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Modulation of the stretch feedback pathway in the cardiac neuromuscular system of the American lobster, *Homarus americanus*

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An Honors Project for the Program of Neuroscience

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Acknowledgements

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Abstract

The cardiac ganglion (CG) is a central pattern generator, a neural network that, when activated, produces patterned motor outputs such as breathing and walking. The CG induces the heart contractions of the American lobster, *Homarus americanus*, making the lobster heart neurogenic. In the American lobster, the CG is made up of nine neurons: four premotor pacemaker neurons that send signals to five motor neurons, causing bursts of action potentials from the motor neurons. These bursts cause cardiac muscle contractions that vary in strength based on the burst duration, frequency, and pattern.

The activity of the CG is modulated by feedback pathways and neuromodulators, allowing for flexibility in the CG's motor output and appropriate responses to changes in the animal's environment. Two feedback pathways modulate the CG motor output, the excitatory cardiac muscle stretch and inhibitory nitric oxide feedback pathways. The CG is an ideal model system to study these modulatory processes because of the few number of neurons, with known connectivity, comprising this system and the understanding that this system receives feedback from cardiac muscle stretch. Despite our knowledge of the modulation of the CG by feedback pathways and neuromodulators separately, little is known about how neuromodulators influence the sensory feedback response to cardiac muscle stretch.

Myosuppressin, an endogenous modulatory neuropeptide, decreases the frequency and increases the amplitude of cardiac muscle contractions. Myosuppressin also decreases the burst frequency and increases the burst duration of the CG. I found that myosuppressin modulates the stretch response differently depending on how the cardiac muscle is being stretched. Myosuppressin modulated the CG's response to cardiac muscle stretch by enhancing the increase in interburst interval during the rising phase, suppressing the stretch response during the hold

phase, and inducing no changes in burst parameters during the release phase. Additionally, NO, a short distance signaling molecule that decreases the CG burst frequency and whole heart contraction frequency, did not modulate the stretch response. On the other hand, CLDH, another endogenous modulatory neuropeptide that increases the CG burst frequency and whole heart contraction frequency, weakly suppressed the stretch response. These results suggest neuromodulators can modulate both the motor output of this cardiac neuromuscular system and the response of this system to cardiac muscle stretch.

Introduction

1.1 Central Pattern Generators

Central pattern generators (CPGs) are neural networks that, when activated, produce rhythmic motor outputs in the absence extrinsic inputs. These rhythmic motor outputs produce patterned movements such as breathing and walking. The ability of CPGs to produce fictive rhythmic activity *in vitro* positions them as useful models to study modulation by neuromodulators and feedback pathways. The intrinsic properties of central pattern generating neurons influence the mechanisms underlying the patterned motor output produced by CPGs and allow for a variety of neuronal activity (Marder & Buchner, 2001). These circuit properties include CPGs that fire bursts of action potentials intrinsically, half-center oscillators which are characterized by the alternating activity of two mutually inhibitory halves of a circuit, and those that maintain rhythmicity by relying on post inhibitory rebound (Katz, 2016; Selverston, 2010). Additionally, CPGs function as dynamic systems, rather than single state networks, that can produce a diverse set of behaviors (Birggman et al., 2008).

1.2 The American Lobster Cardiac Neuromuscular System

The cardiac ganglion (CG) is a CPG that induces heart contractions in the American lobster, *Homarus americanus*, making the lobster heart neurogenic. The CG is made up of nine electrochemically coupled neurons: four premotor neurons that stimulate five motor neurons that synapse directly onto the heart (Figure 1A). These neurons fire synchronous bursts of action potentials that induce heart contractions varying in strength based on the burst duration, frequency, and pattern (Figure 1B). The activity of the CG is modulated by neuromodulators and feedback pathways, allowing for flexibility in the motor outputs and appropriate responses to changes in the animal's environment (Cooke, 2002).

Underlying the motor output of the crustacean CG are driver potentials (DPs) that originate in and near the cell body of the motor neurons (Cooke, 2002). These DPs are slow, sustained calcium (Ca^{2+}) based depolarizations that allow for the bursting pattern of the CG action potentials (Tazaki & Cooke, 1996; Ball et al., 2010) (Figure 1C). Ca^{2+} is known to mediate the inward current underlying DPs as the DP amplitude is dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and independent of the extracellular sodium (Na^+) concentration. Three distinct outward currents underlying CG motor neuron DPs have similar voltage dependences, time courses, and pharmacology to the early voltage-dependent outward current (I_A), delayed voltage-dependent outward current (I_{Kd}), and late Ca^{2+} dependent-outward current (I_C) in molluscan neurons. Analysis of the tail currents for these outward currents showed the reversal potentials to be near -75 mV, suggesting they are K^+ mediated (Tazaki & Cooke, 1996).

The Ball et al. (2010) model of the crustacean CG motor neurons predicted that when a DP first starts, the slow inward calcium current (I_{CaS}) and A-type potassium outward current (I_A), two opposing currents, are the most active. Once the DP starts to depolarize, voltage gated

potassium current (I_{Kd}), I_A, slow calcium current (I_{CaS}), and transient calcium current (I_{CaT}) are activated. At the peak voltage of the DP, I_{CaT} and I_A inactivate before I_{CaS} and I_{Kd}. Repolarization of the DP is mediated by a combination of a calcium-gated potassium current (I_{KCa}) activating and the inactivation of I_{Kd}, I_A, I_{CaT}, and I_{CaS}. This model emphasizes the importance of coregulating ionic currents to preserve the rhythmic motor output of CG motor neurons.

1.3 Feedback Pathways in the American Lobster Heart

The lobster cardiac neuromuscular system is modulated by two main feedback pathways: the excitatory stretch and inhibitory nitric oxide (NO) feedback pathways (Figure 2). The presence of two opposing feedback pathways in the lobster cardiac neuromuscular system led us to ask the question, do the excitatory stretch and inhibitory NO feedback pathways interact in this system?

The excitatory stretch feedback pathway is mediated by stretch-sensitive dendrites that project from the CG and innervate the heart. These stretch sensitive dendrites respond to hemolymph filling and stretching the cardiac muscle (Cooke, 2002). In the whole heart preparation, cardiac muscle stretch increases the passive, active, and total force of heart contractions along the axis of the stretch being applied (Dickinson et al., 2016). Cardiac muscle stretch also increases the whole heart contraction frequency (Chin-Purcell, 2014; Dickinson, 2014). In the isolated CG preparation, the excitatory stretch feedback is induced by applying ramp shaped stretches consisting of a rising, hold, and release phase to the cardiac muscle. The rising phase stretches the cardiac muscle from the baseline length to the final stretch length, the hold phase holds the muscle at the final stretch length, and the release phase brings the stretch length back to the baseline length (Figure 3). The rising phase induces an increase in interburst interval, the hold phase causes an increase in burst frequency and decrease in burst duration, and

the release phase increases the burst duration (Figure 3). The greatest changes in burst frequency and burst duration during the hold phase occur for stretches of greater forces and preparations with a longer baseline burst duration (Qu, 2017).

Nitric oxide (NO) is a short distance signaling molecule that slows the heart rate by decreasing CG burst frequency. NO is thought to be produced and released from the crustacean heart muscle upon contractions because of the high levels of a calcium-sensitive form of nitric oxide synthase found in crustacean heart muscle extracts. Exogenous application of NO to the whole heart preparation and isolated CG decreases the frequency of cardiac muscle contractions and CG bursts. Furthermore, application of L-Arginine (NO synthase blocker) to the whole heart results in an increased heartrate (Mahadevan et al, 2004).

1.4 Mechanosensory Signal Transduction

Different types of stretch responses and mechanosensory transduction have been studied in numerous crustacean models. In the rock louse, *Ligia pallisii*, the CG is composed of six electrically coupled neurons, rather than the nine electrochemically coupled neurons in the lobster CG (Sakurai & Wilkens, 2002; Cooke, 2002). The rock louse CG neurons are endogenous oscillators and glutamatergic motor neurons that innervate the cardiac muscle. In this model, stretching the heart wall decreased the CG burst frequency, while release from the stretch increased the burst frequency. Additionally, brief stretches caused either a phase advance or phase delay, depending on when the stretch was applied, and repeated brief stretches could either increase or decrease the burst frequency. Regardless of the change in burst frequency induced by stretch, the CG hyperpolarized during the muscle stretch (Sakurai & Wilkens, 2002). In another crustacean model, the Chesapeake blue crab, *Callinectes sapidus*, cardiac muscle stretch decreased the contraction amplitude and increased the burst frequency of CG motor

neurons (García-Crescioni et al., 2009). Similar to the stretch response in the American lobster, these changes in cardiac neuromuscular activity in the blue crab were mediated via mechanosensitive dendrites that the CG project onto the myocardial muscle (García-Crescioni et al., 2009; Qu, 2017).

In crayfish, a stretch receptor in the abdomen induces an increase in the stretch sensory neuron burst frequency followed by a lack of activity for a short time. The extent of changes in neuronal activity in response to stretch are a function of stretch length (Rydqvist & Purali, 1993). Variability in the reversal potential of changes in sensory neuron membrane potential caused by stretch has been attributed to the length of dendrites projecting to the abdominal muscle (Brown et al., 1978). Additionally, there are two main categories of stretch receptors in the abdomen of crayfish: slowly adapting (RM_1) and rapidly adapting (RM_2) receptors. While the activation of RM_1 receptor neurons is associated with larger action potentials, there are no differences in the current-voltage relationship of the ion channels responsible for action potentials facilitated by RM_1 and RM_2 receptors. Whereas RM_1 receptor neurons will fire long lasting trains of action potentials in response to depolarizing current injections, RM_2 receptor neurons never produce repetitive action potentials (Nakajima & Onodera, 1969). Overall, changes in neuronal activity in response to the sensory inputs of stretch for the heart and abdomen in various crustacean models provide proprioceptive information of vital organs. Despite the variety of responses to stretch of vital organs in different species of crustaceans, the extent to which this feedback can be modulated is largely unknown. Modulation of this response to stretch across systems would allow these organs to appropriately alter their function in response to changes in the environment on the level of their patterned motor output as well as their response to sensory inputs.

1.5 Neuromodulation of the American Lobster Heart

Neuromodulators are amines, amino acids, gases, and neuropeptides that modulate CPGs, providing CPGs with the ability to produce a variety of patterned motor outputs (Dickinson, et al., 2016). This flexibility in CPG motor outputs allows for individuals to respond appropriately to changes in their environment and generate varied behaviors. Neuromodulatory inputs can modulate a CPG's motor output by altering the synaptic strength and intrinsic membrane properties of CPG network component neurons without physically rewiring the circuit (Katz, 2016; Marder & Bucher, 2001). Neuropeptide families vary in the extent to which they are conserved throughout crustacean species and invertebrates. While the effect of numerous neuromodulators on crustacean CPG networks are well characterized, the receptor types, distribution of these receptors, and mechanisms of neuromodulators binding to receptors are relatively unknown. The possible variation in the distribution of receptors binding to neuromodulators throughout a system has implications for how neurons in the same or different circuits respond to single modulators and multiple modulators at once (Dickinson, et al., 2016).

In the American lobster, there are 17 peptide families consisting of 84 known neuropeptides (Dickinson et al., 2015). qQDLDHVFLRFamide (crustacean myosuppressin) is part of the myosuppressin subfamily of the FMRFamide-like peptides. Myosuppressin decreases the contraction frequency and increases the contraction amplitude of the lobster whole heart. In the isolated CG, myosuppressin decreases the burst frequency and increases the burst duration of the CG (Stevens et al., 2009). The increase in heart contraction amplitude observed in the presence of myosuppressin is thought to be induced by myosuppressin directly affecting cardiac muscle physiology. Petropoulos (2023) showed that myosuppressin increased glutamate evoked contraction amplitude, while having no impact on evoked excitatory junction potentials. These

findings confirmed myosuppressin modulates the lobster cardiac neuromuscular system by acting at both the site of the nervous system and the cardiac muscle itself. This modulation of both the nervous system and the cardiac muscle by myosuppressin led to my goal of determining if myosuppressin modulates the stretch feedback pathway.

GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide (Homam-CLDH/DH-31) is part of the calcitonin-like diuretic hormone (CLDH) family. In insects, the CLDH family of peptides is involved in the regulation of diuresis, a process that involves mixing hemolymph to aid in digestion. In the lobster whole heart, CLDH increases the contraction frequency and amplitude and decreases the contraction duration (Christie et al., 2009). In the isolated CG, CLDH is understood to increase the burst frequency without altering the burst duration (Bowers, 2010). Because CLDH, similar to myosuppressin, modulates distinct features of cardiac output, I wanted to study its effects on the stretch feedback pathway.

Lastly, GYSDRNYLRFamide (GYS) and SGRNFLRFamide (SGRN) are two peptides in the FLRF-like peptide (FLP) family. The FLP peptide family is made up of 19 peptides, most of which have a C-terminal sequence of FLRFamide (Dickinson et al., 2015; Ma et al., 2008). Ma et al. (2008) used mass spectrometry to show that GYS and SGRN are both found in the brain, ventral nerve cord, and stomatogastric ganglion of the American lobster. At low concentrations (10^{-9} M) both SGRN and GYS increase the contraction amplitude and frequency of the whole heart. At high concentrations (10^{-8} M) SGRN has no effect on the contraction frequency but increases the contraction amplitude, while GYS decreases the contraction frequency and increases the contraction amplitude. In the isolated CG, SGRN and GYS do not modulate the CG burst frequency at low concentrations (10^{-9} M and lower). At high concentrations (above 10^{-9} M), despite the similarity in these peptide sequences, only SGRN decreases the burst frequency,

whereas GYS does not alter the CG activity (Dickinson et al., 2015). Since the modulation of the CG by GYS and SGRN do not fully explain the modulation of the whole heart by these neuropeptides, we wanted to understand how these neuropeptides may modulate the stretch feedback pathway.

1.6 Neuromodulation of the Stretch Feedback Pathway

The stretch feedback pathway is an excitatory response to the sensory input of cardiac muscle stretch in the lobster heart. The characterization of this excitatory stretch-feedback pathway and the responses of the cardiac neuromuscular system to different neuromodulators led us to ask the question, how do sensory and modulatory inputs interact to affect the physiology of the lobster cardiac neuromuscular system?

We were interested in uncovering how myosuppressin, a neuromodulator that decreases the CG burst frequency and contraction frequency, and CLDH, a neuromodulator that increases the CG burst frequency and contraction frequency, modulates the stretch response. I hypothesized that myosuppressin 1) would modulate the stretch response because the neuropeptide modulates both the nervous system and the muscle of the cardiac neuromuscular system and 2) would limit the excitation of the system by the stretch feedback pathway and suppress the stretch response because myosuppressin largely inhibits the activity of the cardiac neuromuscular system. I hypothesized CLDH would increase the excitation of the system by the stretch feedback pathway and enhance the stretch response because CLDH excites the activity of the cardiac neuromuscular system.

Additionally, we believed SGRN and GYS would modulate the stretch response because the modulation by both neuropeptides of the CG do not fully explain the effects of the modulators on the whole heart preparation. We hypothesized that some interaction of these

modulators with the stretch response would help us understand a connection between SGRN and GYS's effects on the CG, whole heart, and peripheral sites. Since both SGRN and GYS increase the whole heart contraction amplitude at low and high concentrations, it was hypothesized that both modulators would amplify the increase the contraction amplitude caused by cardiac muscle stretch, therefore enhancing the stretch response.

To answer these questions, I generated a controlled stretch in an isolated CG preparation while leaving a subset of the cardiac muscle intact in both physiological saline and in the presence of a neuromodulator. These results suggest that the modulation of the stretch response by myosuppressin is dynamic and varies depending on the stretch phase, while CLDH weakly suppresses the stretch response, and NO does not modulate the stretch response. Furthermore, SGRN and GYS both suppress the stretch response despite the excitatory effects of both neuropeptides on the whole heart.

Methods

2.1 Animals and Isolated CG Preparation

Male and female adult lobsters were purchased from local fish markets. The lobsters were stored at 10-12 °C in recirculating seawater and kept on a 12:12 dark light cycle. Animals were fed shrimp and mussels on a weekly basis. Each lobster was anesthetized on ice for 30 minutes before the heart was dissected from the animal. For these dissections, the heart was left attached to a section of the overlying carapace. Once the heart was separated from the carapace, the CG along with the muscle surrounding the four small cells was further dissected, transferred to a Sylgard-lined dish, and submerged in chilled physiological lobster saline [479.12mM NaCl, 12.74 mM KCl, 13.67 mM $CaCl_2$, 20.00 mM $MgSO_4$, 3.91 mM Na_2SO_4, 11.45 mM trizma base, 4.82 mM maleic acid, pH = 7.45]. GLUture topical tissue adhesive was used to stabilize muscles

on each side of the CG small cells, with a space approximately 1.8 mm-wide left untouched by adhesive around the small cells. The adhesive ensured that each stretch would be applied to the muscle evenly and prevented the muscle from shredding. The isolated CG and cardiac muscle were transferred to a petri dish filled with chilled saline and pinned in preparation for intracellular and extracellular recordings (Figure 4).

2.2 Application of Myocardial Stretches

Two hooks were attached to each muscle: a SI-TM2 pointed hook tissue mount (World Precision Instruments, Sarasota, FL, USA) attached to the SI-KG4 optical force transducer (World Precision Instrument, Sarasota, FL, USA) and a SI-TM2 pointed hook tissue mount attached to a SI-H linear motor (World Precision Instruments, Sarosota, FL, USA) (Figure 4). The force of each stretch was amplified using a BAM211-LCB amplifier (World Precision Instruments, Sarasota, FL, USA). Ramp shaped stretches consisting of a rising, hold, and release phase were applied to the cardiac muscle. Stretch displacements of 1.3 mm were applied to the cardiac muscle that varied in stretch rate and hold time (Table 1).

Table 1. List of stretch parameters. Motorized trapezoid shaped stretches were applied to the muscle of the isolated CG preparation using the parameters included below. Stretch number refers to different stretch protocols created in Spike2 – (extracel + intracel + motor + current updated stretches).

Stretch Number	Rising and Release Phase Duration (s)	Hold Phase Duration (s)	Length of Stretch (mm)
E	7.2	10	1.3
L	14.4	10	1.3
M	18.0	10	1.3
P	14.4	20	1.3
O	18.0	20	1.3
B	14.4	30	1.3
C	18.0	30	1.3

2.3 Application of NO and Neuropeptides

To compare the stretch response in physiological saline and neuropeptides, the stretches were applied to the isolated CG preparation in physiological saline and relevant concentrations of individual neuropeptides. To study the modulation of the stretch feedback pathway by myosuppressin, we applied 10^{-7} M myosuppressin. To study the modulation of the stretch feedback pathway by CLDH, we applied 10^{-8} M CLDH. Peptides were synthesized from GenScript (Piscataway, NJ) and kept stored at -80°C until dissolved in DI H_2O to create a 10^{-3} M stock solution. Final working concentrations of peptide solutions were generated from these stock solutions and diluted in physiological saline, except for the nitric oxide donor (SNAP) which was made fresh each time. In both conditions, solutions were superfused over the preparation such that the temperature could be maintained at 10°C (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA). A final set of stretches were applied to preparations following a 45-minute wash in physiological saline to confirm that responses returned to control conditions in each case or assess if a prolonged series of stretches and/or a NO application altered stretch responses irreversibly.

