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TEMPERATURE EFFECTS ON AN AXON'S ABILITY TO MAINTAIN PHASING OF A RHYTHMIC MOTOR SYSTEM

Marissa Elaine Cruz

57 Pages

The precise timing of action potentials generated in the nervous system is crucial for generating adequate behavior. Once generated, action potentials travel along axons towards the neurons or muscles they innervate. Axons are also responsible for preserving the temporal fidelity of the generated action potentials. One challenge axons face is that they can be of considerable length, and exposed to changes in internal and external conditions. Temperature fluctuations, for example, affect the ion channels that generate and propagate action potentials causing changes in action potential speed. It is unclear if, and how, the timing of action potentials can be preserved when action potential velocity changes. I used axons in the pyloric central pattern generator (CPG) of the crustacean stomatogastric nervous system to evaluate spike time arrival at different temperatures. CPGs are neural circuits that generate vital rhythmic behaviors including breathing and walking that must be robust to environmental challenges such as temperature fluctuations. To maintain functional behavior, the axons of CPG neurons must thus possess mechanisms to counterbalance detrimental temperature influences.

The pyloric CPG in the crustacean stomatogastric nervous system creates a well-defined triphasic rhythm generated by the lateral pyloric (LP), pyloric dilator (PD), and pyloric constrictor (PY) neurons. The pyloric CPG is robust to temperature fluctuations and produces a triphasic motor pattern that - similar to other rhythmic behaviors - requires a particular sequence

of neuronal activities ('phasing') for adequate functioning. While the CPG itself is temperature compensated, it is unknown how temperature affects the pyloric axons' ability to maintain adequate timing of action potentials. Pyloric axons possess different neuronal identities, indicating that their response to temperature may be heterogenous. I hypothesize that changes in conduction velocities during temperature changes are coordinated such that the functionally adequate phasic activity of the pyloric neurons is preserved in the periphery.

To test this, I first measured the axonal diameters of LP, PD and PY using a voltage-sensitive dye technique. I found that LP's axon is the largest and PY's axon is the smallest (N=6 for all axons, One-Way-ANOVA). I then determined how temperature affects conduction velocity in the different sized axons. Across temperatures, the pyloric axons had different conduction velocities (LP > PD > PY), and conduction velocities increased equally (N=8, One-Way-ANOVA). Consequently, the effects of temperature on action potential travel times differed: at high temperatures, LP's (largest axon) action potentials arrived much earlier than those of PY (smallest axon), disrupting functional behavior in the periphery. However, at lower temperatures the arrival times of LP's and PY's action potentials did not disrupt their phase relationships. In conclusion, phase relationships are maintained at lower temperatures at the muscles; however, at higher temperatures phase relationships were lost.

KEYWORDS: Central Pattern Generator; Axons; Timing; Temperature; Conduction Velocity; Action Potentials

TEMPERATURE EFFECTS ON AN AXON'S ABILITY TO MAINTAIN PHASING OF A
RHYTHMIC MOTOR SYSTEM

MARISSA ELAINE CRUZ

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RHYTHMIC MOTOR SYSTEM

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CHAPTER I: INTRODUCTION

Adequate Timing Of Action Potentials Is A Key Feature Of Neuronal Processing

The precise timing of action potentials arriving at the synapse is the foundation for encoding of neuronal information and pivotal for circuit-level plasticity, development, and learning (Lestienne, 2001). Precise spike time arrival is also crucial in sensorimotor systems. Adequate behavioral responses require the temporally precise processing of cues from sensory neurons and precisely timed motor commands. For example, localizing sound sources in the barn owl (*Tyto Alba*) involves the detection of coinciding action potentials traveling in opposite direction along delay lines in the brainstem (Carr & Konishi, 1988). For coincidence detection, the precise timing of action potentials arriving at the nucleus laminaris neurons is essential.

Motor systems also rely on precisely timed action potentials. For instance, in rhythmic systems, motoneurons generate various forms of bursting activity, and the precise timing within these bursts determines the behavioral output. In the stomatogastric nervous system (STNS) of the crab (*Cancer Borealis*), for example, the lateral gastric (LG) neuron generates differential burst activities under different modulatory conditions (Diehl, White, Stein, & Nusbaum, 2013). In specific, LG's burst is either continuous, or it shows intermittent pauses. LG is a motoneuron and drives the protraction movements of teeth within the stomach. While the different burst activities do not result in significant differences in overall firing frequency, the distinct temporal structures of the bursts are reflected in distinct teeth protraction movements.

Temporal dynamics at the neuromuscular junction may contribute to creating appropriate behavioral responses to distinct timing signals. For example, habituation in the gill-withdrawal reflex in *Aplysia* is a result of a depression of neurotransmitter release from the presynaptic terminal that leads to a progressively weaker activation of motoneurons (Gingrich & Byrne,

1985) . The degree to which this synapse is depressed depends on the interspike interval of action potentials arriving at the presynaptic terminal, with shorter interspike intervals causing stronger depression. Consequently, adequate neuronal signaling relies on the precise timing of action potentials at the synaptic terminal.

The Timing Of Action Potentials Changes As They Propagate Along The Axon

The function of axons is often compared to cable cords that reliably transmit information from the spike initiation zone to the output synapses. Contradictory to this oversimplification, axons have been shown to possess unique and complex dynamics that alter the temporal fidelity of action potential conduction and contribute to the computational capabilities of neurons (Dirk Bucher & Goaillard, 2011). For example, C-fiber axon trunks in the rabbit vagus nerve possess peripheral receptors for acetylcholine. Binding of acetylcholine to these receptors results in a depolarization of the C-fiber, a reduction in spike amplitude, and a slowing of conduction velocity (Armett, 1961).

Axons can also display a hysteresis in that conduction velocity depends on previous action potentials that propagated along the axon. This can be seen in the STNS of the lobster, *Homarus gammarus*, whose pyloric dilator (PD) neurons rhythmically burst once every second, with ~20 spikes per burst. Within these bursts, spike amplitude is diminished as a function of frequency, and spike duration increases over the course of the burst (Ballo & Bucher, 2009). More importantly, the conduction velocity for individual action potentials changes non-linearly within the burst. The first action potential, for example, is always faster while the second is slower. Conduction velocity and its history dependence are further altered in the presence of dopamine (Ballo, Nadim, & Bucher, 2012), indicating that action potential propagation speed is

state dependent. Consequently, depending on axon properties and modulatory conditions, the timing between action potentials, and thus also spike frequency and neuronal activity patterns may change between initiation site and arrival at the axon terminal.

Temperature Is An Environmental Challenge That A Nervous System Must Be Able To Overcome

Another condition that can affect action potential propagation in axons is temperature. Ambient temperature influences all living organisms, but none more than poikilothermic animals that cannot maintain constant body temperatures. Maintaining neural activity at different temperatures is a particularly difficult challenge for the nervous system, since temperature is a global factor that influences all biological processes. These biological processes that depend on temperature, include but are not limited to, ligand- and voltage-gated ion channels involved in action potential generation and propagation. Activation and inactivation rates range between two- and 100-fold with a 10°C increase (Tang, Taylor, Rinberg, & Marder, 2012). Consequently, temperature changes can have dramatic effects on action potential production and the encoding of stimuli in neurons.

However, there are examples where neural activity is temperature compensated. If all of the component processes that govern a system's behavior have identical temperature responses, the process should be temperature compensated (Robertson & Money, 2012). However, knowledge about how temperature responses are coordinated remains rather limited and there is evidence that biological processes that govern signal transduction and neuronal excitability vary substantially in their response to temperature (Tang et al., 2010). Furthermore, since temperature responses generally follow an exponential function, even modest differences in the temperature

responses of two processes may result in significantly different activity (Schmidt-Nielsen, 1997). Therefore, maintaining neural activity and its timing over a considerable temperature range is a challenge that neurons within a nervous system must work in concert to overcome. In the STNS of the crab, *Cancer Borealis*, the relative timing of several motoneurons is well maintained over a broad temperature range (Soofi et al., 2014). This system produces a triphasic motor pattern that control rhythmic muscle movements in the crab's pyloric filter. This pyloric rhythm is generated by three different neurons types that are rhythmically active at distinct phases of the rhythm (Selverston, Russell, & Miller, 1976). The sequence of activities of these neurons allows the pylorus to constrict and dilate in precise order to filter food (Selverston, et al., 1976). The relative timing between the activities of the contributing pyloric neurons (their phase relationships) is well maintained within and across animals and varies surprisingly little (D. Bucher, Prinz, & Marder, 2005). When exposed to higher temperatures, the speed of the pyloric rhythm increases. Despite this increase, the phase relationship of the motoneurons remains constant (Soofi, et al., 2014). Such temperature compensation may be achieved by a matched temperature response of several ionic currents with opposing functional roles in the pyloric central pattern generator (Tang, et al., 2010).

