2019-02-15

Development and Regulation of Two-State Patterned Activity in a Model Serotonin Motor Circuit

Bhavya Ravi
University of Miami, bhavyaravi90@gmail.com

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
https://scholarlyrepository.miami.edu/oa_dissertations/2239

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
DEVELOPMENT AND REGULATION OF TWO-STATE PATTERNED ACTIVITY
IN A MODEL SEROTONIN MOTOR CIRCUIT

by

Bhavya Ravi

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida
May 2019
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

DEVELOPMENT AND REGULATION OF TWO-STATE PATTERNED ACTIVITY
IN A MODEL SEROTONIN MOTOR CIRCUIT

Bhavya Ravi

Approved:

________________________                    ________________________
Kevin M. Collins, Ph.D.                               Laura Bianchi, Ph.D.
Assistant Professor of Biology                    Associate Professor of Physiology
                                                    and Biophysics

________________________                    ________________________
Peter Larsson, Ph.D.                                   Stephen Roper, Ph.D.
Professor of Physiology and                        Professor of Physiology and
                                                    Biophysics

________________________
Brock Grill, Ph.D.                                         Guillerme Prado, Ph.D.
Associate Professor of Neuroscience                  Dean of the Graduate School
The Scripps Research Institute
Neuronal activity accompanies synapse formation and maintenance, but how early circuit activity contributes to behavior development is not well understood. Here, I use the *Caenorhabditis elegans* egg-laying motor circuit as a model to understand how coordinated cell and circuit activity patterns emerge during development and its relationship to animal behavior. The *C. elegans* egg-laying circuit is a simple, well-characterized neural circuit which develops during the fourth larval stage and drives a two-state behavior in adult animals where ~20 minute inactive states are punctuated by ~2 minute active states where eggs are laid. The egg-laying circuit comprises two serotonergic Hermaphrodite Specific Neurons (HSNs) which promote the active state, and six cholinergic Ventral C neurons (VC1-6), which synapse onto a set of vulval muscles that contract to allow release eggs from the uterus. During the L4 larval stage, the cells of the circuit undergo morphological maturation and establish synaptic connections, providing a model system to understand the origin and function of cell activity during circuit development. Because each cell in the circuit develops independently in juveniles, how this circuit goes on to develop the robust pattern of coordinated activity seen in adults remains unclear.
Using calcium imaging in behaving animals, I find the serotonergic Hermaphrodite Specific Neurons (HSNs) and vulval muscles show rhythmic calcium transients in L4 larvae before eggs are produced. HSN activity in L4 is tonic and lacks the alternating burst-firing/quiescent pattern seen in egg-laying adults. Vulval muscle activity in L4 is initially uncoordinated but becomes synchronous as the anterior and posterior muscle arms meet at HSN synaptic release sites. However, coordinated muscle activity does not require presynaptic HSN input. Using reversible silencing experiments, I show that neuronal and vulval muscle activity in L4 is not required for the onset of adult behavior. Instead, the accumulation of eggs in the adult uterus renders the muscles sensitive to HSN input. Sterilization or acute electrical silencing of the vulval muscles inhibits presynaptic HSN activity, and reversal of muscle silencing triggers a homeostatic increase in HSN activity and egg release that maintains ~12-15 eggs in the uterus. Feedback of egg accumulation depends upon the vulval muscle postsynaptic terminus, suggesting a retrograde signal sustains HSN synaptic activity and egg release. Thus, the circuit while not seeming to require early activity, does depend on modulation by sensory feedback to drive activity and the two-state egg-laying behavior in adults.