To compare the stretch response in physiological saline and NO, the stretches were applied to the isolated CG preparation in physiological saline and 10^{-5} *S*-nitroso-*N*-acetyl-penicillamine (SNAP), an NO donor (Mahadevan et al., 2004). Similar to the applications of neuropeptides, solutions were superfused over the preparation such that temperature could be maintained at 10°C (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA). Like the neuropeptide experiments, a final set of stretches were applied to preparations following a 45-minute wash in physiological saline to

confirm if responses returned to control conditions in each case. The experiments focused on the interaction between the stretch and NO feedback pathways were completed in collaboration with Grant Griesman.

2.4 Electrophysiological Recordings

Extracellular recordings were taken from one of the anterior lateral nerves of the CG to measure the changes in motor output induced by the stretch response and myosuppressin (Figure 4). One petroleum jelly well was formed around the end of one of the anterior lateral nerves in order to electrically isolate that section of nerve from the bath. Motor neuron action potentials were sampled using a pair of stainless-steel pin electrodes: one electrode was placed inside the well and another outside of the well (Figure 4). The extracellular potentials were amplified using the differential AC amplifier (A-M Systems model 1700, Sequoia, WA). These extracellular potentials were recorded using a 100 Hz low pass and 1000 Hz high pass filter. Intracellular voltage measurements were taken from one of the motor neurons (cells 1, 2 or 3) to measure changes in the driver potentials underlying the bursts of action potentials. The sampling rate for the intracellular voltage measurements was 5000 Hz. First the protective sheath surrounding the motor neurons was removed. Neurons were then impaled using a bridge-balanced glass microelectrode ($R_{electrode}$=15-35 MΩ) filled with squid cytoplasmic solution [20mM NaCl, 15mM Na_2SO_4, 10mM Hepes, 400mM Potassium gluconate, and 10mM $MgCl_2$] (Figure 4). The large cell membrane voltage was amplified with an AxoClamp 2B (Axon instruments/Molecular Devices, San José, CA). Intracellular and extracellular signals were digitized at 10 kHz using the CED 1401 digitizer and recorded using Spike 2 version 7.03 software (Cambridge Electronic Design, Cambridge, UK) on a Dell PC (Dell, Austin, TX).

2.5 Current Injections

The input resistance of CG motor neurons was measured using 1 nA hyperpolarizing currents injections into a motor neuron during each stretch. Because stretches may induce subthreshold current responses, current injections used to measure changes in input resistance were not a constant duration but instead were terminated once the membrane potential reached steady state.

2.6 Data Analysis

The burst frequency, cycle period, burst duration and interburst interval were extracted from the extracellular recordings using custom scripts written in Spike2 (courtesy of Dirk Bucher, NJIT). The driver potential (DP) frequency, DP period, DP duration, time between DPs, and pacemaker potential (PP) slope were extracted from the intracellular recordings using custom scripts written in Spike2. From the extracellular recordings, I measured the instantaneous burst frequency, cycle period (quantified as the start of one burst to the start of the next burst), burst duration, and the interburst interval. Using the intracellular recordings, I measured the instantaneous DP frequency, DP period, the DP duration, and the time between DPs. The PP slope was calculated the rate of change in subthreshold voltage from the end of one DP and the start of the next DP. Extracellular and intracellular recordings of the stretch response were recorded in myosuppressin and CLDH. Only extracellular recordings of the stretch response were recorded in NO. The plots and statistics in this thesis include pooled data of both extracellularly recorded CG bursts and intracellularly recorded DPs. For experiments that included both extracellular and intracellular recordings, only the DP parameters were included in the final comparison of the stretch response in saline and a modulator.

Changes in input resistance during the stretch in physiological saline and myosuppressin were calculated using the difference in membrane potential in response to each 1 nA hyperpolarizing current injection compared to the membrane potential just prior to each hyperpolarizing pulse.

The GraphPad Prism software (Dotmatics, Boston, MA) was used to conduct the Wilcoxon matched-pairs signed rank test to compare the change in each of these parameters for each phase of the myocardial stretches in saline, myosuppressin, NO, and CLDH. The percent change of the CG parameters in myosuppressin during the rising phase was calculated by dividing the parameters of the burst with the longest interburst interval in the rising phase of the stretch by the average parameters of the control bursts preceding the start of the stretch. The absolute difference in PP slope was calculated by subtracting the PP slope of the burst with the longest interburst interval in the rising phase from the average PP slope of the control bursts. For NO, the percent change in burst parameters during the rising phase compared the average CG burst parameters to the average of the burst parameters of the control bursts. For CLDH, the percent change in the rising phase was calculated using the burst with the longest interval, like in myosuppressin, and the average bursts during this phase, like in NO. For the hold and release phase of the stretches, the percent changes compared the average of the CG burst parameters during these phases to the average parameters of the control bursts preceding the start of the stretch. The absolute difference in PP slope was calculated by subtracting the average PP slope of the bursts during these phases from the average PP slope of the control bursts. GraphPad Prism was also used to conduct the Wilcoxon matched-pairs signed rank test on the average input resistance of motor neurons in saline and myosuppressin. Lastly, Graphad Prism was used to conduct the Wilcoxon matched-pairs signed rank test to compare the average burst frequency,

burst duration, interburst interval, and duty cycle between physiological saline and 10^{-8} M CLDH for the minute during which CLDH induced a maximal response during the 20 minute application.

MATLAB (The MathWorks Inc., Natick, MA) was used to conduct the Levene's test for equality of variances to compare the percent change in each burst parameters for each phase of the myocardial stretches in GYS and SGRN. The percent change of each parameter in saline and these two modulators compared the average burst parameters during each phase of the stretch to the control bursts directly preceding the stretch.

Results were considered statistically significant at $p<0.05$. Additionally, Prism was used to plot the percent changes and absolute differences of the burst parameters in saline and myosuppressin, NO and CLDH.

Results

3.1 Myosuppressin Modulates Each Phase of the Stretch Response Differently

Myosuppressin Enhances the Stretch Response During the Rising Phase

To assess the extent to which myosuppressin modulated the stretch response, the stretch response was induced in physiological saline and 10^{-7} M myosuppressin. Myosuppressin enhanced the stretch response during the rising phase, suppressed the response in the hold phase, and induced no changes in the response during the release phase (Figure 5A). During the rising phase, myosuppressin increased the relative change in interburst interval of the burst with the greatest interburst interval (Figure 5B). The extent to which myosuppressin increased the interburst interval was so great that for 7 out of 21 preparations there were no bursts during the rising phase. Because of this, we timed the stretches to begin right after a burst ended and included the burst directly preceding the stretch in our analysis of the rising phase.

Myosuppressin did not affect the change in PP slope for the rising phase of the stretch (Figure 5C).

Myosuppressin Suppresses the Stretch Response During the Hold Phase

During the hold phase, myosuppressin suppressed the stretch response by decreasing the magnitude of the relative change in burst frequency, burst duration, and interburst interval (Figure 6B-D). Myosuppressin also decreased the change in PP slope during the hold phase of the stretch response (Figure 6E). Interestingly, myosuppressin did not affect the relative change in duty cycle but decreased the magnitude of the absolute difference in duty cycle (6F and G). This indicates that myosuppressin decreased the magnitude of the relative change in burst duration and interburst interval concurrently such that the phase was maintained during the hold phase.

Additionally, myosuppressin modulated the relationship between the baseline burst duration and change in burst duration during the hold phase. In saline, the baseline burst duration and the change in burst duration were significantly correlated. Preparations with longer baseline burst durations showed a greater change in burst duration during the hold phase of the stretch response, a phenomenon seen in Qu's thesis (2017) (Figure 7A andC). In myosuppressin, this relationship between the baseline burst duration and the change in burst duration ceased as these two parameters were no longer correlated (Figure 7B and C). Unexpectedly, the change in burst duration in saline was positively correlated with the change in burst duration in myosuppressin (7D). Therefore, although myosuppressin suppressed the relationship between the baseline burst duration and the change in burst duration we observed in saline, a greater change in burst duration in saline was associated with a greater change in burst duration in myosuppressin.

Myosuppressin Does Not Modulate the Release Phase of the Stretch Response

During the release phase of the stretch feedback pathway, myosuppressin did not alter the changes in burst frequency, burst duration, interburst interval, or PP slope (Figure 8B-E). Interestingly, myosuppressin increased the relative change in duty cycle during the release phase of the stretch response but did not affect the absolute difference of the duty cycle (Figure 8F and G.

Initially, the release phase of the stretch response was analyzed by calculating the average relative change in burst parameters of all the bursts during the release portion of the stretch. However, the main effect of the release phase of the stretch response is an increase in burst duration for the last burst during this phase, but not all the bursts (Qu, 2017). Given this understanding, I then split the release phase of these experiments between early bursts and the last burst to confirm that myosuppressin does not modulate this phase of the stretch response. Myosuppressin did not affect the relative change in burst duration of the early bursts during the release phase (Figure 9B). However, myosuppressin increased the magnitude of the relative change in interburst interval and increased the relative change in duty cycle of the early bursts (Figure 9C and D). On the other hand, myosuppressin did not significantly affect the change in burst duration, interburst interval, or duty cycle of the last burst in the release phase (Figure 10B-D). Overall, the modulation of the stretch feedback pathway by myosuppressin varies by the phase of the stretch. Myosuppressin enhances the stretch response during the rising phase, suppresses the response during the hold phase, and does not modulate the release phase.

The Modulation of the Stretch Response by Myosuppressin is a Result of Myosuppressin Interacting with the Stretch Feedback Pathway

At this point I understood how myosuppressin modulates the stretch response. However, one possibility I had not considered was this modulation being a result of the change in CG bursts parameters induced by myosuppressin during the control period rather than myosuppressin interacting with the stretch feedback pathway to produce a different response. As a result, I re-analyzed my data and normalized the control period in myosuppressin to saline for each stretch. I then normalized the rising phase in myosuppressin to saline and did the same for the hold and release phases. Lastly, I compared the normalized burst parameters between the control period and each phase of the stretch. I found that that for each burst parameter that was significantly modulated by myosuppressin in my initial analysis, there was a significant difference in the relative change of that same burst parameter between the control period and corresponding stretch phase.

First, there was a significant difference between the control period and the rising phase for the relative change in interburst interval from saline to myosuppressin (Figure 11). This confirmed that the increase in the relative change in interburst interval during the rising phase caused by myosuppressin was because of myosuppressin interacting with the stretch response and not myosuppressin increasing the interburst interval of the CG during the control period. For the hold phase, there was a significant difference between the control period and hold phase in the relative change in burst frequency, burst duration, and interburst interval induced by myosuppressin (Figure 11). This suggested that the decrease in the magnitude of the relative change in burst frequency, burst duration, and interburst interval observed during the hold phase in myosuppressin was because of the interaction between myosuppressin and the stretch

feedback pathway rather than the effects of myosuppressin on control CG burst parameters alone. For the release phase, the only significant change between the control period and release phase was in the relative change in duty cycle (Figure 11). This confirmed that myosuppressin did not interact with the release phase of the stretch to produce a distinct response.

Myosuppressin May Increase the Input Resistance of CG Motor Neurons

To determine a possible mechanism by which myosuppressin modulates the stretch response, -1 nA currents were injected into a CG motor neuron to measure possible changes in the input resistance during the stretch in physiological saline and myosuppressin. During the stretch response in saline, there seemed to be no changes in the input resistance of CG motor neurons between the control period and each phase of the stretch (Figure 12). Interestingly, myosuppressin induced an upward trend in the control CG motor neuron input resistance and the input resistance for each phase of the stretch (Figure 13).

3.2 Nitric Oxide Does Not Affect the Stretch Feedback Pathway

To determine if the excitatory stretch and inhibitory NO feedback pathways interact in the lobster cardiac neuromuscular system, the stretch response was recorded in the isolated CG preparation in physiological saline and the NO donor, SNAP 10^{-5} M. The application of NO to the CG largely did not alter the stretch feedback pathway, leading to the conclusion that the stretch and NO feedback pathways do not interact in the lobster CG (Figure 14A). NO did not modulate the relative change in burst duration, interburst interval, and burst frequency during the rising phase of the stretch response (Figure 14B-D). During the hold phase, NO significantly decreased the magnitude of the relative change in burst duration but did not affect the relative change in interburst interval or burst frequency (Figure 15B-D). Lastly, NO did not alter the

relative changes in burst duration, interburst interval, and burst frequency during the release phase of the stretch response (Figure 16B-D).

These results were interesting because they confirm that the modulation of the stretch feedback pathway by myosuppressin are not generalizable to inhibitory neuromodulators. Additionally, this finding suggested that although the lobster cardiac neuromuscular system has two opposing feedback pathways, they do not interfere with each other's feedback.

3.3 CLDH Weakly Suppresses the Stretch Feedback Pathway

To understand the extent to which CLDH modulates the stretch response, I compared the stretch response in saline and 10^{-8} M CLDH. Based on Bowers (2010), I initially planned to study the stretch response in 10^{-7} M CLDH. However, I found 10^{-7} M CLDH to increase the stretch response magnitudes higher than expected from the results in Bowers (2010). As a result, I applied 10^{-8} M CLDH, despite this previous thesis reporting 10^{-8} CLDH to have no effect on the CG motor output. I found 10^{-8} M CLDH to increase the burst frequency, decrease the burst duration, increase the duty cycle, and decrease the interburst interval (Figure 17). These results were surprising because 10^{-8} M CLDH previously had no effect on the CG motor output. Additionally, CLDH had been reported to increase the burst frequency without affecting any other burst parameters, an observation that is not supported by these experiments.

Unexpectedly, CLDH only affected the change in burst parameters during the rising and release phases of the stretch response. During the rising phase, CLDH did not modulate the average relative change in burst duration, interburst interval, burst frequency, or duty cycle (Figure 18). However, during these experiments, I noticed the increase in interburst interval during the rising phase to typically decrease in CLDH. As a result, I analyzed the rising phase once more, this time conducting the same analysis on this phase as I did for myosuppressin

where I compared the burst with the longest interburst interval during the rising phase in saline and CLDH. As I predicted, CLDH decreased the relative change in interburst interval for the burst with the longest interburst interval (Figure 19). For the hold phase, CLDH did not affect the relative change in burst duration, interburst interval, burst frequency, or duty cycle (Figure 20). Notably, CLDH decreased the relative change in burst frequency and decreased the magnitude of the relative change in interburst interval during the release phase (Figure 21B and D). CLDH did not affect the relative change in burst duration or duty cycle during this phase (Figure 21C and E).

GYS and SGRN are two other neuromodulators that stimulated the whole heart preparation and suppressed the stretch response during each phase. These results suggest that excitatory neuromodulators may generally suppress the stretch response but with varying degrees.

Discussion

4.1 Neuromodulators Allow for Flexibility in the Motor Output of the CG's Response to Cardiac Muscle Stretch

Neuromodulators can induce flexibility in the motor output of CPG systems and allow for the organism to respond appropriately to changes in its environment (Dickinson et al., 2016). This understanding has largely been applied to the motor output of CPG systems on their own, but little is known about how neuromodulators affect CPG responses to different stimuli. The results from this project focused on the stretch feedback pathway further support the idea that neuromodulators allow for flexibility in CPG motor outputs on their own and reveal that neuromodulators can be used to produce varied responses to cardiac muscle stretch. We have found one neuromodulator to modulate each phase of the stretch response differently, one

neuromodulator to generally not affect the stretch response, and three neuromodulators to suppress the stretch response.

This project aimed to understand the extent to which neuromodulators and short distance signaling molecules that facilitate flexibility in the CG motor output can also allow for different responses to cardiac muscle stretch. To answer this question, we used the isolated CG preparation. We induced the stretch feedback pathway in physiological saline and neuromodulators of interest to determine if the stretch feedback pathway was modulated.

4.2 Myosuppressin Prevents the Excitation of the Cardiac Neuromuscular System Induced by the Stretch Feedback Pathway

First, I wanted to understand if myosuppressin modulates the stretch response. Given that myosuppressin decreases the CG burst frequency and the whole heart contraction frequency, I predicted myosuppressin would prevent the increase in burst frequency, characteristic of the hold phase of the stretch response, and therefore suppress the stretch response (Steves et al., 2009). Surprisingly, myosuppressin modulated each phase of the stretch response differently. Myosuppressin enhanced the rising phase, suppressed the hold phase, and did not modulate the release phase of the stretch response. Additionally, the effects of myosuppressin on the stretch response were not attributable to the decrease in burst frequency and the increase in interburst interval and burst duration induced by myosuppressin during the control period. Instead, the modulation of the stretch feedback pathway by myosuppressin was a result of myosuppressin interacting with the stretch feedback pathway to produce a different response to cardiac muscle stretch.

Myosuppressin enhanced the stretch response during the rising phase by increasing the change in interburst interval. This result suggests the increase in interburst interval induced by

myosuppressin alone could combine with the mechanisms underlying the increase in interburst interval during the rising phase of the cardiac muscle stretch to cause this greater change in interburst interval during the rising phase. This also indicates that myosuppressin modulates the currents underlying the increase in interburst interval characteristic of the rising phase. However, we found the change in PP slope, the rate of depolarization of motor neurons between driver potentials, to be unaffected by myosuppressin during this phase. This suggested myosuppressin may change the threshold of I_{CaT}, which underlies the initial depolarization of driver potentials, leading to myosuppressin increasing this change in interburst interval without affecting the rate of depolarization between bursts (Ball et al., 2010). Voltage clamp experiments are necessary to confirm any changes in conductance and voltage thresholds underlying the rising phase of the stretch response itself and its modulation by myosuppressin. Overall, myosuppressin modulates the rising phase of the stretch response to further increase the delay in bursting.

Myosuppressin suppressed the stretch response during the hold phase by decreasing the magnitude of the relative change in burst frequency, burst duration, and interburst interval. This result supports the hypothesis that the decrease in burst frequency induced by myosuppressin prevents the excitation caused by the stretch feedback pathway. This modulation of the hold phase of the stretch response suggests that, similar to the rising phase, myosuppressin modulates currents underlying the hold phase. We also found myosuppressin to decrease the change in PP slope during the hold phase, suggesting myosuppressin may modulate the currents responsible for depolarizing the CG motor neurons between driver potentials. Two currents that are thought to be the most active between driver potentials are I_{CaS} and I_A. Myosuppressin could prevent the increase in burst frequency and interburst interval during the hold phase by decreasing the conductance of I_{CaS} or increasing the conductance of I_A in between driver potentials.

Additionally, myosuppressin could limit the decrease in burst duration characteristic of the hold phase by decreasing the conductance of I_{KCa}, the current most active during the repolarization of driver potentials, or by slowing down the rate at which I_{CaT} and I_{CaS} inactivate (Ball et al., 2010). Since myosuppressin does not affect the change in PP slope during the rising phase of the stretch response, it may be more likely that either myosuppressin is modulating the stretch response by altering the voltage thresholds of currents underlying the stretch response or by altering both current conductances and voltage thresholds. Voltage clamp experiments would be needed to confirm any changes in conductance and voltage thresholds underlying the hold phase of the stretch response itself and their modulation by myosuppressin.