While the pyloric rhythm is inherently temperature compensated, this does not seem to be the case for another rhythmic activity produced by the same system: the gastric mill rhythm. The gastric mill rhythm is sensitive to temperature increase and terminates when temperature increases even modestly. In this case, however, the permissible temperature range is broadened by modulatory inputs from descending projection neurons that allow the gastric mill central pattern generator to continue to oscillate at elevated temperatures (Städle, Heigele, & Stein, 2015).

Temperature also affects action potential propagation along axons. Most notably described in the axons of giant squid, this phenomenon has been replicated in many other systems such as spinal cord ventral root fibers of rats (Rasminsky, 1973; Westerfield, Joyner, & Moore, 1978). Axons can have considerable lengths in comparison to their somata and dendrites, and may encounter significant temperature fluctuations if they project to peripheral targets. Changes in conduction velocity may thus affect the timing of action potential arrival at the target cells. This is particularly problematic in cases where multiple neurons with diverse axon morphologies cooperate to generate appropriate output (Otis & Gilly, 1990; Soofi, et al., 2014; Tang, et al., 2010), such as the aforementioned pyloric rhythm. Axons that have large diameters possess conduction velocities that are faster than axons with small diameters (A. Hodgkin, 1954). How temperature affects conduction velocity in axons with different diameters and how such temperature perturbations affect the timing between action potentials propagating along different axons is unknown. However, given that some systems reliably support the generated behavior over a broad temperature range, I hypothesize that axons of different diameters possess temperature responses that maintain the timing of action potentials between them when faced with temperature perturbations.

The STNS Offers Many Advantages To Study Temperature Effects On Axons

I have tested my hypothesis using the axons of pyloric neurons in the STNS of the Jonah crab, *Cancer borealis*. The STNS controls the filtering and chewing of food in all decapod crustaceans (W. Stein, Städele, & Smarandache-Wellmann, 2016). The pyloric rhythm controls the filtering of the food and is comprised of three motoneuron types (lateral pyloric neuron (LP), pyloric constrictor neurons (PYs), and PDs) that are spontaneously and rhythmically active even

when the nervous system is isolated from the animal (Figure 1, Right). The order in which these neurons fire, i.e. their phase relationship, is consistent within and across animals with only small variability (Hamood & Marder, 2015). Together, the three pyloric neurons interact to control the rhythmic dilation and constriction of the pylorus (W. Stein, 2009). The axons extend to the pylorus through the lateral ventricular nerve (*lvn*) and are ~4 cm long. They project dorsally along the external carapace and are thus more exposed to ambient temperature changes than the core parts of the respective neurons that reside in the stomatogastric ganglion, which by itself lies protected within the opthalmic artery (Fox, 2001). Experimentally, these rather long axons are readily isolated to expose the axons to temperature perturbations (large black circle in Figure 1, Left).

I have sought to determine how axons of the pyloric neurons respond to temperature fluctuations and how this affects their phase relationships at their target muscles. It has been shown that the diameter of an axon is proportional to its amplitude on an extracellular recording and can largely influence the conduction velocity of an axon (R. B. Stein & Pearson, 1971). More specifically, axons that have a larger diameter will have larger amplitudes on an extracellular recording and will have faster conduction velocities than smaller axons (R. B. Stein & Pearson, 1971). Given the different amplitudes of action potentials of the pyloric neurons on an extracellular recording, their axonal diameters should be different from one another. It is unknown how temperature affects action potential propagation in axons with distinct diameters. However, given that the central pattern generator itself is temperature-compensated (Tang, et al., 2012), it is sensible to assume that the respective axons of the motoneurons within the central pattern generator would also be temperature-compensated, so as to preserve the phase relationship of the pyloric neurons. My specific hypothesis is thus that the phase relationships of

PD, LP and PY are maintained in the periphery when temperature is changed, despite their different axon diameters.

CHAPTER II: RESULTS

Relating Extracellular Amplitude To Axon Diameter

A consistent characteristic of extracellular recordings from the *lvn* is the difference between the action potential amplitudes of PDs, LP and PYs (Figure A-1). Stein and Pearson [23] determined that the diameter of an axon is proportional to the cubic root of the extracellular action potential amplitude raised to the second power (diameter $\propto \sqrt[3]{amplitude^2}$). This indicates that the axons of PD, LP and PY are different diameters.

To show if the axon diameters of LP, PY and PD were significantly different from each other, I measured their action potentials amplitudes from nine animals and calculated the axonal diameters of LP, PY and PD using the aforementioned formula. Figure A-2 (Top, Left) shows an example extracellular recording from the *lvn* with one cycle of the pyloric rhythm. The amplitudes of the action potentials of LP, PY and PD were noticeably different from each other, which was more pronounced when looking at individual action potentials from LP, PY and PD (Figure A-2, Top, Middle). In all experiments, LP's action potential amplitude was always the largest ($0.052 \text{ mV} \pm 0.028$, N=9), PYs' action potentials amplitude was always the smallest ($0.026 \text{ mV} \pm 0.011$, N=9), and PDs' action potentials amplitude was intermediate ($0.036 \text{ mV} \pm 0.017$, N=9) (Figure A-2, Top, Right). After normalizing to the average amplitude of LP, PY and PD in each experiment, the relationship of LP having the largest amplitude (1.32 ± 0.17) and PY having (0.70 ± 0.12) the smallest amplitude was conserved (Figure A-2, Bottom, Left). I then converted the normalized extracellular amplitude to diameter, using the formula mentioned above. LP had the largest axon diameter (1.538 ± 0.302 , N=9), PD had an intermediate axon diameter (0.961 ± 0.178 , N=9) while PY had the smallest (0.594 ± 0.151 , N=9) (Figure A-2, Bottom, Right). Differences in these estimated diameter measurements between the different

pyloric axons were significant. Given these estimated diameter measurements, LP possessed an axon diameter 2.5 times larger than PY and 1.6 times larger than PD, and PD had an axon diameter 1.6 times larger than PY.

However, the amplitude of an extracellularly recorded action potential is determined by the strength of ionic current flowing through the axonal membrane [23]. Since ion channel conductance levels change with temperature [10], the amplitude of an extracellularly recorded action potential should also change. To test if this is the case for the pyloric axons, I varied the bath temperature in which the axons were kept between 3 and 22°C. I then measured each neuron's amplitude at different temperatures (see Materials and Methods). Figure A-3 (Top) shows three exemplar recordings from the *lvn* (4°C, 10.5°C and 20.5 °C). Individual LP, PD, and PY action potentials are shown below. The comparison of amplitudes at these different temperatures shows that LP's amplitude was always largest and PY's amplitude was always smallest. At 4°C, the amplitudes of all the pyloric neurons action potentials were decreased. At 10.5°C PYs' and PDs' amplitudes were much more similar in size than at 4°C but still much smaller than LP's amplitude, which was increased relative to its 4°C value. At 20.5°C, the difference in amplitude of LPs action potential to that of PD or PY was the largest in comparison to 4°C and 10.5°C. Additionally, the amplitudes of PDs' and PYs' action potentials were decreased relative to their 10.5°C amplitudes. Figure A-3 (Bottom, Left) shows the average amplitudes of all temperatures from one experiment. At any given temperature, LP always had the largest amplitude, and PY always had the smallest. The relationship between temperature and amplitude was overall nonlinear for all the pyloric neurons (Nonlinear regression analysis, LP: Adjusted $R^2=0.92$, $p<0.0001$, $N=5$; PD: Adjusted $R^2=0.67$, $p<0.0001$, $N=5$; PY: Adjusted $R^2=0.56$, $p<0.0001$, $N=5$). However, as temperature was increased from 3.0-12.0°C, the relationship between amplitude and temperature was highly linear

for all the pyloric neurons (Linear Regression analysis, LP: Adjusted $R^2=0.97$, $p<0.001$; $N=5$; PD: Adjusted $R^2=0.96$, $p<0.001$, $N=5$; PY: Adjusted $R^2=0.96$, $p<0.001$, $N=5$).

