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ABSTRACT

REGULATION OF VOLTAGE-GATED K^+ CURRENTS IN MOTOR NEURONS: ACTIVITY-DEPENDENCE AND NEUROMODULATION

by
Dalia Salloum

Neuronal output is shaped by extrinsic modulation as well as modulation of intrinsic properties of individual neurons, mediated by activity-dependent changes in the expression levels of voltage-gated ionic currents. Activity-dependent regulation of ionic currents is a mechanism by which electrical output of a neuron feeds back onto the expression of its own ion channels to alter cellular excitability in response to stimuli. Neurons alter their intrinsic properties to achieve long lasting changes involved in development, learning and memory formation and vital functions of organ systems such as locomotion and digestion. At the same time, plasticity of neuronal excitability driven by previous experience requires mechanisms to promote stability to maintain physiological function, and many examples of this type of homeostatic plasticity changes have been reported. At the same time, neuromodulation can alter electrical output indirectly via ligand-gated receptors and second messenger pathways and potentially affect activity-dependent effects. Activity-dependent regulation of ionic currents functions to allow neurons to track their own electrical activity and adjust their intrinsic properties in response to changing synaptic drive or other inputs to maintain their functional output. This phenomenon has been demonstrated to occur over the course of minutes and is a relatively fast process. Neuromodulators exert long-term effects on ionic currents via activation of cellular signaling pathways that do not directly affect

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ionic current levels. Neuromodulation and activity-dependent effects can alter neuronal networks on different time scales, e.g. over several hours to days to accommodate the needs of the behaving organism such as in transitions between sleep and waking states. However, this is not necessarily so, and the possibility of real-time interactions exist and needs to be examined.

This dissertation demonstrates that activity-dependent regulation of K^+ currents is gated by the neuromodulatory environment and can be altered depending on the activation of a ligand-gated peptide receptor. This study demonstrates novel findings of interactions between metabotropic receptor activation and modulation of highly K^+ currents after acute changes to activity and neuromodulatory input.

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REGULATION OF VOLTAGE-GATED K^+ CURRENTS IN MOTOR NEURONS: ACTIVITY-DEPENDENCE AND NEUROMODULATION

by
Dalia Salloum

A Dissertation
Submitted to the Faculty of
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APPROVAL PAGE

REGULATION OF VOLTAGE-GATED K+ CURRENTS IN MOTOR NEURONS: ACTIVITY-DEPENDENCE AND NEUROMODULATION

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I dedicate this work to my mother, an unwavering source of unconditional love and compassion. And to my father, who instilled in me the desire and the strength to challenge the status quo.

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I am very thankful for the support of the administrative staff that helped me from my first day as a graduate student until this present time: Karen Roach, Shandell Riviera and Neermala Gazi. Their passion, dedication and friendship were essential in making this thesis come to fruition.

My friends, colleagues, and family members made this possible by motivating, supporting, coaching, feeding, sheltering and simply loving me.

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CHAPTER 1

INTRODUCTION

1.1 Objective

The objective of this dissertation is to identify a potential interaction between neuromodulators and activity-dependent regulation of intrinsic properties of single neurons. The pyloric network of the stomatogastric ganglion (STG) of the Jonah crab, Cancer borealis is used as model network to measure intrinsic voltage-gated ionic currents in single identified motor neurons. The particular voltage-gated currents measured are outward K^+ currents, which are involved in regulating membrane excitability and the electrical activity of a neuron. The oscillatory activity of the network is highly dependent on modulatory input from anterior ganglia. Modulators exert long-term effects on the expression of ionic currents by regulating their coordinated expression.

Activity-dependent regulation and neuromodulation function at different time scales and on different molecular targets yet they both have the ability to change the output of a neuron and the entire network in which it functions. In particular, it is known that removal of neuromodulators leads to a cessation of rhythmic activity, which slowly recovers in what has been thought to be an activity-dependent process of regulation of ion channel expression. At the same time, neuromodulators are known to regulate the expression of ion channels as well. Therefore, it is probable that some interaction between the regulation of ionic currents by activity and neuromodulators exists to promote stability of ion channel expression and ultimately of activity itself.

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This thesis is based on the general hypothesis that neuromodulators gate activity dependent regulation of ionic currents in pyloric neurons. The aim is to first characterize and quantify activity-dependent regulation by stimulating isolated neurons with patterned electrical stimulation. To test if and how an interaction occurs, different modulatory substances will be applied and the magnitude and direction of change induced by electrical stimulation will be quantified in different modulatory environments.

1.2 Significance

Neurons integrate many different inputs to maintain a stable output that is appropriate for the environment the network is exposed to at any given time. The particular output of the neuronal networks we study is a rhythmic motor pattern comparable in many ways to a broad class of patterns that control vital behaviors, like respiration, locomotion and digestion. The networks responsible for the generation of such patterns are called central pattern generators (CPGs). Oscillatory activity is seen across a wide variety of neuronal behaviors other than motor patterns, such as learning and memory, sleep and wakefulness, addiction and fear (Nanou, Scheuer et al. 2016). An essential characteristic that many of these vital activity patterns must have is certain degree of stability that guarantees continued function and ultimately the survival of the organism. However, a defining quality of all nervous systems is the ability to adapt to change thanks to their multiple mechanisms of cellular plasticity. With flexibility arises the need for stability. Otherwise, how would neuronal activity be able to control vital behaviors consistently throughout the lifetime of an organism? To accomplish both flexibility and stability, the component neurons in a network must constantly alter their firing properties, synaptic

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strengths and membrane properties in response to physiological changes according to specific regulatory rules (Mee, Pym et al. 2004, Bucher, Prinz et al. 2005, Hengen, Lambo et al. 2013, Butz, Steenbuck et al. 2014, Felix-Oliveira, Dias et al. 2014, Frank 2014, Korotchenko, Cingolani et al. 2014). Stability requires that even with all the incoming stimuli component neurons respond to, the emergent behavior of each individual neuron and the network as a whole is within a prescribed physiological range.

Like long-term synaptic plasticity, long lasting changes in cellular excitability (termed intrinsic plasticity) facilitate learning and memory formation (Turrigiano, Abbott et al. 1994, Marder, Abbott et al. 1996, Daoudal and Debanne 2003, Dityatev, Schachner et al. 2010, Porro, Rosato-Siri et al. 2010, Felix-Oliveira, Dias et al. 2014, Nanou, Scheuer et al. 2016). It is currently almost impossible to discuss neurophysiology without mentioning plasticity. The concept of plasticity has been widely explored and characterized across a large array of cell types, networks and model organisms (Du, Feng et al. 2000, Cudmore and Turrigiano 2004, Tully and Bolshakov 2010, Naude, Paz et al. 2012, Butz, Steenbuck et al. 2014, Felix-Oliveira, Dias et al. 2014, Lee, Soares et al. 2014, Wenner 2014, Karunanithi and Brown 2015, Fauth and Tetzlaff 2016). In STG neurons, plasticity of neuronal excitability has been demonstrated from the cellular level to the network level, rendering this network an excellent model system to explore more complex questions regarding how plasticity is controlled to avoid destabilization of the system by runaway excitation or inhibition. Homeostatic plasticity is a feedback mechanism neurons often use to counteract over excitation or prolonged suppression of neuronal activity (Pozo and Goda 2010).

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Homeostatic plasticity is a mechanism that allows neurons to regulate their intrinsic properties based on their own activity output. In injury and lesion studies, recovery of function after disruption is highly dependent on cell-autonomous mechanisms. In STG neurons, for example, modification of the intrinsic properties of individual neurons contributes to recovery of function after removal of modulatory input that is required for oscillatory behavior (Luther, Robie et al. 2003, Zhang and Golowasch 2007, Zhang, Khorkova et al. 2009, Zhang and Golowasch 2011). Several studies in the STG have demonstrated that neuromodulators exert long-term control on the expression level of ionic currents as well as directly coordinating expression of different types of ionic currents (Thoby-Brisson and Simmers 2002, Temporal, Desai et al. 2012). Ionic current expression is regulated by neuromodulators at the gene level and neuromodulatory input is required to maintain expression levels over long periods of time (Haedo and Golowasch 2006, Khorkova and Golowasch 2007, Temporal, Desai et al. 2012). Because network activity is dependent on modulatory input, many efforts have gone into separating the mechanisms that are directly regulated by activity and those directly dependent on modulatory input.

1.3 The Pyloric Network

The pyloric network of the Jonah crab *Cancer borealis* is an excellent model system to study activity-dependent plasticity. With relatively few component neurons, well-known synaptic connections and well characterized modulatory input from anterior ganglia this system can produce complex outputs and is a useful tool in understanding complex systems. The pyloric network consists of a group of 11 neurons that innervate muscles in

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the pylorus which is responsible for the filtration of macerated food in the crab's foregut. The rhythm produced by this network is a triphasic rhythm involving the alternate bursting of its component neurons (Hooper, O'Neil et al. 1986, Bucher, Prinz et al. 2005, Grashow, Brookings et al. 2009). The pyloric rhythm oscillates at a frequency of 1 Hz *in vivo* and *in vitro* (Hedrich, Diehl et al. 2011). It is driven by the pacemaker kernel which includes one interneuron, the anterior burster (AB), and two motor neurons, the pyloric dilators (PDs) which are all electrically coupled via gap junctions(Selverston, Russell et al. 1976).

The pyloric neurons are located in the stomatogastric ganglion (STG) along with neurons of two other CPGs, the cardiac sac and gastric mill (Buchholtz, Golowasch et al. 1992). All of these neurons receive modulatory input from projection neurons in three anterior ganglia; the esophageal ganglion (OG) and the paired commissural ganglia (CoG) (Swensen, Golowasch et al. 2000, Goldman, Golowasch et al. 2001). These four ganglia along with motor and sensory nerves make up the stomatogastric nervous system (STNS) which controls swallowing, chewing, digestion and filtering of food in the crab's foregut. Although the pyloric rhythm can produce stable rhythmic output without timed sensory feedback, it does depend on modulatory input from the projection neurons which descend down the main input nerve, the stomatogastric nerve (*stn*) (Luther, Robie et al. 2003). Removal of this input by blocking action potential transmission down the *stn* via sucrose block or cutting the nerve results in a cessation of rhythmic activity (Thoby-Brisson and Simmers 1998, Luther, Robie et al. 2003).

1.4 Voltage-gated K^+ Currents

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The activity of a nervous system, from the single neuron to the behaving organism is governed by the abundance and combination of voltage-gated ion channels. While synaptic and other chemical signals drive activity, the inherent excitability of a cell is largely determined by the balance of inward and outward movement of charged particles through transmembrane channel proteins and the resulting activity depends on their relative abundances. More specifically, for the purposes of this work we focus and will discuss voltage-gated K^+ channels and their crucial role in neuronal activity and function.

Pyloric neurons express a variety of distinct outward currents. We focus on three of those currents: the calcium-activated (I_{KCa}), the delayed rectifier (I_{Kd}) and the transient (I_A) potassium current. Each current is encoded by a different gene. I_{KCa} and I_{Kd} are encoded by the BK-KCa and *shab* genes, respectively. I_A is encoded by the *shal* gene. Each current possesses distinct kinetics and temporal dynamics. Therefore, they each play a different role in shaping the electrical identity of a neuron.

I_A is a rapidly inactivating current and controls the latency to first spike in a bursting neuron (Baro, Cole et al. 1996, Baro, Coniglio et al. 1996). It is a highly modulated current and the channel proteins can interact with many different intracellular components (An, Bowlby et al. 2000, Holmqvist, Cao et al. 2002, Rhodes, Carroll et al. 2004, Monaghan, Menegola et al. 2008). The delayed rectifier has been characterized across many preparations. It is a non-inactivating current responsible for fast repolarization following an action potential in spiking neurons (Clark, Mangoni et al. 2004, Bocksteins, Raes et al. 2009). Mammalian studies of the Kv2.1 channels which mediate I_{Kd} suggest that they play a major role in regulating somatodendritic excitability in cortical and hippocampal neurons (Golowasch and Marder 1992, Bekkers 2000,

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Misonou, Mohapatra et al. 2004, Mohapatra, Misonou et al. 2009). I_{KCa} is a voltage- and calcium- gated current. It contributes to the afterhyperpolarization (AHP) current following and action potential (Gu, Vervaeke et al. 2007). Due to its gating properties, it has been suggested as a molecular link between neuronal activity and Ca^{2+} -targeted processes such as gene expression (Li, Jie et al. 2014).

Across many cell types in mammalian nervous systems, K^+ channel dysfunction is associated with disease states such as ischemia, epilepsy, stroke and general dysregulation of cellular metabolism. Voltage-gated K^+ currents generally function to repolarize the membrane potential following an action potential. It is therefore crucial to understand the mechanisms which regulate expression levels of these currents in a behaving nervous system.

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1.5 Neuromodulation

Neuromodulation allows nervous systems to produce different behaviors using the same set of neurons (Grashow, Brookings et al. 2009). Without the presence of neuromodulators nervous systems would not be able to respond appropriately and generate the behavior required to sustain and adapt to a changing environment. Neuromodulators have a wide range of targets some of these being ionic channels which can regulate the excitability of the cell. Neuromodulators can directly target synapses such that there is an increased flexibility in the motor pattern (Marder and Thirumalai 2002). Neuromodulators are present in different concentrations at different stages of the developing nervous system and their role and function may change depending on the expression levels of their targets.

Neuromodulation plays an important role in shaping the motor output of CPGs. Unlike classical neurotransmission, neuromodulation involves the release of multiple chemical signaling molecules onto a group of targets. Where neurotransmitters are released in a small space between the pre- and post-synaptic neuron, neuromodulators diffuse through large areas in the nervous system. Neuromodulation functions in shaping and fine tuning neuronal activity. It allows a circuit with a small number of neurons and synaptic connections to produce many complex behaviors beyond the output of each individual neuron (Marder 2012). Distinct motor patterns in the pyloric network, and other CPGs, result from selective recruitment of cell types by neuromodulators (Meyrand and Marder 1991, Weimann, Meyrand et al. 1991, Meyrand, Simmers et al. 1994). It is possible then, for the motor pattern of a CPG to adjust its output in response to the

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behavioral state of the animal. In respiratory control, bioamines such as 5-HT and norepinephrine (NE) modulate activity during development, normal breathing and gasping (Viemari and Tryba 2009). Modulators can change the output of a network by altering the strength of a synapses (Akcay, Bose et al. 2014), intrinsic membrane properties of single neurons (Golowasch and Marder 1992, Johnson, Peck et al. 1995, Swensen and Marder 2000), firing activity of a neuron (Buchholtz, Golowasch et al. 1992) and even at the level of a whole network (Sharples, Almeida et al. 2014, Sharples, Koblinger et al. 2014).

In the case of the pyloric network, actions of over 20 modulatory substances that project onto these neurons have been studied extensively at the aforementioned levels (Swensen and Marder 2001) although almost 100 modulatory substances in this system have been identified. It is known that normal function of the pyloric network requires continuous modulatory input and that removal of this input results in a cessation of oscillatory activity (Thoby-Brisson and Simmers 1998, Luther, Robie et al. 2003). One mechanism for production of oscillations in pyloric neurons that has been extensively studied is the convergence of multiple peptides onto one inward current, the modulatory inward current (I_{MI}) (Golowasch and Marder 1992, Swensen and Marder 2000). This current has been shown to be sufficient to produce bursting behavior in pyloric neurons that are isolated from modulatory input (Zhao, Golowasch et al. 2010).

While the anatomical composition of a neuronal network is relatively rigid in that new neurons do not extensively grow after development and maturation, neuromodulators have the profound ability to alter the identity, connectivity, and functional output of a network. As mentioned before, they can selectively recruit neurons

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to participate in different networks or silence an entire circuit of neurons (Weimann and Marder 1994). They can have acute effects on voltage-gated currents and change the electrophysiological properties of a neuron. Harris-Warrick and colleagues have done extensive studies on amine modulation in STG neurons (Beltz, Eisen et al. 1984, Harris-Warrick and Kravitz 1984). They demonstrated how amines can modulate network output by targeting single voltage-gated currents. They provide an elegant mechanism for how aminergic modulation changes the activity of a neuron by reducing or enhancing the ionic currents that shape its firing activity (Peck, Nakanishi et al. 2001). Neuromodulators also have long-term effects on intrinsic properties of pyloric neurons. Khorkova & Golowasch (2007) demonstrated that neuromodulators regulate intrinsic properties of these neurons by coordinating the expression of ionic currents (Khorkova and Golowasch 2007). They showed that coordinated expression of ionic currents depends on continuous modulatory input and that removal of the input results in a loss of co-regulation, while the reintroduction of a single neuromodulator, proctolin, was sufficient to prevent the loss of this co-regulation. Neuromodulators have even also been shown to control the co-regulation of ion channels at the gene expression level (Temporal, Desai et al. 2012).

1.6 Activity-Dependent Regulation of Cellular Excitability

Studies in vertebrate and invertebrate model systems have shown that the conductances of voltage-gated ion channels are quite variable between cell types as well as within the same cell types across different animals (Golowasch, Abbott et al. 1999, Golowasch, Casey et al. 1999, Goaillard, Taylor et al. 2010). Although there is variability, there also must be a level of stability allowing neurons to produce stable output, or maintain certain

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levels of plasticity to respond to dynamic inputs. The role of activity-dependent plasticity has been studied extensively at the synaptic level, especially as a cellular paradigm of learning and memory. Here, however, I propose to explore plasticity of a regulatory mechanism: activity-dependent regulation of ionic currents. Even for learning and memory, plasticity of intrinsic excitability has been suggested as a cellular correlate of learning (Marder, Abbott et al. 1996, Daoudal and Debanne 2003). For example, the conditioning of the phototactic response in the marine mollusk *Hermissenda crassicornis,* is associated with a reduction of the transient potassium current, I_A, and the calcium-dependent potassium current, I_{KCa} in photoreceptor cells (Alkon 1984). These changes in neuronal excitability outlasted the stimulus and were seen only in animals that had learned the conditioning response. There are many examples in the literature where changes in ionic current densities have been associated with regulating neuronal output in response to changing inputs (Haedo and Golowasch 2006, Paz, Mahon et al. 2009) In fact, the classical publication that first described long-term synaptic potentiation in granule cells of the hippocampus prominently reported a modification of granule cell excitability together with synaptic potentiation (Bliss & Lomo, 1973)

Golowasch et al. (1999) showed that patterned stimulation regulates voltage-gated potassium currents in isolated STG neurons where neuromodulatory input and activity had been removed. Their results show a reversible increase in the transient potassium current (I_A) and a decrease in the high threshold potassium current (I_{HTK}), a current composed of both the I_{KCa} and the I_{Kd} in these cells, while having no effect on the leak current (I_{LEAK}) in the inferior cardiac (IC) neuron (Golowasch, Abbott et al. 1999). In cultured neurons that have been dissociated from synaptic and modulatory inputs, STG

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neurons transition from tonic firing to bursting activity similar to their endogenous activity and the transition is suggested to be facilitated by up-regulation and down-regulation of ionic currents (Thoby-Brisson and Simmers 1998).

These observations raise two fundamental questions:

1) Are changes in intrinsic currents in decentralized preparations due to a change in activity or the loss of neuromodulation? Or a combination of the two?

2) If so, how do neuromodulation and activity-dependent mechanisms interact to facilitate these changes?

It seems, as suggested from the studies we have discussed here, that removal of either of the signals, activity or neuromodulators, results in a system that is susceptible to regulation independent of the absent signal. In other words, neurons isolated from modulatory input are capable of changing their intrinsic properties in response to activity changes. In other examples, such as the experiments conducted by Zhao et al. (2010), neurons devoid of activity can transition back to oscillatory behavior simply my reintroduction of the modulatory current, $I_{MI.}$

The novel aspect of this study, where few examples exist, is the effect of the interaction of the two regulatory mechanisms. It is unclear how the signaling pathways activated by activity-dependent mechanisms and neuromodulators converge to regulate voltage-gated currents. In visual cortex, for example, activity-dependent plasticity is gated by neuromodulation from cholinergic and adrenergic input. In this type of plasticity, spike-timing-dependent plasticity (STDP), timing of pre- and post-synaptic spikes results in long-term depression (LTD) or long-term potentiation (LTP). Whether

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LTP or LTD occurs depends on which of the modulatory pathways is activated (Seol, Ziburkus et al. 2007). Regulation of ionic conductances has also been shown to depend on activation of a metabotropic membrane receptor such as the TrkB receptor in cortical neurons (Turrigiano, Abbott et al. 1994, Desai, Rutherford et al. 1999).

In this study, we aim to test how neuromodulators affect activity-dependent regulation of intrinsic properties. Our hypothesis is that neuromodulators gate activity-dependent regulation of ionic currents. We aim to understand what actions different modulatory inputs exert on activity-dependent regulation of ionic currents. Specifically, we are interested in how activity-dependent regulation and neuromodulation interact to regulate expression levels of ionic currents in neurons of a CPG.