Another current that is important to consider when we think about mechanisms that may underly the stretch response and the modulation of the stretch response is a chloride (Cl^-) ion-mediated current. One interesting component of the stretch response is that although it is generally excitatory, we occasionally have preparations that either have no response to cardiac muscle stretch or have an inhibitory stretch response (Chin-Purcell, 2014; Dickinson, 2014). This suggests that Cl^- ions may have a role in mediating this response because the reversal potential of Cl^- tends to be very close to the resting membrane potential, possibly allowing for the variation in the stretch responses we recorded. Experiments blocking Cl^- channels could deduce if Cl^- does in fact mediate the stretch response. Additionally, future projects could include using voltage clamp to understand the changes in currents underlying the complex nature in which the generally excitatory stretch feedback pathway has different motor outputs in response to each phase of the stretch. If a Cl^- ion-mediated current does in fact underly the changes in burst parameters we see in response to cardiac muscle stretch, we would expect myosuppressin to have a role in modulating this current as well.

Additionally, during the hold phase, myosuppressin eliminates the relationship between the baseline burst duration and the magnitude of change in burst duration that is observed in saline. As a result, the baseline burst duration in myosuppressin cannot be used to predict the change in burst duration during the hold phase of the cardiac muscle stretch. However, we found the magnitude of change in burst duration in saline to be positively correlated with the magnitude of change in burst duration in myosuppressin. Therefore, even though myosuppressin removes the relationship between the baseline burst duration and the change in burst duration, the amount of change in burst duration in saline remains as an indicator of how much the burst duration will change during the hold phase in myosuppressin.

Lastly, myosuppressin largely did not modulate the release phase of the stretch response. When the entire release phase of the stretch response was analyzed as a whole, myosuppressin only increased the relative change in duty cycle and did not affect the change in burst frequency, burst duration, interburst interval, PP slope, or the absolute change in duty cycle. I then considered Qu, 2017's finding that the main effect of the release phase of the stretch response was an increase in burst duration of the last burst in this phase and decided to analyze the early bursts and the last burst separately. This analysis revealed myosuppressin increased the magnitude of the relative change interburst interval and relative change in duty cycle but did not affect the relative change in burst duration for the early bursts of the release phase. The last burst in the release phase was unaffected by myosuppressin as there were no significant differences in the change in burst duration, interburst interval, or duty cycle. This second analysis of the release phase of the stretch response revealed myosuppressin only alters the early bursts in the release phase.

Overall, these results have shown the decrease in burst frequency caused by myosuppressin further amplifies the increase in interburst interval during the rising phase of the cardiac muscle stretch. The decrease in burst frequency caused by myosuppressin also prevents the increase in burst frequency during the hold phase of the stretch response. Lastly, myosuppressin generally does not affect the stretch response during the relaxation of the cardiac muscle stretch.

4.3 The Excitatory Stretch and Inhibitory Nitric Oxide Feedback Pathways Do Not Interact in the Lobster Cardiac Neuromuscular System

Next, I wanted to understand if the modulation of the stretch feedback pathway by myosuppressin was generalizable across inhibitory neuromodulators. This led me to ask if the inhibitory NO feedback pathway in the lobster heart interacts with the excitatory stretch feedback pathway. Since NO decreases the whole heart contraction frequency and amplitude and decreases the CG burst frequency and interburst interval, we expected NO to inhibit the stretch feedback pathway by preventing the increase in burst frequency characteristic of the hold phase of the stretch response (Mahadevan et al., 2004). Interestingly, we observed NO to generally not alter the stretch response, with the exception of NO decreasing the magnitude of relative the change in burst duration during the hold phase. This led to the conclusion that although the excitatory stretch and inhibitory NO feedback pathways oppose each other in the lobster heart, they do not interact. These results suggest that the mechanism by which NO inhibits the lobster cardiac neuromuscular system does not affect the mechanism underlying the stretch feedback pathway. Alternatively, the stretch feedback mechanism may occlude the mechanism for NO inhibition. Additionally, there may be beneficial aspect to the cardiac system maintaining the stretch response even when NO is released to inhibit cardiac contractions.

4.4 The Excitation of the Cardiac Neuromuscular System by CLDH Does Not Interact with the Stretch Feedback Pathway to Further Excite the System

I then tested if the modulation of the stretch feedback pathway by excitatory neuromodulators is generalizable. Given that CLDH increases the CG burst frequency and increases the whole heart contraction frequency and amplitude, I predicted the excitation of the cardiac neuromuscular system by CLDH combined with the stretch feedback pathway would produce a more prominent stretch response in comparison to saline (Christie et al., 2009, Bowers, 2010). Unexpectedly, I found CLDH to weakly suppress the stretch response, with CLDH decreasing the relative change in interburst interval during the rising phase and CLDH decreasing the magnitude of the relative change in burst frequency and interburst interval during the release phase. This is similar to the modulation of the stretch feedback pathway by GYS and SGRN, two excitatory neuromodulators that suppress each phase of the stretch response. However, the suppression of the stretch response by CLDH is less consistent across each stretch phase. This suggests the modulation of the stretch feedback pathway may be similar across excitatory neuromodulators but to varied degrees.

An interesting aspect of these results is that the CLDH only modulated the rising and release phase of the stretch feedback pathway. This was surprising because I expected the increase in burst frequency caused by the stretch response combined with the increase in burst frequency caused by CLDH to induce an even greater increase in burst frequency in comparison to the increase in burst frequency observed in saline during the hold phase. In contrast, CLDH prevented both the increase in interburst interval during the rising phase and the increase in burst frequency during the release phase without affecting any burst parameters during the hold phase.

One possible explanation for this observation is the frequency dependency of neuromodulation. In the crab stomatognathic ganglion (STG), the amplitude and peak time of a current activated by proctolin, a neuromodulator, depends on the burst frequency of the lateral pyloric (LP) neuron. Notably, when the LP neuron was depolarized and hyperpolarized using a voltage ramp, one proctolin-activated current was independent of the rate and direction of the voltage ramp while an inactivating current was only activated by depolarizing ramps and the amplitude increased with a greater voltage ramp slope (Schneider et al., 2021). This frequency dependency of neuromodulation has implications for the modulation of the stretch feedback pathway because the CG burst frequency changes in response to the stretch feedback. Perhaps the currents activated by CLDH are independent of the changes in burst frequency we observe during the hold phases of the stretch response but are not independent of the change in burst frequency during the rising and release phases. This theory can be used to understand why myosuppressin does not modulate the release phase of the stretch response and suggests that we should expect neuromodulators to modulate each phase of the stretch response differently.

Additionally, we observed variability in the stretch response in saline with some preparations showing greater or lesser changes in burst frequency in response to myocardial stretches. If the neuromodulation of the CG is dependent on the burst frequency, the neuromodulation of the stretch feedback pathway could also depend on the extent to which individuals respond to cardiac muscle stretch feedback.

4.5 The Combined Modulation of the Nervous System and Peripheral Sites May Underly the Modulation of the Stretch Response

The distinct modulation of the stretch feedback pathway by myosuppressin and NO I observed in this paper tells us the modulation of the stretch response by inhibitory

neuromodulators is not generalizable. If this were case, I would find NO and myosuppressin to alter the stretch feedback pathway in similar ways. Instead, I have observed NO to have no effect on stretch response and myosuppressin to modulate each phase of the stretch response differently. One possible explanation for this difference in NO and myosuppressin's modulation of the stretch response could be the differences in how both modulators affect the entire cardiac neuromuscular system. While NO acts directly on the CG and does not act on the cardiac muscle itself, myosuppressin acts on both the CG and the cardiac muscle (Mahadevan et al., 2004; Petropoulos, 2023). In the case of SGRN and GYS both modulators act on both the cardiac muscle or neuromuscular junction and the nervous system and suppress the stretch response (Dickinson et al., 2015). This is notable because NO is the only modulator that did not modulate the stretch response and is the only modulator we have confirmed to not act outside of the nervous system in the lobster heart. This means that while myosuppressin, SGRN, and GYS may induce variable responses at the nervous system and peripheral sites, NO will only induce a response at the nervous system. This could affect how each modulator could induce flexibility in the nervous system's response to cardiac muscle stretch because the stretch response requires information from the cardiac muscle stretch to provide feedback to the nervous system. Without the modulation of the cardiac muscle or neuromuscular junction, a modulator may fail to affect the muscle stretch information feeding back to the nervous system. Experiments focused on the effect of CLDH on the peripheral sites of the cardiac neuromuscular system are necessary to confirm this hypothesis that the combined modulation of both the nervous system and the peripheral sites of the lobster heart produce flexibility in the response to stretch feedback.

4.6 Future Directions

Here, we have shown that neuromodulators can act to produce flexibility in a CPG's motor output, allowing the system to respond appropriately to changes in an organism's environment, and allow for variation in CPG responses to different stimuli. Future directions of this project could include deducing a mechanism underlying the stretch feedback pathway. In addition to focusing on the involvement of Cl^- ions modifying the currents underlying DPs to produce the response to cardiac muscle stretch, we could also focus on specific mechanosensitive channels that have been established in other models. Qu (2017) used a mathematical model incorporating the TREK and Piezo mechanosensitive channels to deduce their potential involvement in the stretch response. This model successfully showed the increase in interburst interval during the rising phase, the increase in burst frequency during the hold phase, and the increase in burst duration during the release phase. The TREK and Piezo mechanosensitive channels were chosen for this model because of their presence in the transcriptome of lobster tissues and the possibility of producing the complex stretch response when activated together. TREK mechanosensitive channels were selected because they respond to both force and are mediated by potassium ions (K^+) (Brohawn et al., 2014). Therefore, TREK channels activated by cardiac muscle stretch in the lobster heart could be responsible for the increase in interburst interval characteristic of the rising phase of the stretch. Piezo mechanosensitive channels were chosen because when they respond to changes in force, they activate inward cation currents (Coste et al., 2012). The inward cation currents activated by Piezo channels were hypothesized to mediate the increase in burst frequency during the hold phase of the stretch. Future experiments could use immunohistochemistry to localize TREK and Piezo mechanosensitive channels within the lobster CG and block these channels to confirm their involvement in the stretch response.

Another future experiment could be focused on how the lobster cardiac neuromuscular system responds to the sensory inputs of cardiac muscle stretch and temperature changes at the same time. We know from previous work that the lobster heart contraction frequency and CG burst frequency increase as temperature increases until a critical temperature is reached at which the system no longer functions (Owens, 2014; Powell, 2023). It would be interesting to determine how the system responds to cardiac muscle stretch when the contraction frequency and burst frequency are increased due to an increase in saline temperature. Additionally, we could start to understand how increasing ion channel kinetics via temperature changes affects the system's response to cardiac muscle stretch.

Figures

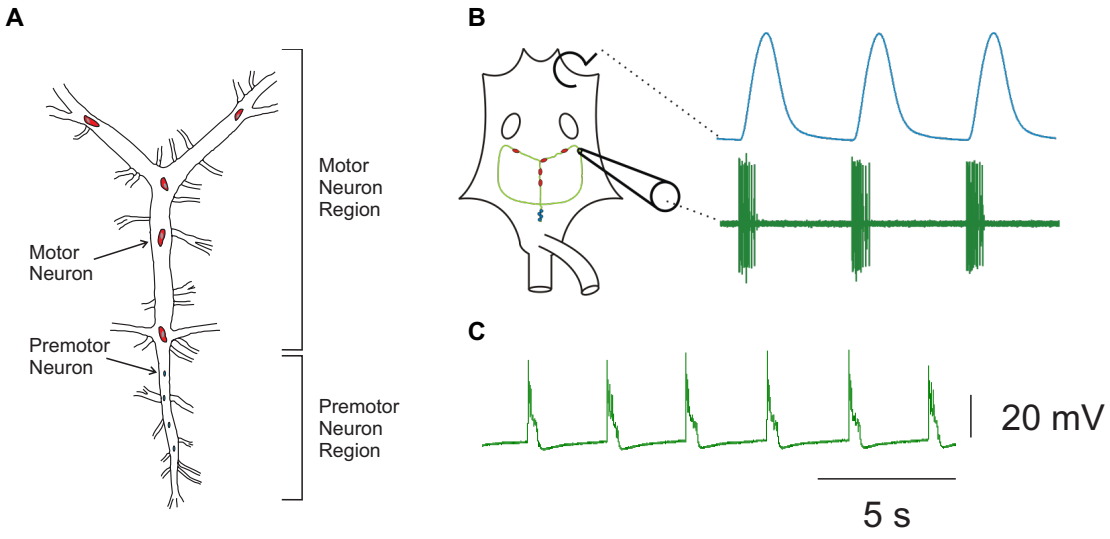

Figure 1. Overview of the cardiac neuromuscular system in *H. americanus.* (A) Schematic of the cardiac ganglion: The cardiac ganglion is composed of nine electrochemically coupled neurons: 4 premotor neurons or small cells (blue) that send signals to 5 motor neurons or large cells (red). (B) Cartoon of the whole heart preparation demonstrating that neuron action potentials can be simultaneously recorded with muscle contractions. Bursts of action potentials from the motor neurons induce cardiac muscle contractions. (C) Intracellular recording of a CG motor neuron showing that underlying the CG bursts of action potentials are slow and sustained calcium-based depolarizations called driver potentials.

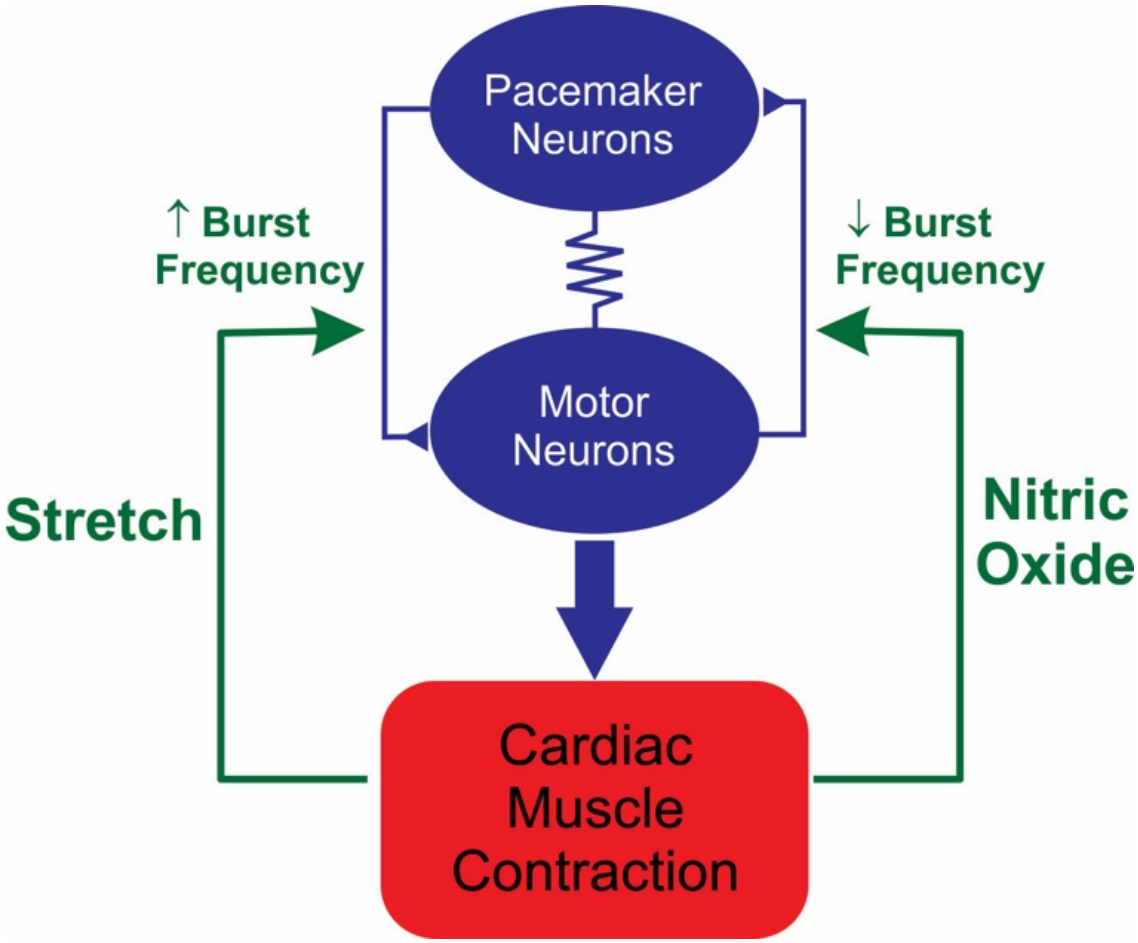

Figure 2. The positive stretch feedback pathway opposes the negative nitric oxide feedback pathway in the cardiac neuromuscular system of the lobster. Feedback from cardiac muscle stretch increases the burst frequency and whole heart contraction frequency. Nitric oxide, a short distance signaling molecule, is thought to be produced in the crustacean heart muscle upon heart contractions. NO decreases the burst frequency and contraction frequency of the whole heart.

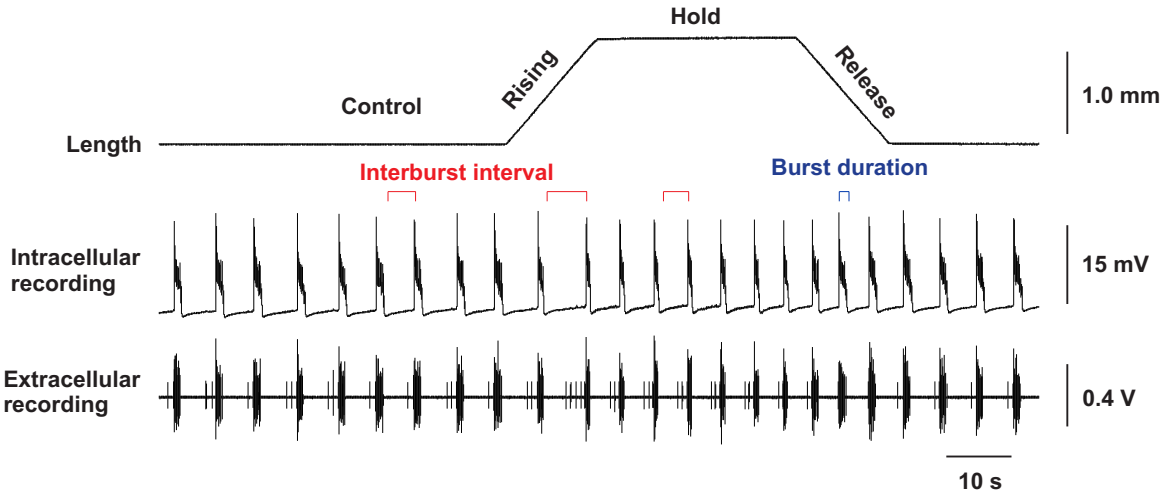

Figure 3. The three phases of cardiac muscle stretch are the rising, hold, and release phase. Extracellular anterior lateral nerve recordings and intracellular motor neuron driver potential (DP) recordings show the increase in interburst interval during the rising phase, the increase in burst frequency and decrease in burst duration during the hold phase, and increase in burst duration during the release phase.