To better assess how temperature differentially affects the amplitude of LP, PDs, and PYs action potentials, I normalized all amplitudes of a given neuron to its respective amplitude at 10°C. At 10°C the amplitude of action potentials for all three pyloric neurons is defined as “1” (Figure A-4 left). The normalized amplitude values for each neuron is a measure of difference in comparison to its 10°C value. For example, in cases where PYs’ normalized amplitude is largest, indicates that the PY action potential amplitude increased more than that of LP or PD. In order to assess the magnitude of amplitude change between the different neurons, I calculated their change in amplitude as a percentage (Figure A-4, Right). At lower temperatures (3.0-4.5 °C), the amplitudes of all the pyloric neurons were decreased by a similar magnitude. At temperatures ranging from 5.0 °C to 7.5°C, PY’s amplitude decreased the most relative to its 10°C value, while PD and LP’s amplitudes were more similarly decreased. In the temperature range of 13.5-16 °C, the changes in amplitude between the different neurons yielded no observable trend. However, at temperatures ranging from 16.5-22.5°C, LP always had the largest increase in amplitude, while PD always had the largest decrease in amplitude. Overall, the change in LP’s amplitude was significantly different from the amount of change in PY’s or PD’s amplitude (Two Way ANOVA of temperature ($F(39,119) = 18.140$, $p<0.001$) and neuron type ($F(2,119) = 30.189$, $p<0.001$) LP vs PY($p<0.001$, $N=5$) and LP vs PD($p<0.001$, $N=5$) but a non-significant difference for PD vs PY($p=0.276$, $N=5$)). Collectively, my results indicate that the extent of change in the amplitude, because of temperature perturbations, was dependent on the neuronal identity.

To test whether temperature altered the amplitudes of the action potentials of pyloric neurons enough to affect their estimated axonal diameters. I normalized the amplitude of the action potential for each neuron at a given temperature to the average amplitude of the action potentials for all three neurons. I then took the normalized amplitude measurements and calculated their relative axonal diameters using the formula: diameter $\propto \sqrt[3]{\text{amplitude}^2}$. At lower temperatures, the changes in amplitude (Figure A-4) resulted significant differences between the calculated axon diameters of PD, LP and PY. (Figure A-5, Left). However, at higher temperatures, PD's and PY's calculated axonal diameter were no longer significantly different (Figure A-5, Left). The different estimated axonal diameters (Figure A-5, Left) caused the ratios of the diameters of the pyloric axons to be different at different temperatures and highly variable within a given temperature (Figure A-5, Right). For example, at 20.5 °C LP's calculated diameter was 5.3 times larger than that of PY's and 3.1 times larger than PD's, while PD's calculated diameter was 2.2 times larger than PY's. However, at 6.5 °C LP's calculated diameter was 7.2 times larger than PY's and only 1.8 times larger than PD's, and PD's calculated diameter was 3.8 times larger than PY's. Because of the variability seen in their estimated relative axon diameters (Figure A-4, Left), it was necessary to obtain actual axonal diameter measurements. Actual axonal diameter measurements were obtained by applying a voltage sensitive dye (Di-4-ANEPPDHQ) to the *l_{vn}*, which allowed me to stain and identify the axons [24] of the three motoneuron types and subsequently measure their diameter.

Voltage-Sensitive Dye Application Allows For Identification Of Pyloric Axons

In order to measure the diameters of the pyloric axons, I identified pyloric axons by measuring fluorescence changes in the stained axons contained in the *l_{vn}* (see Materials and

Methods). For this, fluorescence changes were averaged using the extracellularly recorded action potentials of LP, PY and PD as a time trigger (similar to (Städle & Stein, 2016)). This allowed me to identify the axons of the pyloric neurons based on the temporal correlation between action potentials in the optical and electrical recording (Figure A-6, Left and Right). Once the axons were identified, I then measured their diameters (Figure A-6, Middle) using photos of the stained axons (see methods).

I obtained diameters for all of the pyloric axons and found they differed according to neuron type (Figure A-7). To account for the natural variability of axonal diameter between animals, I normalized each neuron's axonal diameter to the average diameter of all three neurons within an experiment. After I obtained the normalized axonal diameter for each experiment, I then averaged across experiments for the individual neurons. Throughout all experiments, LP had the largest axon diameter (1.3 ± 0.18 N=6), PDs had an intermediate sized axon (0.94 ± 0.24 , N=6), and PYs had the smallest axon diameter (0.69 ± 0.21 , N=6). The axonal diameters of LP, PYs and PDs were significantly different from one another (1 Way RM ANOVA $F(2,16)=0.004$, LP vs. PY: $p=0.001$, LP vs PD: $p=0.017$, PY vs PD: $p=0.05$). In conclusion, it seems that larger diameter axons always have larger extracellular action potential amplitudes than smaller diameter axons (Figure A-3, right). However, the estimated axonal diameters for the pyloric neurons (Figure A-2, Bottom, Left) did not match the axonal diameter values I obtained in my voltage-sensitive dye imaging experiments (Figure A-7). More specifically, the estimated axonal diameters for LP (1.5 vs 1.3) and PD (0.96 vs 0.94) were larger than the actual diameter measurements. The opposite holds true for PY, the estimated axonal diameter for PY was smaller than the actual diameter measurement (0.59 vs 0.69). Overall, it seems that the formula relating

axonal diameter to extracellular action potential amplitude slightly overestimates how different axon diameters are based on differences seen in their extracellular action potential amplitude.

Conduction Velocity Increases Linearly With Temperature Increases For All Pyloric Neurons

To test how temperature affects the conduction velocity of action potentials of different axon diameters, I exposed a portion of the *lvn* to temperatures ranging from 4-23°C and measured the conduction velocity for LP, PY and PD (see Materials and Methods). My experiments show that the axons of the pyloric neurons had different conduction velocities at a control temperature of 13°C, with LP being the fastest (1.04 ± 0.23 m/s, N=6), PY (0.76 ± 0.14 m/s, N=8) being the slowest and PD having an intermediate speed (0.89 ± 0.09 m/s, N=8) (Figure A-8, Left). When I exposed the axons to different temperatures, conduction velocities in the axons of all three pyloric neuron types increased significantly with temperature (Figure A-8, Left). Across all temperatures, the relationship of LP having the fastest conduction velocity and PY having the slowest conduction velocity was maintained. Additionally, the conduction velocities increased equally between the three axons showing that they have similar responses to an increase of temperature. Thus, it seems that the relative velocity increase of an action potential from a temperature increase was independent of the size of the axon (Figure A-8, Right).

Because the conduction velocities of all the pyloric axons increased similarly, their arrival times at the axon terminal will be altered. The reason for this is that arrival times change with the reciprocal of the velocity. Consequently, with the same relative increase in velocity, the arrival times for already faster axons will change more than those of slower axons. As a result,

the timing of action potentials arriving at the axon terminal may be altered due to the difference axonal diameters of the pyloric neurons. The phase relationship of the pyloric neurons is measured using action potential timing between neurons. If arrival times change differentially between neurons, then a change of phasing in the pyloric rhythm is expected. To test whether could have a significant effect on the motor output, however, it is also necessary to determine if the expected change in phase is larger than the natural variability.

Pyloric Axons Maintain Phase Relationships At Lower Temperatures

I measured the arrival times of LP and PY relative to PD and used these arrival times to calculate how the phase relationship of the pyloric neurons changes when action potentials are exposed to temperature changes in the axon (see Materials and Methods). I then compared the resulting changes in phase to the narrow but nonetheless present range of variability of the phasing in the pyloric rhythm (Figure A-9). I measured the variability of the pyloric rhythm in each animal by taking the standard deviation of the onset of LP and PY bursts' at a control temperature of 13°C and then averaged the standard deviation across all animals (N=8) (see Materials and Methods). At any given temperature, a change in phase that exceeded two standard deviations was considered to be detrimental to the axons ability to maintain phase. I found that at all temperatures tested, the effect of the imposed temperature changes were too small to significantly affect PY (the smallest axon) onset phase. However, the change in LP's (the largest axon) onset phase exceeded two standard deviations of the natural variability at higher temperatures (19 and 23°C). In conclusion, at higher temperatures larger axons are more likely to discontinue phase maintenance compared to smaller axons.

CHAPTER III: DISCUSSION

The Pyloric Axons Have Distinct Diameters

The pyloric neurons that comprise the pyloric circuit are responsible for different aspects of the rhythmic dilation and constriction of the pylorus. The electrical activity patterns generated by the pyloric neurons provide a means to distinguish the different pyloric neurons from one another (W. Stein, et al., 2016). Their distinct electrical activity patterns are driven by their respective neuropils possessing different relative magnitudes of ionic conductances (Daur, Nadim, & Bucher, 2016), different connections to other pyloric neurons within the STG, as well as different receptors to neuromodulators (Marder & Thirumalai, 2002). The neuropile structure of the pyloric neurons are morphologically indistinguishable with respect to neurite location and fine dendritic structures (Otopalik et al., 2017). This indicates that pyloric neurons do not rely on their specific neuropile morphologies to support their respective functions.