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CHAPTER 2

METHODS: SULFATE AS AN ELECTRODE FILLING SOLUTION CAUSES A DECAY IN PEAK I_{HTK} IN PD NEURONS

2.1 Reliable Methods in Electrophysiology

In order to ask questions about how nervous systems function and attempt to uncover the answers, we ensure that experimental techniques are reliable to quantify parameters of neuronal excitability and behavior. Using sharp electrode electrophysiology enables intracellular recording of the electrical activity of single neurons. The two electrode voltage clamp (TEVC) method allows us to measure voltage-gated ionic currents. Many studies exploring drug therapies for an array of diseases use ion channels as a target since ion channel dysfunction is associated with many diseases including epilepsy, ischemia, and stroke (Du, Haak et al. 2000, Monaghan, Menegola et al. 2008, Butz, Steenbuck et al. 2014, Frank 2014, Pribiag, Peng et al. 2014, Swann and Rho 2014). It is, therefore, critical that the physiology of these channels can be accurately and reliably measured.

Coincidentally, Hooper and colleagues (2015) also demonstrated that electrodes filled with high salt concentration caused changes in intrinsic properties of lobster and leech neurons (Hooper, Thuma et al. 2015). Multiple solutions with different salt concentrations were tested and it was found that ion leak into the cell due to high concentrations in the electrode cause a change in neuron properties. In this chapter, we discuss general methods for electrophysiology as well as how we reliably measured K^+ currents in pyloric neurons.

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2.2 General Methods

In Chapter 2, general preparation methods used for all experiments discussed in this dissertation are presented. Methods pertaining to specific experiments and statistical analysis are described further in each chapter.

2.2.1 Preparation of the Stomatogastric Nervous System

Jonah crabs, *Cancer borealis,* were obtained from local seafood markets in Newark, NJ and kept in salt water tanks maintained at 10 – 13°C. Prior to dissection of the foregut, animals were anesthetized on ice for 30-40 minutes. The stomatogastric nervous system (STNS) was dissected and pinned to a Sylgard-lined Petri dish as described by (Selverston, Russell et al. 1976). Briefly, the STNS was dissected out of the foregut which included the stomatogastric ganglion (STG), the paired commissural ganglia (CoGs), the esophageal ganglion (OG) and the lower motor nerves. Motor nerves included the medial (*mvn*) and lateral (*lvn*) ventricular nerves as well as the pyloric dilator (*pdn*) nerves.

Typically, dissections were completed and stored in the fridge overnight at ~10°C on the day before the experiment was performed. Immediately before beginning experiments, a sheath of tissue over the STG was removed to expose somata for intracellular recording. Preparations were perfused with cold physiological saline (10-13° C) at 6-10 ml/min depending on the ambient temperature. Temperature around the STG was recorded with a thermometer probe placed near the ganglion (~1 cm) and for all experiments the temperature was regulated to 10-13° C.

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Normal *Cancer* saline had the following composition (in mM): 11.0 KCl, 440.0 NaCl, 13.0 $CaCl_2$, 26.0 $MgCl_2$, 11.2 Trizma base, 5.1 Maleic acid; pH= 7.4-7.5 Tetrodotoxin (TTX) obtained from Sigma-Aldrich was added to the saline at a concentration of 10^{-7}M to block action potentials and remove endogenous activity. Unless otherwise stated, TTX was bath perfused for at least 30 minutes prior to any experimental manipulations.

2.2.2 Electrophysiology

Extracellular recordings were obtained using stainless steel electrodes placed into Vaseline wells built around motor nerves. Extracellular recordings were amplified using A-M Systems differential amplifiers (Carlsborg, WA). Data were recorded on a computer hard drive using Clampex 10.2 software and Digidata 1322 digitizer boards (Molecular Devices). Intracellular recordings were made using glass microelectrodes filled with 1M KCl or 0.6M K_2SO_4 + 20 mM KCl. For two electrode voltage clamp (TEVC) experiments, voltage recording electrodes were 18-25 MΩ and current injection electrodes were typically 12-18 MΩ. TEVC was performed using an Axoclamp 2B amplifier (Molecular Devices). At the end of each experiment, if an electrode offset larger than $\pm$5mV was observed, the experiment was discarded.

2.2.3 Stimulation Protocols

Depolarization stimulations were square step voltage pulses (from -60mV to -10mV) injected into the neurons using TEVC at a rate of 1 Hz with a 50% duty cycle.

2.2.4 Voltage-gated Current Protocols

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The total potassium current (I_K) is defined as the total outward current measured from a holding potential of -80mV. To measure total I_K, neurons were held at -80mV for 500msec to deinactivate outward currents, then voltage steps from -60mV to +20mV were applied for 1sec in +10mV increments. The high threshold potassium current (I_{HTK}) was measured with the same voltage steps from a holding potential of -40mV and leak subtracted. I_{HTK} can be pharmacologically separated into two currents: calcium activated potassium current (I_{KCa}) and the delayed rectifier current (I_{Kd})(Golowasch and Marder 1992). Because the experimental protocols involved repeated measurements of different currents, no pharmacology was used to separate these two components. Rather, they were separated into an inactivating transient component, peak I_{HTK}, and a steady state component, steady state I_{HTK}, measured in the last 100msec of the test pulse. The transient potassium current (I_A) is calculated by digital subtraction of I_{HTK} from total I_K.

2.2.5 Statistical Analyses

Current-voltage plots were created using Matlab, OriginLab 8.5, and Corel Graphics Suite X8 software. Sigmaplot 12 software was used to perform statistical tests.

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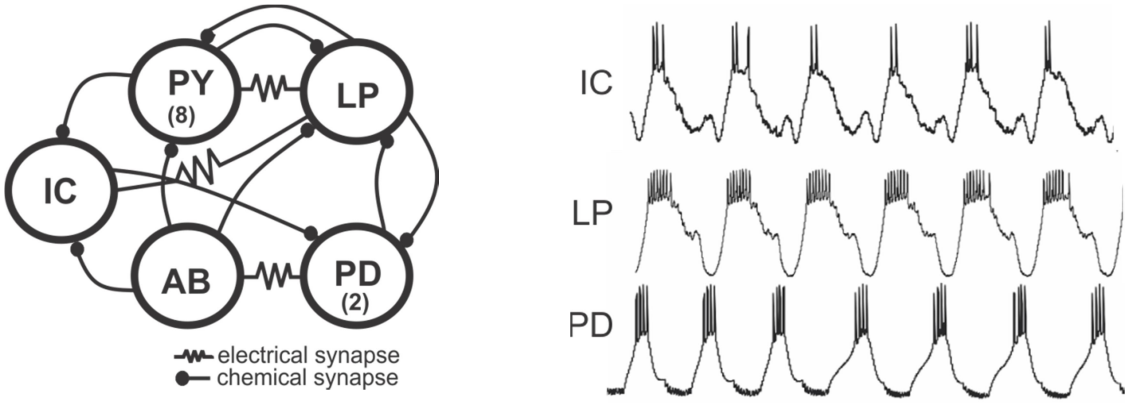

Figure 2.1 The pyloric network**.** *Left*: Connectivity diagram of the pyloric network of the crab *Cancer borealis. Right:* Examples of intracellular recordings from the three neuron types in this study. Inferior cardiac (IC), lateral pyloric (LP), and pyloric dilator (PD) neurons.

`

2.3 Results

In this dissertation, we explored the interaction between neuromodulation and activity-dependent regulation of ionic currents in pyloric neurons of the crab stomatogastric ganglion (STG). However, we were unable to measure activity-dependent effects on I_{HTK} and I_A due to an artifact in the measurement technique in our first attempts to characterize the phenomenon. We measured and quantified four currents: Peak $I_{HTK,}$ - steady state $I_{HTK,}$ I_A, and total I_K (Figure 2.2).

We found that peak I_{HTK} decayed over time when sulfate filled electrodes were used but not when KCl filled electrodes were used. In control experiments, K^+ currents were measured in PD neurons before and after holding the membrane potential at -60 mV to establish the effect of measurement over time. We found that the peak of I_{HTK}, which is predominantly I_{KCa}, disappeared with or without stimulation. Figure 2.5 shows I_{HTK} and I_A current recordings after 30 minutes of holding at -60 mV. The peak current of I_{HTK} disappears while the peak of I_A is unaffected.

After discovering the decay of I_{HTK} due to sulfate, we began measuring currents using KCl filled electrodes. Stimulation experiments were usually conducted over 120 minutes. However, it was found that the stimulation induced change in the currents occurred after 30 minutes and did not change further in both I_{HTK} and I_A (Figure 2.4). In KCl experiments, I_{HTK} decreased in neurons that were stimulated with depolarizing square steps (Figure 2.3) and not in neurons that were clamped at -60 mV (Figure 2.6). The sulfate- induced decay in I_{HTK} did not occur in LP or IC neurons (Figure 2.7). We therefore concluded that this effect was a cell-type specific effect.

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2.4 Discussion

Voltage-gated ion channels are major targets for drug therapies in treating many diseases such as epilepsy, heart attacks, stroke and ischemia. It is therefore crucial to understand how experimental techniques affect reliability of measurements. While this was not the main goal of this dissertation, we were unable to test our hypotheses about activity-dependent regulation of ionic currents without first establishing control measurements.

K^+ currents were measured using K_2SO_4 filled electrodes under many different conditions. Qualitatively, we noticed that I_{HTK} did not have a peak current. At first, we suspected a contamination issue. Experimental setups were cleaned thoroughly and experiments were repeated. In more cases than not, we could not measure the HTK current reliably. We tried measuring currents with KCl electrodes and found that the current was no longer showing decay. We were able to reliably measure all four currents in PD, LP, and IC neurons for 120 minutes in normal saline during an ongoing rhythm. We were also able to measure stable currents over this same time course in TTX-treated preparations. Coincidentally, Hooper el al. (2015) published their findings that high ionic concentrations in sharp electrode physiology affected passive properties of neurons in the STG and leech.

Since both electrode filling solutions we used contained relatively high ionic concentrations, we suggest that the mechanism of action on I_{HTK} is more specific. We did not directly test this hypothesis but comparing results between the two solutions suggests that sulfate could be blocking either I_{KCa} or the underlying I_{Ca} in PD neurons. Many of the results we obtained using sulfate filled electrodes are not presented in this study because of the artifact we discovered. Further experimentation on the effect of sulfate could

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reveal a novel pharmacological agent to selectively block $I_{KCa.}$ Golowasch et al (1999) used both solutions and stated that no significant differences were observed. However, in those experiments, the amount of current inject was controlled because neurons were stimulated using current clamp, whereas our experiments did not control for current injection but rather the voltage of the membrane. Using TEVC allows large amounts of current to be injected into neurons to achieve the desired voltage commands. The results of this study definitely raise many important questions about how physiological measurements are made in this system and many other model systems. We did not observe any alarming changes in measurements using KCl electrode fill. Hooper et al. (2015) demonstrate that one electrode fill which does not result in any changes to passive properties is squid axoplasm, which closely resembles ionic concentrations of lobster STG neurons. A more comprehensive study on crab STG neurons will be required to establish the effect of ionic concentrations on passive properties.

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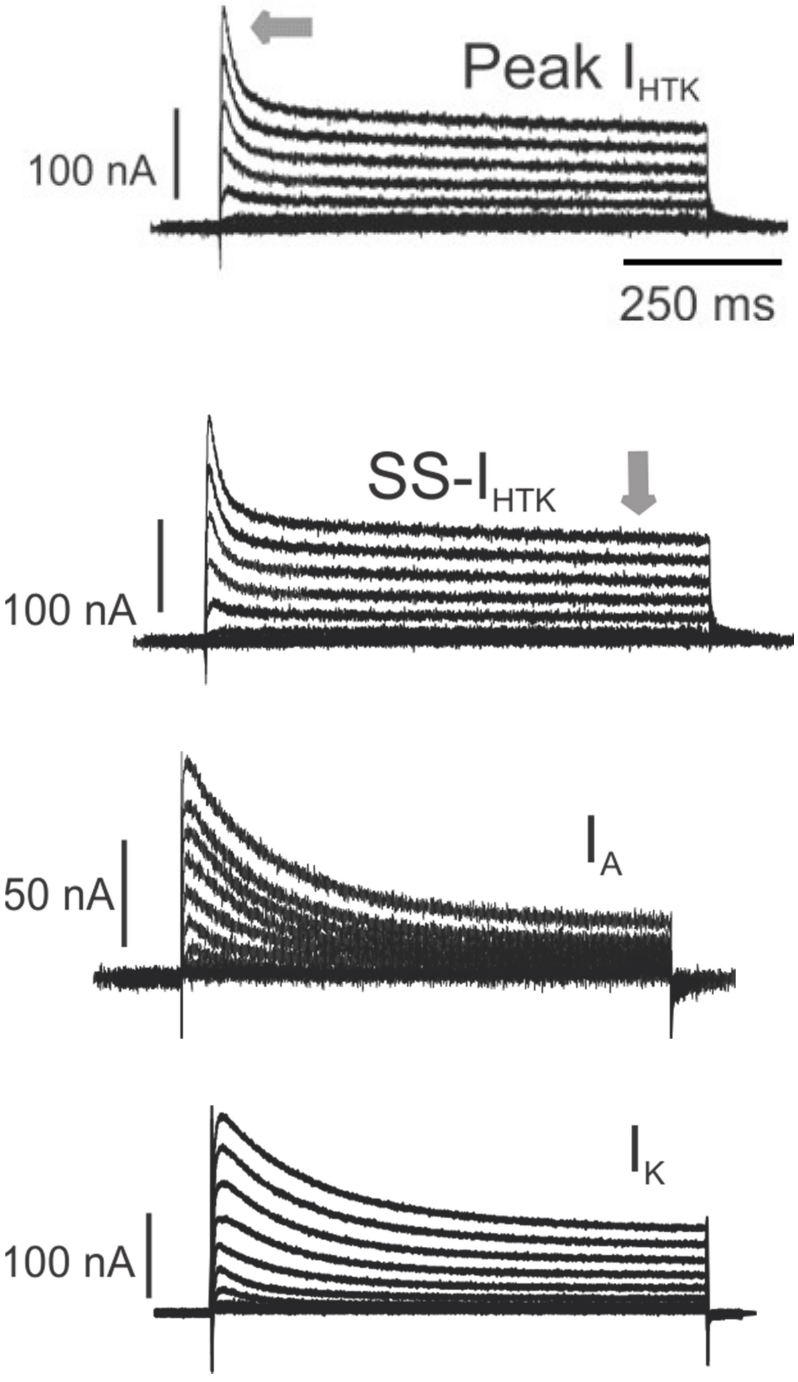

Figure 2.2 Voltage-gated K^+ currents measured in pyloric neurons.

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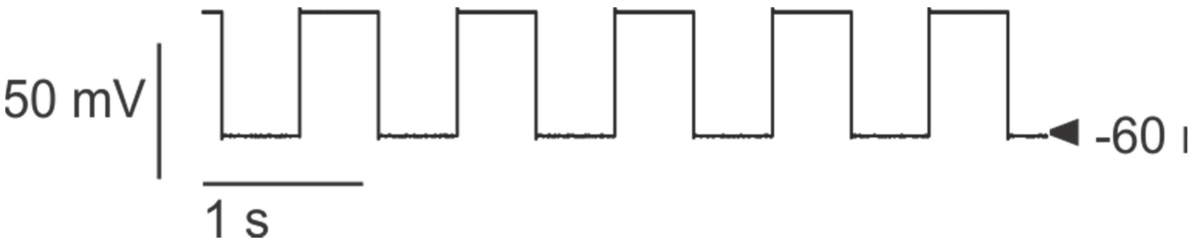

Figure 2.3 Depolarizing stimulation voltage trace.

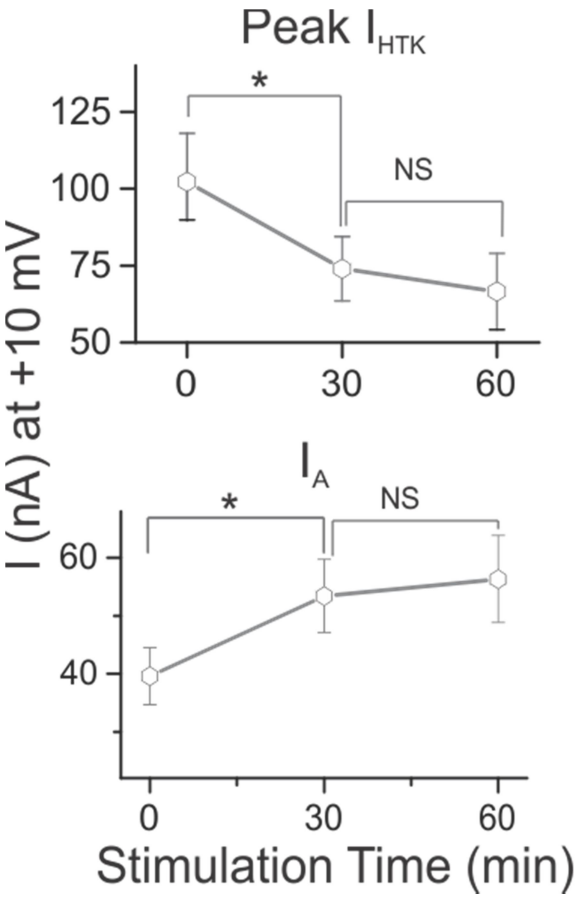

Figure 2.4 Stimulation induced changes in I_{HTK} and I_A occur in 30 minutes in PD neurons. Mean current amplitude ±SEM (n = 9) in response to +10 mV test pulse. One-way Repeated Measures ANOVA shows both I_{HTK} ($p = 0.02$) and I_A ($P = 0.04$) change after 30 minutes.

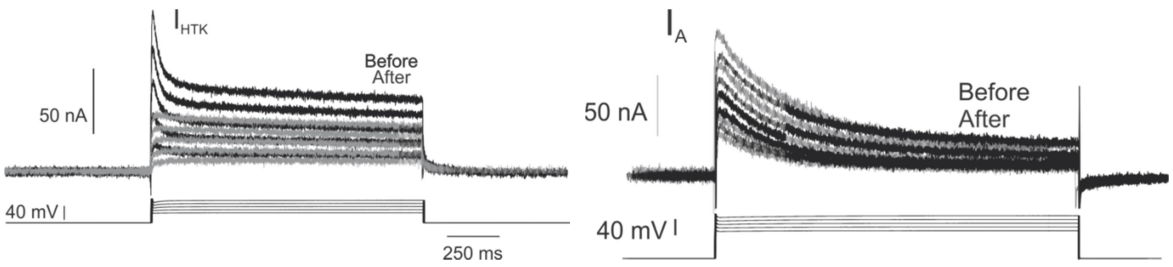

Figure 2.5 K_2SO_4 filled electrodes cause a decay in peak I_{HTK}**.** *Left:* I_{HTK} before (black traces) and after (gray traces) clamping the neuron at -60mV. The peak current disappears completely after 30 minutes of voltage clamp. *Right:* I_A before and after voltage clamping. The peak is unaffected.

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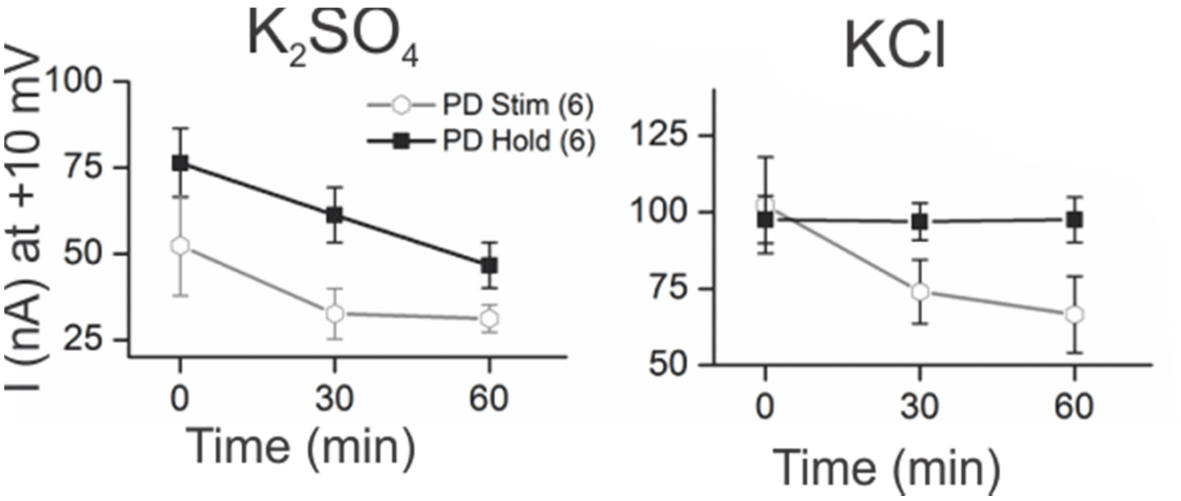

Figure 2.6 Sulfate filled electrodes effect Peak I_{HTK} in PD neurons independent of stimulation. I_{HTK} measured over time in PD neurons using two electrode filling solutions. Mean current amplitude ±SEM. Two-way RM ANOVA with Friedman *post hoc* analysis reveals decrease occurs as a result of time ($P < 0.001$) and not whether neurons were stimulated or not ($P = 0.13$). In KCl filled electrode measurements, results show decrease in peak I_{HTK} in stimulated neurons ($n = 9$), but not held neurons ($n= 11$) an activity-dependent manner. Neurons clamped at -60mV showed no change over time while stimulated neurons decreased significantly ($P = 0.04$, 2-way ANOVA with Holm-Sidak *post hoc* analysis).