In adults, the signaling mechanisms which maintain the egg-laying inactive behavior state are not well understood. The Pertussis toxin sensitive G alpha subunit, Gαo, is the most abundantly expressed alpha protein in both the central and peripheral nervous systems, comprising about 1% of all membrane-bound proteins in the mammalian brain. Knockout of Gαo in the nematode C. elegans,
which shares more than 80% sequence identity with its mammalian orthologue, leads to hyperactive locomotion and reproductive behaviors. In order to test the effects of Gαo signaling on circuit activity and in maintaining the inactive behavior state, I recorded the long-term egg-laying behavior in wildtype animals and animals with altered Gαo signaling. Mutants with decreased Gαo signaling and animals expressing Pertussis Toxin in the HSN neurons showed early behavior onset, frequent active states, and a reduction in the duration of inactive behavior states compared with wild type animals. Mutants with increased Gαo signaling showed a delay in behavior onset, a lowered frequency of active states, and a significant increase in the duration of inactive behavior states compared with wild type animals. Additionally, I used approaches to cell-specifically perturb Gαo signaling combined with Ca2+ imaging to visualize the effects on cell and circuit activity. Gαo null mutants and animals expressing Pertussis Toxin in the HSN neurons showed a dramatic increase in HSN Ca2+ burst firing and vulval muscle activity. These results point to a key role played by Gαo in controlling neuronal activity and neurotransmitter release, providing a genetically tractable system to understand the underlying physiological mechanisms of Gαo signaling.
This dissertation is dedicated to my parents for their love and unwavering support.
Acknowledgements

This work would not have been possible without the support of several key people. First, I would like to thank my mentor, Dr. Kevin Collins for his guidance, support, and feedback. He is a truly excellent mentor who has helped me immensely in improving my skills as a scientist and I will greatly miss working in his lab. His dedication in helping me make sense of my data during weekly one-on-one meetings, encouragement towards presenting my work at scientific conferences, and feedback to improve my presentation skills have helped me tremendously. I would like to thank my committee members: Drs. Laura Bianchi, Peter Larsson, and Stephen Roper for their insight, support, and advice throughout my training. I would like to thank Dr. Brock Grill for serving as the external examiner for my dissertation defense. I would like to thank Addys Bode and Michael Scheetz for technical assistance and help with strain construction, Jessica Garcia for behavioral assays, and Collins lab members for helpful discussions and feedback. I would like to thank the C. elegans Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440) for providing worm strains used in this study. I would also like to thank Dr. Michael Koelle, Dr. Alexander Gottschalk, Dr. Yuichi Iino, and Dr. Cori Bargmann for providing strains and plasmids. This work was funded by a grant from National Institute of Neurological Disease and Stroke (NINDS) to Dr. Kevin M. Collins (R01 NS086932). Last, but not least, I would like to thank my family and my boyfriend Rajan Dasgupta for supporting me every step of the way.
# TABLE OF CONTENTS

List of Figures ........................................................................................................................ vii

List of Tables ........................................................................................................................... ix

Chapter 1 ................................................................................................................................... 1

Introduction ............................................................................................................................ 1

1.1. Communication Between Cells in Neural Circuits .................................................. 1

1.2. Development of Activity in Motor Circuits in the Context of Animal Behavior ................................................................. 9

1.3. Neuromodulation of Motor Circuit Activity and Behavioral Output ............ 13

1.4. Morphology of the *Caenorhabditis elegans* Egg-laying Behavior Circuit .................................................................................. 18

1.5. Development of the Egg-laying Circuit ................................................................. 22

1.6. Overview of Expression Patterns of Neurotransmitters, Neuropeptides, Their Receptors, Gap Junctions and Where They Function in the Egg-laying Circuit ......................................................................................... 28

Chapter 2 ................................................................................................................................... 45

Materials and Methods ........................................................................................................ 45

2.1. Nematode Culture and Developmental Staging .................................................. 45

2.2. Plasmid and Strain Construction ........................................................................ 50

2.3. Fluorescence Imaging ............................................................................................ 61

2.4. Behavior Assays and Microscopy ........................................................................ 64

2.5. Experimental Design and Statistical Analysis .................................................. 67

Chapter 3 ................................................................................................................................... 68

Development of Mature Patterns of Activity in the *C. elegans* Egg-laying Behavior Circuit ......................................................................................................................... 68

3.1. Rationale and Introduction to Experiments ...................................................... 68
3.2. Results ................................................................................................................. 69

3.3. Major Findings and Discussion ......................................................................... 89

Chapter 4 .................................................................................................................... 94

**Mature Patterns of Activity are Driven by Sensory Signals That Detect the Accumulation of Unlaid Eggs in the Uterus** ......................................................... 94

4.1. Rationale and Introduction to Experiments ...................................................... 94

4.2. Results ................................................................................................................. 96

4.3. Major Findings and Discussion ......................................................................... 114

Chapter 5 .................................................................................................................... 119