In contrast, their axons possess quite distinct morphologies and propagation properties. The action potentials of the pyloric neurons have distinct amplitudes on extracellular recordings, providing an easy means to distinguish one neuron from another. The relationship of extracellular action potential amplitude and axonal diameter has been described in many animal model systems, such as giant squid, cockroaches and locust (R. B. Stein & Pearson, 1971). More specifically, these studies demonstrate that neurons that possess larger diameter axons have larger amplitudes on an extracellular recording in comparison to neurons with smaller diameter axons. This is suggested by the assumption that when axons maintain a set density of ion channels (R. B. Stein & Pearson, 1971), larger diameter axons will have more ion channels than smaller diameter axons. Consequently, the current flow across the axon membrane is increased, and so is the electrical field surrounding the action potential. The latter is then measured by

extracellular recordings. The relationship between axonal diameter and extracellular action potential amplitude is described using the formula: diameter $\propto \sqrt[3]{amplitude^2}$ (R. B. Stein & Pearson, 1971). My results indicate that one of the factors that affect the relationship between diameter and amplitude is the temperature of the axon. I showed this by directly measuring the diameters of the pyloric axons and comparing these diameters to the extracellular amplitudes of the pyloric axons. My results demonstrate that temperature significantly affects the amplitudes of extracellular action potentials of the pyloric neurons in a nonlinear fashion. The nonlinear relationship between temperature and amplitude is separated into a rising phase and a plateau phase. The rising phase occurred at low temperatures, while the plateau phase occurred at higher temperatures. This finding may be explained by the fact that extracellularly recorded action potential amplitudes are generated by temperature-sensitive ionic conductances (A.L. Hodgkin & Katz, 1949). The sensitivity of these ionic conductances to temperature changes has been characterized with intracellular recordings of axons and somata (Frankenhaeuser & Moore, 1963; A.L. Hodgkin & Katz, 1949; Städele, et al., 2015; Xu & Robertson, 1994). Intracellular action potential shape is mostly dependent on sodium and potassium currents, but also on leak in the axon (A. L. Hodgkin & Huxley, 1952). The sodium current causes a depolarization of the membrane potential while an increase in leak current will shunt the depolarizing effects of the sodium current. Leak and sodium currents have opposing effects on the membrane potential.

Extracellular recordings reflect the ionic currents that contribute to action potential generation. The peak amplitude is mostly dependent on the inward sodium current (A. L. Hodgkin & Huxley, 1952). As temperature increases, however, the sodium current is augmented due to an increased diffusion and faster gating (Frankenhaeuser & Moore, 1963). Similarly, the leak increases. However, the leak current does not have any gating properties and thus the

temperature effects on leak are dependent on diffusion speed. Studies in giant squid support this idea and indicate that the resting membrane potential (reflective of the leak current) of an axon appears less affected than sodium currents until higher temperatures ($\sim 20^{\circ}\text{C}$) at which point it becomes hyperpolarized (A.L. Hodgkin & Katz, 1949). Assuming similar dynamics in pyloric neurons, action potentials would be shunted at higher temperatures as a result of the leak current reducing intracellular action potential amplitude and thus the sodium current. The plateau phase of LP seen in my results may be a result of the leak current not being strong enough to effectively shunt the axonal membrane. My results are consistent with the hypothesis that the linear increase in amplitude with temperature seen during the rising phase is due to increasing permeability for sodium in the axon membrane. However, during the plateau phase, amplitudes no longer increased, and two of the pyloric neurons showed even a slight decrease (PD and PY). A potential explanation as to why action potential amplitudes did not further increase with temperature may be reflective of the gating properties of sodium channels being altered by temperature in such a way that they are no longer able to support the increased diffusion of sodium. At the same time, the increase in leak current may have been large enough that it effectively shunted the axonal membrane, leading to smaller and shorter action potentials, with accordingly less sodium current. It should be noted that studies in the somata of motoneurons in locust and in crabs, have shown that even modest temperature increases causes the leak conductance to increase (Städele, et al., 2015; Xu & Robertson, 1994). However, my results indicate that the effects of temperature on the leak conductance is more similar to the axons of the giant squid given that the plateau phase did not occur until higher temperatures.

The amplitude measurements taken from the pyloric neurons were segregated into either the rising phase or the plateau phase, the change in amplitude was different between the pyloric

neurons. Each ion channel has a given Q_{10} , which describes how sensitive a specific channel is to temperature changes. Q_{10} 's for ion channels such as those belonging to the TRPV family, for example, can be as high as 21 (Benham, Gunthorpe, & Davis, 2003). However, the Q_{10} s for the ion channels that are important for the phase at which LP and PY recover from inhibition (I_A and I_H), range from 1.2-1.5—rendering them temperature compensated (O'Leary & Marder, 2016). The total amount of current flow through a membrane is determined by both the Q_{10} of an ion channel and how many ion channels are present. Thus, the effects of temperature on current flow over an entire membrane will not be the same as the temperature effects of a specific ion channel. When taking into account the number of ion channels (which is not the same between different axonal diameters), the effects of temperature on the magnitude of current flow through the axonal membrane will be different between axons that have different diameters. This seems to be the case for my experiments, as the changes in an action potentials amplitude were different between the pyloric neurons.

Linear Vs. Nonlinear Temperature Effects On Action Potential Conduction Velocity And Amplitude

The extracellular amplitude of action potentials is proportional to the diameter of the axon along which the action potential propagates. Similarly, action potential conduction velocity is proportional to axonal diameter in non-myelinated axons. This relationship was first demonstrated in the stellar nerves of squid and cuttlefish (Pumphrey & Young, 1938). Specifically, axons in the cuttlefish are smaller and have slower conduction velocities than those in squid. Pumphrey and Young concluded that conduction velocity was proportional to diameter as: $velocity \propto \sqrt{diameter}$. This relationship has been confirmed in studies that related

conduction velocity to axonal diameter within a single species such as: axons within the stellar nerve of giant squid, axons within the extensor nerve in insect legs, and the axons involved in the movement detector pathways in locust (Card, 2012; A. Hodgkin, 1954; Hoyle, 1977). Thus both the amplitudes and conduction velocities of action potentials in non-myelinated axons are related to the diameter of the axon along which the action potential is conducted.

However, mammals have evolved an alternative strategy to increase their axonal diameter in order to increase conduction velocity, namely by wrapping segments of their axons in a non-conductive myelin sheath (Hartline & Colman, 2007). Wrapped segments are separated by Nodes of Ranvier, where the axon is not wrapped in myelin sheath. At the Nodes of Ranvier, action potentials propagate the length of an axon through saltatory conduction (Hartline & Colman, 2007). While some types of mammalian axons are larger than others to ensure conduction velocity is increased (Gasser, 1941), it seems that mammals do not increase their axonal diameter to the extent that invertebrates do (Hartline & Colman, 2007).

Temperature perturbations has been shown to cause conduction failure in both myelinated and non-myelinated axons in mammals. This has been demonstrated in the saphenous nerve of cats, which contains both myelinated and non-myelinated axons. Axons within the saphenous nerve are responsible for transmitting sensory information from the legs and are more likely to go through conduction failure as temperature is decreased (Franz & Iggo, 1968). This study shows that myelinated axons are less robust to colder temperatures than non-myelinated axons and are more likely to go through conduction failure as temperature is decreased. However, some axons are more resistant to temperature changes and do not display conduction failure over a broad temperature range (Young, Peck, & Matheson, 2006). In a study that compared the conduction velocities of action potentials in non-myelinated axons of four squid

species over a broad temperature range, conduction velocity increased linearly as temperature increased- for all species (Rosenthal & Bezanilla, 2002). My results are consistent with these findings for different neurons in the same species, since LP, PD and PY propagation velocities also increased linearly as temperature was increased.

In contrast, temperature had nonlinear effects on the extracellular amplitude of these neurons, despite the fact that both, amplitude and conduction velocity are related to axonal diameter. These differences may be attributed to the underlying processes of how temperature affects conduction velocity and amplitude. Conduction velocity increases when the transition rate constants of the activation and inactivation gates of sodium channels increases (α value describes the gates transition from closed to open, β value describes the gates transition from open to closed (Fohlmeister, 2015)) or by decreasing membrane capacity and is relatively insensitive to changes in leak conductances alone (R. B. Stein & Pearson, 1971). Hodgkin and Huxley assumed the α and β values possessed Q_{10} s of 3.5 for both the activation and inactivation gates (A.L. Hodgkin & Huxley, 1951). However, modeling of mammalian retinal ganglion cells conclude that the α and β values for both the activation and inactivation gates possess Q_{10} values of 2.2 and 14.4, respectively (Fohlmeister, 2015). In giant squid axons, it was shown that an increase in temperature causes membrane capacitance to increase (Taylor, 1965). However, action potential amplitude is largely affected by the amount of leak conductance, but is relatively insensitive to changes in the gating rate constants, or capacitance (R. B. Stein & Pearson, 1971). Studies in the axons of giant squid demonstrate that the leak conductance increases as temperature is increased (A.L. Hodgkin & Katz, 1949). Thus, the conflicting effects of temperature on conduction velocity and amplitude seen in my results could be a consequence of

conduction velocity and amplitude being governed by a distinct set of parameters that possess a unique response to temperature changes.