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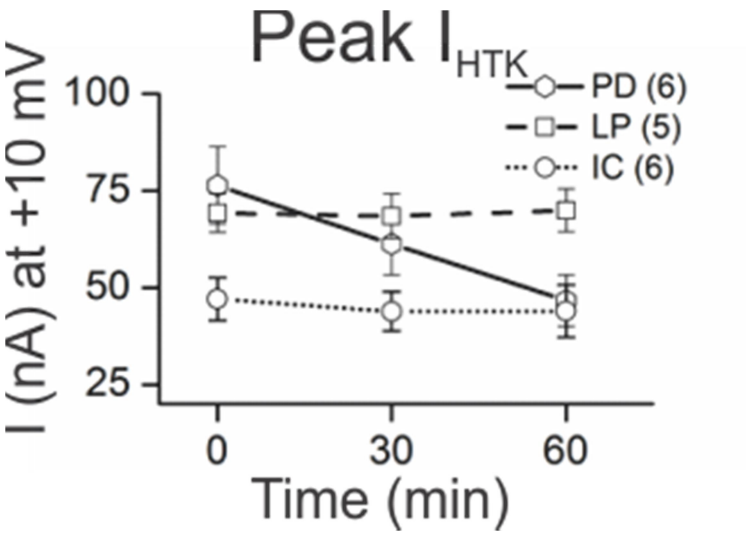

Figure 2.7 K_2SO_4 filled electrodes cause a decay in Peak I_{HTK} in a cell-type specific manner. Mean current amplitude ±SEM in PD (n = 6), LP (n = 5), and IC (n = 6). Neurons voltage clamped at -60mV for 60 minutes. Effect of time is significant in PD (P = 0.003) revealed by One Way ANOVA but not in LP (P = 0.5) or IC (P = 0.2).

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CHAPTER 3

ACTIVITY-DEPENDENT REGULATION OF K^+ CURRENTS ACROSS IDENTIFIED CELL TYPES

3.1 Introduction

Nervous systems produce a wide variety of activities that allow organisms to respond to changing environments. With the capacity to be plastic, neuronal networks must also be able to have some rigidity or constraint on neuronal firing. Incoming stimuli can drive neurons to become more or less excitable, depending on whether the input is an excitatory or inhibitory one. Neurons are capable of tracking their history of activation and adjusting their levels of excitability in order to maintain their output within a stable physiological range. One of the most prominent mechanisms that neurons use to alter their excitability is to alter K^+ conductances (Turrigiano, Abbott et al. 1994, Turrigiano, LeMasson et al. 1995, Debanne, Daoudal et al. 2003, Misonou, Mohapatra et al. 2004). The variety and localization of K^+ channels allows neuronal networks to target changes in their excitability to specific compartments and functions of the network. In hippocampal and pyramidal neurons, for example, the K_{V2} channel, which carries the delayed rectifier current, I_{Kd}, has been shown to suppress neuronal excitability after glutamate stimulation (Mohapatra, Misonou et al. 2009) in the somatodendritic region of these neurons.

In the last decade, many studies have investigated the mechanisms employed by neurons to promote stability after periods of increased activity. Long-term changes in activity of neuronal circuits as a result of long-term potentiation (LTP), facilitation (LTF) and neuromodulation act to drive neurons to a state of activity different than their baseline. The concept of homeostatic plasticity has been characterized as a driving factor

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that targets voltage-gated channels to alter cellular excitability and drive neuronal activity back to baseline (Ransdell, Nair et al. 2012;Becker, 2014 #118). Homeostatic mechanisms allow neurons to track their own output and adjust their intrinsic properties in order to increase or decrease excitability (Golowasch, Abbott et al. 1999, Surmeier and Foehring 2004, Haedo and Golowasch 2006, Ransdell, Nair et al. 2012). Increased K^+ conductance is a widely used mechanism which functions to suppress cellular excitability (Ransdell, Nair et al. 2012) across many neuronal cell types (Turrigiano, LeMasson et al. 1995, Turrigiano, Leslie et al. 1998, Turrigiano and Nelson 2004, Wenner 2014, Whitt, Petrus et al. 2014).

Pyloric neurons have very well characterized intrinsic properties (Golowasch and Marder 1992) and exhibit a wide range of variability across animals while maintaining the same activity, at least with respect to one crucial activity feature, phase relationships (Bucher, Prinz et al. 2005, Goaillard, Taylor et al. 2010, Golowasch 2014). Each neuron produces a distinct activity pattern as a result of the levels of ionic currents it expresses (Golowasch, Buchholtz et al. 1992), synaptic input it receives, and chemical receptors it expresses (Marder and Thirumalai 2002, Prinz, 2003 #189). Previously, it was reported that pyloric neurons have different sensitivity to activity. It was demonstrated that IC neurons exhibited activity-dependent regulation of K^+ currents while LP neurons apparently did not (Golowasch, Abbott et al. 1999). We suggest that the activity profile of a particular neuron dictates what activity will trigger homeostatic mechanisms. We define activity profile as the characteristic electrical activity produced by a particular neuron as a result of its intrinsic properties and inputs it receives from within the network it is a part of. For example, we hypothesized that PD neurons would respond to the same

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electrical stimulation as IC neurons and are more sensitive to activity than LP neurons. In Chapter 2, we demonstrated a cell-type specific effect of sulfate used as an electrode filling solution. PD neurons were sensitive to sulfate and showed decay in peak I_{HTK} while LP and IC did not. PD neurons are distinct in the fact that they are coupled to the pacemaker neurons and can oscillate in isolation. Sensitive and rapidly activating homeostatic mechanisms in neurons that drive network frequency could certainly promote network stability. Since each neuron produces a very specific activity pattern with tightly regulated temporal properties, and across all individuals, we predicted that possess distinct regulatory mechanisms.

We investigated activity-dependent regulation of K^+ currents across three different motor neurons within the pyloric network of the crab stomatogastric ganglion (STG). We tested the hypothesis that regulation of intrinsic excitability may be a cell-type specific mechanism and that neurons possess different sensitivity to the same electrical activity. In cultured STG neurons for example, activity-dependent regulation of K^+ conductance was observed in neurons that underwent a change in activity (Haedo and Golowasch 2006). Subpopulations of neurons were able to adjust their K^+ conductance in response to hyperpolarizing stimulation while others were not. In the present experiments, we applied the same electrical stimulation on isolated pyloric dilator (PD), lateral pyloric (LP) and inferior cardiac (IC) neurons (i.e. in the absence of synaptic and modulatory input) to establish to what extent they all adjusted their K^+ currents and how they differed in this capability.

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3.2 Methods

Methods for preparation and electrophysiology of the stomatogastric nervous system are described in Chapter 2.

3.2.1 Statistical Analysis

Current-voltage plots were created using Matlab and OriginLab 8.5 software. Sigmaplot 12 software was used to perform statistical tests. Statistical analyses involved comparing ionic current parameters using Student's paired t-tests. Two-way repeated measures ANOVA were used to compare current-voltage relationships (I-V curves) before and after stimulation. We adjusted I-V plots to obtain ionic current parameters by fitting the current-voltage values obtained experimentally to the Boltzmann equation

$$I_{\mathrm{K}=\, g_{max}} m_{\infty} (V_m \, - \, E_K)$$

where g_{max} is the maximal conductance, V_m is the membrane potential in the test pulse and E_K is the reversal potential of K^+ (set at -80 mV). m_{∞} is the steady state of activation gate described by

$$m_{\infty} \, = \frac{1}{1+e^{\frac{-(\mathrm{V_m}-V_{\frac{1}{2}})}{k}}},$$

where $V_{1/2}$ is the voltage of half-maximal activation and k a measure of the slope of m_{∞}.

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3.3 Results

All three different pyloric neurons altered their K^+ currents in response to depolarizing stimulation similarly. In each neuron type, K^+ currents were measured before and after 30 minutes of patterned stimulation consisting of a 50% duty cycle depolarization at 1 Hz from -60 to -10 mV.. We refer to the control measurement as the 'before' condition. The measurement was taken after preparations were perfused with 10^{-7}M TTX for 30 minutes and before stimulation began. In the control condition, neurons were isolated from activity and modulatory input. The 'after' measurement was taken immediately after 30 minutes of depolarizing stimulation.

In PD neurons, all four currents measured were affected by stimulation (results shown in Figure 3.1 and Tables 3.1- 3.4). Figure 3.1 shows the four currents measured before and after stimulation in 9 PD neurons. Each row contains an example of a raw trace (left panels) and a current-voltage plot (right panels). These data confirmed our hypothesis that PD neurons adjust their K^+ current levels after being exposed to depolarizing stimulation. Both peak I_{HTK} ($P < 0.001$, $n = 9$) and steady state I_{HTK} ($P < 0.001$, $n = 9$) showed significant reduction in amplitude between the 'before' and 'after' conditions as determined by a Two-way RM ANOVA. Unless indicated otherwise, statistical significance of changes in current amplitudes was determined using 2-way RM ANOVA with Holm-Sidak post hoc analysis. I_A on the other hand, showed a significant increase ($P < 0.001$, $n = 8$), which we predicted since I_A and I_{HTK} have been shown to be co-regulated in a compensatory manner in these neurons (Golowasch, Abbott et al. 1999, Ransdell, Nair et al. 2012). We measured total I_K to demonstrate that changes in I_{HTK}

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and I_A were indeed compensating for each other and that there were no changes in total K^+ current levels of the cell. Surprisingly, a significant decrease in I_K was observed ($P < 0.001$, $n = 8$). This suggests that the effect of stimulation is dominated by I_{HTK}. Secondly, a decrease in total I_K could mean that this stimulation protocol may be a inducing an increase cellular excitability.

We tested LP neurons with the prediction that they would be insensitive to activity. LP neurons responded similarly to PD neurons. Results for current measurements and activation parameters before and after LP stimulation are reported in Figure 3.2 and Tables 3.5-3.8. A significant decrease in peak I_{HTK} was observed at voltages where the current was activated ($P < 0.001$, $n = 9$). The activation parameters we obtained from fitting the I-V plots changed significantly in response to stimulation. The maximal conductance decreased from 1.24 ± 0.07 to 1.08 ± 0.06 us ($P = 0.0004$, $n = 9$, paired Student's t-test). The voltage dependence of activation was affected by stimulation, $V_{1/2}$ increased significantly to a more depolarized voltage ($P = 0.002$, $n = 9$, paired Student's t-test). I_A amplitude showed a significant increase across the voltage range it is activated after stimulation ($P = 0.045$, $n = 8$). Total I_K also decreased significantly after stimulation at 0 mV, +10 mV and +20 mV ($P < 0.001$, $n = 8$).

Results from IC neurons are reported in Figure 3.3 and Tables 3.9-3.12. IC neurons responded similarly to PD neurons. Peak and steady state I_{HTK} amplitude decreased significantly ($p < 0.001$, $n = 5$) after stimulation (Figure 3.3). I_A increased (Figure 3.3) after stimulation ($p = 0.04$, $n = 5$). Unlike in PD neurons, total I_K (Figure 3.3) was not affected by stimulation in IC neurons ($p = 0.084$, $n = 5$).

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To test whether there were differences in sensitivities to activity across cell types, and considering that each cell types expresses different levels of each current (Golowasch, Abbott et al. 1999), we quantified the percent change for each current after stimulation (Figure 3.4). Each panel in Figure 3.4 shows the mean percent change $\pm$SEM for one current in each cell type. We compared the three means of peak I_{HTK} ($P = 0.7$) and I_A ($P = 0.7$) using a One-way ANOVA. All three neurons adjusted their K^+ currents similarly and with the same magnitude in response to stimulation.

3.4 Discussion

The results of this study demonstrate cell-autonomous regulation of intrinsic properties across cell types within an oscillatory motor network. K^+ currents in PD, LP and IC neurons are regulated by depolarizing activity in the absence of synaptic and modulatory input. In all three cell types, I_{HTK} (peak and steady state) decreased while I_A increased after thirty minutes of depolarizing patterned stimulation. These findings indicate that neurons possess the capability of tracking their activity and adjusting intrinsic membrane properties in response to their own electrical output. We also demonstrate that the three cell types regulate K^+ currents with similar sensitivity to the same activity pattern (Figure 3.4). The opposing changes in these two outward currents have been describes as a compensatory homeostatic mechanism (Ransdell, Nair et al. 2012).

While we did not test membrane excitability directly, our results suggest that when a neuron begins to oscillate in an excitatory manner, K^+ current levels change in response. We propose that the mechanisms which drive changes in K^+ currents involve both homeostatic and Hebbian plasticity. The homeostatic force which acts on multiple

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voltage-gated currents to stabilize cellular excitability allows neurons to respond to destabilizing inputs rapidly to control intrinsic excitability. Depolarizing voltage steps may be activating calcium-dependent reduction in I_{HTK} (Misonou, Mohapatra et al. 2004) which then leads to a compensatory increase in I_A. When neurons are isolated in TTX, current levels are sitting at some steady state as a result of lack of activity. Once depolarization begins, calcium-activated pathways (Liu, Golowasch et al. 1998, Misonou, 2006 #192, Misonou, 2004 #89) lead to reduction in $I_{HTK.}$ The transition from silent to either tonic or bursting activity has been shown to be accompanied by a reduction in K^+ conductance in STG neurons (Haedo and Golowasch 2006, Turrigiano, 1994 #70). Interestingly, our results demonstrate that this process occurs at a very short time scale. Studies investigating activity-dependent regulation of neuronal properties have involved chronic changes that occur on the magnitude of hours to days (Thoby-Brisson and Simmers 2002, Temporal, 2014 #224, Desai, 1999 #225, Roceri, 2004 #226). The effect of electrical stimulation we observed occurred in 30 minutes.

We also observed a decrease in the total K^+ current which suggests an increase in excitability of the membrane. Hebbian plasticity drives changes in nervous systems with a positive feedback from experience while homeostatic mechanisms generally involve a negative feedback on intrinsic currents in response to increased activity. The compensatory shift between I_{HTK} and I_A has been suggested to be a homeostatic response to changes in cellular excitability (Ransdell, Nair et al. 2012). While our results do not directly demonstrate, the change in current levels in opposite directions in response to electrical stimulation does not contradict the theory of compensation. The decrease observed in total K^+ current suggests an increase in cellular excitability and a

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nonhomeostatic response. Nonhomeostatic or Hebbian mechanisms(Toyoizumi, Kaneko et al. 2014) which rely heavily on presynaptic scaling result in increased excitability in response to excitatory inputs. Periods of increased activity lead to increased Kv 2.1 (I_{Kd}) via dephosphoryaltion of channel proteins and suppression of excitability in mammalian neurons (Misonou, Menegola et al. 2006). Indeed, many studies have demonstrated an overall increase in K^+ conductance in response to increased activity especially in disease models such as seizure and stroke (Bekkers 2000, Du, 2000 #288). Since there were changes in activation of K^+ currents (Tables 3.1-3.12), it is likely that changes in current amplitude were due to changes in voltage dependence due to changes in phosphorylation states. It is unlikely that channel density was altered since the effect occurred at such a short time scale.

In this study, we performed the first measurements in isolated or activity-deprived neurons and the second measurement in neurons which were driven at a frequency similar to their endogenous activity. We suggest that the two measurements represent very different physiological conditions that the neurons sense. The activity-deprived state involves a blockade of Na^+ channels. Little is known about the regulation of these fast channels in these neurons since their kinetics render them difficult to measure. After stimulation, it can be assumed that activation of calcium currents and calcium-dependent pathways occurs.

The fact that all three neuron types responded similarly to the same activity was surprising. Golowasch et al (1999) showed that IC neurons were sensitive to depolarizing patterned stimulation while LP neurons were not. We suspect that this discrepancy may be due to the experimental technique used to stimulate the cells. Using TEVC to

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stimulate neurons rather than current clamp may be more effective in changing membrane potential and causing cells to adjust their intrinsic properties. Our original hypothesis was that activity-dependent regulation is cell-type specific. However, one major caveat in our experiments is the fact that the neurons were all isolated from synaptic input and tested in the absence of their endogenous activity. It is possible that isolating the neurons removed any cell-type specific differences that exist and could explain why they all had similar responses to the same electrical stimulation.

Our observations raised the question of whether different types of activity drove the current levels in a different direction. The square step pulses we used to stimulate were meant to be proxies for endogenous activity. Do the neurons sense these voltage changes as similar to their endogenous activity? Do different types of activity patterns have the same effect? Understanding how neurons deal with changes in electrical activity and how they regulate voltage-gated currents which can either cause or compensate for these changes can lead to valuable insight on the contribution of different regulatory mechanisms working to stabilize neuronal function. This will be the focus of the next chapter.

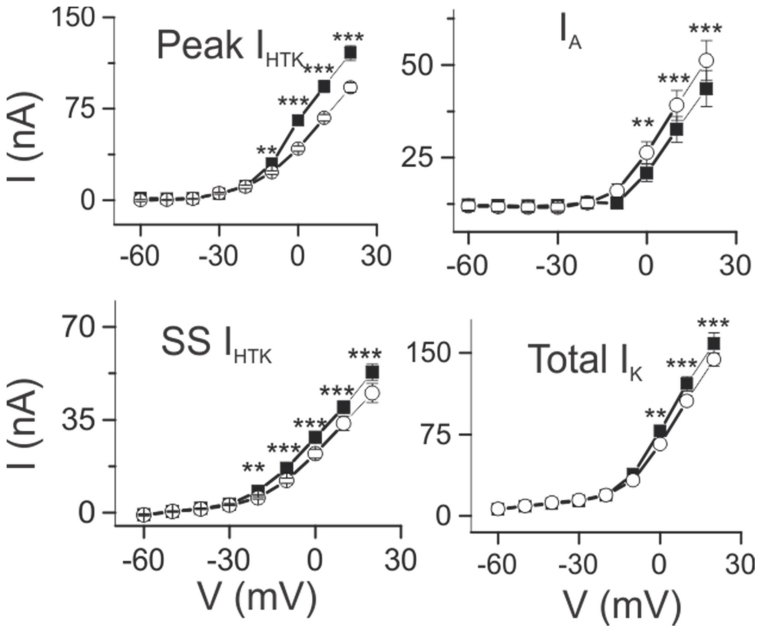

Figure 3.2 Depolarizing stimulation affects K^+ currents in LP neurons. Left panels: examples of current responses to +10mV test pulses before (black trace) and after (gray trace) depolarizing stimulation. Currents are (from top to bottom) Peak I_{HTK}, steady state I_{HTK}, I_A and total I_K. Right panels: current-voltage plots of the four currents before (solid square symbols) and after (open circle symbols) depolarizing stimulation. Statistical analysis was done by performing pairwise multiple comparisons using a 2-way RM ANOVA and Holm-Sidak post hoc analysis. **P < 0.01 ***P < 0.001

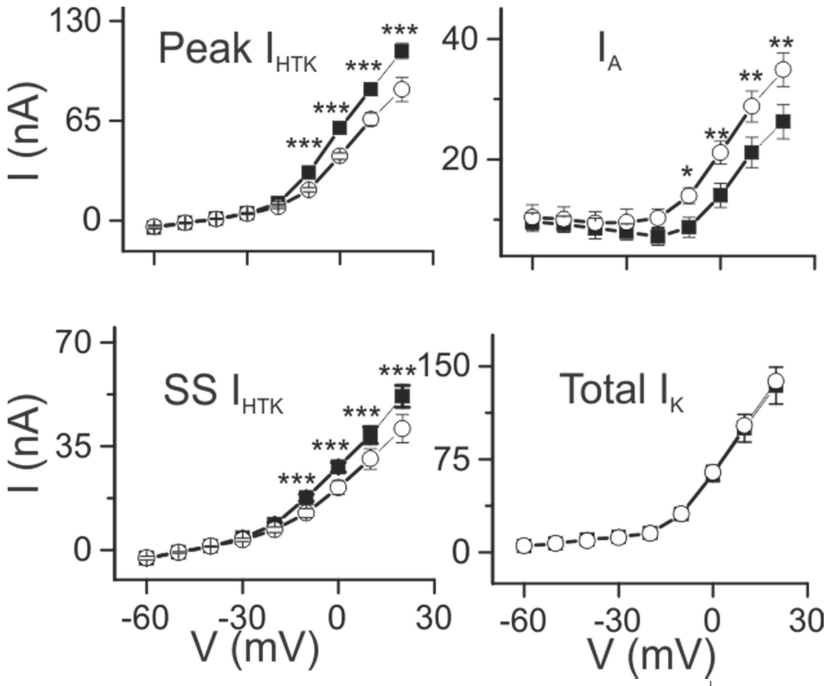

Figure 3.3 Depolarizing stimulation in IC neurons affects K^+ currents. Left panels: examples of current responses to +10mV test pulses before (black trace) and after (gray trace) depolarizing stimulation. Currents are (from top to bottom) Peak I_{HTK}, steady state I_{HTK}, I_A and total I_K. Right panels: current-voltage plots of the four currents before (solid square symbols) and after (open circle symbols) depolarizing stimulation