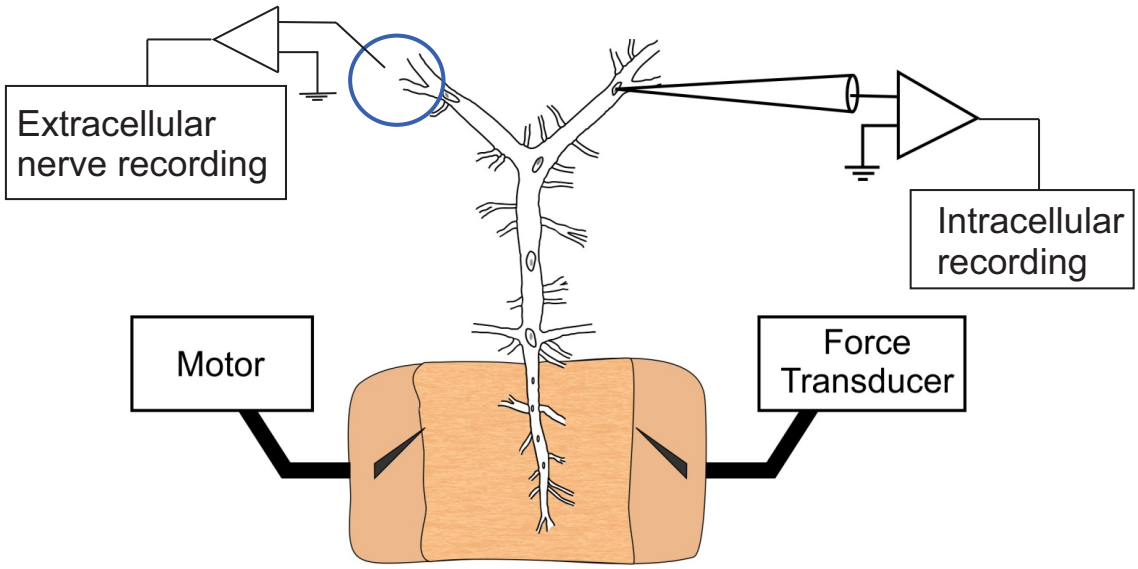

Figure 4. Experimental preparation with lower muscles left intact. To induce the stretch feedback pathway, the CG was isolated along with the muscle surrounding the small cells. Two hooks, one connected to the motor that stretched the muscle and one connected to the force transducer that measured the force of each stretch, were placed into the muscle on each side of the CG. Extracellular and intracellular recordings were taken to measure changes in the CG motor output in response to the stretch feedback pathway and neuromodulators.

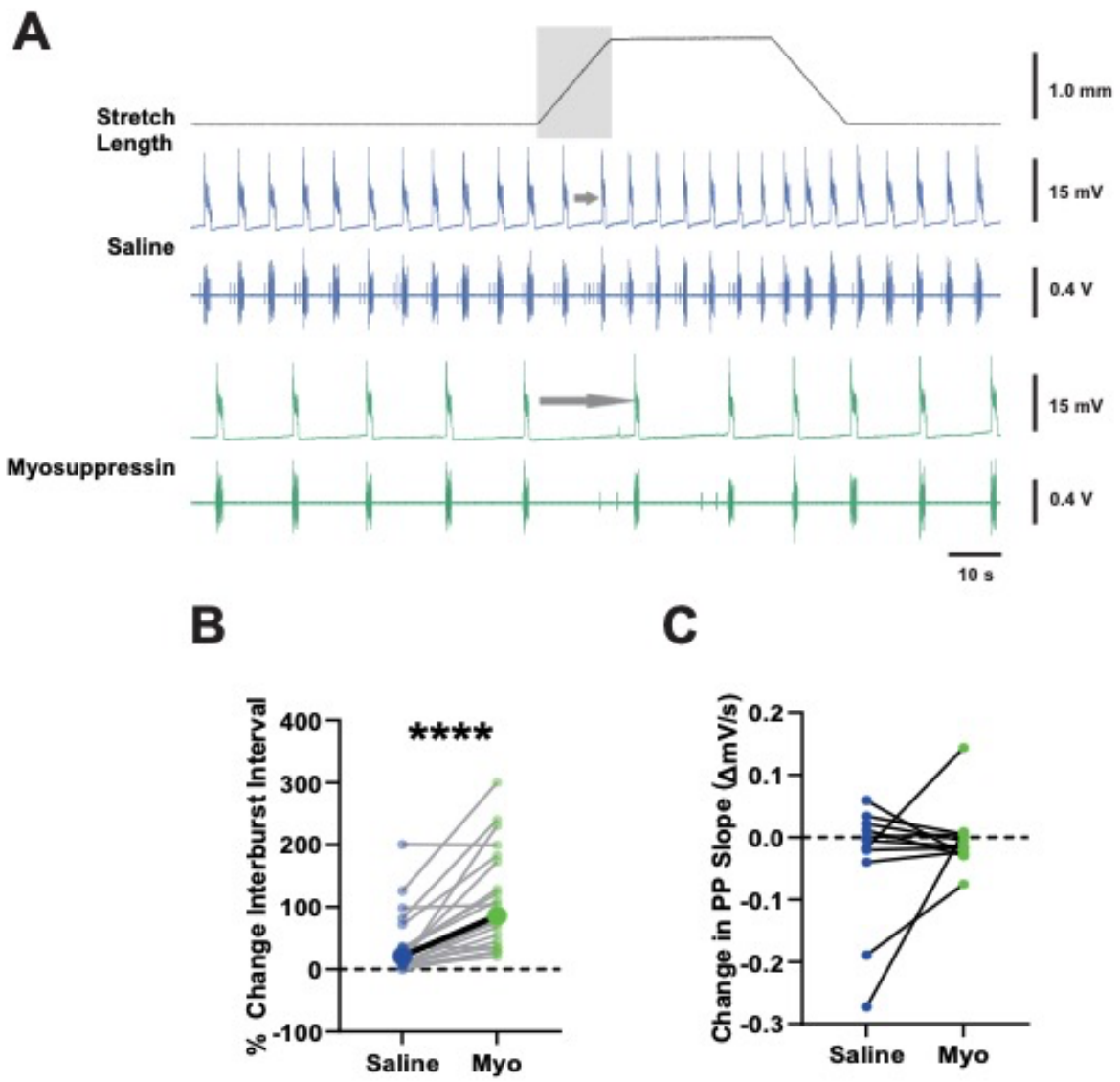

Figure 5. Myosuppressin enhances the rising phase of the stretch response. (A) Sample trace with grey arrows indicating the bursts with the longest interburst interval in saline (blue) and myosuppressin (green) that will be compared. (B) The application of myosuppressin significantly increased the change in interburst interval (Wilcoxon matched-pairs signed rank test, $p<0.0001$, $N=21$). (C) Myosuppressin did not affect the change in pacemaker potential (PP) slope (Wilcoxon matched-pairs signed rank test, $p=0.7646$, $N=11$). Bolded points in panel B and C represent median relative change across preparations.

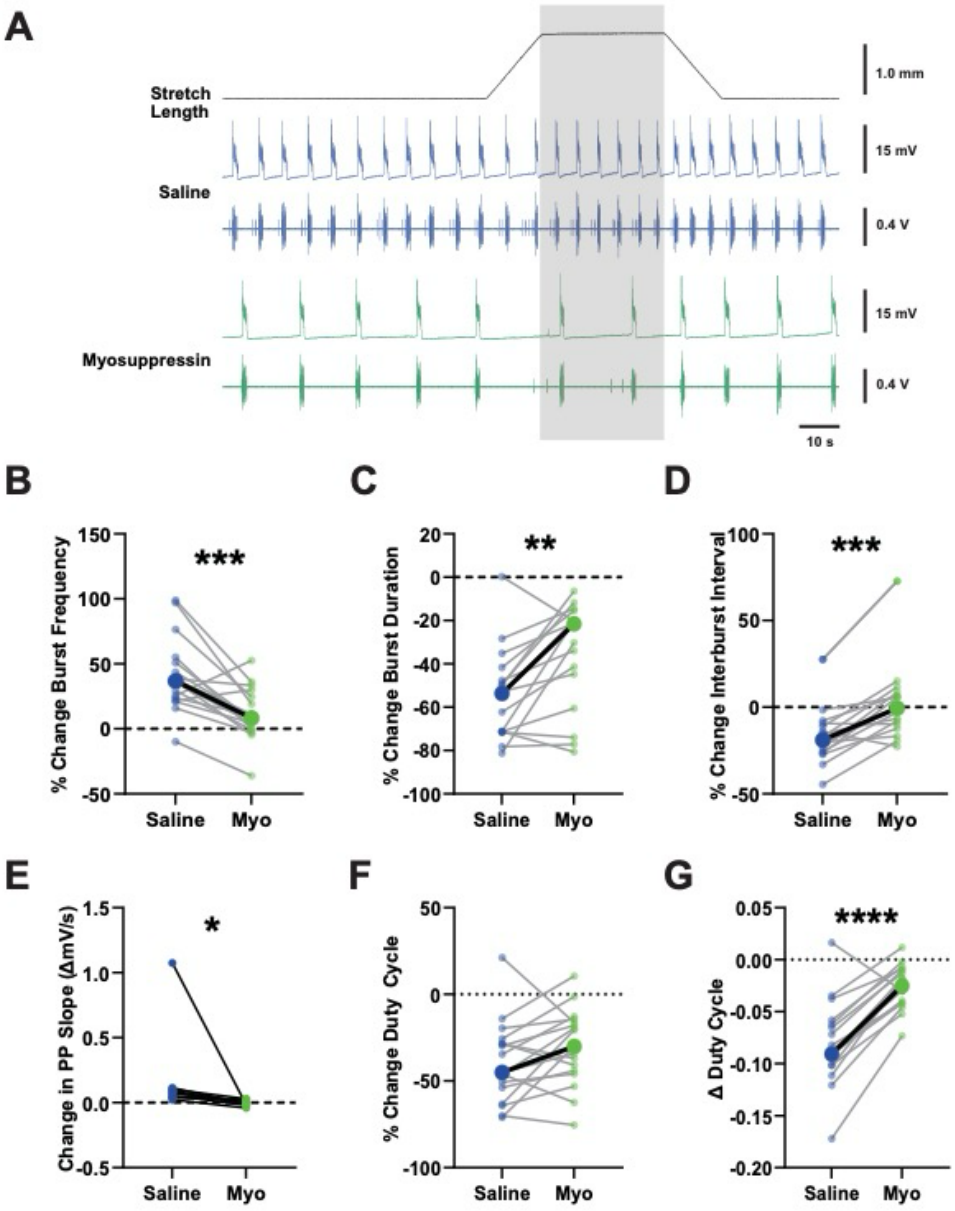

Figure 6. Myosuppressin suppresses the stretch response during the hold phase. (A) Sample trace with a grey box indicating the hold phase of the stretch. (B) Myosuppressin decreased the magnitude of the relative change in burst frequency (Wilcoxon matched-pairs signed rank test, p=0.0007, N=17), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.0021, N=17) and (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.0002, N=17) during the hold phase. (E) Myosuppressin decreased the change in pacemaker potential (PP) slope (Wilcoxon matched-pairs signed rank test, p=0.0020, N=10). (F) Myosuppressin did not significantly alter the relative change in duty cycle (Wilcoxon matched-pairs signed rank test, p=0.0984, N=17) during the hold phase. (G) Myosuppressin decreased the magnitude of the absolute difference in duty cycle (Wilcoxon matched-pairs signed rank test, p<0.0001, N=17). Bolded points in panels B-D, F, and G represent median relative change across preparations.

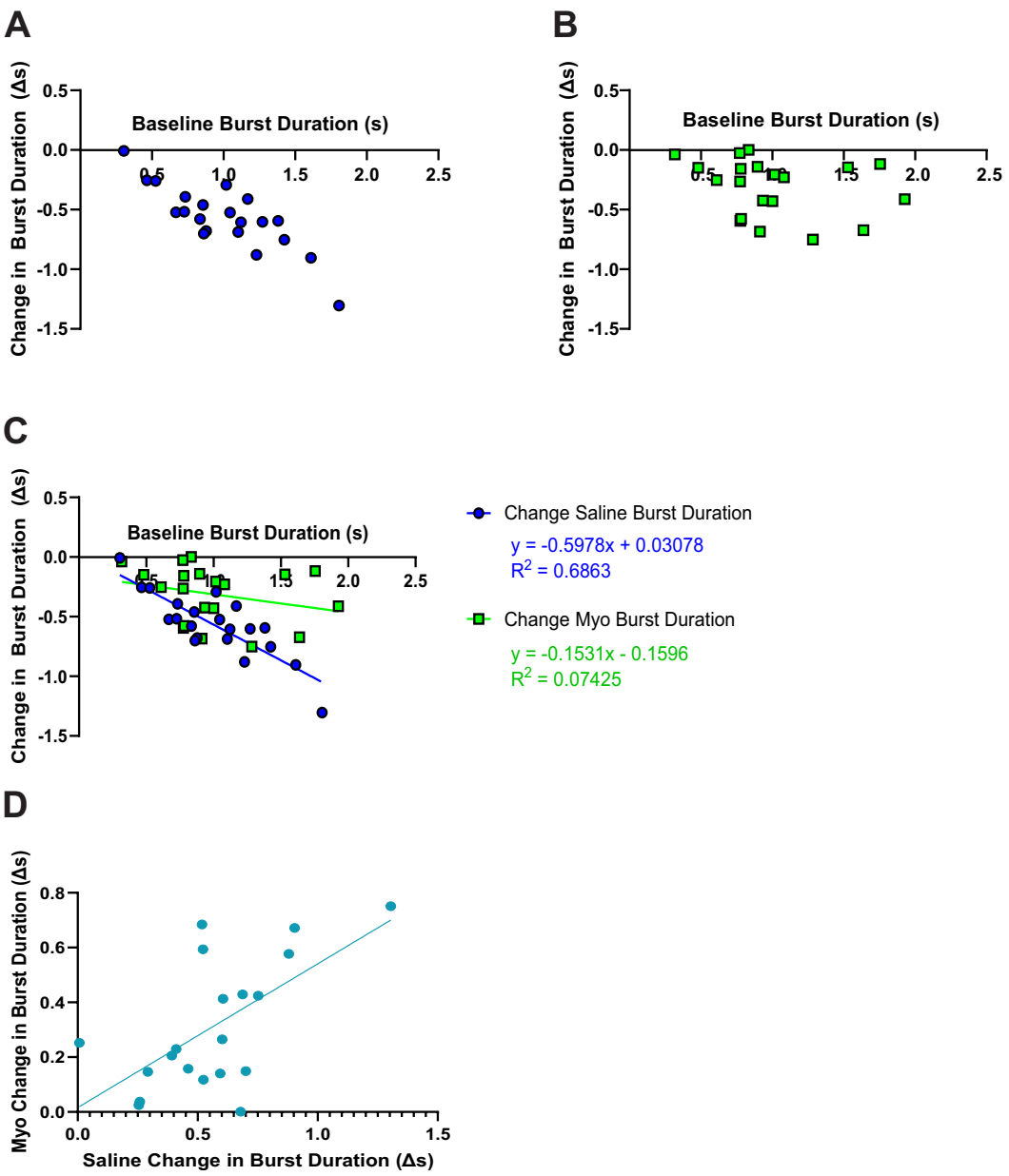

Figure 7: Myosuppressin modulates the relationship between the baseline burst duration and the absolute change in burst duration during the hold phase. (A). In physiological saline (blue) there is a significant correlation between the baseline burst duration and the absolute change in burst duration during the hold phase (Spearman, r=-0.7753, p<0.0001, N=21). (B) In myosuppressin (green) there is no relationship between the baseline burst duration and the absolute change in burst duration during the hold phase (Spearman, r=-0.2827, p=0.2272, N=20). (C) Saline and myosuppressin plots overlayed to emphasize the loss of the relationship between the baseline burst duration and the absolute change in burst duration in myosuppressin. (D) The absolute value of the change burst duration in saline is positively correlated with the absolute value of the change in burst duration in myosuppressin (Spearman, r=0.5053, p=0.0231, N=20).

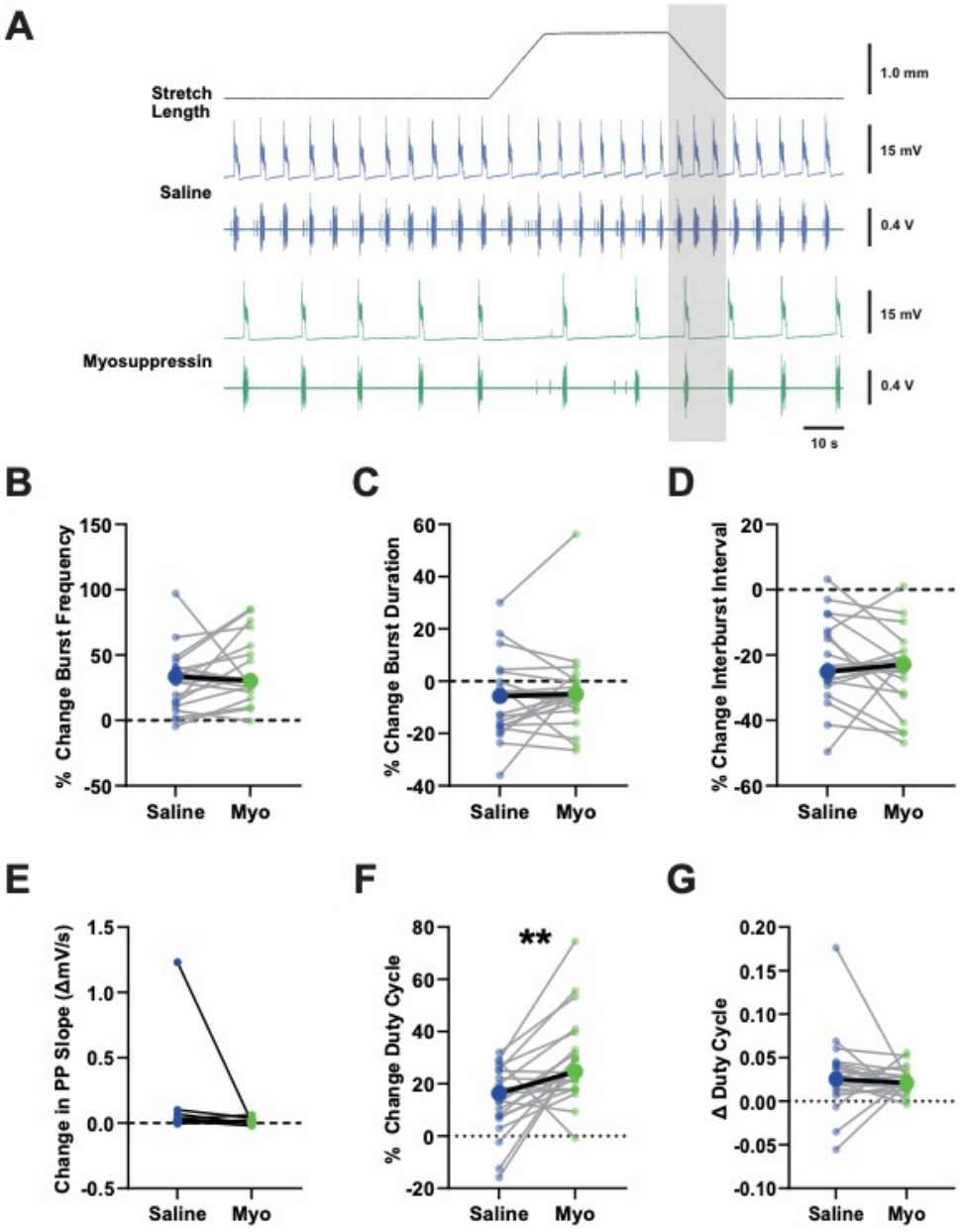

Figure 8. Myosuppressin does not modulate the stretch response during the release phase. (A) Sample trace with a grey box indicating the release phase of the stretch. Myosuppressin did not affect the relative change in (B) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.0822, N=21), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.5621, N=21), (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.3926, N=21), and (E) pacemaker potential (PP) slope (Wilcoxon matched-pairs signed rank test, p=0.1230, N=11). (F) Myosuppressin increased the relative change in duty cycle during the release phase (Wilcoxon matched-pairs signed rank test, p=0.0014, N=21). (G) Myosuppressin did not affect the absolute difference in duty cycle (Wilcoxon matched-pairs signed rank test, p=0.2290, N=21). Bolded points in panels B-D, F, and G represent median relative change across preparations.