In the pyloric axons used in my study, the changes of these parameters with temperature are unknown, and measuring these variables is experimentally difficult. Nevertheless, because conduction velocity increased as temperature increased, it is unlikely that capacitance accounts for my results. Capacitance is not a likely explanation for my result because capacitance and conduction velocity have an inverse relationship (R. B. Stein & Pearson, 1971) while temperature and capacitance have a direct relationship (Taylor, 1965). Instead, my results indicate that the increase in conduction velocity may be due to the temperature-sensitive gating rate constants. The Q_{10} values for both α and β values and for both gates, imply that conduction velocity would increase as temperature is increased. However as some systems have demonstrated, the Q_{10} of the beta value can be much larger than the Q_{10} of the alpha value (Fohlmeister, 2015), which suggests that the amplitude would be decreased at higher temperatures because the channel is inactivated sooner and at a more hyperpolarized membrane potential. This is consistent with the amplitudes of PD and PYs action potentials being decreased at higher temperatures. In conclusion, my results suggest that the extracellular action potential amplitude is likely determined by sodium and leak currents, while the conduction velocity of an action potential is likely determined by the gating properties of sodium channels.

Conduction Velocity Increased Linearly For All Pyloric Axons

It is well established that conduction velocity is proportional to axonal diameter, and action potential conduction velocity for a given axon diameter increases with temperature (Card, 2012; A. Hodgkin, 1954; Hoyle, 1977; Pumphrey & Young, 1938). However, these studies

provide little insight into how temperature-induced changes in conduction velocity compare between neurons with different axon diameters.

My results show that the conduction velocity of LP's (largest diameter axon) action potential is fastest, and the conduction velocity of PY's (smallest diameter axon) action potential is slowest, respectively, at all temperatures. Conduction velocity increased in all neurons, regardless of diameter. The rate at which conduction velocities increased was similar for each neuron. This is at least partly surprising, given that LP, PD and PY neurons have quite distinct ionic conductance profiles: When measured in the STG, the levels of mRNA expression of different ion channels is significantly different between PD and LP. For example, PD expresses more mRNA for shab I_{Kd} and I_A than LP and LP expresses more mRNA for I_h channels (hyperpolarization-activated inward current), shaw I_{Kd} , and Na^+ channels than PD (Schulz, Goaillard, & Marder, 2007). Similarly, the ionic currents corresponding to these mRNA profiles are correlated to conductance densities, where higher expression levels for a given ion channel results in a higher ion density (Schulz, Goaillard, & Marder, 2006). These differences in ion channel expression levels between the pyloric neurons suggest that while they possess the same complement of channels, the ion channel densities are quite distinct. However, whether this distinctiveness holds true for their axons is unknown. Fast sodium currents, for example, have never been measured in STG neurons, and there is no data available for postassium currents in the respective axons. However, since the rate at which conduction velocity increased was similar for all neurons, pyloric axons are unlikely to possess vastly different ion channel densities.

Phase Maintenance

LP, PD, and PY neurons are part of the pyloric CPG, - a class of neuronal circuits responsible for the generation of rhythmic behaviors such as breathing, walking and chewing (Wolfgang Stein, 2015). Within CPGs, single neurons will alternate between periods of quiescence and periods of generating many action potentials (constituting one burst). More importantly, different neurons burst at different phases within a CPGs cycle (Marder & Calabrese, 1996). Many studies have demonstrated how a change in phase relationships produced by CPGs directly impacts animal behavior. For example, studies in mice show that breathing requires the continuous rhythmic output of a CPG, located within the pre-Botzinger complex (PBC) of the medulla (Lieske, Thoby-Brisson, Telgkamp, & Ramirez, 2000). PBC neurons are divided into expiratory neurons, early inspiratory neurons, and late inspiratory neurons, and are active at distinct phases relative to another. When environmental conditions are optimal these neurons generate activity that allow for eupneic (normal) breathing. Under hypoxic conditions, the phase relationship of the respiratory neurons is altered, the late inspiratory neurons are active earlier and all the neurons are active more. The hypoxic-induced changes in bursting activity of the respiratory neurons is directly reflected in the breathing behavior, which is marked by more frequent and more shallow breaths (Lieske, et al., 2000). Effects of phase relationship changes on behavior are also seen in the gastric mill CPG of the STNS. Here, the gastric mill motoneurons innervate muscles that drive rhythmic protractions and retractions of the medial and paired lateral teeth within the stomach. The pro- and retractor neurons are typically active in alternation (i.e. at an about 50% phase offset) during spontaneous chewing. This results in a chewing movement where all teeth protract together, followed by their simultaneous retraction (*squeezemode*;(Heinzel, 1988)). Occasionally, however, the phase

relationship of the lateral tooth protractor and the medial tooth retractor neurons changes, leading to simultaneous bursts rather than alternating ones. This has marked consequences for the chewing behavior in that the lateral teeth are now protracted while the medial tooth is retracted, leading to a quite distinct cut and grind movement (Heinzel, 1988)

Because changes in phase relationships affect behavior, systems have developed strategies to ensure that phase relationships are maintained when environmental conditions change. In the pyloric CPG of the STNS, the pyloric CPG generates phasic activity between 4-30°C, both *in vivo* and *in vitro* (Tang, et al., 2010; Tang, et al., 2012) Across this wide temperature range, the phase relationships of LP, PY and PD) are held constant (Tang, et al., 2010). Modeling studies suggest that temperature compensation is achieved by having a matched temperature response of ionic currents with opposing functional roles, such as I_A and I_H ((Tang, et al., 2010)). However, the aforementioned study focused on the generation of phasic activity (at the CPG) and not how phase relationships are maintained as action potentials propagate towards the periphery. It is surprising that the effects of temperature on the axons of CPGs are not well described because axons are primarily responsible for transmitting information from the somata (where phasic activity is generated) to the axon terminal.

My results indicate that the pyloric axons maintain phase relationships of the pyloric neurons from temperatures ranging from 4-19°C. At temperatures below 19°C all the pyloric neurons' action potentials had arrival times at their distal axonal regions that maintained the phase relationships generated at the pyloric CPG. What properties of the pyloric neurons support phase maintenance? In lobsters, neuromodulatory influences have been implicated in antagonizing detrimental temperature effects. For example, force production of the muscle innervated by PD becomes compromised at 15°C, suggesting that the pylorus is no longer able to

filter food at elevated temperatures. One explanation for the loss of force production at elevated temperatures could be that transmitter release at the presynaptic terminal is compromised at higher temperatures similar to temperature effects at the neuromuscular junction of frogs (Barrett & Stevens, 1972). Another explanation for the loss of force production at higher temperatures could be due to an increase in leak at the muscle structure, which would shunt the motor end plate potential at the neuromuscular junction. Regardless of the mechanism of the loss of force production, exogenous application of dopamine restores muscle force production at all temperatures and abolishes the temperature dependence of the neuromuscular system (Thuma, Hobbs, Burstein, Seiter, & Hooper, 2013). In the STNS there are no known modulatory neuronal inputs to any of the pyloric muscle structures. This indicates that dopamine would likely exist in the hemolymph. Humoral release was unlikely to play a role in my experiments however, since the STNS was isolated from the animal and thus free from humoral influences (Zhang, Buchberger, Gajanthan, & Lingjun, 2015). Neuronal release of peptides from neuroendocrine neurons in the STNS, such as the post-oesophageal commissure (*poc*) seem similarly unlikely. *Poc* activity initiates gastric mill rhythms and increases the frequency of the pyloric rhythm (Blitz et al., 2008). In my experiments, I did not see any gastric mill related activity and did not see an increase in the frequency of the pyloric rhythm from any temperature manipulation.

Modeling suggests that the pyloric circuit of the STG is able to maintain phase relationships across a broad range of temperatures through specific ionic currents with opposing functional roles having similar Q_{10} 's. More specifically, the transient outward current, I_A and the hyperpolarization-activated inward current I_h are known to largely contribute to the phase of LP and PY's recovery from inhibition (Harris-Warrick, Coniglio, Barazangi, Guckenheimer, & Gueron, 1995; Harris-Warrick, Coniglio, Levini, Gueron, & Guckenheimer, 1995; Tierney &

Harris-Warrick, 1992). The model implemented in this study shows that temperature compensation of phase is best achieved when I_A and I_H have similar and low Q_{10} values. In my experiments, the conduction velocity of all three pyloric neurons increased similarly with temperature, meaning their conduction velocities have similar Q_{10} s. Additionally, the increase in conduction velocity was not significant enough to alter phase relationships in the periphery at temperatures below 19°C —indicating that their Q_{10} 's were relatively low. However, the Q_{10} 's of their conduction velocities were not low enough to ensure phase was maintained at higher temperatures.

