Another possibility that has been suggested is that multiple related peptides may exert complementary effects, with two or more peptides together enabling the system to produce a response that none alone can produce (Vilim et al. 2010). Such effects are seen in response to several FLPs that act on the feeding system of *Aplysia*. Thus FMRFamides and FRFamides are complementary: they modulate different components of the feeding motor program. Both tend to move the motor pattern from an ingestive toward an egestive pattern; together they result in a fully egestive pattern. At the same time, both peptides enhance contraction of the same muscle, but they do so using different mechanisms, with one subfamily modulating presynaptic sites and the other acting postsynaptically (Cropper et al. 1994; Vilim et al. 2010). However, within each of these groups of peptides, members of the subfamilies appear to be largely redundant.

The functional roles that different members of peptide families play may be in part determined by their patterns of release, particularly relative to one another (Brezina and Weiss 1997; Vilim et al. 2010). Thus, if two peptides are encoded by the same gene, and thus synthesized and released by the same neurons or endocrine cells, they are likely to be co-released in the same ratios under most, if not all, conditions, and thus may tend to be redundant. If, however, they are released separately, they are more likely to increase behavioral flexibility.

The crustacean heart is modulated by a wide variety of peptides as well as amines (reviewed in Christie et al. 2010). In the lobster, these include several members of the FLP family, two of which were examined here. The three that have been examined extensively in the lobster, myosuppressin (pQDLDHVFLRFamide; Stevens et al. 2009), GYS, and SGRN, are members of separate subfamilies within the larger FLP family. Myosuppressin is the only FLP encoded by its gene (Stevens et al. 2009); the genes encoding GYS and SGRN have not yet been identified, but it is likely that these peptides are encoded by two different genes. This suggests that the three peptides could be complementary or have unrelated effects rather than being redundant. Interestingly, however, all three cause increased contraction amplitude at the periphery (though the mechanisms by which they do so are not known), while having differing effects on the CG. Thus they appear to have effects that are partially, but not fully, overlapping.

Conclusions

Taken together, these data indicate that the modulation and control of even this relatively simple pattern generator-effector system are actually quite complex. Contraction frequency, while determined by the cycle frequency of the motor output from the CG, is altered when neuromodulators, such as the FLPs studied here, directly modulate the output of the pattern generator. In addition, cycle frequency is modulated when the same peptides alter contraction amplitude at the periphery, which in turn alters the feedback from the cardiac muscle to the pattern-generating neurons. Perhaps most strikingly, our data also suggest that these feedback loops by themselves are not sufficient to explain the changes in cycle frequency that result from peptide application. Instead, we must postulate that at least one of the two peptides modulates the feedback itself, or that the two peptides modulate feedback differentially.

Second, our data confirm that contraction amplitude is modulated by changes at the periphery as well as in bursting parameters, and that these interactions are mediated by the nonlinear neuromuscular transform. What is not yet clear is the extent to which the neuromuscular transform itself may be modulated by the peptides. This is currently under investigation.

Finally, our data suggest that, although these two peptides are members of the same neuropeptide family, i.e., the FLPs, they likely do not act on the same receptors. They have similar, but not identical effects, and it appears that even these similar effects are mediated by different mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.S.D., A.C., and J.S.S. conception and design of research; P.S.D., A.C., and J.S.S. performed experiments; P.S.D., A.C., and J.S.S. analyzed data; P.S.D., A.C., and J.S.S. interpreted results of experiments; P.S.D. and A.C. prepared figures; P.S.D. and A.C. drafted manuscript; P.S.D., A.C., and J.S.S. edited and revised manuscript; P.S.D., A.C., and J.S.S. approved final version of manuscript.

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