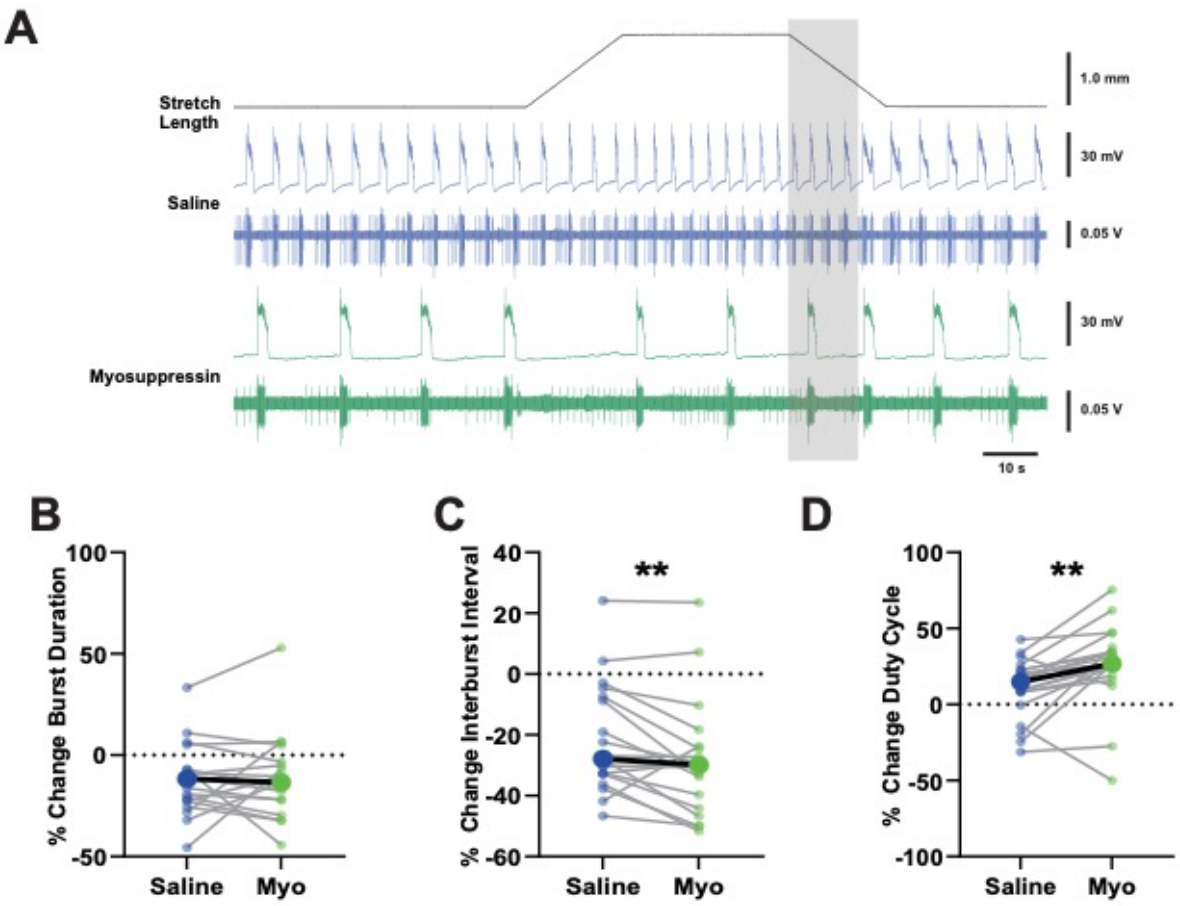

Figure 9. Myosuppressin modulates the early bursts during the release phase of the stretch response. (A) Sample trace with a grey box indicating the early bursts in the release phase of the stretch. (B) Myosuppressin did not affect relative change in burst duration during the release phase (Wilcoxon matched-pairs signed rank test, p=0.7680, N=19). (C) Myosuppressin increased the magnitude of the relative change in interburst interval (Wilcoxon matched-pairs signed rank test, p=0.0095, N=19). (D) Myosuppressin increased the relative change in duty cycle (Wilcoxon matched-pairs signed rank test, p=0.0046, N=19). Bolded points in panels B-D represent median relative change across preparations.

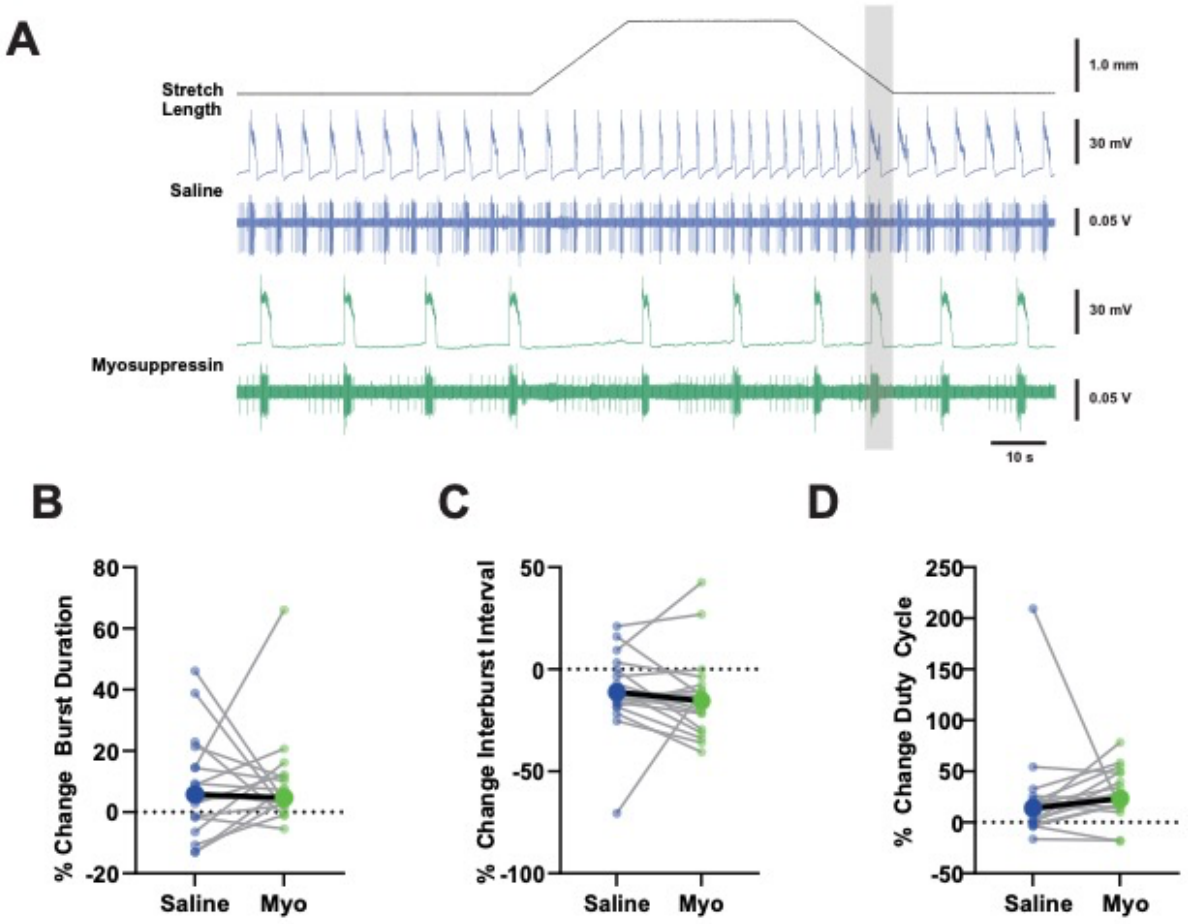

Figure 10. Myosuppressin does not modulate the last burst during the release phase of the stretch response. (A) Sample trace with a grey box indicating the last burst in the release phase of the stretch. Myosuppressin did not affect relative change in (B) burst duration (Wilcoxon matched-pairs signed rank test, p=0.8906, N=19), (C) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.2253, N=19), and (D) duty cycle (Wilcoxon matched-pairs signed rank test, p=0.0546, N=19). Bolded points in panels B-D represent median relative change across preparations.

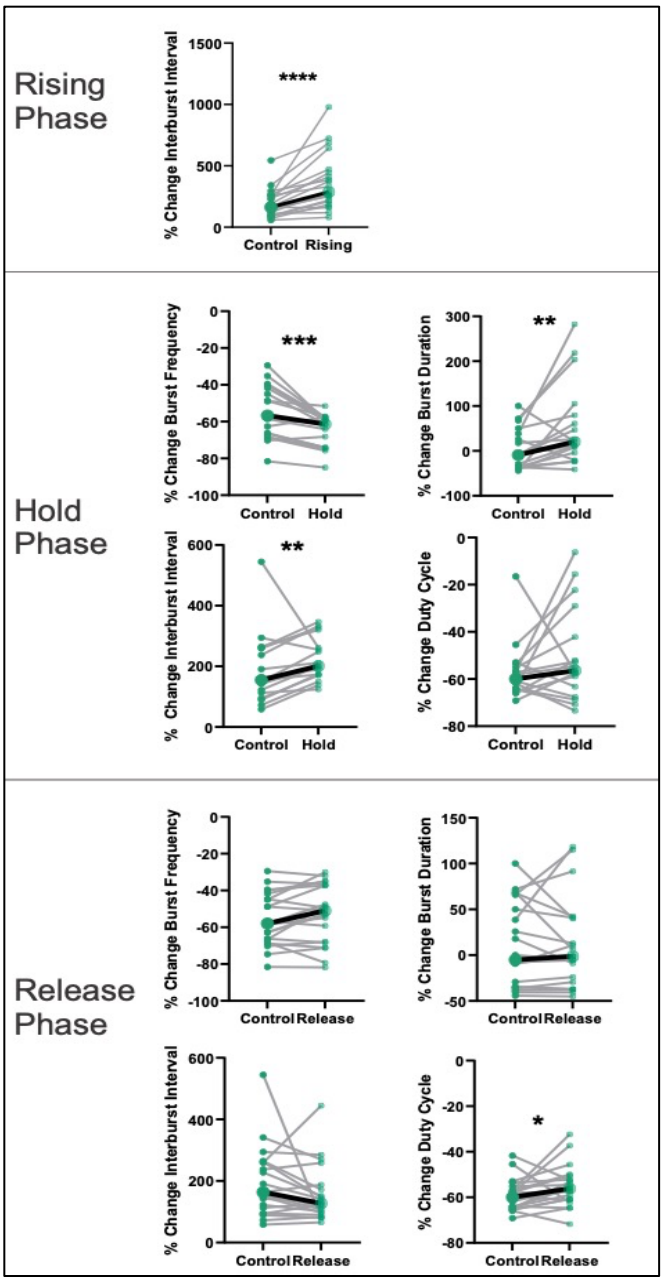

Figure 11. The modulation of the stretch response by myosuppressin is a result of myosuppressin interacting with the stretch feedback pathway. During the rising phase, the relative increase in interburst interval between saline and myosuppressin is significantly greater than the control period (Wilcoxon matched-pairs signed rank test, $p<0.0001$, N=21). During the hold phase, the relative change in the burst frequency (Wilcoxon matched-pairs signed rank test, $p=0.0002$, N=17), burst duration (Wilcoxon matched-pairs signed rank test, $p=0.0093$, N=17), and interburst interval (Wilcoxon matched-pairs signed rank test, $p=0.0079$, N=17) between saline and myosuppressin is significantly different from the control period. During the release period, the relative change in duty cycle is significantly different from the control period (Wilcoxon matched-pairs signed rank test, $p=0.0312$, N=21).

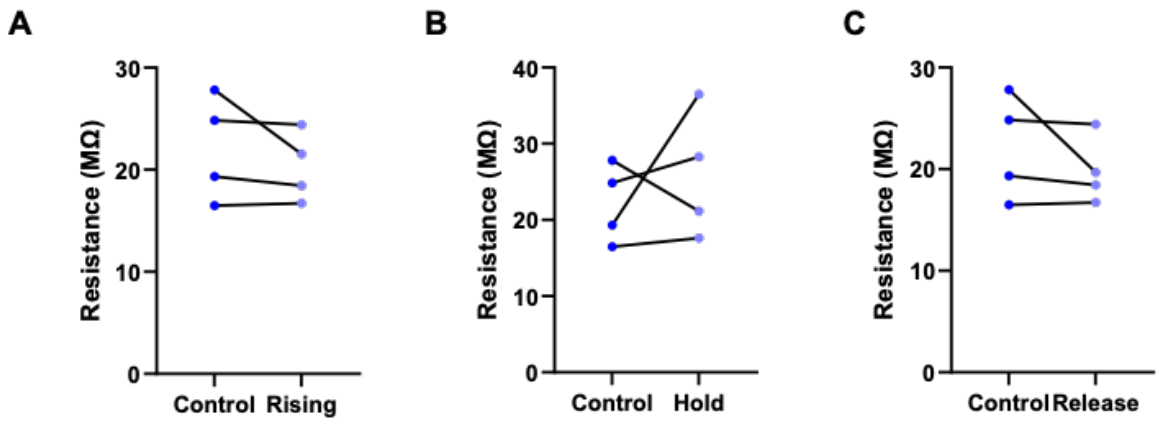

Figure 12. The stretch response does not seem to alter the input resistance of CG motor neurons. There were no trends in the resistance of CG motor neurons during the (A) rising, (B) hold, and (C) release phase of the stretch response.

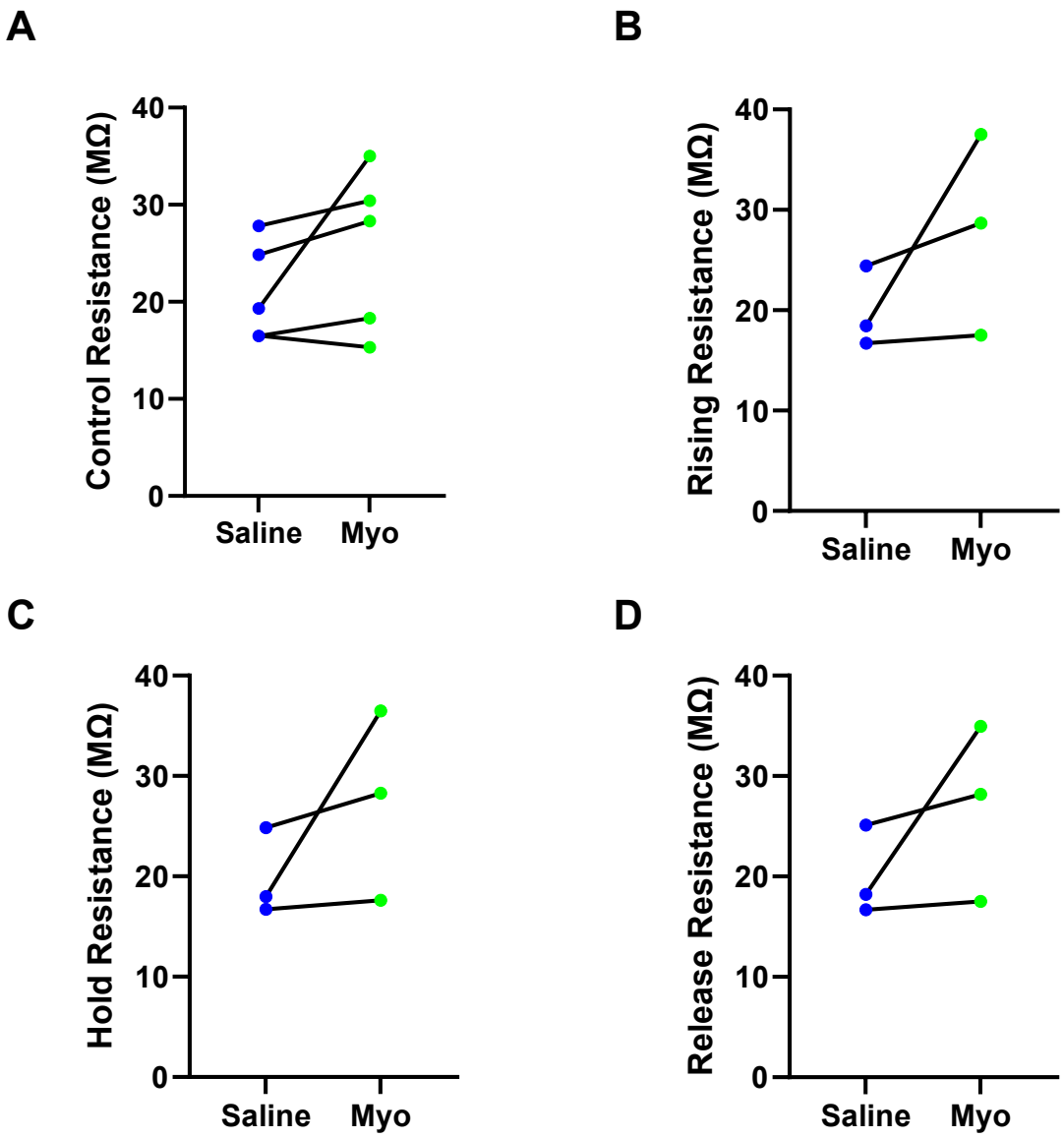

Figure 13. Myosuppressin may increase the input resistance of CG motor neurons. (A) Myosuppressin induced an upward trend in the resistance of the CG motor neurons during the control period of the stretch. Myosuppressin induced an upward trend in the resistance of CG motor neurons during the (B) rising, (C) hold, and (D) release phase of the stretch response.