In conclusion, my results indicate that the intrinsic properties of pyloric axons ensure that the timing of action potentials between them is maintained at lower temperatures. At 19°C and above, the conduction velocity of LP's (largest diameter axon) action potential increased significantly enough to cause LP's arrival time in the periphery to be too early in relation to PD or PY's phase relationship.

One reason why LP's axon fails to maintain phase relationships at higher temperatures may be its axonal diameter. LP is the largest axon (Figure A-7) and thus likely possesses more ion channels (R. B. Stein & Pearson, 1971). Specifically, sodium and potassium channels are the ion channels that play an important role in determining the conduction velocity of an action potential (Zhang, Bucher, & Nadim, 2017). Like all ion channels, sodium and potassium channels are temperature-sensitive (Tang, et al., 2010). Consequently, the LP axon possesses more temperature sensitive (R. B. Stein & Pearson, 1971) sodium and potassium ion channels than those of the smaller diameter PD or PY axons. Thus, LP's conduction velocity may be more sensitive to temperature changes.

Precise Timing Of Action Potentials

The precise timing of action potentials arriving at the synapse is the foundation for encoding of neuronal information, and has shown to be pivotal for circuit-level plasticity, development, and learning (Lestienne, 2001). In owls, for example, the ability to localize sound is an example of how sensorimotor integration is dependent on the timing of action potentials arriving at the post-synaptic site to be precise (Carr & Konishi, 1988). Specifically, barn owls are able to pinpoint the source of a sound by means of coinciding action potentials traveling in opposite direction along delay lines in the brainstem (Carr & Konishi, 1988). In order for coincidence detection to occur, action potentials must arrive at nucleus laminaris in a precise manner. Neural circuits that largely serve to generate motor output, such as the STNS, also rely on the temporal coding of information to adjust muscle and movement patterns. An example would be the LG motoneuron. LG displays different modes of bursting activity (with a fixed firing frequency): it is either continuous or it shows intermittent pauses. The different firing patterns of LG are directly matched in the teeth movements (Diehl, et al., 2013). In *Aplysia*, the accessory radula closer (ARC) muscle is innervated by a pair of B15 and a pair of B16 motoneurons (Cropper, Kupfermann, & Weiss, 1990). Innervation of the ARC muscle allows the animal to produce an egestive response along with a multitude of ingestive responses (Cropper, et al., 1990). B15 and B16 can produce bursts that are highly variable or have irregular timing of individual action potentials (Zhurov & Brezina, 2006). The variability in the timing of action potentials of these motoneurons is responsible for both the detailed contraction of the ARC muscle and in maintaining the overall combined amplitude of the ARC muscle contraction (Zhurov & Brezina, 2006). In order for any of these aforementioned behaviors to occur, the

axons within a nervous system must maintain the temporal fidelity of action potentials as they travel to their pre-synaptic terminal.

In my study, I sought to understand whether axons can maintain precise spike timing despite temperature changes. The behavioral output of the pyloric CPG is the rhythmic constriction and dilation of the pylorus. This CPG maintains its phase relationships over a broad range of temperatures. However, if the temporal sequence of action potentials is lost between the CPG and the axon terminals that innervate the muscles behavior will be compromised. My results indicate though that this is not the case. Rather, the axons are able to preserve phase relationships over a 15 °C temperature range. However, at higher temperatures the axons are not able to preserve phase relationships. Thus, phase relationships at the muscles are preserved at muscles at lower temperatures but are lost at higher temperatures.

Rhythmic behaviors must often function over a wide temperature range. In locusts, for example, the flight motor pattern is generated by a neural circuit comprised of peripheral and central components (Robertson & Pearson, 1985; Wolf, 1993; Wolf & Pearson, 1988). Locusts are able to generate flight between 20-40°C (Xu & Robertson, 1994; Xu & Robertson, 1996). While the wing beat frequency increases temperature, the phase relationship of peripheral input and motoneuronal output is conserved (Xu & Robertson, 1994). This may be partly due to phasically active peripheral sensors such as the tegula that reset the timing of the rhythm (Robertson & Pearson, 1985; Wolf, 1993; Wolf & Pearson, 1988). Furthermore, an increase of conduction velocity of sensory stretch receptors and motoneurons may contribute to the increase in the frequency of the centrally generated flight rhythm. The increase in conduction velocity likely affects wing beat frequency by reducing time delays between the central and peripheral elements of the circuit and by increasing the efficiency of summation of synaptic activity from

different presynaptic sources onto individual neurons (Xu & Robertson, 1994). This study is thus a good example of how temperature effects an axons contribution to the generation of rhythmic behaviors. In contrast to locust flight patterns, my results contribute to how axons are involved in the maintenance of an already temperature compensated motor pattern (Rinberg, Taylor, & Marder, 2013; Tang, et al., 2010; Tang, et al., 2012). However, it remains unknown if the pylorus is able to adequately constrict and dilate at elevated temperatures. As the PD muscle has been shown to not function at higher temperatures (Thuma, et al., 2013), it is likely that muscle output in the periphery needs some form of additional compensation—either humoral or neuronal.

CHAPTER IV: MATERIALS AND METHODS

Dissection

I performed *in vitro* optical recordings from the *lvn* and extracellular recordings from the *lvn*, *pdn*, *pyn*, and the lateral pyloric nerve (*lpn*). Crabs were purchased from Fresh Lobster (Gloucester, MA) and housed in tanks containing artificial seawater at 12°C for a minimum of one week before use. Because animals can homeostatically adapt ion channel expression in different external and internal conditions (O'Leary & Marder, 2016; Soofi, et al., 2014), animals were kept at a fixed temperature for at least one week before dissection. For dissection, crabs were first anesthetized on ice for 30-50 minutes (Gutierrez & Grashow, 2009). After removal of the carapace, the stomach was removed from the animal and the dorsal and lateral teeth were subsequently be removed (Gutierrez & Grashow, 2009). The inside of the stomach was placed in a large, black, Sylgard-coated dish and secured with pins. For the fine dissection I isolated the commissural ganglia, stomatogastric ganglion (STG), and the previously listed nerves of interest. Once the fine dissection is complete, the nervous system was transferred into a Sylgard-coated petri-dish and pinned down using insect pins.

Temperature Effects On The Amplitudes Of Extracellular Recorded Action Potentials Of

LP, PY And PD

I performed experiments to measure the effects of temperature on the amplitudes of extracellularly recorded action potentials of LP, PY and PD. During these experiments, the temperature at upper half of the dish contained the STG and the paired commissural ganglia was held constant (10-12°C). The lower half of the dish contained the *lvn*, pyloric dilator nerve (*pdn*) and the pyloric nerve (*pyn*). In between the upper and lower half of the dish, I constructed a large

petroleum jelly wall that spanned from one side of the dish to the other. I then exposed the nerves to different temperatures by manually exchanging saline with temperatures ranging from 4-23°C. Amplitude measurements of all three neurons were taken from the *lvn*, which was isolated by a small well made of petroleum jelly. Temperature was continuously recorded through two thermocouplers that were placed inside the bath of the lower portion of the dish and, also inside a reference well which was used as a proxy for the temperature inside the recording well. The reference well was placed close to the recording and contained no nerves, but was similar in size, height and shape to the recording well. Extracellular nerve recordings and thermocoupler recordings were filtered and amplified through an amplifier from AM systems (Model 1700, Carlsborg, WA, USA). Data was collected on a computer using Spike2 (CED, Cambridge, UK). In order to measure the pyloric neurons' amplitudes, I detected action potentials using an enhanced trigger detection script in Spike2, which created trigger events that correlated to each action potential. A separate script in Spike2 used these trigger events as a time cue to measure the amplitude of an action potential at the recording site. LP action potentials were triggered from the *lvn* because it was the largest unit and was the easiest to detect. Action potentials of PD and PY were also simultaneously recorded on the *pdn* and *pyn*, respectively. The action potentials recorded on these nerves were used as trigger events in my data analysis and the script took into account the time delay between a trigger event created on the *pdn/pyn* and where the action potential of interest would be on the *lvn*. The script was set to average amplitudes and the temperature, for each neuron, within a 1 second period. After each experiment, I removed the thermocoupler from the bath and placed it inside the recording well and performed the same temperature ramp. I then measured how tightly correlated the temperatures inside the recording well and the reference well were. The average slope between

the two wells was 0.885 ± 0.095 (N=5) and the average $r^2=0.986 \pm 0.009$ (N=5). Temperature values plotted on the x-axis in Figures A-3, A-4, and A-5 were adjusted by 1°C to account for the ~10% difference seen between the recording well and reference well temperatures.