**Major G-protein, GOA-1 (Gαo), Regulates Neuronal Excitability of the Serotonergic HSN Neuron in Caenorhabditis elegans** .................................................. 119

5.1. Rationale and Introduction to Experiments ...................................................... 119

5.2. Results ................................................................................................................. 123

5.3. Major Findings and Discussion ......................................................................... 140

Chapter 6 .................................................................................................................... 147

**Perspectives and Future Directions** .................................................................... 147

6.1. Role of Early Activity in the Egg-laying Circuit .............................................. 147

6.2. The Egg-laying Circuit Functions as a Stretch Homeostat ............................ 151

6.3. Candidate Retrograde Signaling Molecules in C. elegans ........................... 155

6.4. Gαo Signaling in Regulating Neural Excitability in Circuits .......................... 159

References ............................................................................................................... 168
LIST OF FIGURES

Figure 1.1. Motor circuit development in the larval zebrafish ......................... 13

Figure 1.2. Egg laying is a two-state behavior .................................................. 19

Figure 1.3. Morphology of the egg-laying behavior circuit .............................. 21

Figure 3.1. Morphological development of the C. elegans egg-laying circuit .... 71

Figure 3.2. HSN neurons show tonic Ca²⁺ activity during the late L4 stage and burst firing during the egg-laying active state ....................................................... 75

Figure 3.3. HSN Ca²⁺ activity during the late L4 stage is not correlated with animal locomotor state ............................................................................................. 77

Figure 3.4. HSN regulates the defecation motor program ................................. 79

Figure 3.5. Development of coordinated vulval muscle Ca²⁺ transients in the L4.9 stage does not require presynaptic HSN input .................................................. 82

Figure 3.6. Early HSN and vulval muscle activity is not required for the onset of egg-laying behavior .............................................................................................. 87

Figure 3.7. Timeline of key developmental events and the onset of Ca²⁺ activity in the C. elegans egg-laying circuit .............................................................................. 90

Figure 4.1. Vulval muscle responsiveness to HSN input correlates with egg accumulation .................................................................................................................... 98

Figure 4.2. Sterilization decreases vulval muscle responsiveness to optogenetic HSN activation .............................................................................................................. 100
Figure 4.3. Germ line activity is required for HSN burst firing and the active state ................................................................. 103

Figure 4.4. Vulval muscle activity and egg accumulation promote HSN burst firing .............................................................................................................. 105

Figure 4.5. The vm2 muscle arms are required for vulval muscle feedback to HSN and burst firing .............................................................. 107

Figure 4.6. VC neurons show a homeostatic increase in Ca^{2+} activity with increased egg accumulation ................................................................. 110

Figure 4.7 VC neurotransmission inhibits HSN burst firing .................................................. 112

Figure 4.8. Working model of how retrograde signals from the postsynaptic vulval muscles modulate burst firing in the presynaptic HSNs .......................... 115

Figure 5.1. Gαo signaling maintains the inactive egg-laying behavior state ..... 127

Figure 5.2. Gαo signaling regulates the inactive state by inhibiting HSN Ca^{2+} activity ........................................................................................................ 132

Figure 5.3. Gαo signaling in HSN reduces vulval muscle excitability ............... 134

Figure 5.4. Gαo signaling functions in the vulval muscles and uv1 neuroendocrine cells .............................................................................................. 139

Figure 5.5. Increased Gαs signaling causes hyperactive egg laying ............... 145

Figure 6.1. Effect of blocking synaptic transmission in vulval muscles ............ 157
LIST OF TABLES

Table 1. Genes affecting egg-laying behavior and function ............................... 38

Table 2. Strain names and genotypes for all animals used in this study (behavior assays and calcium imaging).............................................................................. 45

Table 3. Intra-cluster and inter-cluster intervals in wildtype animals and mutants with altered Gαo signaling ................................................................. 129
Chapter 1