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Table 3.1 Ionic Current Parameters for Peak I_{HTK} in Depolarized PD Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	1.5	1.2	0.16	0.15
$V_{1/2}$	-5.9	-0.9	1.0	1.8
Slope Factor	8.69	9.94	0.51	0.87

Table 3.2 Ionic Current Parameters for Steady State I_{HTK} in Depolarized PD Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.76	0.59	0.12	0.12
$V_{1/2}$	-3.2	-1.9	1.02	0.9
Slope Factor	12.4	12.1	0.8	0.6
Input Resistance	11.2	16.5	2.1	4.4

Table 3.3 Ionic Current Parameters for I_A in Depolarized PD Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.6	0.7	0.08	0.1
$V_{1/2}$	-1.7	-4.9	1.9	2.08
Slope Factor	11.2	11.8	1.3	1

Table 3.4 Ionic Current Parameters for Total I_K in Depolarized PD Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	2.2	2.0	0.18	0.13
$V_{1/2}$	-4.02	-2.2	0.8	1.6
Slope Factor	10.3	11.6	0.73	1.3

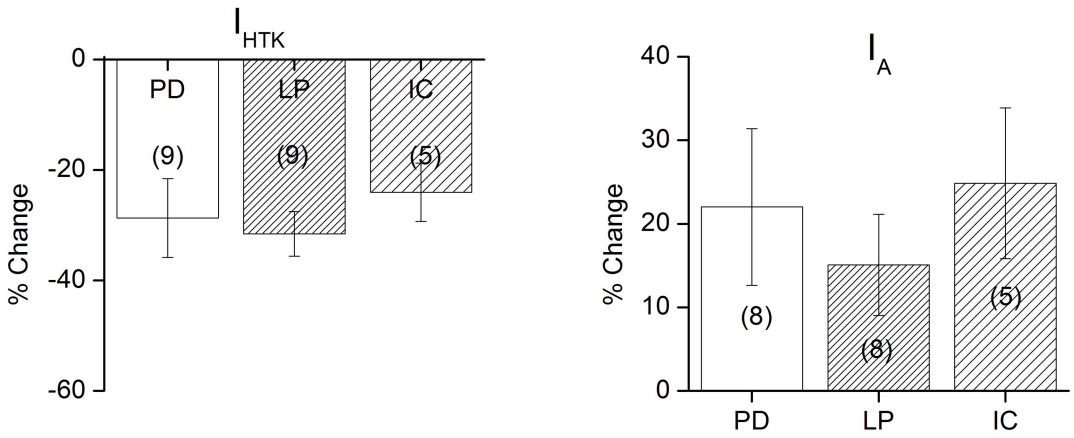

Figure 3.4 Activity-dependent change in K^+ currents across pyloric neurons. Changes in K^+ currents in response to depolarizing stimulation are similar in PD, LP and IC neurons. Current measurements taken from data shown in Figures3.1-3.3. Plots show mean percent change in current amplitude (measured at +10mV) $\pm$ SEM after 30 minutes of stimulation. Numbers in parenthesis represent sample sizes. No significant differences in sensitivty across the three cell types were observed using a one-way ANOVA (I_{HTK}, P = 0.7, I_A, P = 0.7).

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Table 3.5 Ionic Current Parameters for Peak I_{HTK} in Depolarized LP Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	1.2	1.1	0.07	0.06
$V_{1/2}$	-3.5	3.1	1.2	1.8
Slope Factor	8.4	11.6	0.4	0.7

Table 3.6 Ionic Current Parameters for Steady State I_{HTK} in Depolarized LP Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.6	0.5	0.03	0.04
$V_{1/2}$	-1.6	0.36	1.2	0.6
Slope Factor	12.4	11.7	1.01	0.2
Input Resistance	17.1	24	2.7	4.3

Table 3.7 Ionic Current Parameters for I_A in Depolarized LP Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.4	0.5	0.05	0.05
$V_{1/2}$	5.8	3.5	0.6	0.98
Slope Factor	6.02	7.4	0.43	0.5

Table 3.8 Ionic Current Parameters for Total I_K in Depolarized LP Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	1.7	1.63	0.12	0.09
$V_{1/2}$	-0.6	2.6	0.5	0.6
Slope Factor	9.1	10	0.5	0.4

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Table 3.9 Ionic Current Parameters for Peak I_{HTK} in Depolarized IC Neurons

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	1.1	0.99	0.05	0.09
$V_{1/2}$	-6	-0.5	1.2	1.7
Slope Factor	9.3	10.6	0.4	0.4

Table 3.10 Ionic Current Parameters for Steady State I_{HTK} in Depolarized IC Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.5	0.4	0.04	0.05
$V_{1/2}$	-0.99	1.8	1.7	1.2
Slope Factor	13.7	13.7	0.6	0.2
Input Resistance	7.9	9.6	1.7	2.01

Table 3.11 Ionic Current Parameters for I_A in Depolarized IC Neurons

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	0.2	0.3	0.02	0.05
$V_{1/2}$	3.2	-0.3	1.03	2.4
Slope Factor	4.9	6.6	0.3	0.5

Table 3.12 Ionic Current Parameters for Total I_K in Depolarized IC Neurons.

	Mean		SEM	
	Before	*After*	*Before*	*After*
G_{max}	1.4	1.4	0.05	0.09
$V_{1/2}$	-1.5	0.5	1.1	2.04
Slope Factor	11.5	11.6	0.5	0.97

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CHAPTER 4

K^+ CURRENTS ARE REGULATED BY DEPOLARIZING PATTERNS OF ACTIVITY IN RHYTHMIC MOTOR NEURONS OF THE PYLORIC NETWORK

4.1 Introduction

Plasticity and long-lasting changes in strength and structure of synapses have been extensively characterized. These studies have demonstrated that growth, development, learning and memory require that intrinsic properties of neurons also be plastic in order to respond to changing synaptic drive. The response and subsequent output of a neuron depends highly on the intrinsic membrane properties. The combination and balance between inward and outward voltage-gated currents plays a big role in regulation of excitability and providing neurons the capability to be both flexible and stable in a history and experience-dependent manner (Turrigiano, LeMasson et al. 1995, Golowasch, Abbott et al. 1999, Misonou, Menegola et al. 2006, Fauth and Tetzlaff 2016). The cellular mechanisms driving this cell-autonomous regulation are not well understood. Many phenomena involving neurons producing a reliable output by adjusting levels of voltage-gated currents to compensate for decreased synaptic excitation have been observed (Golowasch, Abbott et al. 1999, Marder, 2002 #335, Karunanithi, 2015 #308, Nelson, 1998 #218, Turrigiano, 2004 #334).

Much of the experimental evidence demonstrating regulation of voltage-gated currents suggest that states of electrical activity are tracked by

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cell autonomous mechanisms that allow neurons to sense their electrical output and modify intrinsic properties to maintain stable output(Desai, Rutherford et al. 1999, Thoby-Brisson and Simmers 2000, Haedo and Golowasch 2006). One example of this occurs in seizures in rats *in vivo.* Increased neuronal activity induced by glutamate excitation leads to modifications in the voltage activated potassium channel, Kv2.1, which carries the delayed rectifier current, I_{Kd} (Misonou, Mohapatra et al. 2004, Misonou, Menegola et al. 2006) and facilitates suppression of action potential firing. Increased activity for extended periods of time is destabilizing to neuronal networks. Therefore, activity-dependent regulation of voltage-gated currents allows neurons to link intrinsic excitability to synaptic excitation. In many studies of activity-dependent changes to intrinsic currents, drastic changes in neuronal activity have been demonstrated to activate these pathways, such as in extended periods of activity deprivation (Hengen, Lambo et al. 2013, Felix-Oliveira, Dias et al. 2014) or extended excitation (Leslie, Nelson et al. 2001, Swanwick, Murthy et al. 2006). Activity-dependent phosphorylation of Kv2.1 channels in rat hippocampal neurons has been shown to be bidirectional and sensitive to both excitation and suppression of neuronal activity (Misonou, Menegola et al. 2006). These changes represent a cell autonomous response after a dramatic change to overall change in excitability and functional output. Here, we ask whether neurons have the capacity to sense subtle changes in activity, such as a modification of the polarity of oscillatory behavior.

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To examine these processes we measured activity-dependent regulation of K^+ currents in single motor neurons of the crab pyloric network of the stomatogastric ganglion (STG). The electrical output of these neurons is very reliable; they fire bursts of action potentials with a constant phase at a similar network frequency across many preparations (Bucher, Prinz et al. 2005, Hamood and Marder 2014). The same constancy is observed for each identified neuron in the network across many preparations and the network activity is similar to that in the intact animal. These observations suggest that the stability of activity output is very tightly regulated. In crustacean cardiac neurons, pharmalogical blockade of each of the two outward currents, I_{KCa} and I_A, resulted in increased excitability of the neurons. However, over time excitability returned to baseline and it was shown that I_A and I_{KCa} rapidly compensate for each other to maintain stable neuronal output (Ransdell, Nair et al. 2012).

In the present study, we tested whether K^+ current regulation was sensitive to patterned voltage changes with different polarity and temporal properties. We predicted that K^+ currents are tuned to respond to a range of activity patterns thus giving the neuron flexibility to vacillate between different electrical outputs yet remain within a functional physiological range. By driving the membrane potential of a neuron with an imposed activity and measuring intrinsic currents, we can infer what the effects of different synaptic drives are on cellular excitability in the absence of modulatory input.

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4.2 Methods

Preparation and electrophysiology of the stomatogastric nervous system is described in Chapter 2.

4.2.1 Stimulation Protocols

We exposed pyloric neurons to four different modes electrical stimulation: 1) Depolarization 2) Hyperpolarization 3) Holding (or no stimulation) 4) Realistic waveforms. Depolarizing stimulations consisted of square step pulses injected into the neurons using TEVC. The membrane potential was clamped from -60mV to -10mV at a rate of 1 Hz with a 50% duty cycle. Hyperpolarization stimulations consisted of square step pulses from -60mV to -110mV. Holding involved continuously clamping the membrane potential at -60mV. Realistic waveforms were injected into PD neurons using prerecorded intracellular activity obtained from a PD neuron that had a slow wave oscillation from -60mV to -40mV and bursts of action potentials reaching -30mV. All experiments using realistic waveforms utilized the same PD recorded waveform. All stimulation protocols were applied for 30 minutes followed by current measurements.

4.2.2 Statistical Analyses

Current-voltage plots were created using Matlab, OriginLab 8.5, and Corel Graphics Suite software. Sigmaplot 12 software was used to perform statistical tests. Two-way repeated measures ANOVA were used to compare current amplitudes across different voltages before and after stimulation. *Post hoc* tests are indicated in figure legends. For normally distributed data, the Holm-

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Sidak method was used to determine where differences occurred for significant differences in treatments. For data that was not normally distributed, usually in small sample sizes, we used a nonparametric test for an analysis of variance.

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4.3 Results

The prediction was that K^+ current levels were tuned to respond to activity within a certain voltage range. It was expected that realistic waveforms would produce similar or more enhanced changes in current levels as depolarizing square steps. Whether or not hyperpolarizing steps would produce the opposite changes was not determined *a priori*. Figure 4.1 shows the four currents measured before and after stimulation in 9 PD neurons which were presented previously (see Chapter 3). Briefly, our findings showed that I_{HTK} (peak and steady state) decreased significantly ($P< 0.001$, $n = 9$) while I_A increased ($P < 0.001$, $n = 8$) in response to depolarizing stimulation. The total K^+ decreased as well ($P < 0.001$, $n = 8$). To control for time in these experiments and test whether changes were observed in 'resting' neurons, we measured currents before and after holding the membrane potential at -60 mV. Preparations were treated with 10^{-7} M TTX for 30 minutes before the first measurement. After the first measurement, the membrane potential was clamped at -60 mV continuously for 30 minutes. Figure 4.2 shows raw traces (left panels) and I-V plots (right panels) of K^+ currents recorded before and after clamping at -60mV. No significant changes were observed in peak I_{HTK} ($P = 0.96$, $n = 12$), steady state I_{HTK} ($P = 0.56$, $n = 12$), I_A ($P = 0.41$, $n = 10$) and I_K ($P = 0.6$, $n = 10$).

Figure 4.3 shows current responses to depolarizing stimulation in LP neurons (discussed in Chapter 3). When LP neurons were held at -60 mV, K+ currents remained stable (Figure 4.4). Statistical comparisons showed no

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significant difference before and after holding the membrane potential at -60 mV: Peak I_{HTK} (P = 0.8, n = 8), steady state I_{HTK} (P = 0.2, n = 8), I_A (P = 1, n = 8), I_K (P = 0.7, n = 8). In IC, we observed similar results (statistical results for Figure 4.5 discussed in Chapter 3). Currents remained stable when the membrane potential was clamped at -60 mV (Figure 4.6): Peak I_{HTK} (P = 1, n = 4), steady state I_{HTK} (P = 0.5, n = 4), I_A (P = 0.35, n = 4), I_K (P = 0.5, n = 4).

In order to test the main hypothesis of this study, PD neurons were stimulated with two other patterns of activity: hyperpolarizing square steps and prerecorded realistic waveforms. Hyperpolarizing square steps were applied (-60 mV to -110 mV) at a rate of 1 Hz. Current levels did not change after 30 minutes of hyperpolarizing. Figure 4.7 shows the four currents measured before and after stimulation: Peak I_{HTK} (P = 0.9, n = 11), steady state I_{HTK} (P = 1, n = 11), I_A (P = 0.09, n = 11), total I_K (P = 0.9, n = 11). In neurons stimulated with a prerecorded PD waveform (Figure 4.8), no significant change in current levels was observed after 30 minutes of stimulation: Peak I_{HTK} (P = 0.9, n = 9), steady state I_{HTK} (P = 0.7, n = 9), I_A (P = 0.24, n = 9), total I_K (P = 1, n = 9).

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4.4 Discussion

In the present study, we characterized how single neurons regulate voltage-gated ionic currents under different voltage fluctuation conditions, or electrical activity output. Numerous studies have demonstrated that neurons can transition between different states of electrical activity (i.e., single spikes, tonic firing, bursting, etc.) as a consequence of shifts in expression levels of ionic currents (Haedo and Golowasch 2006, O'Leary, 2014 #337). To examine how ionic current levels respond to experimentally induced changes in electrical activity we subjected multiple populations of PD neurons to distinct activity patterns. We found that PD neurons express stable levels of K^+ currents under three different conditions: patterned hyperpolarization, realistic waveforms and holding. Only depolarizing pulses had an effect on the currents. It is interesting that certain paradigms exist where there is robustness of current levels.

Our results demonstrate that K^+ currents are very sensitive to changes in the electrical activity of a neuron with a very high resolution. These results demonstrate that neurons isolated from modulatory and synaptic input can maintain stable levels of ionic currents. In all the experiments, currents were measured with the same starting conditions and the only variable that changed in each condition was the membrane potential over time. The activity pattern we imposed on the neurons effectively caused a transition from a silent to oscillatory-like state. In the realistic waveform experiments, PD neurons were indeed oscillating as they would in an intact preparation. My observations

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indicate that neurons are capable of distinguishing temporal properties in patterns of activity with great acuity. In square step pulses the voltage change from the resting potential is virtually instantaneous. When stimulating with realistic waveforms, the depolarization of the membrane happens with a slower time course. This result demonstrates that neurons can track their activity with precision that is sensitive to how slow or fast voltage fluctuations occur within a cycle of an oscillation, or the period of the oscillation. K^+ currents only change when the membrane potential is driven with depolarizing square step pulses in all three cell types measured. we suggested in Chapter 3 that depolarizing square steps maybe activating both homeostatic mechanisms and Hebbian plasticity simultaneously. Within one cycle of the step, the membrane potential spends a considerable amount of time (500 msec) at a depolarized voltage (-10 mV) where calcium currents are activated (Fisher, Gray et al. 1990, Johnson, Kloppenburg et al. 2003, Catterall 2011). During realistic waveforms on the other hand, the membrane potential is not as depolarized (-40 mV) and firing bursts of action potentials during that depolarization (to -30 mV). Although we did not measure calcium currents, we suspect that increased Ca^{2+} during square steps mediated changes in current levels. This is conclusion is consistent with previous findings in STG neurons as well as mammalian neurons (Turrigiano, Abbott et al. 1994, Liu, Golowasch et al. 1998, Golowasch, Abbott et al. 1999, Misonou, Mohapatra et al. 2004, Haedo and Golowasch 2006, Misonou, Menegola et al. 2006). Since changes in current levels are calcium-dependent (Golowasch, Abbott et al. 1999), it is

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possible could be that realistic waveforms do not activate this pathway. More interestingly, we suspect that the information encoded in the realistic waveform (i.e., slope of the voltage change, bursts of action potentials, etc.) promotes robustness or suppresses the need for homeostatic mechanisms.

The fact that we did not observe changes in K^+ current levels in response to hyperpolarizing pulses does not necessarily mean that intrinsic properties were unaffected by this activity. In hyperpolarizing the membrane potential (-110 mV) with the same time course as depolarizing pulses (500 msec), it is likely that inward currents, such as I_h or I_{Ca}, which are active in this voltage range, were either upregulated or downregulated. If homeostatic rules apply, then we predict that inward currents were upregulated (O'Leary, Williams et al. 2013) during hyperpolarizing pulses. In PD neurons, I_h is very small and difficult to detect therefore we did not measure it. Since it was established that the effect of stimulation occurs after 30 minutes, in future experiments, inward currents can be pharmacologically isolated and measured to examine whether they are involved in homeostatic, activity-dependent mechanisms.

The fact that current levels did not change in holding experiments was exactly what was predicted. The hypothesis was that currents are regulated in an activity-dependent manner. Therefore, holding experiments were performed as a time-dependent control. These experiments demonstrate how acute changes of inputs to oscillatory neurons can still result in stability of intrinsic properties. Changes in intrinsic properties after removal of neuromodulation

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has been demonstrated to occur of the course of several days in STG neurons (Thoby-Brisson and Simmers 2000, Thoby-Brisson and Simmers 2002, Zhang, Khorkova et al. 2009, Zhang and Golowasch 2011). Moreover, this study was limited to the functional consequences of changes in activity and only measured a small sample of voltage-gated currents. It is interesting that a certain range, or repertoire, of activity patterns that these currents are tuned to exists. The three oscillatory patterns tested in these experiments all had a similar frequency (1 Hz) and duty cycle (50%). To explore the effect of activity patterns on K^+ currents, frequencies and spiking behaviors different than those tested here need to be examined, such as high frequency repetitive spiking. Comparing Ca^{2+} currents in these different protocols may reveal more about activity-dependent regulation.