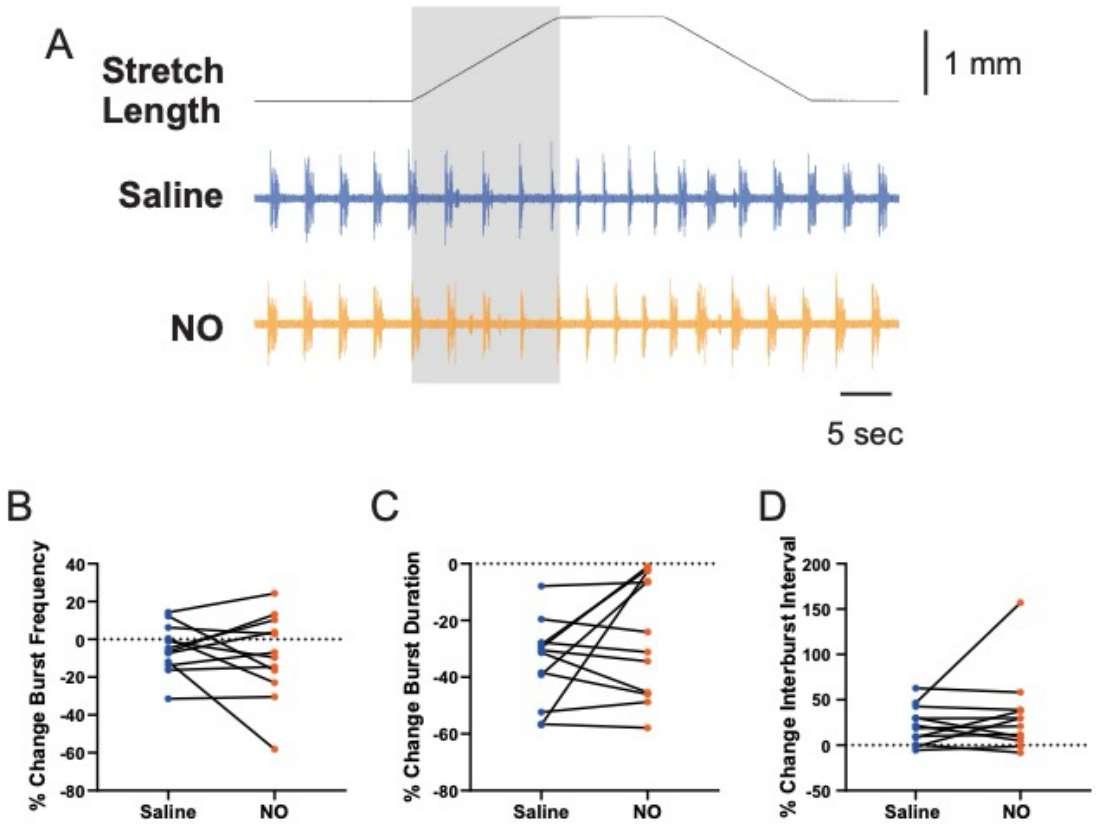

Figure 14. NO does not alter the stretch response during the rising phase. The application of NO did not affect the relative change in (B) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.9097, n=12), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.5693, n=12), or (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.7334, n=12) during the rising phase of the stretch response.

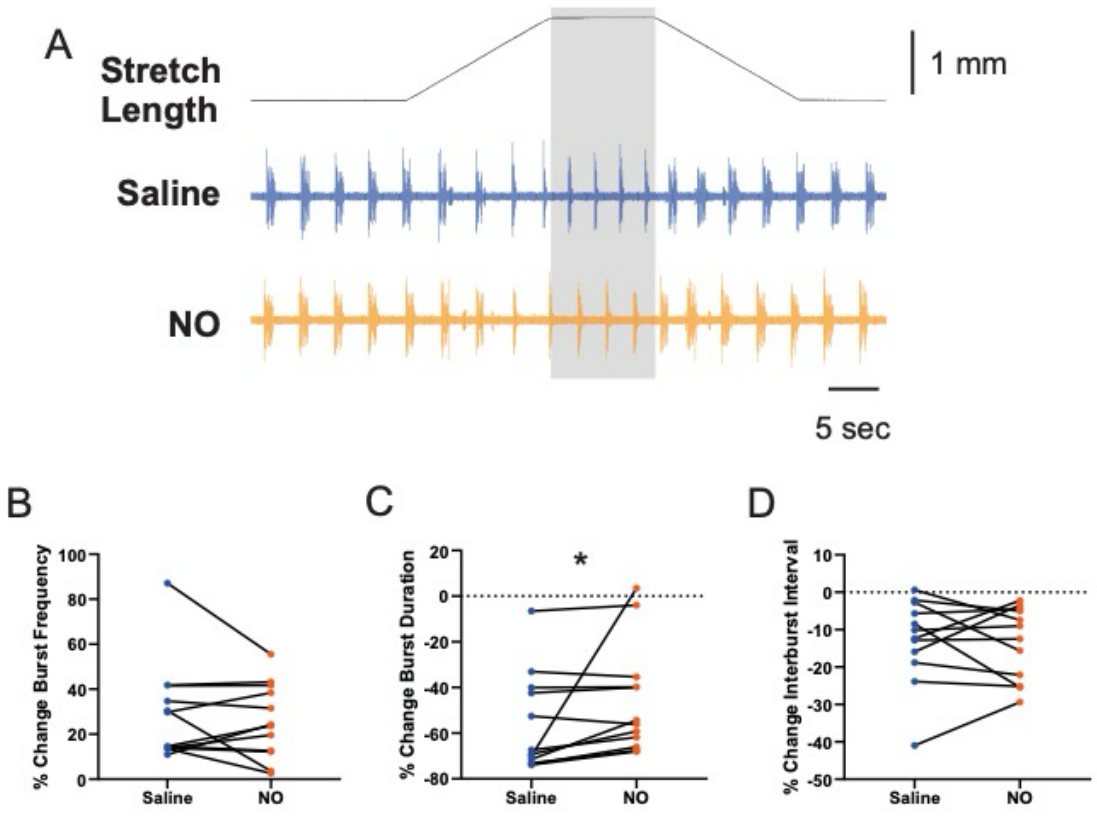

Figure 15. NO does not alter the stretch response during the hold phase. (B) The application of NO did not affect the relative change in burst frequency during the hold phase of the stretch response (Wilcoxon matched-pairs signed rank test, p=0.7334, n=12). (C) NO significantly decreased the magnitude of the relative change in burst duration (Wilcoxon matched-pairs signed rank test, p=0.0093, n=12). NO did not affect the relative change in (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.6772, n=12).

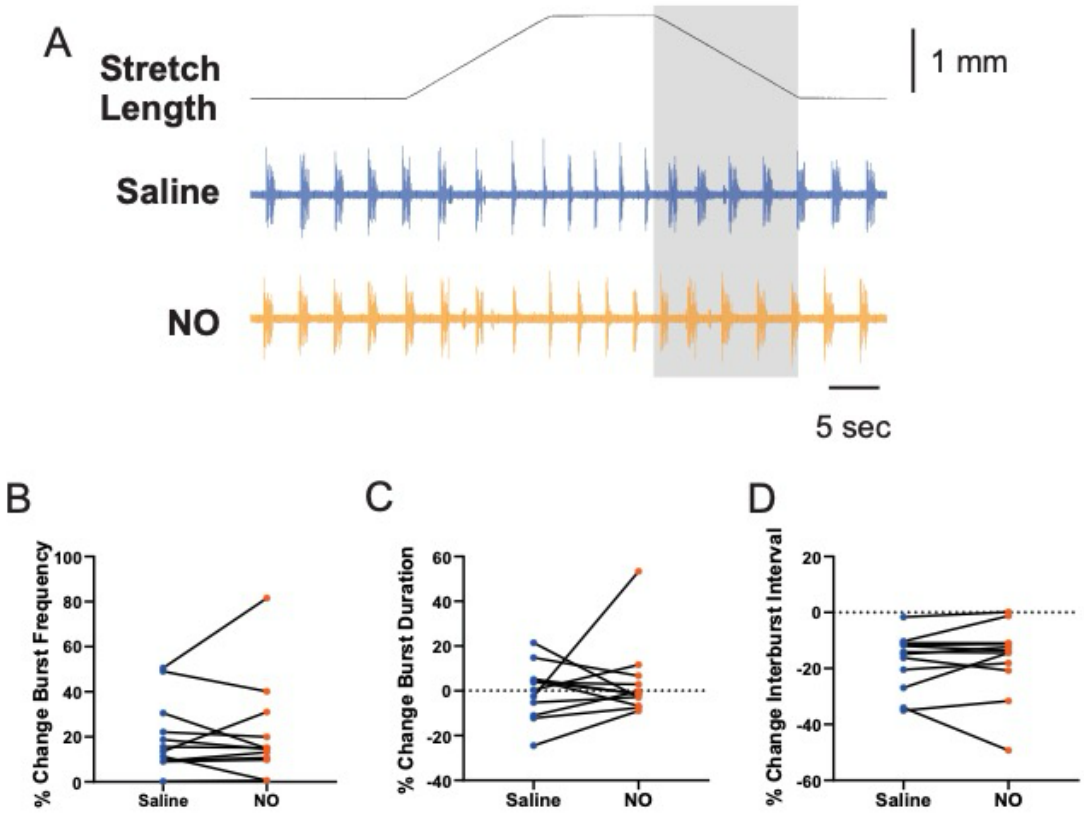

Figure 16. NO does not alter the stretch response during the release phase. The application of NO did not affect the relative change in (B) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.9097, n=12), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.8507, n=12), or (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.4238, n=12) during the release phase of the stretch response.

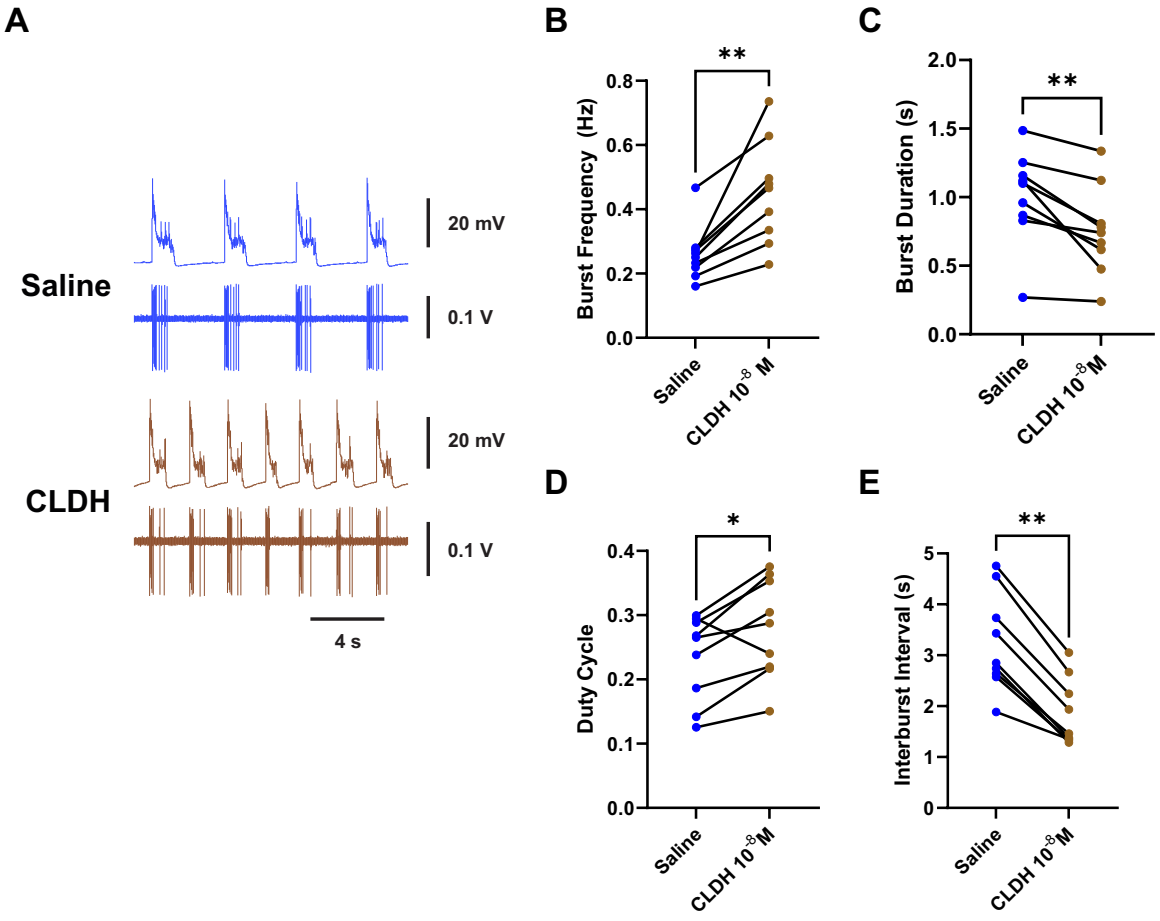

Figure 17. CLDH increases the burst frequency at 10^{-8} M in the isolated cardiac ganglion. CLDH increased the (A) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.0039, N=9), decreased the (B) burst duration (Wilcoxon matched-pairs signed rank test, p=0.0039, N=9), increased the (C) duty cycle (Wilcoxon matched-pairs signed rank test, p=0.0273, N=9), and decreased the (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.0039, N=9).

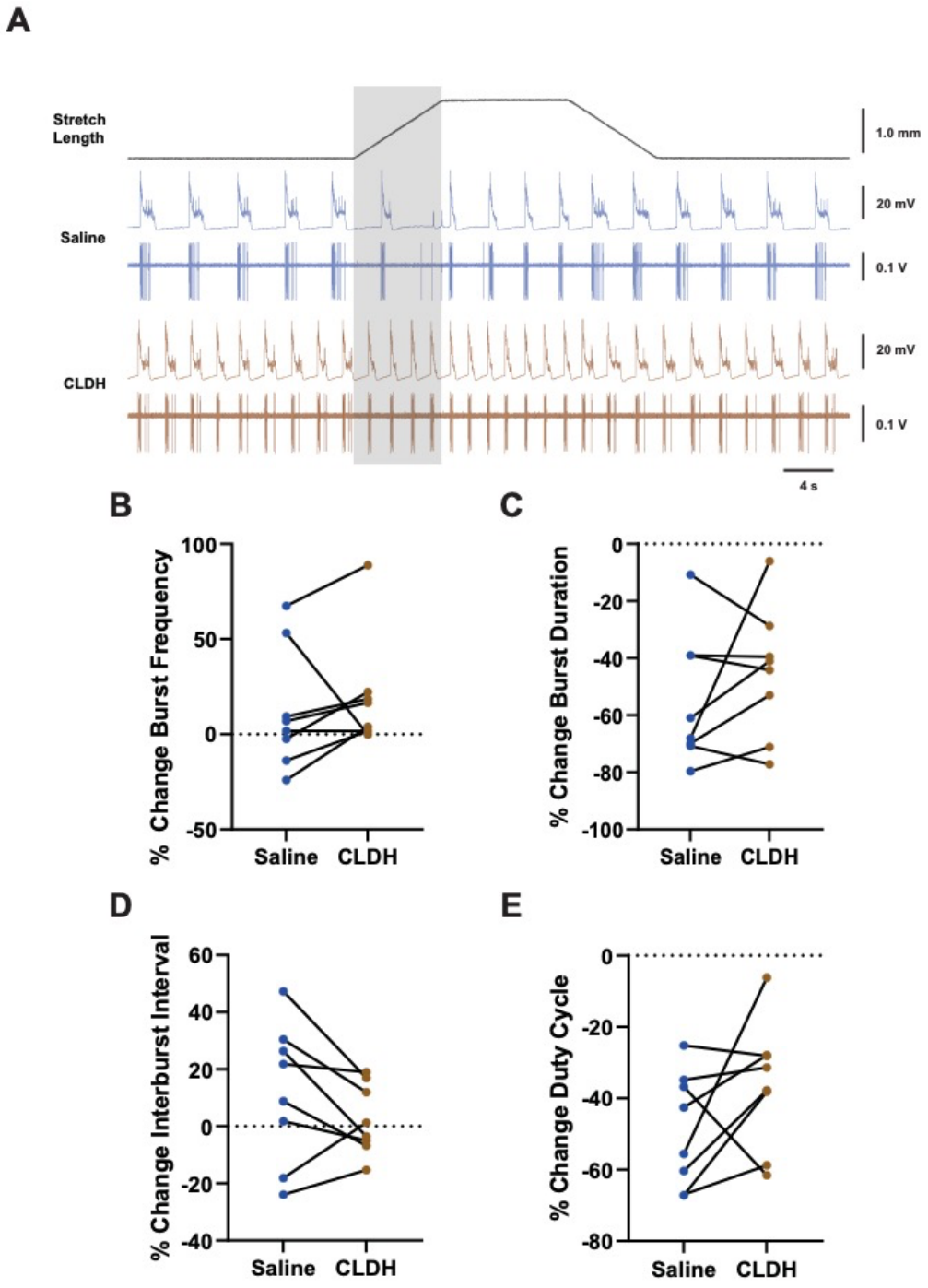

Figure 18. CLDH does not modulate average burst parameters during the rising phase of the stretch response. (A) Sample trace with a grey box indicating the rising phase of the stretch. CLDH did not affect the relative change in (B) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.25, N=8), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.4609, N=8), (D) interburst interval (Wilcoxon matched-pairs signed rank test, p=0.2500, N=8) or (E) duty cycle (Wilcoxon matched-pairs signed rank test, p=0.1484, N=8) during the rising phase.

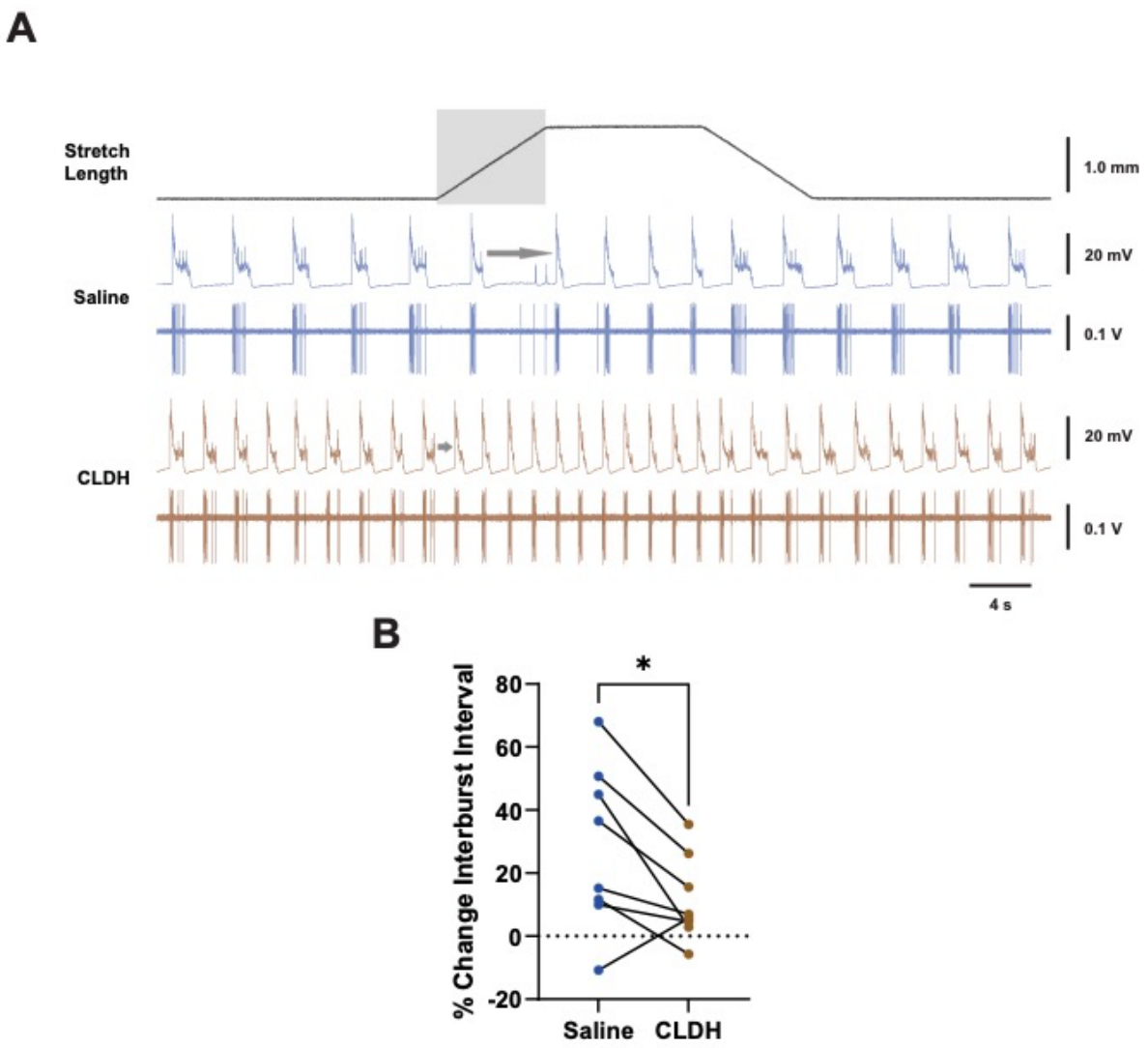

Figure 19. CLDH suppresses the increase in interburst interval of the burst with the longest interburst interval during the rising phase. (A) Sample trace with grey arrows indicating the bursts with the longest interburst interval in saline (blue) and CLDH (brown) that will be compared. (B) CLDH decreased the relative change in interburst interval for the burst with the longest interburst interval during the rising phase (Wilcoxon matched-pairs signed rank test, p=0.0391, N=8).