Introduction

1.1 Communication Between Cells in Neural Circuits

Neurons or nerve cells are the basic units of the nervous system and can be categorized into at least a thousand different types (Kandel et al., 2013). The complexity of behaviors is not generated by the diversity of neuron types, but rather by the ways in which they connect to perform specific functions. Understanding the nervous system and how it generates behaviors has focused on identifying: 1) The molecular components of individual nerve cells, 2) The mechanisms that allow neurons to communicate electrically and chemically, 3) The patterns of connections between neurons and other cells such as muscles, and how these connections allow neural circuits to generate behaviors, and 4) The mechanisms of neural circuit plasticity which modifies neuronal connections through experience (Kandel et al., 2013). Understanding how simple neural circuits drive behavior is a critical first step in neuroscience. Currently, there is no neural circuit for which the following are clear: a) All synaptic connections and signaling events occurring through neurotransmitters and neuromodulators are all defined, b) How such signaling events produce and modulate the dynamic patterns of activity in neural circuits to drive behaviors, and c) How altered synaptic connections and signaling at the level of the neural circuit affects behaviors. Before we address these questions, it is important to review some major ways by which neurons in circuits communicate. I will start by defining the molecular interactions and signaling
events that allow apposed cells to communicate and develop into functional neural circuits.

**Synaptic transmission:** There are two classes of synapses in the nervous system: chemical synapses and electrical synapses (also referred to as gap junctions). In chemical synapses, the presynaptic neurons that release neurotransmitter and postsynaptic cells bearing the neurotransmitter receptors are separated by the synaptic cleft. In electrical synapses, the cytoplasm of connected cells has electrical and small molecule continuity through gap junction molecules. In chemical synapses, the depolarizing stimulus from the presynaptic cells results in release of neurotransmitters from vesicles which fuse with the plasma membrane in the presynapse which bind to postsynaptic receptors to initiate a postsynaptic response (unidirectional) and incurs a synaptic delay (1-5 ms or longer), whereas in gap junctions, a depolarizing stimulus can pass between cells connected via gap junctions (bidirectional) with no synaptic delay (Hormuzdi et al., 2004).

Gap junctions allow a depolarizing current to pass between the pre- and postsynaptic cells directly though specialized channels in the membranes connecting the cytoplasms of the two cells. Weak, subthreshold depolarizing stimuli and hyperpolarizing current into the presynaptic neuron can then be transmitted to the postsynaptic neurons independent of presynaptic action potentials or Ca\(^{2+}\). The separation between the cell membranes of neurons that form gap junctions is 4 nm, essentially the combined width of the two plasma membranes, and the gap junction is formed as a bridge by specialized channels
which form a conductive pore for the flow of ion channels (Evans and Martin, 2002; Hormuzdi et al., 2004). A pair of hemichannels (also called a connexon), one on the presynapse and the other in the postsynapse, form the pore which has a diameter of approximately 1.5 nm which allows ions and also small organic molecules to pass between cells (Evans and Martin, 2002). Each hemichannel is composed of six subunits called connexins which are encoded by a family of about 20 genes (Beyer et al., 1990; Willecke et al., 2002). Hemichannels can be formed by combinations of different connexin proteins with different sensitivities to cytoplasmic Ca$^{2+}$ levels and intracellular pH (Hermans et al., 1995; Peracchia, 2004). As the transmission of electrical information is rapid across gap junctions, gap junctions function in circuits that mediate fast motor responses (Kiehn and Tresch, 2002). Some specialized gap junctions called rectifying gap junctions also possess voltage-dependent gates which allows for conduction only in one direction (Marder, 2009; Liu et al., 2017).

In chemical synaptic transmission, presynaptic action potentials lead to the activation of voltage-gated Ca$^{2+}$ channels into the synaptic boutons, leading to a Ca$^{2+}$ influx and the fusion of specialized vesicles containing several thousand neurotransmitter molecules into the synaptic cleft (20-40 nm wide). These neurotransmitters bind to specialized ionotropic neurotransmitter receptors on the postsynaptic neuron. Fast neurotransmitter receptors are ion channels that directly elicit postsynaptic electrical responses by altering membrane conductance (Hormuzdi et al., 2004). Despite chemical transmission being comparatively slow (0.3 ms) compared to electrical synapses (near instantaneous), the coincident,
quantal release of thousands of neurotransmitter molecules with each vesicle fusion event can bind to and open thousands of ionotropic receptor channels in the target cell, thereby allowing a weak electrical presynaptic current to depolarize a large postsynaptic cell (Kandel et al., 2013).