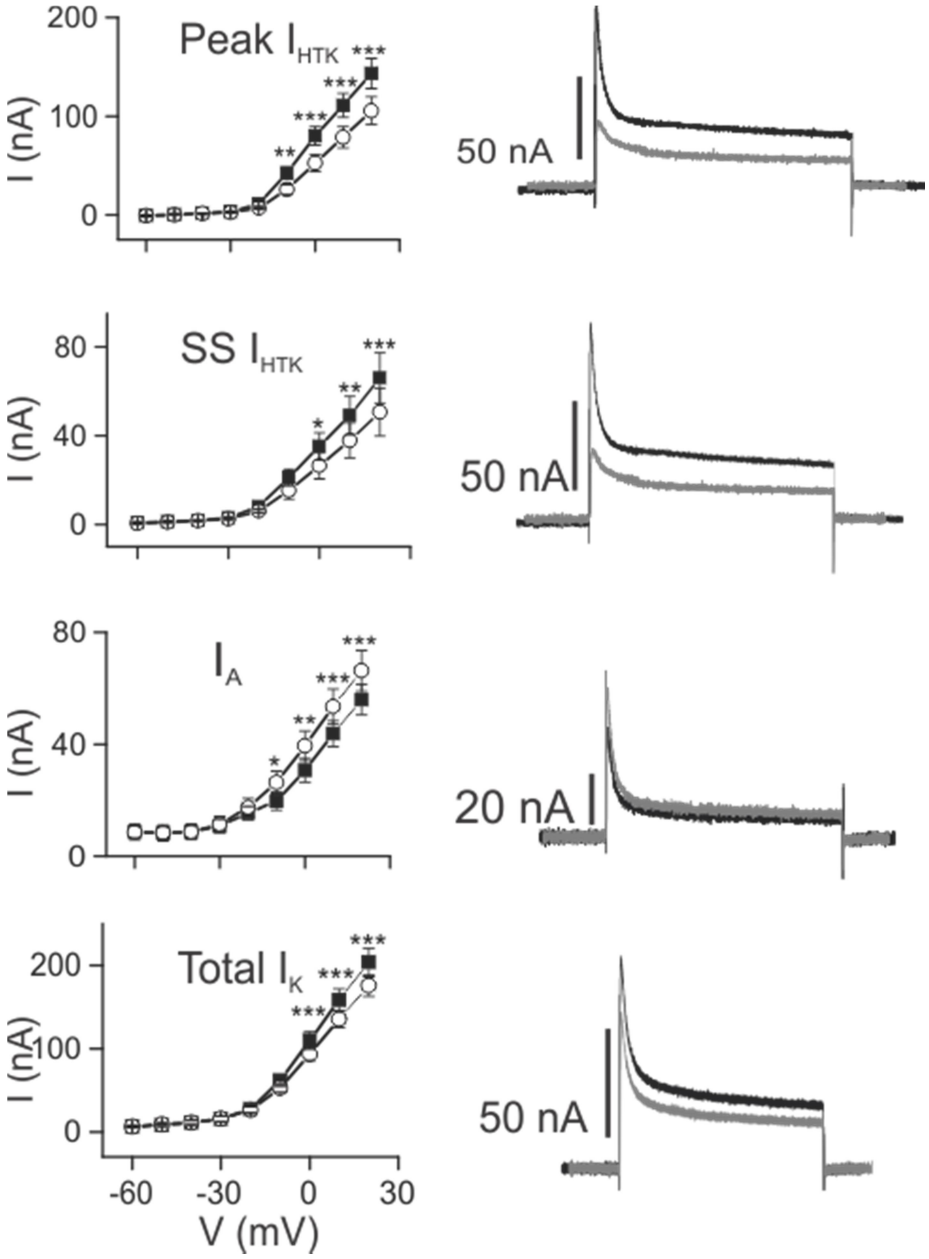

Figure 4.1 Depolarizing stimulation affects four K^+ currents in PD neurons. Right panels: ɛamples of current responses to +10mV test pulses before (black trace) and after (gray trace) imulation. Currents are (from top to bottom) Peak I_{HTK}, steady state I_{HTK}, $I_{A,}$ and total I_K. eft panels: current-voltage (I-V) plots of the four currents. Solid square symbols are control ɹrrents measured 30 minutes after TTX treatment and before stimulation begins. Open circle ɣmbols are currents measured after 30 minutes of depolarizing stimulation. Statistical ɑalysis was done by performing pairwise multiple comparisons using a 2-way RM ANOVA ɑd Holm-Sidak *post hoc* analysis (n = 9). **P < 0.01 ***P < 0.001

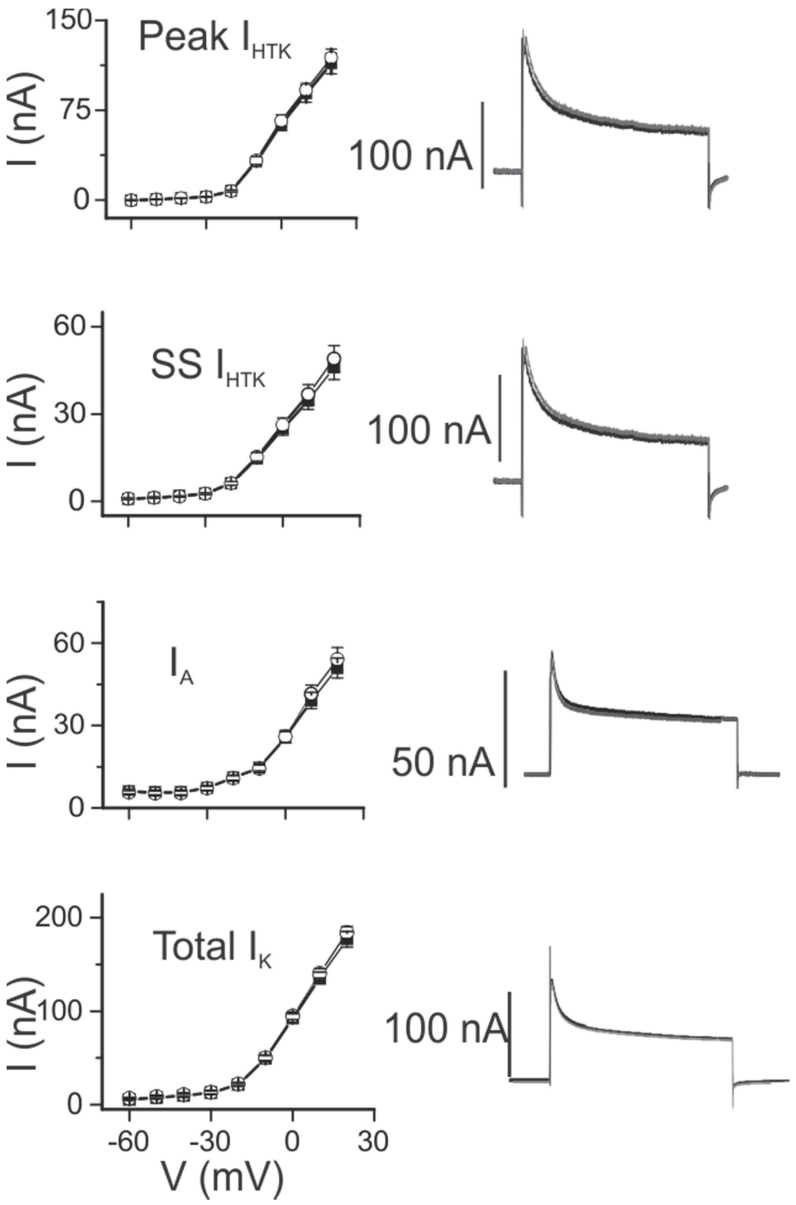

Figure 4.2 K^+ currents do not change after holding PD neurons at -60 mV. Right panels: examples of current responses to +10mV test pulses before (black trace) and after (gray trace) holding. Currents are (from top to bottom) Peak I_{HTK}, steady state I_{HTK}, I_A and total I_K. Right panels: I-V plots of the four currents. Solid square symbols are currents measured 30 minutes after TTX treatment. Open circle symbols are currents measured after 30 minutes of voltage clamping at -60mV. Two-way RM ANOVA reveals no significant differences occur. Total I_K and I_A(n=10). Peak and steady state I_{HTK} (n= 12)

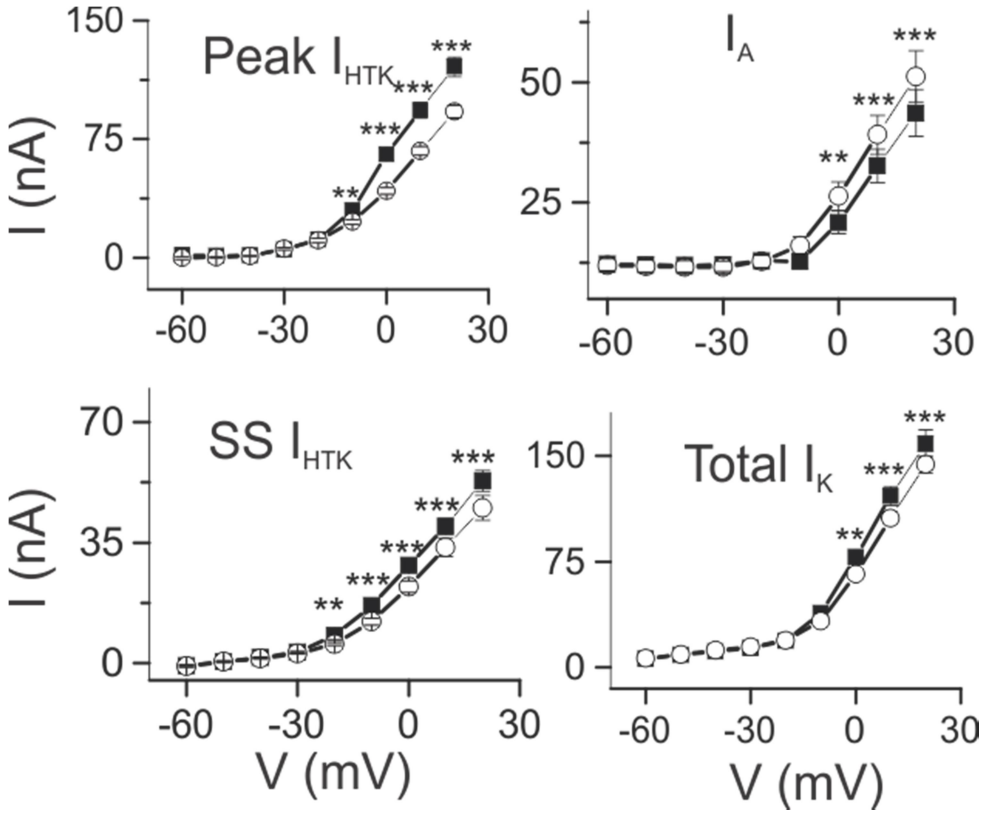

Figure 4.3 Depolarizing stimulation affects K^+ currents in isolated LP neurons. Mean current amplitude ±SEM. I-V plots of the four currents before (solid square symbols) and after (open circle symbols) depolarizing stimulation on LP neurons (n= 9). Statistical analysis was done by performing pairwise multiple comparisons using a 2-way RM ANOVA and Holm-Sidak *post hoc* analysis. **P < 0.01 ***P < 0.001

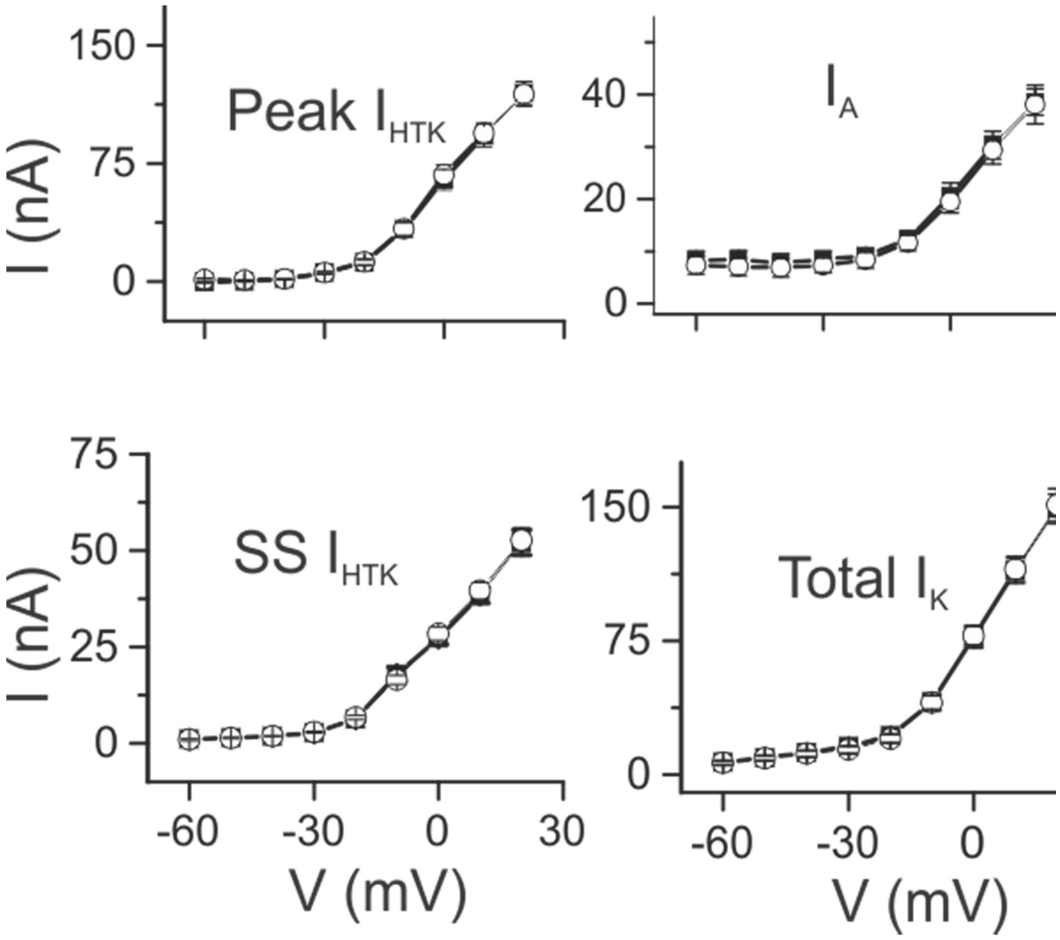

Figure 4.4 K^+ current levels do not change in isolated LP neurons clamped at -60 mV. Mean current amplitude ±SEM (n = 8). I-V plots of the four currents before (solid square symbols) and after (open circle symbols) holding the membrane potential at -60 mV in LP neurons. Two-way RM ANOVA reveals no significant differences.

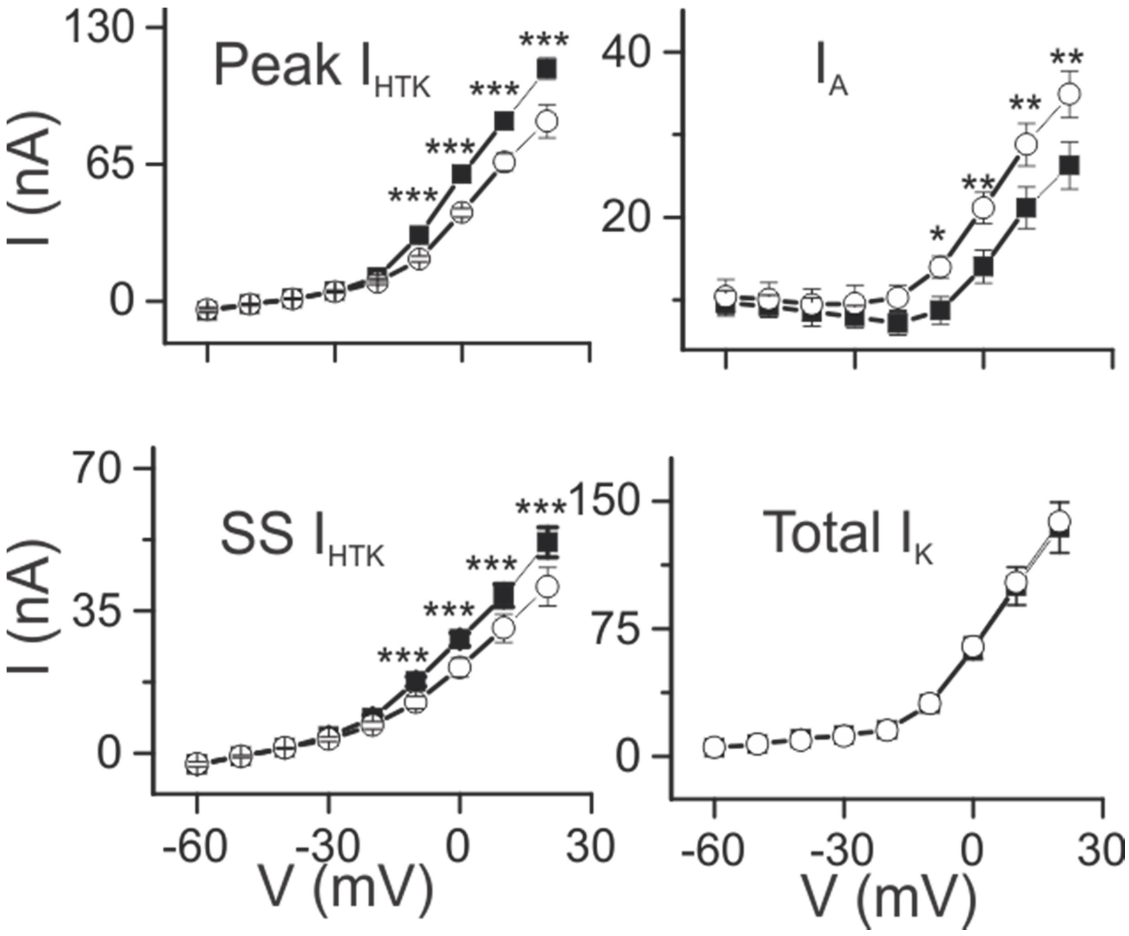

Figure 4.5 Depolarizing stimulation in isolated IC neurons affects K^+ currents. Mean current amplitude ±SEM (n = 4). I-V plots of the four currents before (solid square symbols) and after (open circle symbols) depolarizing stimulation. Statistical analysis was done by performing pairwise multiple comparisons using a 2-way RM ANOVA and Holm-Sidak *post hoc* analysis. **P < 0.01 ***P < 0.001

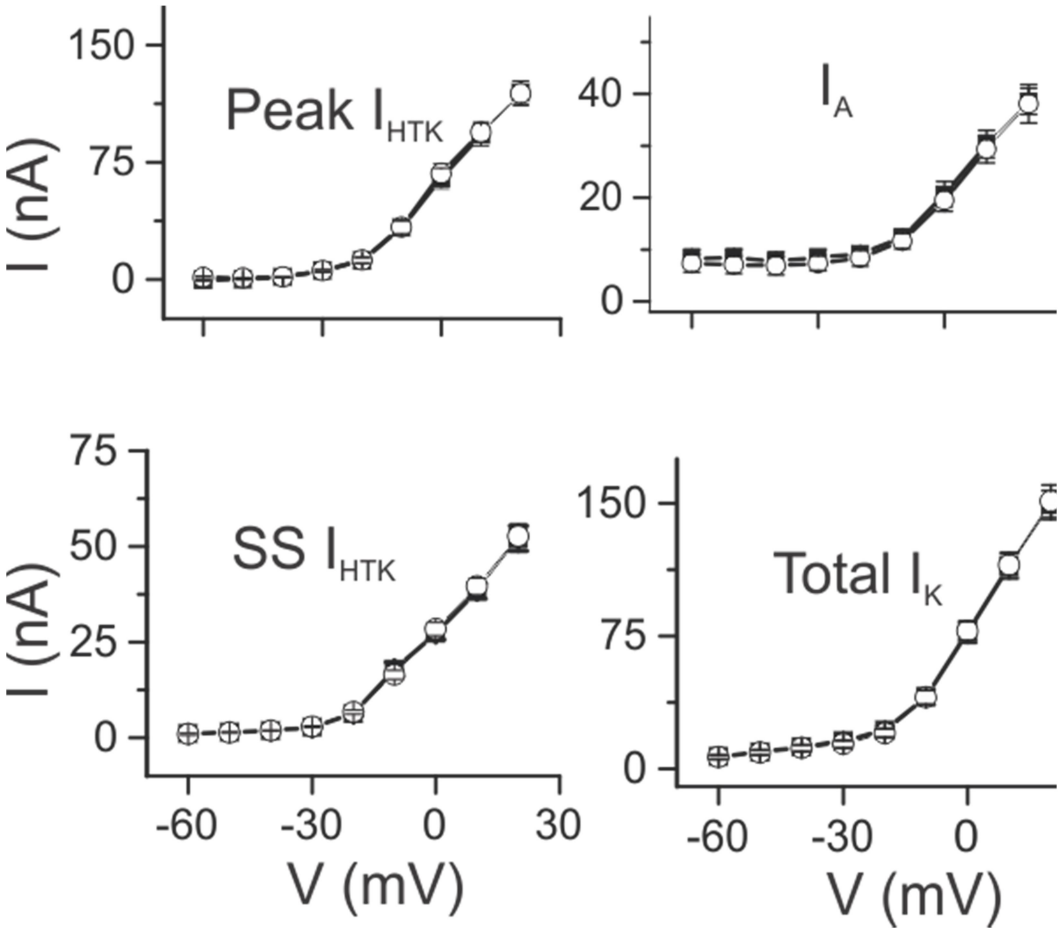

Figure 4.6 K^+ currents do not change in isolated IC neurons. Mean current amplitude ±SEM (n = 4). I-V plots of the four currents before (solid square symbols) and after (open circle symbols) holding the membrane potential at -60 mV in IC neurons.

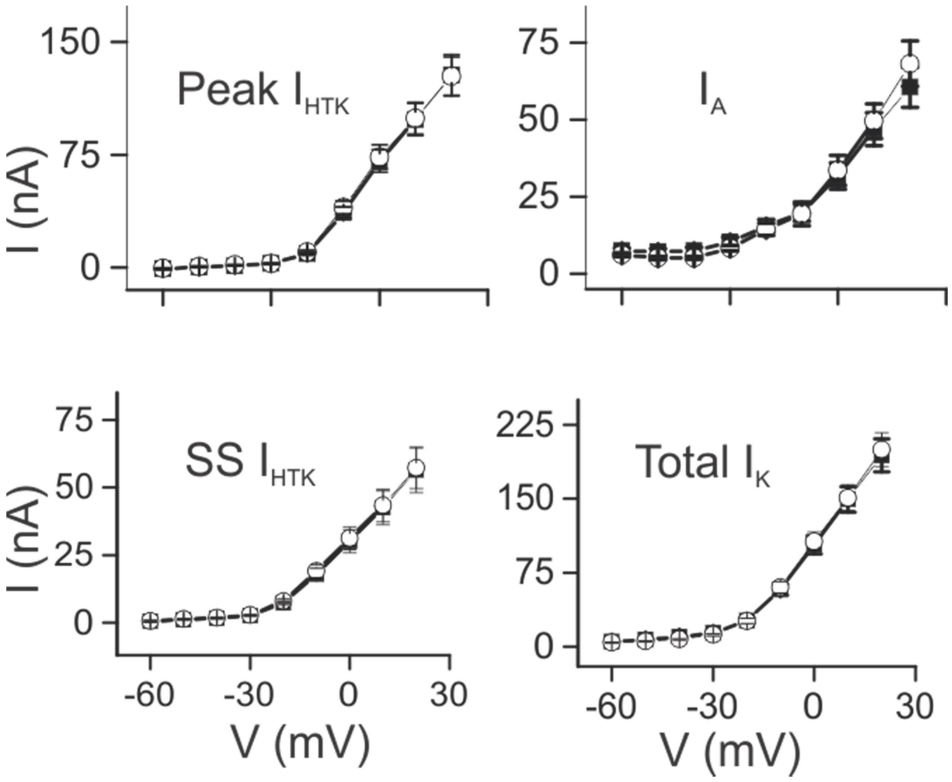

Figure 4.7 K^+ currents do not change in response to hyperpolarizing stimulation in PD. Mean current amplitude ±SEM (n = 11). I-V plots of the four currents before (solid square symbols) and after (open circle symbols) hyperpolarizing stimulation.