Data Transformations For Amplitude Measurements

All amplitude measurements were taken as temperature was increased from 4-23°C. For each neuron within each experiment, I binned amplitude values into 0.5°C bins. If there were multiple amplitude values for a given temperature bin I then averaged the amplitudes within that bin (Figure A-3, Left). The temperature-binned amplitudes were used for all further calculations. I then pooled the binned amplitudes for all my experiments and averaged each temperature bin across experiments (Figure A-3, Right). The normalized values plotted in Figure A-4 (Left) were calculated by dividing each amplitude for each neuron by its amplitude at 10°C for each experiment. After I had normalized each neurons amplitude to its value at 10°C, I pooled all of the values from the different experiments together and averaged their normalized values, for each temperature. To calculate the percent change in amplitude (Figure A-4, Right), I subtracted the normalized value from 1 and multiplied the difference by 100, for each neuron and for each experiment. I then pooled the percent change in amplitude for each neuron and at each temperature and averaged across experiments.

Temperature Effects On Conduction Velocities Of LP, PY And PD

I performed experiments to measure the effects of temperature on conduction velocities of the axons of LP, PY and PD. I measured these effects by isolating a portion of the *lvn* using petroleum jelly and by manually exchanging saline with temperatures of 4, 7, 13, 19 and 23°C.

During these experiments the temperature close to the STG was monitored to ensure that the pyloric rhythm and the phase relationship of its neurons were not affected by temperature. To measure conduction velocities, I also had extracellular recording wires placed on the *lvn* proximal to the STG (before the site of temperature perturbation) and on the posterior *lvn* (after the site of temperature perturbation) (Figure A-1). By measuring the delay of action potentials between the two recording sites, I measured the conduction velocity and phase relationships of the pyloric neurons. The saline in the petri dish equilibrated to 13°C and was thus used for normalization (Figure A-8, Right).

Calculating The Natural Variability In The Phase Relationships Of The Pyloric Rhythm

The PD neurons' offset phase, and the onset phase and offset phase of LP and PY neurons display a natural variability at a control temperature of 13°C. The pyloric period is the reciprocal of the pyloric frequency and was calculated by measuring the delay from the beginning of one PD burst (on an extracellular recording) to the beginning of a subsequent PD burst. The onset phase of LP and PY bursts' was calculated by measuring the time delay between the beginning of a PD burst to the beginning of the LP or PY burst and normalizing their respective delays to the pyloric period. To calculate the natural variability of the phasing the pyloric rhythm, I calculated the standard deviation of the onset phase of LP and PY for an individual animal and then averaged the standard deviations across animals.

Calculating Deviations In Phasing Of The Pyloric Rhythm Using Arrival Times At The Axon Terminal

I used the arrival times of LP's and PYs' action potentials at the axon terminal to calculate changes in phase. The arrival times of PD, LP and PY were calculated by taking the average length of the pyloric axons (4 cm) and dividing it by the conduction velocities of PD, LP and PY at temperatures ranging from 4-23°C. At any given temperature, I calculated the deviation in phase by first subtracting PD's arrival time from those of LP or PY. The deviation in phase was then calculated by dividing these values by the cycle period. The cycle period of the pyloric rhythm is known to increase with temperature with $Q_{10} = 2.32 \pm 0.2$ (Tang, et al., 2010). For any given temperature this equation was used to calculate the cycle period necessary for calculating the deviation in phase.

Voltage Sensitive Dye Application On The *lvn* For Determining Axon Diameters

To determine axon diameters of the pyloric neurons of interest (LP, PD, and PY), I used a combination of electrophysiology and optical imaging on the *lvn*. After dissecting and desheathing the *lvn*, the desheathed portion was then isolated using petroleum jelly. I then bath applied a fluorescent voltage-sensitive dye (VSD, Di-4-ANEPPDHQ, 50 μ M in saline) and allowed it to stain the *lvn* axons for 30-60 minutes [24]. After this period, I removed the well and superfused cool saline to wash out residual dye that was not bound directly to axonal membrane; the nerve was then subjected to this superfusion for the remainder of the experiment. Fluorescence changes (optical imaging) of the stained axons was collected using the MiCam02 imaging system and software (Brain-Vision Analyzer, BV-ANA, Version 11.08.20; SciMedia Ltd, Tokyo, Japan) with the HR (High Resolution) camera (6.4x4.8 mm actual sensor size).

Fluorescent excitation Light used to excite the VSD is provided by a narrowband LED with 525 nm (CoolLED pE 4000, Yorktown Heights, NY, www.coolled.com) and fluorescence emission is detected using a quadband filter cube (Semrock, Inc, Rochester, NY). Excitation light intensities and imaging exposure time vary and were adjusted to the individual preparation. I used a 20x objective (XLUMPlanFL N, NA 1.0, WD 2.0 mm, cc=water; Olympus Corporation, Tokyo, Japan) mounted on an upright epifluorescence microscope (modified BX51, Scientifica, East Sussex, UK). Each focal region was imaged for 100 s (broken up into 5 x 20 s photos) using a 333 Hz frame rate and spatial resolution of 96x64 pixels. I repeated this protocol for several regions along the *lvn*. Single axons was defined manually as individual regions of interest (ROIs) in the BVAna program. The fluorescence (dF/F_0) values of all pixels contained in a given ROI were averaged to yield a single trace over time for each ROI (axon). These traces were compared to the simultaneously acquired extracellular recordings of the *dvn*. The axons of PD and PY were selectively stimulated, extracellularly, by stimulating the *pdn* and *pyn*, respectively. The axon of LP was selectively stimulated from a lower region of the *lvn*. The LP axon has the lowest threshold for an action potential to be elicited. Each axon in each focal plane was stimulated in 5 trains at 8 Hz for 4 seconds, in between each train was a 5 second pause. After I compiled all the images and extracellular data into Spike2 software (version 7.12, Cambridge Electronic Design, Cambridge, UK), I then compared the timing of action potentials on the optical recording with that on the extracellular recordings, using multisweep and spike-triggered average analyses (Städle & Stein, 2016). This approach allowed me to identify the imaged axons. After identification, I measured their diameter by importing the images of the axons into Coreldraw (version 17.1.0.572 for Windows, Corel Corporation, Ottawa, ON, Canada) and measuring the distance from one side of the axon to the other with an appropriate scaling factor. In a separate

set of analyses, I measured the variability of axonal diameter for each neuron type by taking nine diameter measurements within the field of view. The coefficient of variation for PD (N=5), LP (N=4), and PY (N=2) was 6.85%, 12.16% and 9.19%, respectively. While it is possible that axonal diameters change throughout the length of the *lvn*, axonal diameter measurements and amplitude measurements were taken at approximately the same location on the *lvn*. Thus, the measured amplitude ratio are likely representative of the diameter ratios I have reported in my voltage-sensitive dye experiments.

Preparation Of Voltage-Sensitive Dye (Di-4-ANEPPDHQ)

The 5 mM stock solution of dye is prepared by diluting 1 mg of dye in 300 μ l of DMSO, aliquotting for single-staining use, and kept in darkness at -20 °C. Preceding bath application the stock solution was diluted 1:1 with pluronic acid F-127 (20% solution; Biotium, Hayward, CA) DMSO solution, and mixed with saline to the final concentration of 50 μ M.

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APPENDIX A: FIGURES

Figure A-1. Schematic of the stomatogastric nervous system with the axons of the lateral pyloric (LP), the four pyloric constrictor (PY) and the two pyloric dilator motoneurons (PD) that extend from their somata to the periphery to innervate muscles of the pylorus.

The two smaller black circles located on the *dvn* and the *lvn* represent the recordings wells made with petroleum jelly. The wells were used to measure delays between action potentials before and after temperature perturbation. The larger asymmetrical well situated between the two recording wells depicts where temperature was altered. An example extracellular recording of the pyloric rhythm from the lateral ventricular nerve (*lvn*) depicts the stereotypical rhythm (LP neuron in green, multiple PY neurons in purple and multiple PD neurons in blue). *pdn*= pyloric dilator nerve, *pyn*= pyloric nerve

Figure A-2. The axonal diameter of the pyloric neurons are significantly different based on their extracellular action potentials amplitudes (Top Left) Extracellular recording of the pyloric rhythm from the *lvn* shows that LP had the largest amplitude, PD had an intermediate amplitude and PY had the smallest amplitude. (Top Middle) Single action potentials of LP, PY, and PD obtained from extracellular recordings of the *lvn*. (Top Right) Extracellular amplitude values within individual experiments shows the relationship of extracellular amplitudes of PD, LP and PY. LP, PD and PY amplitudes were significantly different from one another (RM ANOVA, $F(2,26)=13.613$, $p<0.001$, $N=9$, post-hoc test= Student-Newman-Keuls Method, LP vs.