Neurotransmitters can regulate ion channel opening either directly or indirectly and mediate fast synaptic transmission and slow modulation, respectively. Direct activation of ionotropic receptor channels by neurotransmitters causes a conformational change which opens the channel’s conducting pore. Activated receptors can remain in the conducting state for milliseconds. The second class of neurotransmitter receptor often indirectly gate ion channels by first binding to and activating a metabotropic, G protein coupled receptor (GPCR). Neurotransmitter binding to GPCRs stimulates the nucleotide exchange of a coupled heterotrimeric G protein which leads to the separation of the $\alpha$ and a $\beta\gamma$ subunits of the trimer, both of which are bound to the plasma membrane by lipid modification and bind to downstream effectors to signal in the responding cell. A major consequence of GPCR signaling is the production of secondary messengers such as cAMP or diacylglycerol which can directly bind to and modulate channel activity or can subsequently activate secondary effectors like kinases which phosphorylate ion channels or lipids, affecting cell activity. The synaptic actions of metabotropic receptors are comparatively slow in onset but often last longer, often seconds or minutes driving more profound changes in the excitability of neurons. Despite what is known about these two modes of synaptic transmission and their effects on neuronal excitability, we lack a fundamental understanding of how fast-
acting neurotransmitters and slow neuromodulators, which may even be co-released from the presynaptic neuron, act together in-vivo to regulate synaptic communication in intact circuits that drive behaviors.

The G protein activity cycle is completed when the Gα-GDP re-associates with the Gβγ to re-form the inactive heterotrimer Gαβγ. A novel family of molecules called the Regulators of G protein signaling (RGS) were discovered through genetic screens for mutations affecting reproductive and other behaviors in C. elegans. These proteins were found to enhance the GTPase activity of specific heterotrimeric G proteins (Koelle and Horvitz, 1996; Hajdu-Cronin et al., 1999). Similarly, another novel mechanism involving a receptor-independent pathway by a GPR domain containing protein called RIC-8 has been shown to prolong signaling by reactivating Gα-GDP release for nucleotide exchange, although it can also function as a chaperone (Reynolds et al., 2005; Gabay et al., 2011). Genetic models such as C. elegans allow us to identify and study the conserved in-vivo roles of neurotransmitter and G protein signaling pathways by their modulation of synaptic transmission and its regulation of specific behaviors. This basic mechanistic understanding informs the understanding for how these conserved molecules function in the more complex circuits of higher animals.

Mammals have approximately 20 types of α-subunits, 5 types of β-subunits, and 12 types of γ-subunits which can form different heterotrimers which produce different downstream effects (Milligan and Kostenis, 2006; Syrovatkina et al., 2016). The most well understood downstream signaling cascade is the cyclic AMP pathway which is initiated when a transmitter binds to a receptor which activates a
specific protein- Gαs (Godinho et al., 2015). Activated Gαs stimulates the integral membrane protein adenylyl cyclase to catalyze the conversion of ATP to cAMP. The major target of cAMP is the cAMP-dependent protein kinase A (PKA) which targets voltage-gated and ligand-gated ion channels, synaptic vesicle proteins, gene transcription, and neurotransmitter biosynthesis, to produce long-term physiological and biochemical changes in neurons. Two other major classes of Gα proteins are the excitatory Gαq and the inhibitory Gαi/o class. Second messengers diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3) are the chief downstream effectors of the Gαq pathway, but the effectors of the Gαi/o pathway which regulate synaptic release are unknown (Koelle, 2016).

**Trans-cellular signaling:** The communication between neurons and postsynaptic cells can be further classified into anterograde and retrograde signaling based on the direction in which information traverses between the two compartments and both forms of communication are required for synapse development and plasticity (Marques and Zhang, 2006). Anterograde signaling encompasses the various ways in which the presynaptic neuron signals to the postsynaptic targets through synaptic transmission (Marques and Zhang, 2006). Additional, retrograde trans-cellular signals are universally employed in the nervous system and can be defined as “a substance that is released “on demand” from the postsynaptic cell and diffuses to the presynaptic bouton where it regulates some aspect of synaptic transmission” (Regehr et al., 2009). In mammals, retrograde signaling has roles in neuroprotection and synaptic homeostasis (Plomp et al., 1992; Hughes et al.,
A wide variety of molecules have been shown to function as retrograde signals and are listed below:

1. Trophic factors such as Brain-derived neurotrophic factor (BDNF) and Nerve growth factor function as retrograde signals (Levi-Montalcini, 1987; Ito and Enomoto, 2016).