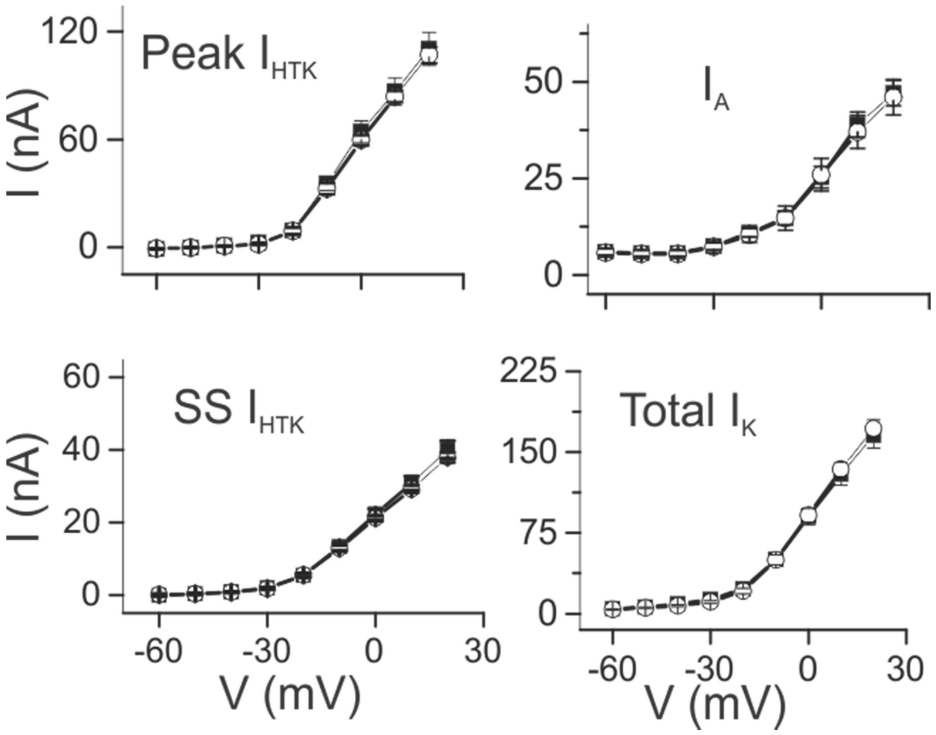

Figure 4.8 PD Neurons stimulated with realistic waveforms do not change K^+ current levels before and after stimulation. Mean current amplitude ±SEM (n = 10). I-Vplots of the four currents before (solid square symbols) and after (open circle symbols) stimulation with realistic waveforms.

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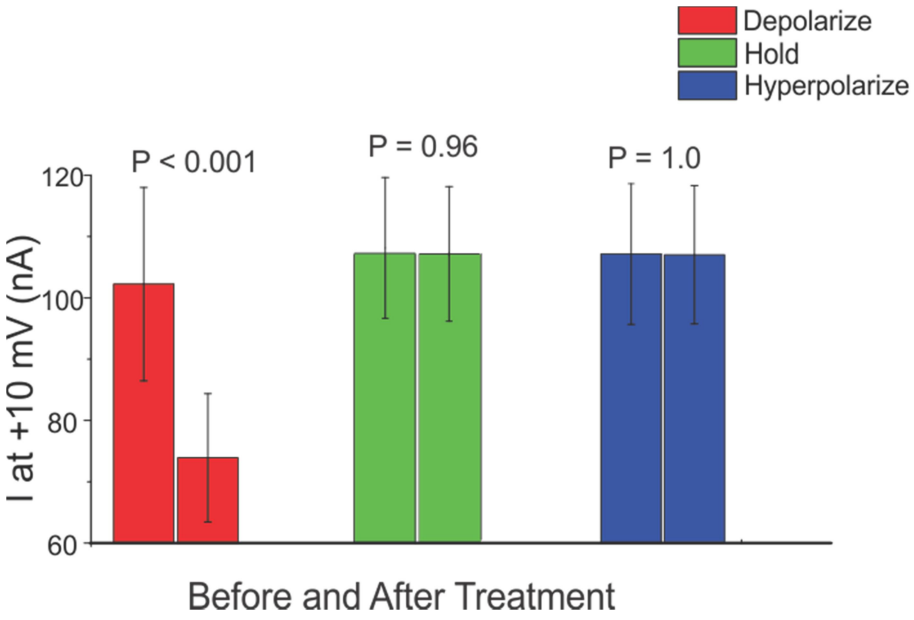

Figure 4.9 I_{HTK} before and after three different stimulation protocols. I_{HTK} peak current only decreases after depolarizing stimulation and not holding or hyperpolarizing. Bars represent current amplitude at +10 mV before (left bar) and after (right bar) 30 minutes of stimulation. P values are results of a paired t-test.

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CHAPTER 5

THE NEUROMODULATOR PROCTOLIN GATES ACTIVITY-DEPENDENT REGULATION OF IONIC CURRENTS IN PYLORIC NEURONS

5.1 Introduction

Neuronal activity, that is the electrical output of a neuron, is the result of the interaction of many ionic currents and their relative expression levels. Neurons can produce many different patterns of electrical output *and* maintain a stable function because of their ability to control and modify these intrinsic currents. Modification of intrinsic currents can occur in an activity-dependent (Turrigiano, Abbott et al. 1994, Turrigiano, LeMasson et al. 1995, Golowasch, Abbott et al. 1999, Misonou, Mohapatra et al. 2004, Temporal, Lett et al. 2014, Gainey, Tatavarty et al. 2015) or activity-independent (Khorkova and Golowasch 2007, Temporal, Desai et al. 2012) manner. Activity-dependent mechanisms usually involve calcium-mediated modifications (Liu, Golowasch et al. 1998, Haedo and Golowasch 2006, Frank 2014, Nanou, Scheuer et al. 2016) and can be either homeostatic (Felix-Oliveira, Dias et al. 2014, Frank 2014, Howard, Rubenstein et al. 2014, Korotchenko, Cingolani et al. 2014, Swann and Rho 2014, Whitt, Petrus et al. 2014) or nonhomeostatic (Daoudal and Debanne 2003, Sourdet, Russier et al. 2003, Cudmore and Turrigiano 2004, Turrigiano 2004, Kim, Jung et al. 2007, Temporal, Desai et al. 2012, Toyoizumi, Kaneko et al. 2014). While nonhomeostatic or Hebbian plasticity drives excitability away from baseline, it can result in a new baseline but can

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also be destabilizing if left unconstrained. Homeostatic plasticity functions to drive neuronal excitability back to some set-point value after a perturbation.

Ionic current density describes the amount of electrical current in a given area of plasma membrane. Homeostatic plasticity promotes stability by changing current densities to decrease cellular excitability in response to an excitatory stimulus by changing the abundance or voltage-dependence of depolarizing or hyperpolarizing channels (Mee, Pym et al. 2004, Temporal, Lett et al. 2014). Conversely, increased excitability can cause the opposite response and activate pathways which perpetuate increased excitability, such as in long-term potentiation (LTP) models (Froemke, Debanne et al. 2010, Wiltgen, Royle et al. 2010, Lee and Chung 2014, Wenner 2014). Numerous studies have demonstrated how changes in ionic current densities occur after perturbations, such as activity blockade(Konishi 1994, Golowasch, Abbott et al. 1999, Golowasch, Casey et al. 1999, Brackenbury and Djamgoz 2006, Haedo and Golowasch 2006, Kim, Jung et al. 2007, Hammond, Lin et al. 2008, Ben Fredj, Hammond et al. 2010, Temporal, Lett et al. 2014, Zhang, Picton et al. 2015), thus implying that the regulatory mechanisms are not necessarily dependent on synaptic input and can be cell autonomous.

We have previously examined activity-dependent regulation of intrinsic currents in pyloric neurons of the crustacean stomatogastric ganglion (STG) and demonstrated that K^+ currents change in response to depolarizing electrical stimulation in three cell types, but not in response to hyperpolarizing stimulation (see Chapter 4). We demonstrated that activity-dependent

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mechanisms are not cell-type specific (see Chapter 3) or dependent on polarity of the stimulus (see Chapter 4) in the STG. It has been demonstrated that neuromodulators can regulate ionic currents over the course of several days: removal of modulatory input results in a change in five different voltage-gated currents in PD neurons (none of which are directly activated or inhibited by proctolin) after 5 days, and the effect could be prevented by exogenous application of proctolin (Khorkova and Golowasch 2007). This result demonstrates, along with others (Thoby-Brisson and Simmers 2002, Zhang, Khorkova et al. 2009), that neuromodulators such as proctolin can have very complex effects on neuronal activity and activity regulation in the STG.

Proctolin is a pentapeptide (Arg-Tyr-Leu-Pro-Thr) located in neuronal somata and axons of commissural ganglia (CoG) and esophageal ganglion (OG) neurons but not pyloric neurons (Marder, Hooper et al. 1986). It is released onto the neuropil of the STG and elicits a pyloric rhythm in quiescent preparations (Nusbaum and Marder 1989, Nusbaum and Marder 1989) by activating an inward current carried mainly by Na^+ (Golowasch and Marder 1992, Swensen and Marder 2000). The current activated by proctolin is also activated by at least 5 other modulators that are released onto the STG and it is expressed by all pyloric neurons (Swensen and Marder 2000). Proctolin targets all three pyloric neurons we studied: lateral pyloric (LP), pyloric dilator (PD), and inferior cardiac (IC) neurons (Swensen and Marder 2001). We used proctolin to test the effects of modulators on regulation of ionic currents because it activates the same current as many other modulators and was shown

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to prevent changes due to removal of the endogenous modulatory input (Khorkova and Golowasch 2007). The modulatory inward current (I_{MI}) activated by proctolin was demonstrated to be sufficient to induce oscillatory activity in pyloric neurons (Zhao, Golowasch et al. 2010).

While there may be many several players influencing how neuronal activity regulates intrinsic properties, neuromodulators likely play a major role in gating activity-dependent regulation of ionic currents (Thoby-Brisson and Simmers 2002, Zhang, Khorkova et al. 2009). It has been shown that the transient potassium current (I_A) is modulated by amines in lobster pyloric neurons (Zhang, Rodgers et al. 2010). Overexpression of I_A via injection of *Shal* mRNA regulates the expression of the separate voltage-gated ionic current, I_h, via an activity-independent mechanism (MacLean et al., 2003). Since both activity-dependent mechanisms, activity-independent mechanisms and neuromodulators have the propensity to alter neuronal excitability via changes in intracellular calcium concentrations, it is plausible that the two mechanisms converge onto a single point along the two signaling pathways.

Here, we explore whether neuromodulators can alter the way activity regulates ionic currents. Specifically, we asked whether proctolin would oppose or enhance the effects of activity on K^+ current levels in PD neurons of the pyloric network of the STG. We focused on PD neurons for the reason that they exist as identical pairs in each preparation, which allows one to be used as control for manipulations of the other, and because they are electrically coupled to the anterior burster (AB) neuron, which is the pacemaker of the

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pyloric network (Marder 1984). To examine the interaction between neuromodulators and activity, we manipulated the two by either applying the peptide proctolin in isolated neurons from endogenous activity and modulatory input using TTX, or using the intact preparation (control). By performing these treatments in each preparation, we could observe if those acute changes had any effect on current levels. By applying TTX to the bath, neurons are deprived of activity due to direct blockade of Na^+ channels, and isolated from modulators by cessation of activity from modulator releasing neurons. We predicted that modulators can gate activity-dependent regulation and that the interaction depends on the modulatory input.

We performed depolarizing stimulations (described in Chapter 3) after treatment with, proctolin. Then, we conducted experiments in preparations in normal *Cancer* saline (Control), i.e., without applying any modulators or ion channel blockers, and measured currents in PD neurons every thirty minutes while the pyloric rhythm was active. Next, we tested how a more naturalistic stimulation affected currents by stimulating with prerecorded waveforms from PD neurons. Square step pulses have been used as imposed activity protocols because it is generally agreed that they are good approximations of normal pyloric activity in two main parameters, frequency and amplitude of the slow wave.

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5.2 Methods

Preparation and electrophysiology of the stomatogastric nervous system is described in Chapter 2.

5.2.1 Experimental Conditions

We tested effects of activity on K^+ currents in PD neurons in several different conditions which we describe as follows: **(1) Depolarizing stimulation:** As described in previous studies (see Chapter 3 and Chapter 4), 10^{-7} M TTX was applied for 30 minutes then neurons were depolarized using square step pulses from -60mV to -10mV for 30 minutes at a rate of 1 Hz. (**2) Holding in TTX:** 10^{-7} M TTX was applied for 30 minutes to functionally isolate PD neurons by abolishing endogenous activity and the release of modulators, then neurons were voltage clamped at -60 mV continuously for 30 minutes. (**3) Depolarizing stimulation plus proctolin:** the same condition as (1) with 10^{-7} M proctolin was added to the bath at the same time TTX treatment was started. **(4) Holding plus proctolin**: the same condition as (2) with 10^{-7} M proctolin added at the same time as TTX. **(5) Realistic waveform stimulation**: 10^{-7} M TTX was applied for 30 minutes and neurons were stimulated for 30 minutes using prerecorded waveforms from a control PD neuron. Neurons were stimulated with realistic waveforms rather than square step pulses to test how a more naturalistic voltage fluctuation regulated currents. **(6) Realistic waveforms plus proctolin**: same condition as (5) with 10^{-7} M proctolin added at the same time as TTX. **(7) Normal saline:** Currents were measured during an ongoing rhythm in an intact preparation in PD, LP, and IC neurons. **(8)**

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Hyperpolarization in normal saline: both PD neurons were clamped at -60mV to abolish bursting activity typically observed in an intact preparation in normal saline. During this hyperpolarization, network activity was monitored by recording from the lower motor nerves. The voltage of both neurons was monitored closely so that the membrane potential was hyperpolarized enough to sufficiently abolish bursting activity.

5.2.2 Statistical Analyses

Current-voltage plots were created using Matlab and OriginLab 8.5 software. Sigmaplot 12 software was used to perform statistical tests. Statistical analyses involved comparing ionic current parameters using Student's paired t-tests. Two-way repeated measures ANOVA were used to compare current amplitudes before and after stimulation. *Post hoc* tests are indicated in figure legends. For normally distributed data, the Holm-Sidak method was used. To compare current changes across time, we used a one-way RM ANOVA. For data that was not normally distributed, usually in small sample sizes, we used a nonparametric test for an analysis of variance. One-way Repeated Measures ANOVA was used to compare current amplitudes over time. To compare results of depolarization experiments with and without proctolin application, we used the respective dataset from Chapter 3. The percent change at +10mV was quantified for each current in both conditions. A Student's t-test corrected for multiple hypothesis testing was used to compare groups. The significance reported in Figure 5.2 uses adjusted significance level of $\alpha = 0.025$ (Sigmaplot 12.0).

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5.3 Results

We tested the hypothesis that neuromodulators gate activity-dependence directly by adding the peptide modulator proctolin to isolated preparations treated with TTX and stimulating the cells with square step depolarizations. We compared these results to those obtained in Chapter 3 where only TTX was used. For comparison purposes, TTX treated preparations are referred to as control experiments here. Figure 5.1 (left) shows depolarizing patterned stimulations caused a 28.6% ± 7.1 decrease in peak $I_{HTK,}$ a 13.9% ± 4.7 decrease in steady state I_{HTK} and a 22.0% ± 9.4 increase in I_A. Total I_K decreased 13.9% ± 3.3 in control experiments. In proctolin treated preparations (Figure 5.1, right), the effect of stimulation was strengthened in three currents: peak I_{HTK} (49.3% ± 8.3), steady state I_{HTK} (41.4% ± 4.7) and I_K (27.7% ± 5.4). The increase in I_A was not enhanced significantly by proctolin. Figure 5.2 more clearly illustrates this effect of the neuromodulator for each ionic current. Statistical significance was measured using a Student's t-test with power corrected for multiple comparisons ($\alpha = 0.025$) (Curran-Everett 2000). Significant difference in treatment was observed in peak I_{HTK} ($P = 0.04$), steady state I_{HTK} ($P < 0.001$), and I_K ($P = 0.02$). The effect of stimulation on I_A was not enhanced by proctolin ($P = 0.44$).

We performed a control experiment to confirm the hypothesis that during ongoing rhythmic activity, interactions between endogenous activity and neuromodulators regulate intrinsic properties to maintain stable levels of K^+ currents. We measured current levels for 120 minutes in LP, PD, and IC

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neurons in normal saline and without the use of Na^+ blockers. This did not affect our ability to accurately record the K^+ currents (Figure 5.3). We observed no significant changes in current amplitude at +10mV over the 90-120 minutes time period (Figure 5.4-5.6) using One-way RM ANOVA tests in any of the three cell types (see figure legends). Figures 5.5 and 5.6 show 90 minutes of measurement due to the fact that some cells died or the voltage clamp failed at 120 minutes.

Next, to attempt to separate the effects of activity from those of neuromodulators on K^+ currents, we abolished rhythmic activity by hyperpolarizing both PD neurons and left modulatory inputs intact by avoiding the use of TTX. We voltage clamped both PD neurons to a hyperpolarized level of -60mV simultaneously, which was sufficient to abolish rhythmic network activity. Inhibiting pyloric activity for 30 minutes in this way in an otherwise intact preparation did not have a significant effect on current amplitudes. All K^+ currents measured remained unchanged before (Peak I_{HTK}: 98.5 ± 10.6 nA, steady state I_{HTK}: 42.5 ± 6.2 nA, I_A: 46.9 ± 5.4 nA, totak I_K: 149.1 ± 12.9 nA) and after hyperpolarization (Peak I_{HTK}: 99.1 ± 10.6 nA, steady state I_{HTK}: 43.3 ± 6.04 nA, I_A: 49.6 ± 5.7 nA, total I_K: 151.1 ± 13.3 nA). Figure 5.7 shows I-V plots for currents before and after hyperpolarizing stimulation. Two-way RM ANOVA was used to test for differences across voltages: Peak I_{HTK} ($P = 0.9$, $n = 7$), steady state I_{HTK} ($P = 0.8$, $n = 7$), I_A ($P = 0.8$, $n = 7$), I_K ($P = 0.9$, $n = 7$).

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Finally, to test how currents respond to acute changes in activity and the modulatory environment, and to evaluate if the square step stimulation indeed resembles natural stimuli that the cells may receive, K^+ current levels were measured in PD neurons that were functionally isolated by applying 10^{-7} M TTX. Only results for peak I_{HTK} and I_A are shown. Next, neurons were stimulated in the absence and in the presence of 10^{-7} M proctolin with realistic waveforms prerecorded from PD neurons. Surprisingly, neither realistic waveforms nor proctolin application had an effect on current amplitudes of any of the K^+ currents recorded (Figure 5.8).

5.4 Discussion

Activity-dependent regulation of ionic currents allows neurons to regulate their intrinsic excitability in response to changing environments. In some instances, increased neuronal activity can drive a positive feedback mechanism, such as Hebbian synaptic plasticity, which causes cells to become more excitable in response to an excitatory input such as in long-term potentiation (LTP) (Felix-Oliveira, Dias et al. 2014). In other cases, increased electrical activity can drive a negative feedback mechanism where neurons modify intrinsic ionic currents to decrease cellular excitability, called homeostatic plasticity, to promote neuronal stability. Our prediction was that changes in response to activity or electrical stimulation would drive current levels in a direction to promote a homeostatic decrease in excitability. We observed a decrease in the total potassium current, suggesting an increase in excitability. If neurons are driven to increase excitability, what prevents this phenomenon from causing

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runaway excitation and destabilizing the network? We hypothesized that neuromodulators play a role in constraining this phenomenon.

In the present study, we tested the hypothesis that neuromodulators gate activity-dependent regulation of ionic currents in STG neurons. We predicted that neuromodulators promote stability in the network by adjusting the strength of the effect of activity-dependent regulation. We found that K^+ currents are robust to acute changes in activity and neuromodulatory input and that proctolin enhances the effect of stimulation on both I_{HTK} and the total I_K in PD neurons. Interestingly, proctolin had no significant effect on activity-dependent regulation of I_A.