PY $p < 0.001$, LP vs. PD $p < 0.01$, PD vs. PY $p < 0.05$)). **(Bottom Left)** Average values of normalized extracellular amplitudes of LP, PD and PY. Amplitudes were normalized to the average amplitude of LP, PD and PY in each experiment. LP, PD, and PY were significantly different from one another (RM ANOVA, $F(2,26) = 30.340$, $p < 0.001$, post-hoc test= Student-Newman-Keuls Method, LP vs. PY $p < 0.001$, LP vs. PD $p < 0.001$, PD vs. PY $p < 0.01$) . **(Bottom Right)** Bar graph displaying the calculated axon diameters from normalized extracellular amplitudes, using the formula: diameter $\propto \sqrt[3]{amplitude^2}$. The relative axon diameters of LP, PD and PY were significantly different from each other (RM ANOVA, $F(2,26) = 30.340$, $p < 0.001$, $N=9$, post-hoc test= Student-Newman-Keuls Method, LP vs. PY $p < 0.001$, LP vs. PD $p < 0.001$, PD vs. PY $p < 0.01$). ***Significantly different groups at a significance level of $p \leq 0.001$; significantly different groups at a significance level of ** $p \leq 0.01$; significantly different groups at a significance level of * $p \leq 0.05$

Figure A-3. Temperature alters the amplitude of extracellularly recorded action potentials.

(Top) Extracellular recording of the pyloric rhythm from the *lvn* at 4.0°C (left), 10.5°C (middle) and 20.5 °C (right). The topmost extracellular traces are two complete cycles of the pyloric rhythm over a period of 15 s. The bottom extracellular traces of the *lvn* are individual action potentials selected from the upper extracellular traces, of the LP, PY and PD neurons (PD, blue; LP, green; PY, purple). **(Left)** Amplitudes of extracellularly recorded action potentials of the three pyloric neurons from one animal are different from each other across all temperatures (3-22°C). Data points were sorted into 0.5°C bins. The different colors of the circles represent different neurons (PD, blue; LP, green; PY, purple). Regions in the graph that are highlighted in

pink denote the temperatures where extracellular traces of the *lvn* placed above the graph were taken. **(Right)** The amplitudes of extracellularly recorded action potentials are affected non-linearly by temperature (Nonlinear regression analysis, LP: Adjusted $R^2=0.92$, $p<0.0001$, $N=5$; PD: Adjusted $R^2=0.67$, $p<0.0001$, $N=5$; PY: Adjusted $R^2=0.56$, $p<0.0001$, $N=5$). Amplitude values were sorted into 0.5°C bins and then averaged across experiments. Temperature had a significant effect on the amplitudes of action potentials for all three pyloric neurons (RM One Way ANOVA, PD: $F(37,184)=2.219$, $p<0.001$, $N=5$, LP: $F(39,236)=3.016$, $p<0.001$, $N=5$, PY: $F(39, 226)=4.272$, $p<0.001$, $N=5$) The different colors of the circles represent different neurons. PD, blue; LP, green; PY, purple. Note that these values are paired data and the large standard deviations for individual neurons are due to animal-animal variation in amplitudes.

***Significantly different groups at a significance level of $p \leq 0.001$

Figure A-4. Differential effects of temperature on the amplitude of action potentials. (Left)

The change in amplitude of extracellularly recorded action potentials from the axons of LP (green), PY (purple) and PD (blue) was non-uniform. Each amplitude value for each neuron was normalized to their respective amplitudes at 10°C within one experiment and then averaged across experiments (N=5). **(Right)** Percent change from the 10°C. The change in amplitude observed in LP is significantly different from the change in amplitude observed in PY (purple line above graph) and PD (blue line above graph) ((Two Way ANOVA of temperature ($F(39,119)=18.140$)($p<0.001$) and neuron type($F(2,119)=30.189$)($p<0.001$) LP vs PY($p=<0.001$,N=5) and LP vs PD($p=<0.001$,N=5) but a non-significant difference for PD vs PY($p=0.276$, N=5)). Significantly different groups at a significance level of $** p \leq 0.01$

Figure A-5. Temperature alters the relative axonal estimates and the axonal diameter

ratios. (Left) Histogram displaying the calculated axon diameters given the extracellular amplitudes taken at different temperatures. The different colors of the rectangles represent different neurons. PD, blue; LP, green; PY, purple. Asterisks denote where axon diameters were significantly different from one another at different temperatures using 1-way RM ANOVA statistical analysis. Statistics for these values are in Table B-1. **(Right)** The ratios of axon diameters were calculated by taking the axon diameter sizes from the left graph and dividing their respective values. The different colors of the rectangles represent different axonal diameter ratios. LP/PY, pink; PD/PY, orange; LP/PD, yellow. ***Significantly different groups at a significance level of $p \leq 0.001$; significantly different groups at a significance level of ** $p \leq 0.01$; significantly different groups at a significance level of * $p \leq 0.05$

Figure A-6. The pyloric axons were identified and diameters were measured using a Voltage-Sensitive Dye. (Top) Image of the desheathed *lvn* after bath application of Di-4-ANEPPDHQ. The shaded green and blue regions denotes the regions of interest selected for the optical imaging of the LP and PD axons, respectively. Axon diameters were measured at indicated sites (black lines). **(Bottom Left)** Multisweeps of extracellularly (n=160) (top trace) and optically (n=32) recorded action potentials (bottom trace) from the axon of PD. n= number of sweeps. The extracellularly detected action potentials of LP were used to trigger both averages. The colored lines in both the optical and extracellular trace represent the average

waveform of their respective optical and extracellular multisweeps. **(Bottom Right)**

Multisweeps of extracellularly (n=160, N=1) (top) and optically (n=32, N=1) (bottom) recorded action potentials from the axon of LP. The extracellularly detected action potentials of LP and PD on the *dvn* were used to trigger both averages. The colored lines in both the optical and extracellular trace represent the average waveform of their respective optical and extracellular multisweeps.

Figure A-7. Diameter measurements from VSD experiments show that the pyloric axons possess distinct diameter. To account for variability between animals, axonal diameter measurements for each neuron was normalized to the average axon diameter of all three pyloric axons. All axon diameters were significantly different from one another (1 Way RM ANOVA $F(2,16)= 0.004$, LP vs. PY: $p= 0.001$, $N= 5$, LP vs PD: $p=0.017$, $N=6$, PY vs PD: $p=0.05$, $N=5$). The different colors of the rectangles represent different neurons. PD, blue; LP, green; PY, purple. ***Significantly different groups at a significance level of $p\leq 0.001$; significantly different groups at a significance level of $* p\leq 0.05$

Figure A-8. The conduction velocities of the pyloric axons respond similarly to temperature perturbations. (Left) Conduction velocities of the three pyloric axons are different from each other across all temperatures, and increase significantly when subjected to temperature perturbations (4-23 °C). The different colors of the lines represent different neurons. PD, blue; LP, green; PY, purple; The slopes of these lines were obtained with a linear regression and are significantly different from zero (Linear Regression Analysis, PD: $r^2=.984$, $p<0.001$, PY: $r^2=0.987$, $p<0.001$, LP: $r^2=0.988$, $p<0.001$, $N=5$ for LP, PY and PD). **(Right)** A comparison of slopes shows that the change in conduction velocities at different temperature is similar for the three pyloric axons (One Way ANOVA of LP, PD and PY slopes, $F(2,20)=0.499$, $p=0.615$, $N=8$). The percent change of conduction velocity was calculated by normalizing each of the axon's conduction velocities at temperatures ranging from 4-23 °C to the conduction velocity measured at a control temperature of 13 °C. ***Significantly different groups at a significance level of $p\leq 0.001$

Figure A-9. Pyloric axons maintain phase relationships at lower temperatures. The ‘calculated deviation in phase’ was calculated by normalizing the arrival times of LP and PY to PD. The individual standard deviations (SD) of the onset of the bursts of LP (light green = 1 SD and dark green = 2 SD regions) and PY (light pink = 1 SD and dark pink = 2 SD regions) taken at a control temperature of 13°C were used as a measure of the natural variability of the phasing of the pyloric rhythm.

APPENDIX B: TABLES

	4°C	6.5°C	10.5°C	18°C	20.5°C
	F(2,12)= 31.33 p<0.001	F(2,14)= 40.60 p<0.001	F(2,13)= 33.70 p = 0.002	F(2,12)= 20.97 p = 0.002	F(2,14)= 22.24 p<0.001
PD vs LP	p=0.003 N=4	p= 0.001 N=5	p= 0.015 N=5	p=0.004 N=4	p=0.002 N=5
LP vs PY	p <0.001 N=4	p <0.001 N=5	p <0.001 N=4	p<0.001 N=4	p<0.001 N=5
PY vs PD	p=0.041 N=3	p=0.004 N=5	p=0.001 N=4	p=0.206 N=3	p= 0.106 N=5

Table B-1. RM ANOVA of axonal diameter at 4°C, 6.5°C, 10.5°C, 18°C, 20.5°C