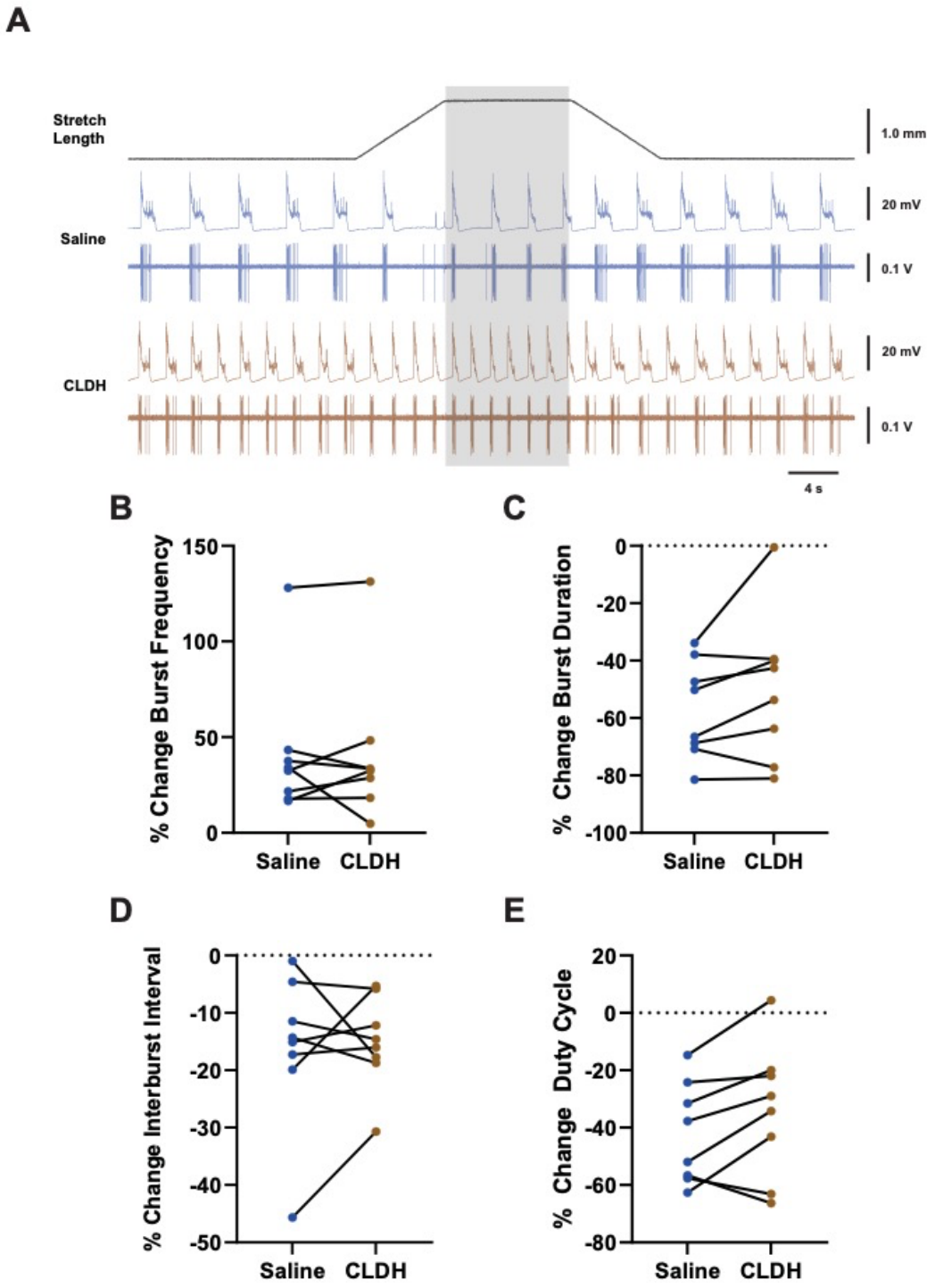

Figure 20. CLDH does not modulate the stretch response during the hold phase. (A) Sample trace with a grey box indicating the hold phase of the stretch. CLDH did not affect the relative change in (B) burst frequency (Wilcoxon matched-pairs signed rank test, p=0.0.8438, N=8), (C) burst duration (Wilcoxon matched-pairs signed rank test, p=0.1484, N=8), (D) interburst interval (Wilcoxon matched-pairs signed rank test, p>0.9999, N=8), (E) or duty cycle (Wilcoxon matched-pairs signed rank test, p=0.1094, N=8).

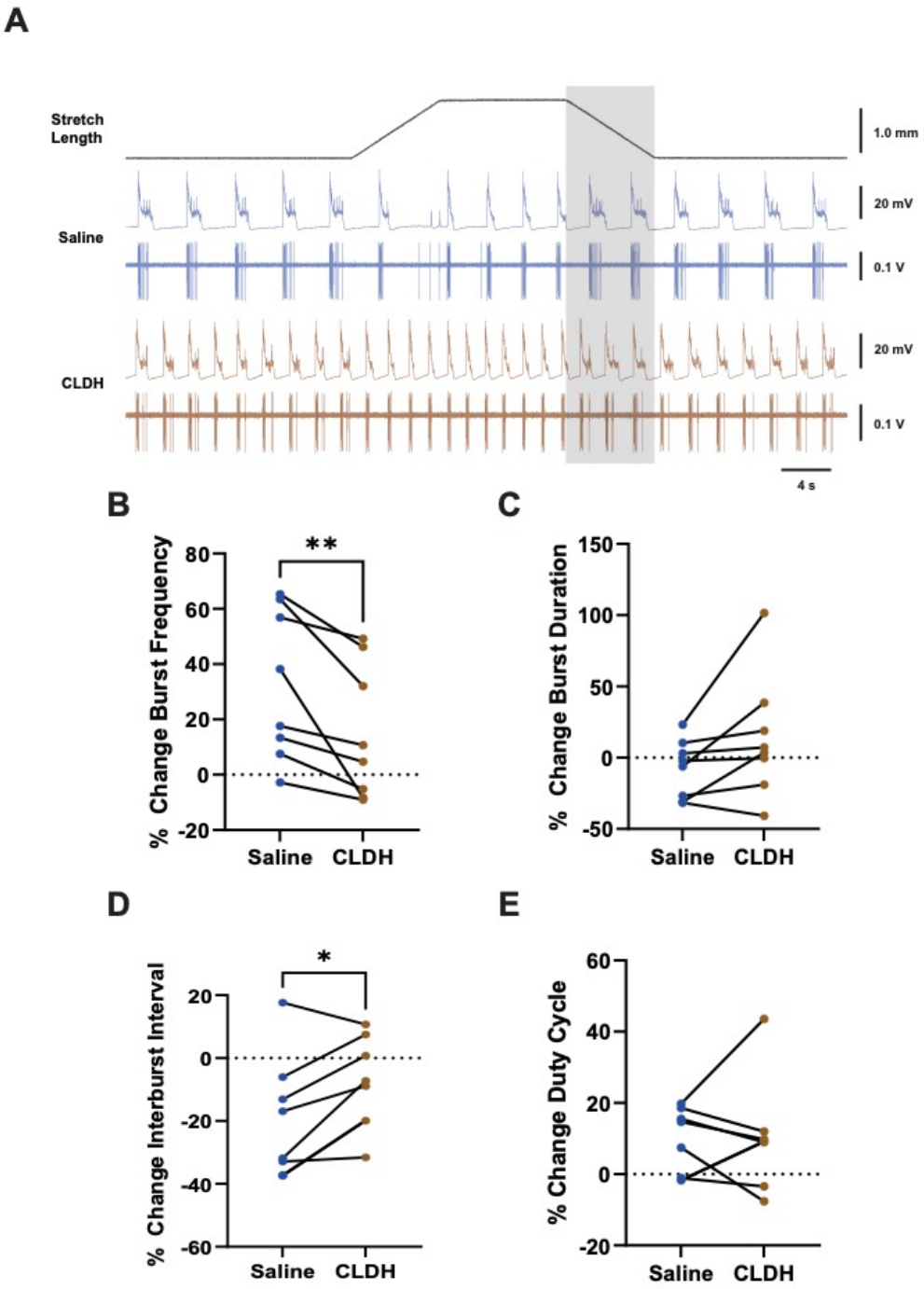

Figure 21. CLDH suppresses the stretch response during the release phase. (A) Sample trace with a grey box indicating the release phase of the stretch. (B) CLDH decreased the relative change in burst frequency (Wilcoxon matched-pairs signed rank test, p=0.0078, N=8). (C) CLDH did not affect the change in burst duration (Wilcoxon matched-pairs signed rank test, p=0.0781, N=8). (D) The magnitude of the relative change interburst interval decreased in CLDH (Wilcoxon matched-pairs signed rank test, p=0.0234, N=8). (E) CLDH did not affect the relative change in duty cycle (Wilcoxon matched-pairs signed rank test, p=0.9141, N=8).

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Appendix

To understand the effects of SGRN and GYS on the stretch response, previous students compared the stretch response in physiological saline, SGRN, and GYS. Students used 10^{-9} M and 10^{-8} M SGRN and GYS to compare the effects of lower and higher concentrations of both modulators on the stretch response. SGRN and GYS were expected to enhance the stretch response because both of these neuromodulators increase the contraction amplitude of the whole heart preparations at low and high concentrations (Dickinson et al., 2014). An increase in contraction amplitude caused by SGRN or GYS in combination with an increase the contraction amplitude induced by the stretch response was hypothesized to produce an even greater response to cardiac muscle stretch. Surprisingly, SGRN and GYS both suppressed the stretch response at 10^{-9} M and 10^{-8} M.

Here, we are presenting the data from the experiments that used 10^{-8} M SGRN and GYS. Previously, the relative changes in burst duration and interburst interval in saline and these modulators for each stretch phase were compared using paired *t*-tests. However, because of the variation in the relative change in the burst parameters, we believed a more useful test would focus on comparing the variation in the relative change of burst parameters between conditions. As a result, I used to Levene's test for equality of variances to compare the variances in the relative change in burst duration and interburst interval between physiological saline, SGRN, and GYS.

This updated analysis of these experiment shows that during the rising SGRN decreased the variability in the relative change in burst duration and interburst interval, bringing the relative changes in both of these parameters closer to zero (Figure 1). During the hold phase, SGRN decreased the variability in the relative change in burst duration and interburst interval, bringing

these changes closer to zero (Figure 2). During the release phase, SGRN decreased the variability in the relative change in burst duration, bringing the change in burst duration closer to zero, without affecting the variability in the change in interburst interval (Figure 3). GYS, on the other hand, decreased the variability in the relative change in burst duration, bringing this change closer to zero, but did not affect the variability in the relative change in interburst interval during the rising phase (Figure 4). Similar to SGRN, during the hold phase GYS decreased the variability in the relative change in burst duration and interburst interval, bringing these changes closer to zero (Figure 5). Lastly, GYS decreased the variability in the relative change in burst duration, bringing this change closer to zero, without affecting the variability in the change in interburst interval during the release phase (Figure 6). Combined, these results show that both SGRN and GYS suppressed the stretch response.

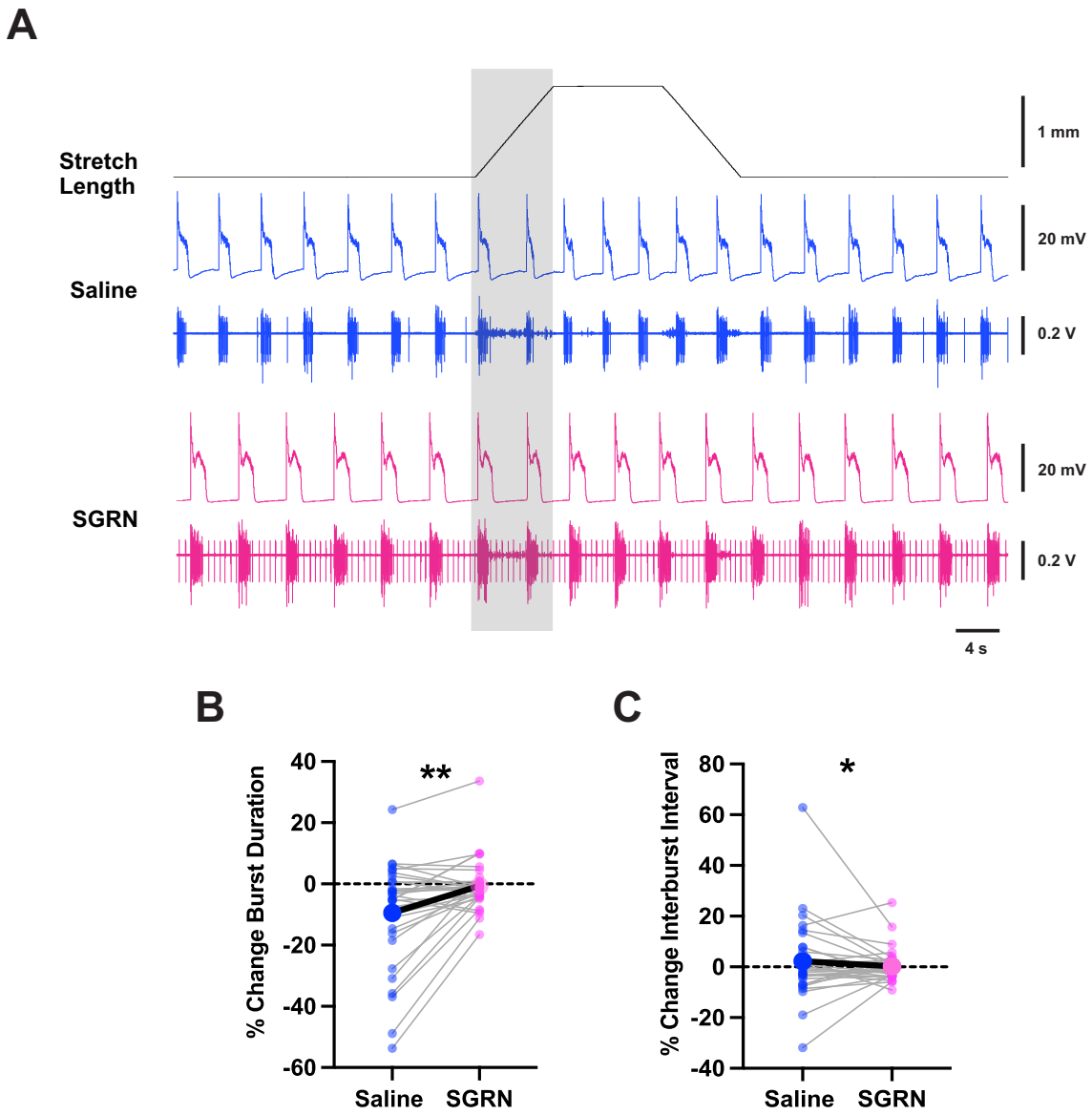

Figure 1. SGRN suppresses the rising phase of the stretch response. (A) Sample trace with a grey box indicating the rising phase of the stretch. SGRN decreased the variance in the change in (B) burst duration (Levene's test for equality of variances, p=0.0023, N=32) (C) and interburst interval (Levene's text for equality of variances, p=0.0260, N=32), bringing the change in these parameters closer to zero. Bolded points represent the mean relative change in burst parameters across preparations.