In order to test the hypothesis that modulators gate activity-dependent regulation of ionic currents, preparations were treated with 10^{-7} M proctolin and then neurons were stimulated with depolarizing square step pulses. These results were compared to results obtained in Chapter 3 where PD neurons were stimulated in preparations treated with TTX. We found that the effect of depolarizing stimulation on PD neurons was significantly enhanced by proctolin application on peak I_{HTK}, steady state I_{HTK} and I_K but not on I_A. Proctolin activates a mixed cation inward current, I_{MI}. Our findings that acute application of proctolin enhances the activity-dependent decrease in I_{HTK} in PD neurons suggest an interaction between neuromodulators and activity-dependent plasticity. This mechanism is a novel interaction between two independent pathways that may alter neuronal excitability. Proctolin activates a G-protein coupled receptor and activates intracellular signaling cascades that

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are calcium dependent (Johnson, Garczynski et al. 2003, Gray and Golowasch 2016). In fact, Gray and Golowasch showed evidence that Ca-dependent pathways, involving Ca^{++} influx and activation of calmodulin dependent kinases (Gray and Golowasch 2016), and earlier Haedo and Golowasch (Haedo and Golowasch 2006)and Golowasch et al(1999) showed that intracellular Ca^{++} is clearly involved in activity-dependent regulatory mechanisms of activity and neuromodulation. I_{HTK} is the result of activation of voltage- and calcium-gated ion channels. The underlying calcium current activated by depolarizing the membrane potential induces elevation of intracellular calcium. It is likely that the bridge between the two signaling pathways involves a signaling molecule that is activated downstream of intracellular calcium release. Modifications to delayed rectifier channels, Kv2.1, occur via the calcium-dependent phosphatase calcineurin in hippocampal neurons (Misonou, Mohapatra et al. 2004, Misonou, Menegola et al. 2006). If Ca^{++} mediates the activity-dependent effects as well as the effects of activating I_{MI} the delayed rectifier current may then also be target of activity and neuromodulator-mediated regulation.

Because the square pulses used in this study for stimulation are clearly a rough approximation to the endogenous oscillatory activity, we tested several combinations of stimulation involving realistic activity patterns. To test the most realistic patterns, we measured currents in pyloric neurons expressing their endogenous activity. We measured K^+ currents in three different pyloric STG neurons during ongoing network activity (normal saline) over a period of two hours in order to determine how ionic current levels are regulated across

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time in a functioning nervous system. We expected that transient changes over the 120 minute period as a functional consequence of homeostatic regulatory mechanisms of these channels would be observed. However, K^+ current amplitude in all four voltage-gated currents remained stable over the course of the experiment. In subsequent experiments, we manipulated activity of PD neurons without blocking endogenous activity. Therefore, we wanted to establish a reliable control of how current levels were maintained in a condition with minimal experimental manipulation. It has been suggested by many studies in the STG that the maintenance of intrinsic excitability is due to long-term effects of neuromodulators and feedback from activity. In this set of experiments, we demonstrated that in the presence of both the descending input from anterior ganglia *and* the activity of the network interact to maintain stable levels of ionic currents.

After observing consistent levels of ionic currents during an ongoing pyloric rhythm, we tested whether abolishing network activity by hyperpolarizing both PD neurons would cause a change in current levels. This experiment was crucial in separating the effects of neuromodulators and activity. By abolishing network activity and voltage clamping PD neurons, changes due to patterned fluctuations in membrane potential were abolished and the input due to neuromodulators could be assessed. Surprisingly, while the pyloric rhythm was inhibited for 30 minutes, no changes in current levels were observed. If this change in activity followed homeostatic rules, a decrease in I_K would have been expected to facilitate an increase in cellular

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excitability. It is possible that 30 minutes of suppression was not sufficient to induce a homeostatic response. It was demonstrated by many studies that neurons respond to long-term activity deprivation by altering their intrinsic properties to promote increased membrane excitability over the course of several days to weeks (Turrigiano, Abbott et al. 1994, Desai, Rutherford et al. 1999, Cummings and Belluscio 2010, Felix-Oliveira, Dias et al. 2014, Mendoza Schulz, Jing et al. 2014, Gainey, Tatavarty et al. 2015). In one study, activity deprivation via removal of descending modulatory input for 5 days in culture caused a decrease in outward currents and an increase in an inward current in STG neurons (Thoby-Brisson and Simmers 2002). Whether activity-deprivation occurs via removal of a permissive signal, such as the descending modulatory input from anterior ganglia in the STG, or it occurs via blockade of Na^+ channels, seems to result in the same shift in ionic conductances to promote neuronal excitability. Results of long-term decentralization studies do suggest that intrinsic currents are regulated by neuromodulators (Thoby-Brisson and Simmers 2002, Sourdet, Russier et al. 2003, Khorkova and Golowasch 2007).

The fact that current levels remained unchanged after the neurons are inhibited from oscillating suggests that the neuromodulators provide an active, complex and continuous input to maintain expression of intrinsic properties of target neurons. It also demonstrates that suppression of activity has no consequence on K^+ current levels and, by extension, cellular excitability. However, this clearly contradicts the result reported in Chapters 3 and 4, which

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demonstrated a clear effect of activity on current amplitude. We think that it is also possible that 30 minutes is simply not a long time period for neurons to activate or deactivate homeostatic mechanisms when activity is inhibited. It is likely that these neurons remain silent for 30 minutes or longer on numerous occasions throughout an animal's life as a consequence of neuromodulation of the network (Weimann, Meyrand et al. 1991). By contrast, strong square depolarizing pulses appears to be an effective enough stimulus to override the safety margins built into the STG neurons. It was demonstrated that changes in intrinsic properties of pyloric neurons after removal of modulatory input occurs on the order of days (Turrigiano, Abbott et al. 1994, Thoby-Brisson and Simmers 2000, Thoby-Brisson and Simmers 2002, Luther, Robie et al. 2003). This long time scale of modulators on ionic currents makes activity-dependent regulation a good substrate for neuromodulation to alter intrinsic currents more quickly.

It was surprising that acute changes in activity and application of proctolin had no effect on current levels. Again, it is likely that 30 minutes was not long enough to induce a homeostatic response. However, it is also possible that K^+ channels are more robust to perturbations than what was expected, and that square depolarizing pulses are an overwhelming stimulus. Functionally, isolating PD neurons by applying TTX and blocking Na^+ channels had no effect on K^+ current levels. This is surprising because it is generally agreed that different ion channels are regulated in a compensatory fashion (Ransdell, Nair et al. 2012, Temporal, Desai et al. 2012, Temporal, Lett

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et al. 2014) and that neuronal identity arises from a consistent difference across cell types in the balance between inward and outward currents (Schulz, Baines et al. 2006)despite variability of expression levels from animal to animal (O'Leary, Williams et al. 2013, Marder, O'Leary et al. 2014, O'Leary, Williams et al. 2014). Stimulating with realistic waveforms had no effect on K^+ current levels even after application of proctolin. In the case of the experiments in this study, PD neurons were stimulated with their natural activity pattern. Yet, when depolarizing stimulations were used, a decrease in I_{HTK} was likely the result of calcium influx which was amplified by proctolin.

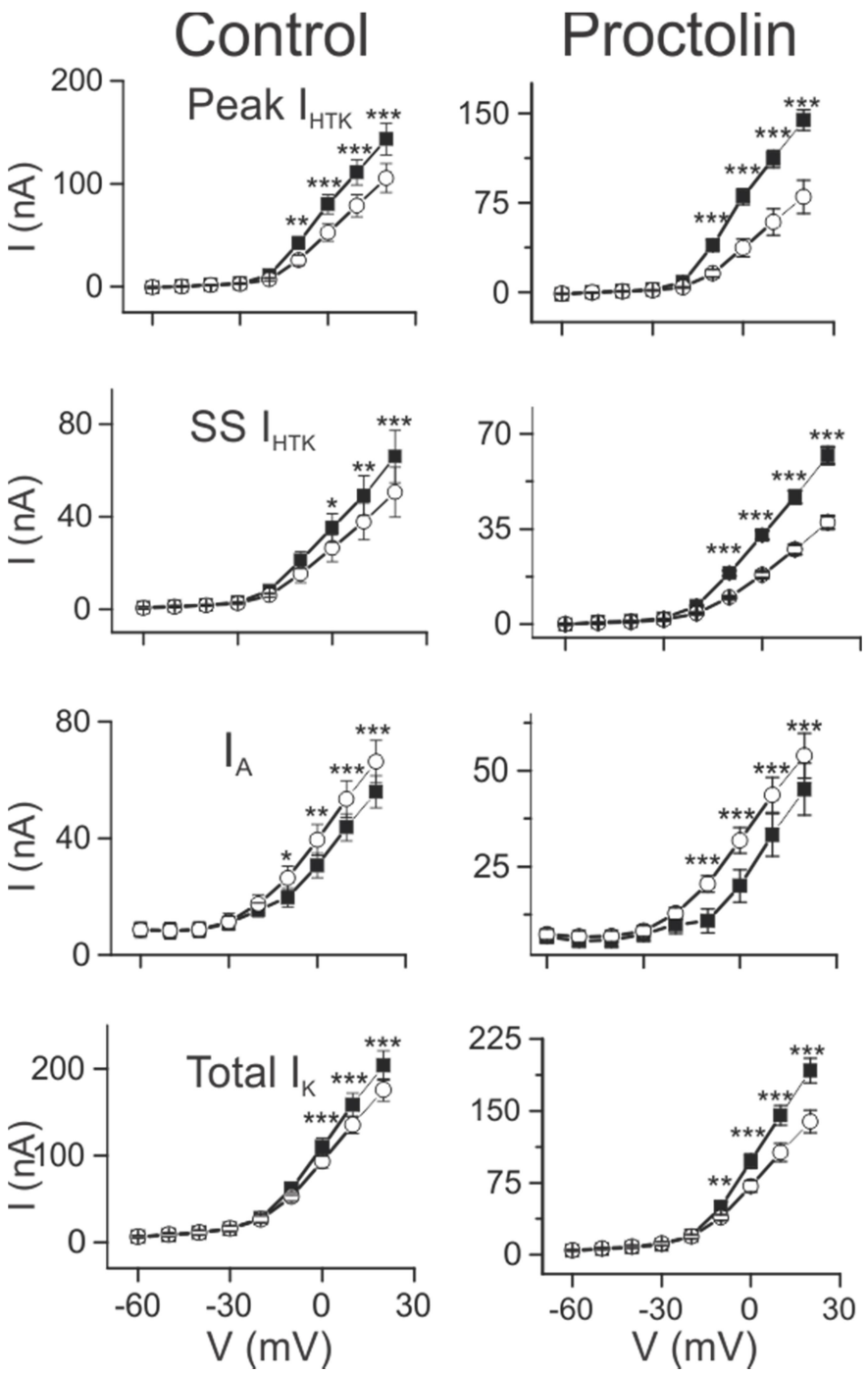

Figure 5.1. Proctolin enhances activity-dependent regulation of K^+ currents in PD neurons. Left panels: I-V relationships measured in 10^{-7} M TTX. Right panels: I-V relationships of currents measured in 10^{-7} M TTX + 10^{-7} M proctolin before (solid squares) and after (open circles) depolarizing stimulation. Currents are (from top to bottom) Peak I_{HTK}, steady state I_{HTK}, I_A and total I_K. Stars indicate comparisons from a two-way RM ANOVA. *P<0.05, **P<0.01, ***P<0.001.

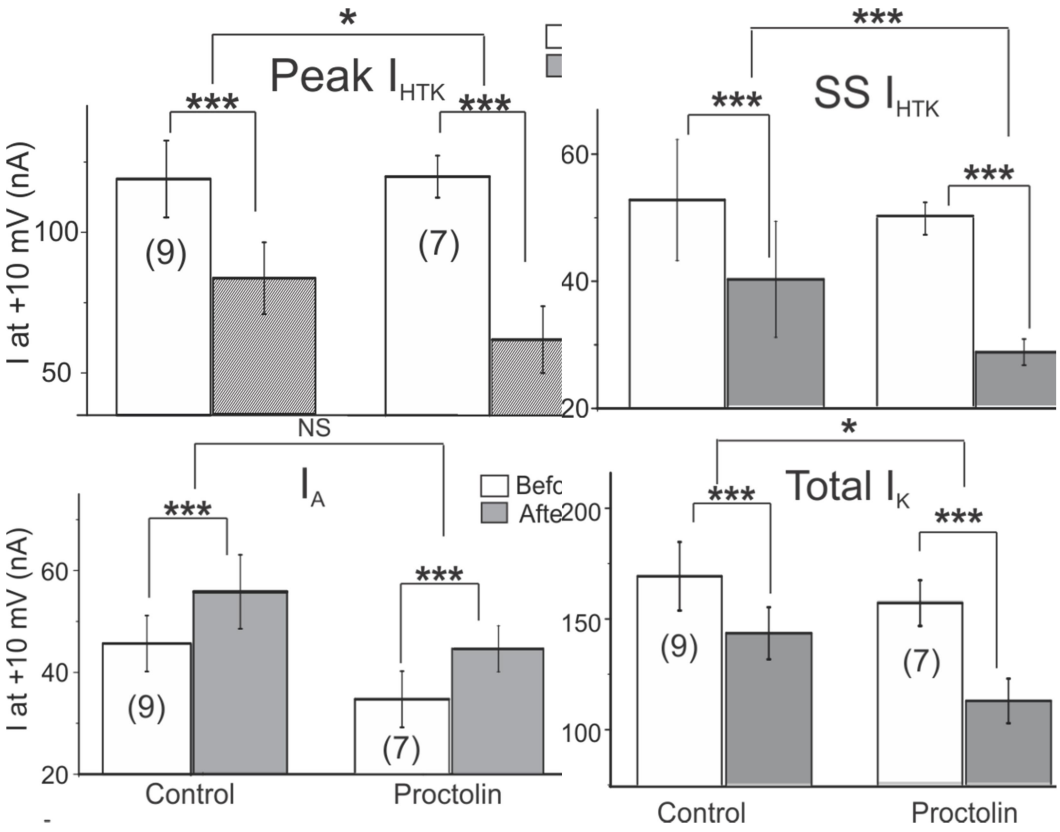

Figure 5.2. Proctolin enhances the effect of depolarizing stimulation in PD neurons. Mean ± SEM percent change of ionic current amplitudes measured at +10 mV after depolarizing stimulation in preparations treated with proctolin (white bar) and preparations treated only with TTX (gray bar). Increase in I_A after stimulation was not further enhanced by proctolin. Sample sizes are shown in parenthesis in bars. Statistics: Student's t-test beetween TTX-treated and TTX+Proctolin-treated conditions. * $P<0.05$, *** $P<0.001$. Values insides boxes indicate sample numbers.

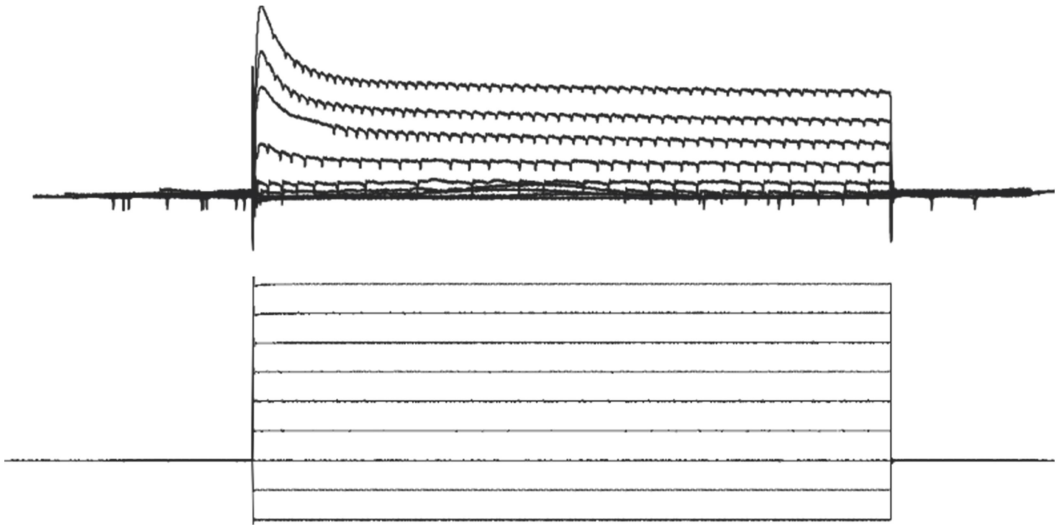

Figure 5.3. I_{HTK} recorded in PD neurons in normal saline. Currents can be appropriately recorded in the absence of Na^+ current blockers. Bottom: Voltage steps from -40mV to +20 mV in 10 mV increments. Bottom: Current responses to test pulses.

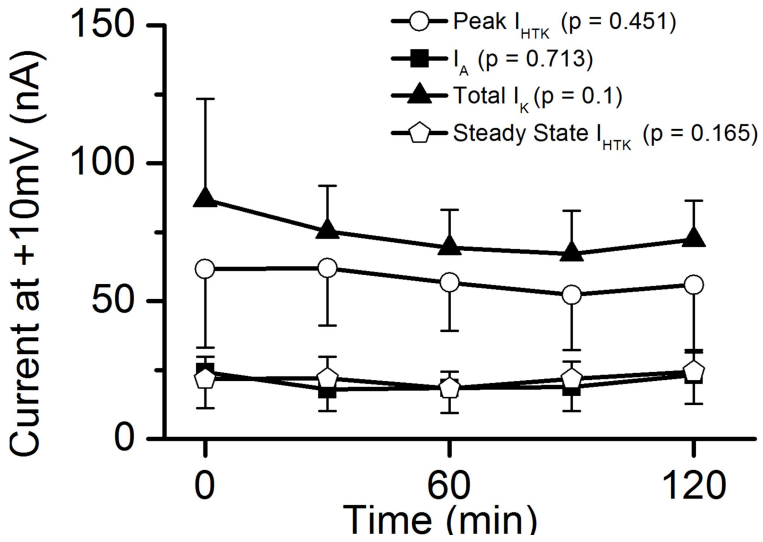

Figure 5.4 K^+ currents are stable in PD neurons in an intact preparation. Mean amplitudes at +10mV of the 4 K^+ current measurements over two hours. Results from 8 PD neurons. Error bars represent SEM. P values obtained from One-way Repeated Measures ANOVA on each separate current.

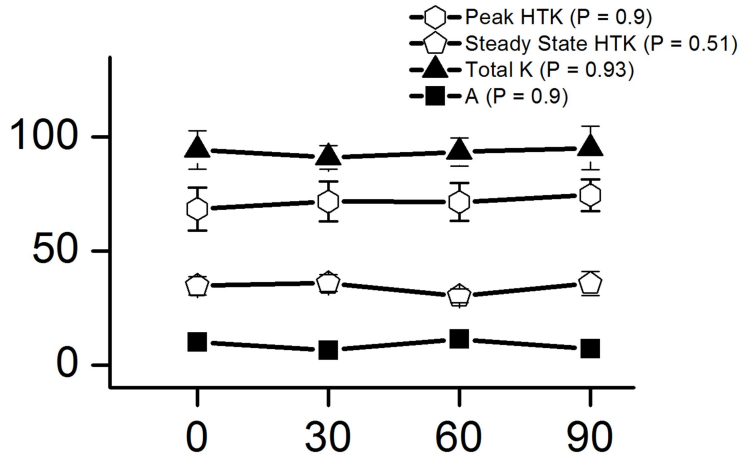

Figure 5.5. LP Currents in Normal Saline. K^+ current levels are stable in LP neurons over 90 minutes while pyloric rhythm is ongoing (n = 9). Symbols represent mean current amplitude ± SEM at +10mV measured every 30 minutes. Statistical comparisons were performed using a One way RM ANOVA on each of the currents (Peak I_{HTK}, p = 0.8. steady state I_{HTK}, P = 0.5, I_A, P = 0.7, total I_K, P = 0.9).

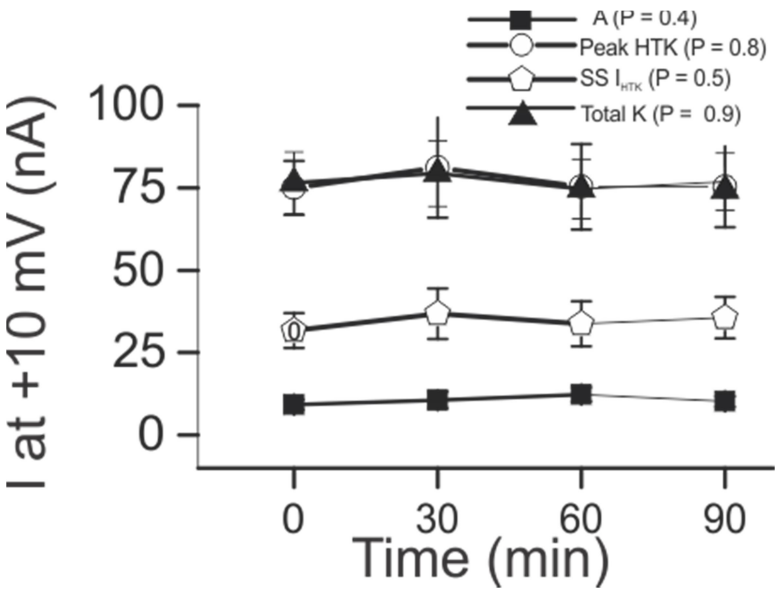

Figure 5.6. IC Currents in Normal Saline. K^+ current levels are stable in IC neurons over 90 minutes during an ongoing pyloric rhythm (n = 5). Symbols represent mean current amplitude ± SEM at +10mV measured every 30 minutes. Statistical significance of effect of time determined using a One-way RM ANOVA.

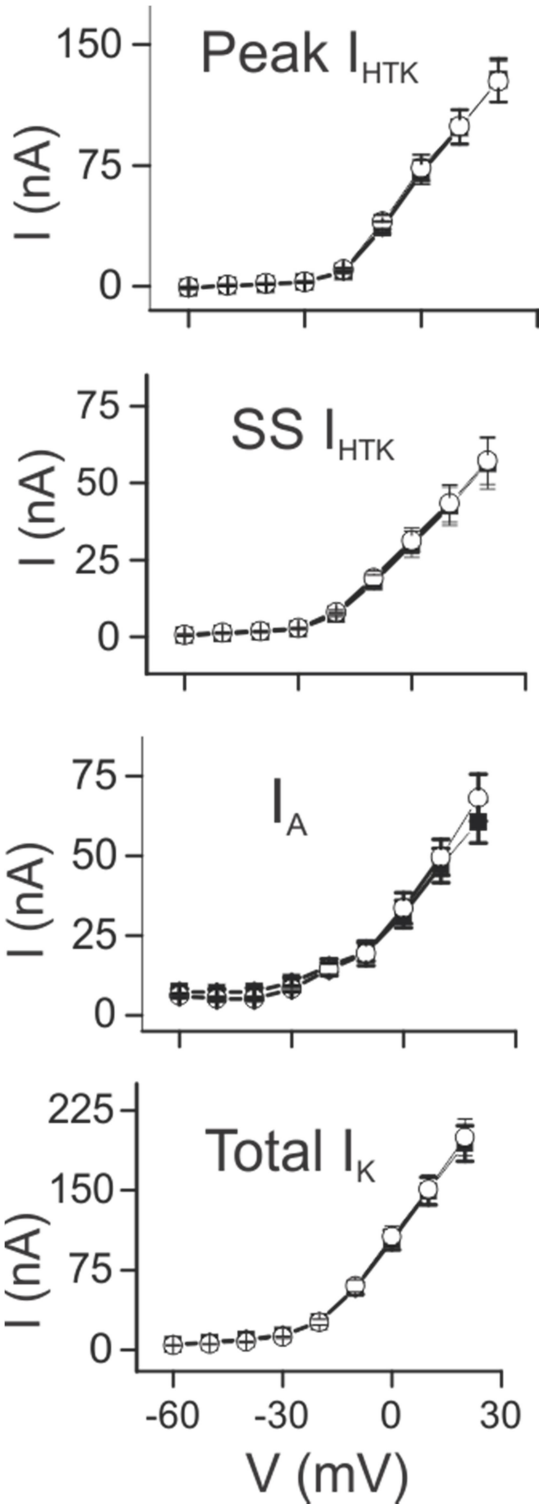

Figure 5.7. Currents in PD neurons hyperpolarized in normal saline remain stable after 30 minutes of activity-deprivation. Mean current amplitude ±SEM (n= 11). Currents are (from top to bottom): Peak I_{HTK}, steady state I_{HTK}, I_A, I_K.

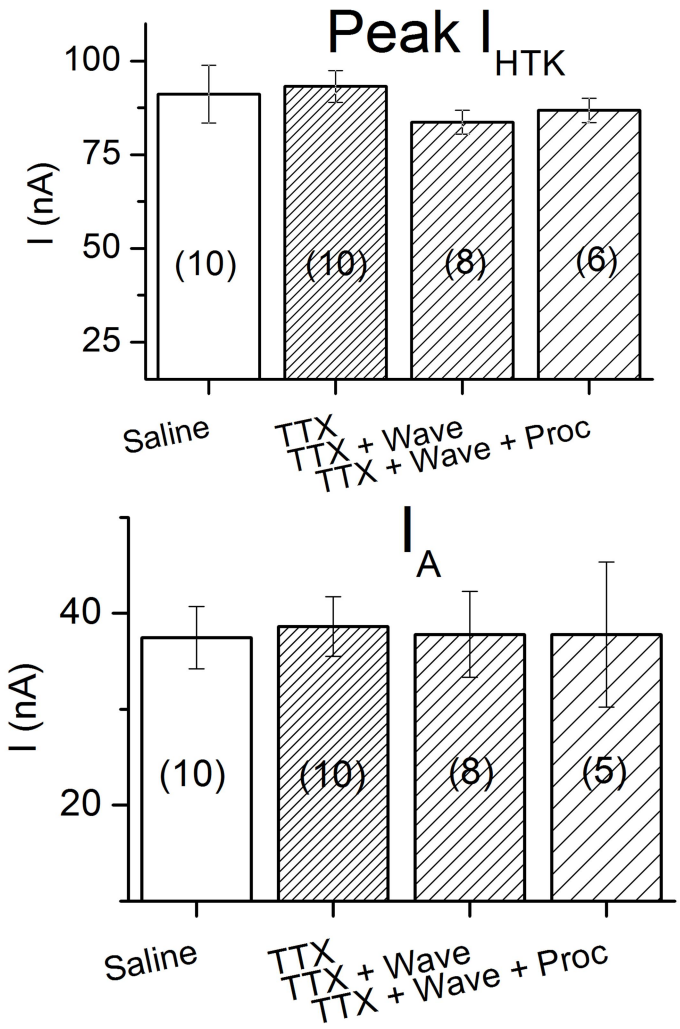

Figure 5.8. K^+ currents in PD neurons stimulated with realistic waveforms. Mean current amplitude ±SEM. Currents measured in normal saline + 10^{-7} M TTX, after 30 minutes of stimulation with realistic waveforms (TTX + Wave), after 30 minutes of stimulation with waveforms and treatment with TTX + 10^{-7} M proctolin. No significant differences were observed in any of the conditions as determined by a One way RM ANOVA (I_{HTK}, P = 0.7, $I_{A,}$ P= 0.1). Numbers in parenthesis indicate sample sizes.

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CHAPTER 6

CONCLUSION

6.1 Significance

Neurons and networks can maintain stable electrical outputs despite constantly changing inputs. Electrical activity of a single neuron can change drastically depending on extrinsic inputs such as neuromodulators and synaptic feedback. While responding to these inputs involves changes in intrinsic excitability, neurons must also be able to maintain some stability to return to a predetermined set point so that they can remain functional. For example, thalamocortical neurons transition from bursting to spiking activity during slow wave sleep and waking states, respectively (Steriade, McCormick et al. 1993). A growing body of evidence supports the hypothesis that neurons change their intrinsic ionic currents in response to altered activity levels and the most direct evidence of this was demonstrated in cortical (Turrigiano, Abbott et al. 1994) and STG neurons (Golowasch, Abbott et al. 1999).

Central pattern generating networks that control vital behaviors such as respiration and locomotion must be able to return to a predetermined level of activity after injury or perturbation to ensure an organism's survival. Therefore, it is crucial to identify what mechanisms these networks use to track their own activity and how these mechanisms regulate intrinsic properties of component neurons to maintain robust behavior.

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6.2 Summary of Results

The goal of this dissertation was to investigate regulation of voltage-gated ionic currents via activity-dependent mechanisms and to characterize how neuromodulators gate this mechanism. Since activity of the pyloric network is dependent on modulatory input, and neuronal activity regulates ionic current levels, it was the primary focus of this investigation to determine how and whether an interaction between the two mechanisms exists on the same time scale.

To accomplish the goal of this dissertation, ionic current levels were measured in three different pyloric neurons to first establish whether cell-type specific effects occurred. The three neurons tested were: lateral pyloric (LP), pyloric dilator (PD), and inferior cardiac (IC) neurons. We then stimulated neurons with different patterns of activity and found that current levels changed in response only to depolarizing square step pulses but remained stable over time when other patterns were used such as hyperpolarizing and realistic waveforms. Finally, it was found that the neuromodulator proctolin enhances the effect of stimulation on two K^+ currents.

The contribution of this dissertation to the understanding of how neurons in a rhythmic network regulate their excitability in response to different inputs is outlined in this chapter.

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6.2.1 Establishing Reliable and Accurate Methods to Investigate Activity-Dependent Regulation of K^+ Currents

After conducting many experiments to test multiple hypotheses about the regulation of K^+ currents in PD neurons, we observed that the peak I_{HTK} current always decayed. We found that the results of these experiments were unreliable due to an experimental artifact which we suggest was due to the electrode filling solution. Hooper et al (2015) tested the effect of several different ionic solutions in lobster STG and leech neurons. They found that high ionic concentrations in filling electrodes caused changes in passive cell properties such as resting membrane potential (V_m) and input resistance (R_n). With high molarity fills, an outward current was shown to decrease as a function of the amount of current injected through the electrodes. These changes were suggested to be due to ion leak from the electrode into, and volume changes of, the cell. In our experiments, we were able to measure stable level of ionic currents using 1 M KCl electrode filling solution. Our control experiments involved measuring currents over time with little to no experimental manipulation (discussed in Chapter 5). Currents were measured in normal saline every 30 minutes for 120 minutes. No changes over time were observed. Given these results we performed all the experiments reported in this thesis using the low ionic strength 1 M KCl solution, and conclude, like Hooper et al (2015) that all studies using high ionic strength solutions should be interpreted with caution.

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Another observation of this work is that depolarizing patterned stimulation caused a change in K^+ current levels after 30 minutes of stimulation (Chapter 2). At first, we attempted to stimulate neurons for 2 hours. However, when comparing current levels after 30 minutes of stimulation to current levels after 60, 90, and 120 minutes, there were no significant differences. This result demonstrates that alterations in current expression levels happen at a relatively short time scale. Many studies that have explored activity-dependent regulation used stimulation protocols much longer than 30 minutes and in many cases, at least 1 hour stimulations were used to investigate changes in cellular excitability (Turrigiano, Abbott et al. 1994, Haedo and Golowasch 2006). Haedo and Golowasch (2006) demonstrated a clear calcium-dependence of the effects of stimulation on ionic current and activity changes. The K^+ current changes we observed in our study could therefore also be mediated by calcium-dependent processes.

To test whether this fast effect of stimulation is calcium-dependent, we suggest reducing intracellular calcium by using a calcium chelator such as BAPTA ((1,2-bis(*o*-aminophenoxy)ethane-tetra acetic acid) to measure current responses to stimulation with reduced intracellular calcium. Intracellular calcium-signaling pathways have been demonstrated to regulate ionic channel densities in pyramidal neurons (Misonou, Mohapatra et al. 2004, Misonou, Menegola et al. 2006). In STG neurons, voltage-dependent properties of the modulator-activated current I_{MI} are suggested to be regulated by calcium via a calcium sensing receptor (CaSR) and intracellular calcium-dependent enzymes

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(Gray and Golowasch 2016). If the decrease observed in I_{HTK} is dependent on an increase in intracellular calcium concentration, treating cells with BAPTA should reduce the effect of stimulation on ionic current levels. While this experiment would not completely explain how proctolin enhances the stimulation-induced decrease in I_{HTK}, it could identify a potential target protein that would link activity-dependent regulation and neuromodulation. In fact, the proctolin-induced current (I_{MI}) has been hypothesized to be at least in part carried by calcium itself (Gray and Golowasch 2016) Zhao et al (2011), which could then explain a possible role of proctolin in facilitating the activity-dependent effects on K^+ currents.

6.2.2 Cell-Type Specific Regulation of K^+ Currents

While the phenomenon of activity-dependent regulation was previously tested in IC neurons (Golowasch, Abbott et al. 1999), we repeated the experiments to compare results we obtained in other STG neurons and test the hypothesis that different cell types adjusted their ionic current levels differently in response to the same stimulation protocol. We were able to replicate the IC results using both K_2SO_4 and KCl electrode fills. LP, PD, and IC neurons were all recorded from using both electrode fills. Surprisingly, the only neuron that showed a sensitivity to sulfate was PD. While this was not the main goal of this dissertation, this revealed a cell-type specific effect of ionic solutions. Hooper et al. (2015) demonstrated that neuron properties (i.e. R_n, V_m, and transient currents) changed as a function of the amount of current injected when using the voltage clamp technique. It is possible that PD neurons required more

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current injection to clamp at the same voltages as LP and IC neurons. This is plausible since PD neurons appear to be bigger than LP neurons (Golowasch, Thomas et al. 2009), exist in electrically coupled pairs and are further gap junctionally coupled to at least 3 other cell (AB, and two LPG neurons) (Marder 1984). The mechanism of action of sulfate on current levels is not clear. We did not explore this phenomenon but one important observation is that only Peak $I_{HTK,}$ and not $I_{A,}$was affected. One possibility is a direct modification of the voltage-gated calcium channel. It is possible that sulfate could be interacting with signaling molecules to modify calcium channel conductance and thus decreasing calcium influx which would explain a decreased calcium-activated potassium current. Measuring I_{Ca} before and after stimulation would reveal whether a change in this underlying current cause a decrease in I_{HTK} and not I_A. If a decrease in I_{Ca} is observed after stimulation, it would explain the specific effect on I_{HTK}. Since both ionic solutions used were relatively high molarity (0.6 M K_2SO_4 and 1 M KCl) the decay of peak I_{HTK} is likely specific to sulfate. Hooper et al (2015) showed that intrinsic properties could be measured accurately with an ionic solution closely matching the molarity of neuronal cytoplasm.

In our experiments, LP showed a change in both I_{HTK} and I_A in response to depolarizing stimulation. This result was not what we expected based on what Golowasch et al. (1999) demonstrated. One major difference between our results and those of Golowasch and colleagues is that we used voltage clamp to stimulate the cells while they used current clamp. We suspect that

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TEVC was a more effective method of controlling membrane potential of the cell and that current clamp results simply did not cause a change in currents because the membrane potential was not changed sufficiently for activity-dependent mechanisms to occur.

6.2.3 K^+ Currents Respond to Specific Patterns of Activity

Hyperpolarizing stimulation did not result in any change in K^+ current levels. The voltage range that was used to drive the membrane potential does not activate any of the currents measured. If we measured an inward current activated by hyperpolarizing such as $I_{h,}$ we would be able to distinguish or hypothesize about the response of outward versus inward currents. It has been established that long-term changes in neuronal networks associated with learning and memory formation occur as a result of *both* changes in synaptic strength (Feldman 2009, Madronal, Gruart et al. 2010, Shouval, Wang et al. 2010, Tully and Bolshakov 2010, Wiltgen, Royle et al. 2010) and modifications to cellular excitability (Lebel, Grossman et al. 2001, Gasque, Labarca et al. 2005, Naude, Paz et al. 2012). In Chapter 2-4, we explored the phenomenon of changes in intrinsic excitability by measuring K^+ currents after exposing motor neurons to patterned electrical stimulation. By using the TEVC method, we drove the membrane potential and imposed a patterned activity pattern on the neurons without actually providing a chemical signal. Therefore, we were able to test how K^+ currents were regulated in response to the activity output of the neuron, rather than synaptic or chemical input.

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The observation that current levels did not respond to hyperpolarizing stimulation was surprising. The fact that current levels in a neuron resting at -60 mV were similar to current levels in a neuron oscillating from -60 mV to -110 mV at 1 Hz was puzzling since modeling and experimental studies have demonstrated that modifications to ionic current levels are correlated with distinct activity outputs. Here, we only measure one subset of ionic currents expressed in pyloric neurons. A key indicator of activity in neurons is calcium. Therefore, measuring Ca^{2+} currents may reveal more about the regulatory mechanisms involved in how neurons utilize activity-dependent regulation. Another possibility that was not explored is the idea that hyperpolarizing stimulation mimicked the patterned synaptic inhibition these neurons receive in the endogenous environment. Turrigiano et al (1994) demonstrated that hyperpolarizing patterned stimulation in cultured STG neurons could in fact reverse the long-term effects of removing synaptic input. In Turrigiano's work neurons were isolated in culture and rhythmic activity ceased. In two days, the neurons transitioned from tonic firing to bursting behavior by adjusting the balance of ionic conductances to compensate for the loss of modulatory and synaptic input. This transition could be reversed and bursting activity abolished by patterned hyperpolarizing stimulation within 1 hour (see also Haedo and Golowasch, 2006). In our experiments, neurons were exposed to patterned hyperpolarizations only after blocking endogenous bursting activity. If a transition from one type of behavior to another has an effect on current

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levels, a good experiment may involve testing how neurons respond to patterned hyperpolarization without using TTX to block sodium channels.

In the absence of synaptic drive and modulatory input, current levels measured in all three neuron types were stable. Clamping the membrane potential at -60 mV for 30 minutes resulted in no changes in ionic current levels. Comparing this result to results obtained from depolarizing stimulation demonstrates that this indeed is a mechanism that responds to fluctuations in the membrane potential. To test whether the effect is reversible, we would impose these different activity patterns within one experiment i.e. neurons would first be stimulated then held at -60 mV to test whether stimulation-induced changes were reversible. As discussed earlier, depolarizing patterned stimulation involved a brief but sustained depolarization of the membrane potential at -10 mV. This could have allowed a very large calcium influx into the cell and thus induced activity-dependent changes in the expression levels of the currents we measured. Using realistic waveforms did not involve such sustained depolarizations which explains the reason no changes were observed.

6.2.4 Neuromodulators Gate Activity-Dependent Regulation

The effects of activity and neuromodulators on intrinsic excitability of neurons have been characterized with great distinction. Many studies have either examined activity-dependent (Konishi 1994, Turrigiano, Abbott et al. 1994, Turrigiano, Leslie et al. 1998, Golowasch, Abbott et al. 1999, Brackenbury and Djamgoz 2006, Swanwick, Murthy et al. 2006, Temporal, Lett et al. 2014) or neuromodulator-dependent pathways (Thoby-Brisson and Simmers 1998,

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Skiebe 2001, Billimoria, DiCaprio et al. 2006, Khorkova and Golowasch 2007, Marder 2012, Temporal, Desai et al. 2012, Marder, O'Leary et al. 2014) leaving many questions open about the possible interaction between the two mechanisms. Because neuromodulators generally activate longer lasting and slower cellular pathways, it is generally assumed that they function on different time scales. However, in this study we observed a preliminary finding that there is a pathway were both the modulatory environment, and activity of a neuron converge onto a cellular signal to regulate the expression levels of outward K^+ currents. In spike timing dependent plasticity (STDP) it was demonstrated that activation of adenylyl cyclase and phospholipase C by neuromodulator coupled receptors controls whether neurons will undergo potentiation or depression (Seol, Ziburkus et al. 2007). We also hypothesized that the neuromodulatory input would gate activity-dependent plasticity by either enhancing or suppressing the effect. In our experiments, the excitatory peptide proctolin enhanced the effect of activity on changes in current levels. The enhancement of activity-dependent regulation could be a priming mechanism to maintain a longer perpetuation of excitation in response to a stimulus.

How would this phenomenon function in a behaving organism? The pyloric network controls the rhythmic movement of the pylorus which filters macerated food. It is plausible to assume that activity-dependent mechanisms function to provide a feedback system about what the network is doing during feeding on a short and fast time scale. Neuromodulators on the other hand,

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could function to change activity-dependent regulation on a longer time scale. For example, neuromodulation could function to enhance or suppress activity-dependent regulation depending on the circadian rhythms of the animal. In a sleeping, non-feeding animal for example, spontaneous activity of the pyloric network may need to be suppressed by activity-dependent regulation so that feeding behavior is reserved only when the animal needs it.

6.3 Future Directions

One key to understanding the functional relevance of these experiments would be to test how excitability actually changes after these changes occur. For example, the decrease in total I_K suggests an increase in excitability yet we have not tested that. To test the functional consequence of such changes in response to intrinsic and synaptic plasticity, we would test excitability by looking at neuronal responses to current injection either in cultured neurons or synaptically isolated neurons.

Another future set of experiments would be to explore the intracellular mechanisms involved in changing current levels. It is known that ion channel activity can be altered in many different ways included phosphorylation and dephosphorylation, translocation, and dispersal of ion channel clusters. These processes not only change the amount of current moving through the membrane but also the voltage-dependent and temporal properties of the channels. We also aimed to test the effect of acute versus long-term removal of neuromodulators. Since it has been described that neuromodulators provide

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a continuous signal f or maintaining intrinsic excitability, it would be interesting to test how activity feeds back onto neurons that can burst independent of modulatory input, such as those in decentralized preparations. The work of this dissertation provides many avenues to further this research and has established some significant findings such as the existence of an interaction between activity and modulatory substances, the short-term action of activity and the importance of using proper measurement techniques.

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