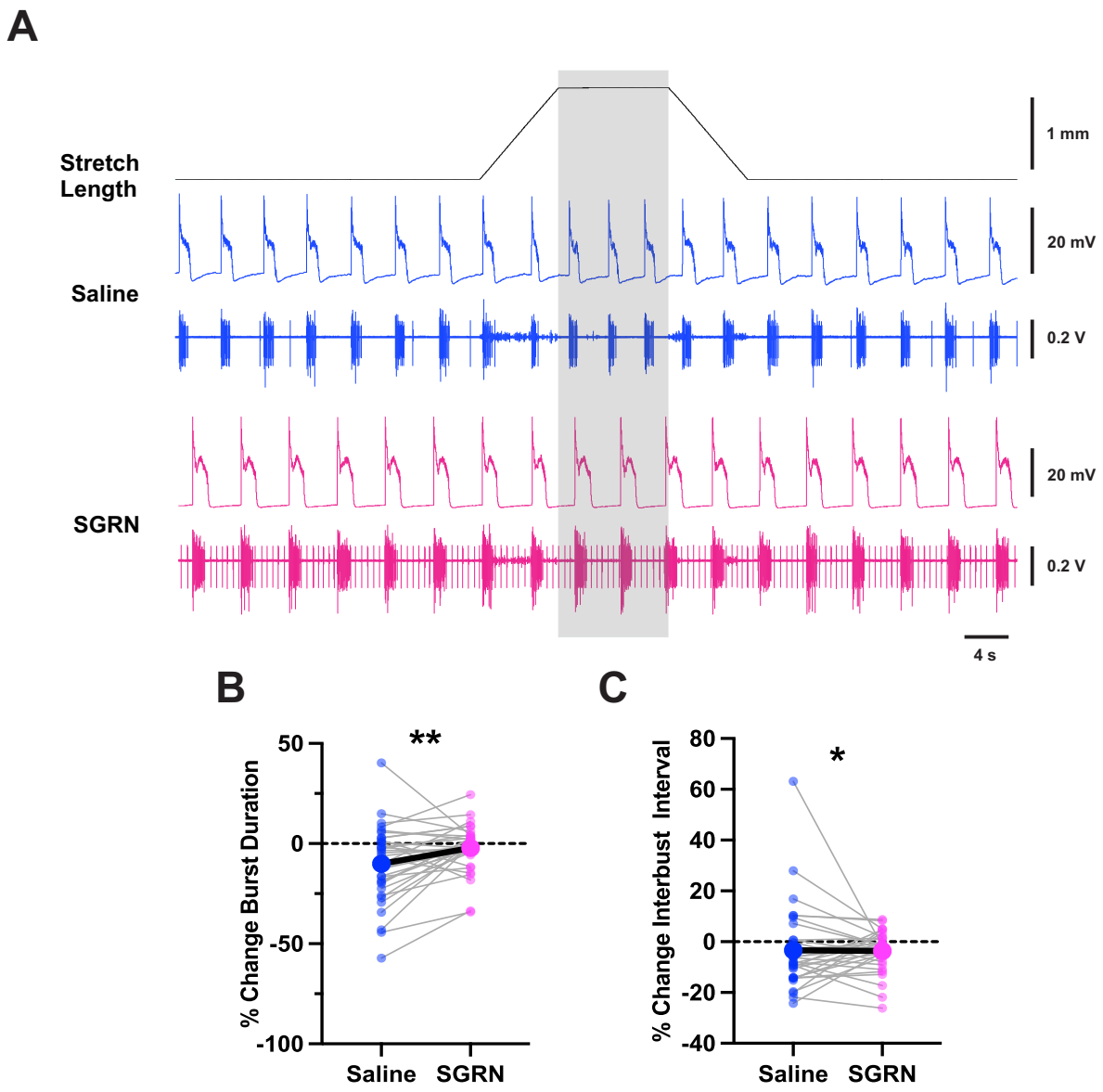

Figure 2. SGRN suppresses the hold phase of the stretch response. (A) Sample trace with a grey box indicating the hold phase of the stretch. SGRN decreased the variance in the change in (A) burst duration (Levene's test for equality of variances, p=0.0093, N=34) (B) and interburst interval (Levene's text for equality of variances, p=0.0309, N=34), bringing the change in these parameters closer to zero. Bolded points represent the mean relative change in burst parameters across preparations.

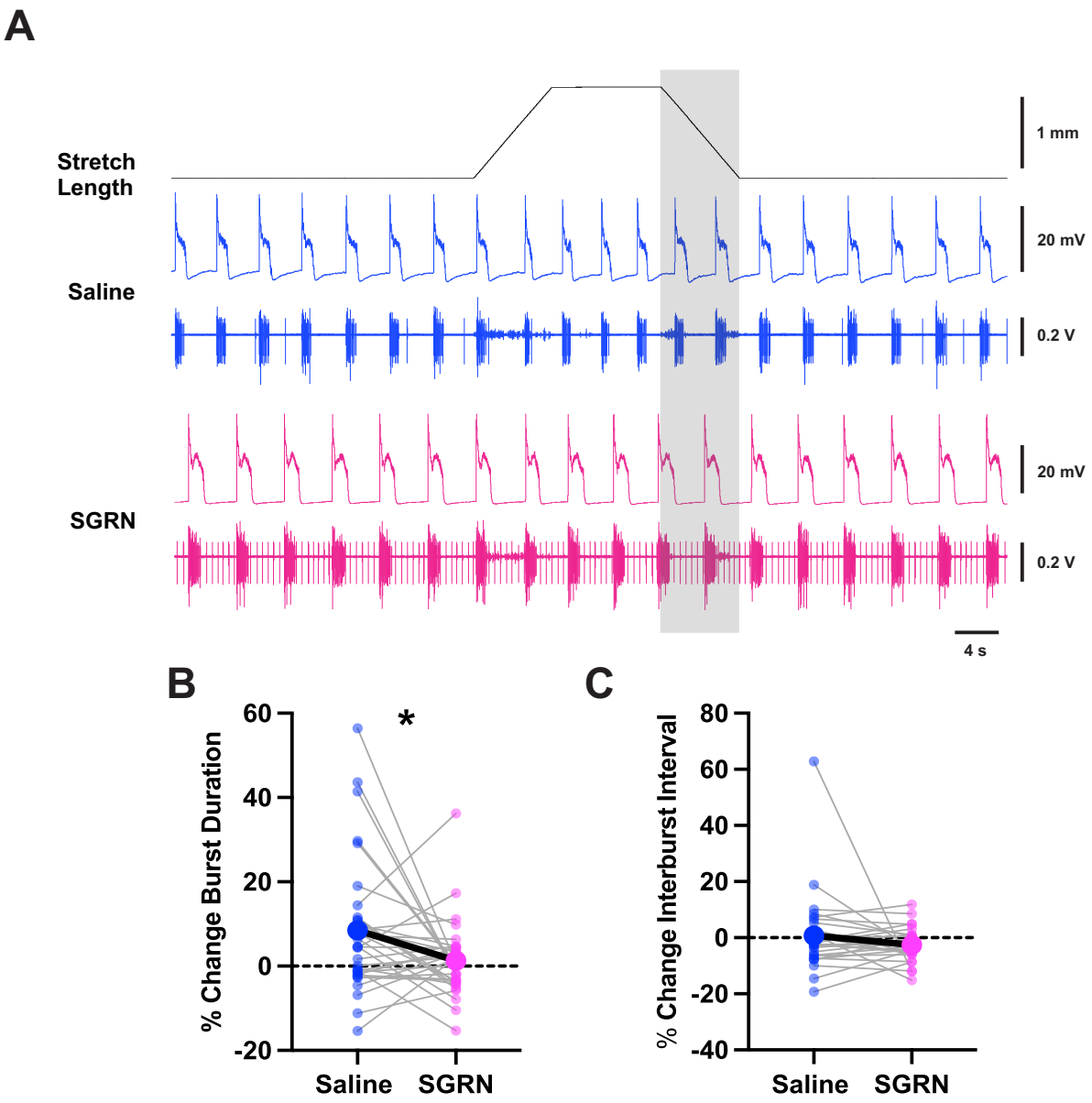

Figure 3. SGRN suppresses the release phase of the stretch response. (A) Sample trace with a grey box indicating the release phase of the stretch. SGRN decreased the variance in the change in (B) burst duration (Levene's test for equality of variances, p=0.0189, N=33), bringing the change in these parameters closer to zero. (C) SGRN did not affect the variance in the change in interburst interval (Levene's text for equality of variances, p=0.1283, N=32). Bolded points represent the mean relative change in burst parameters across preparations.

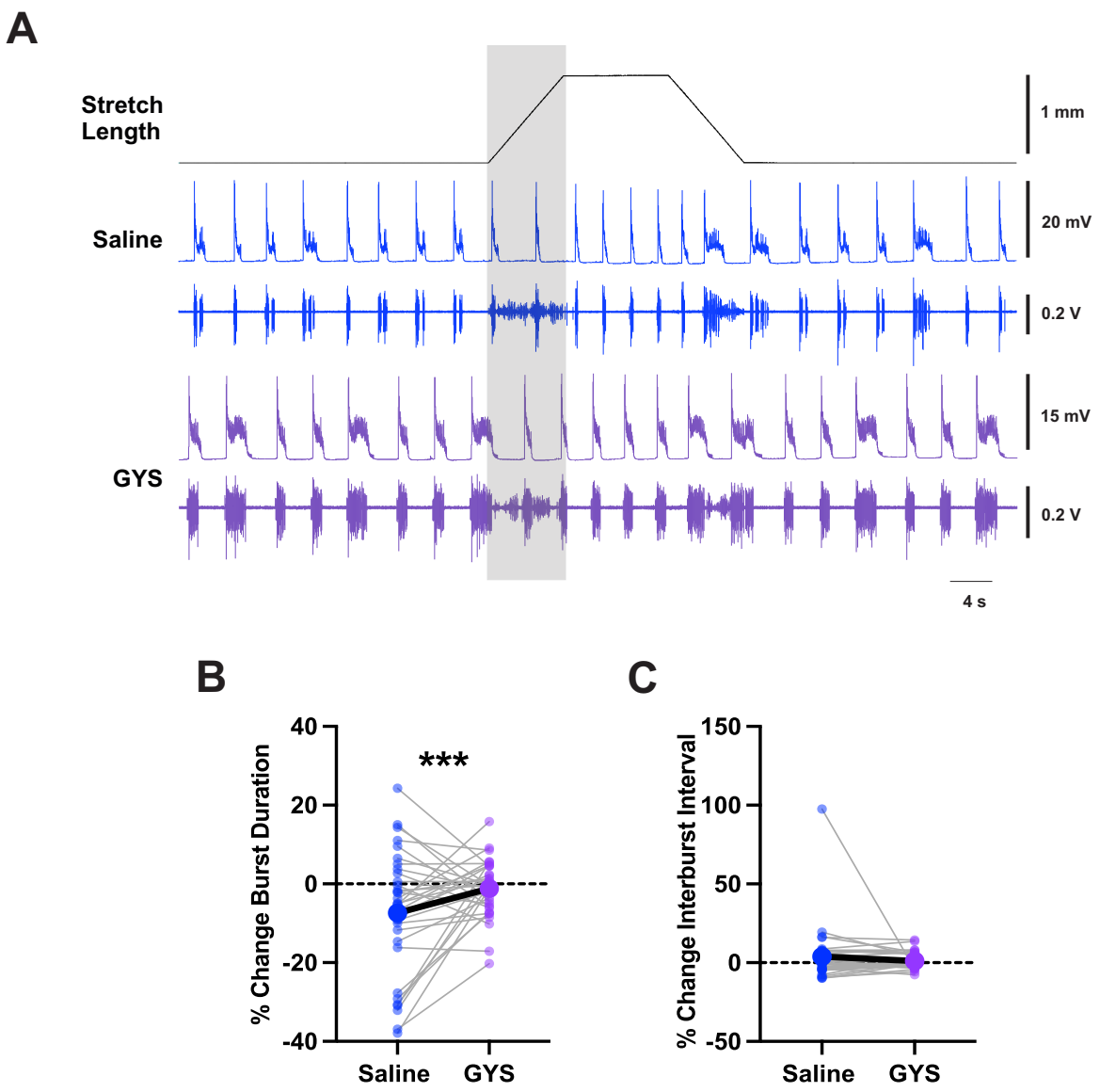

Figure 4. GYS suppresses the rising phase of the stretch response. (A) Sample trace with a grey box indicating the rising phase of the stretch. (B) GYS decreased the variance in the change in burst duration, bringing the change in burst duration closer to zero (Levene's text for equality of variances, p=9.55e-04, N=34). (C) GYS did not affect the variance in the change in interburst interval during the rising phase (Levene's text for equality of variances, p=0.0506, N=34). Bolded points represent the mean relative change in burst parameters across preparations.

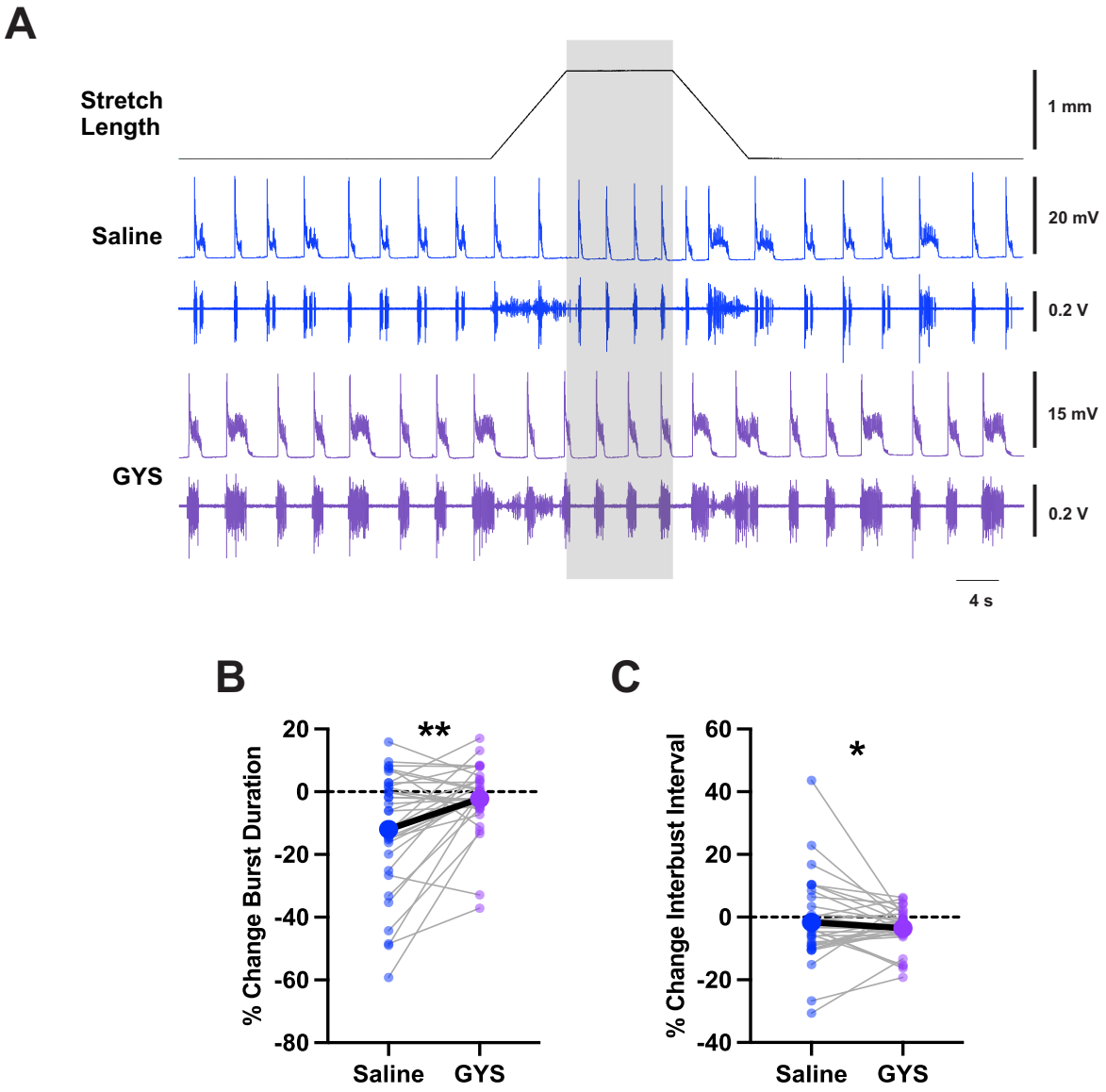

Figure 5. GYS suppresses the hold phase of the stretch response. (A) Sample trace with a grey box indicating the hold phase of the stretch. GYS decreased the variance in the change in (B) burst duration (Levene's test for equality of variances, p=0.0020, N=34) (C) and interburst interval (Levene's text for equality of variances, p=0.0120, N=34), bringing the change in these parameters closer to zero. Bolded points represent the mean relative change in burst parameters across preparations.

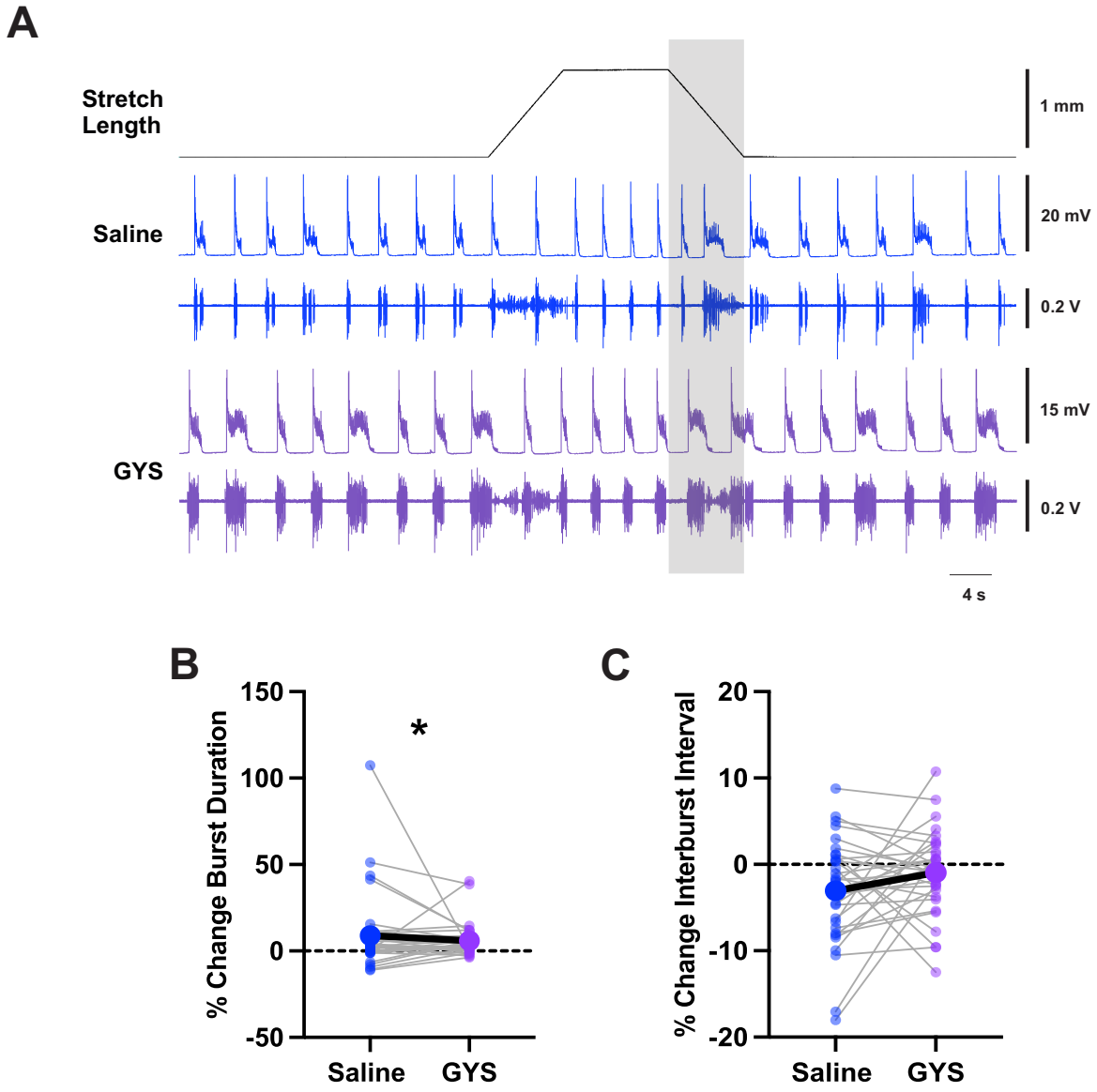

Figure 6. GYS suppresses the release phase of the stretch response. (A) Sample trace with a grey box indicating the release phase of the stretch. GYS decreased the variance in the change in (B) burst duration (Levene's test for equality of variances, p=0.0408, N=33), bringing the change in these parameters closer to zero. (C) GYS did not affect the variance in the change in interburst interval (Levene's text for equality of variances, p=0.2512, N=32). Bolded points represent the mean relative change in burst parameters across preparations.