2. Trans-synaptic adhesion molecules such as the Neurexin-Neureelin complex and the Semaphorin(postsynaptic)-Plexin(presynaptic) complex. The former is a key regulator of reciprocal synaptic communication during development (Sudhof, 2017) and the latter is involved in the homeostatic regulation of presynaptic release (Orr et al., 2017).

3. Lipid messengers such as endocannabinoids, eicosanoids, and arachidonic acid are released during elevations in postsynaptic Ca^{2+}. After release, the molecules bind to specific receptors in the presynaptic terminals where they regulate neurotransmitter release (Alger, 2002).

4. Gaseous messengers such as nitric oxide (NO) and carbon monoxide (CO) which stimulate cGMP synthesis. cGMP signals by opening cyclic nucleotide-gated channels and by activating cGMP-dependent protein kinase (PKG) (Hawkins et al., 1994).

The time-course and impacts of retrograde signaling can be quite variable (Marques and Zhang, 2006). Signals such as endocannabinoids produce immediate effects on presynaptic cells while neurotrophins exert long-lasting effects often requiring retrograde transport from nerve processes back to the cell nucleus followed by changes in gene expression.
The *Drosophila* larval neuromuscular junction (NMJ) has served as a model synapse to understand the mechanisms of retrograde signaling (Marques and Zhang, 2006). Retrograde signaling molecule Bone Morphogenic Protein (BMP) is released by muscles and acts via two BMP receptors (type I and type II) to regulate presynaptic neurotransmitter release. Changes in retrograde signaling drive homeostatic plasticity in response to genetic perturbations that affect postsynaptic glutamate receptors (Petersen et al., 1997; McCabe et al., 2003; Berke et al., 2013). Changes in postsynaptic excitability and membrane resting potential serves can also serve as a monitor for triggering retrograde signaling (Petersen et al., 1997). Molecules of the Wnt signaling family are key mediators of activity-dependent signaling between the pre and postsynaptic terminals during development in CNS synapses and in the Drosophila NMJ (Budnik and Salinas, 2011). At the Drosophila NMJ, Wnt is released by the synaptic boutons (Packard et al., 2002) in a process requiring interactions with exosome-like vesicles (Koles et al., 2012). Wnt receptor (DFz2) are expressed in the pre and postsynapse and mediate the proliferation of presynaptic boutons and development of postsynaptic specializations (Packard et al., 2002; Ataman et al., 2008; Mosca and Schwarz, 2010). Wnt is also required to mediate activity-dependent plasticity (Ataman et al., 2008). In the vertebrate NMJ, Wnt mediates AChR clustering in the postsynapse during NMJ development (Henriquez et al., 2008). Therefore, Wnt seems to function as a trans-synaptic anterograde signal but whether it could also function as a retrograde signal is not known. In *C. elegans*, a novel C2 domain protein AEX-1 functions in a retrograde exocytic signaling pathway in the muscles that
regulates presynaptic neuronal excitability and synaptic maintenance (Doi and Iwasaki, 2002). Despite the progress in the identification of retrograde signaling molecules and the signaling pathways through which they exert their effects, the effects of retrograde and other trans-synaptic signals on cell and circuit activity in the behavioral context has not been examined.

1.2. Development of Activity in Motor Circuits in the Context of Animal Behavior

Developing neural circuits in the cortex, hippocampus, cerebellum, retina, and spinal cord show spontaneous neural activity (Wong et al., 1995; Garaschuk et al., 1998; Garaschuk et al., 2000; Watt et al., 2009; Warp et al., 2012). In contrast, mature neural circuits show coordinated patterns of activity required to drive efficient behaviors. The long-standing view had been that the early steps of neural development such as neuronal migration, axonogenesis, and synapse stabilization were dependent solely on cellular transcriptional programs (Spitzer, 2006). However, more and more, it has been shown that activity-dependent events play key roles in neural circuit formation and development (Shatz and Stryker, 1988; Komuro and Rakic, 1992; Gu et al., 1994; Catalano and Shatz, 1998; Liao et al., 2001; Wong and Ghosh, 2002). Ca\textsuperscript{2+} dynamics are required for cellular differentiation programs that affect morphology, neurotransmitter expression, and the expression of ion channels in cultured neurons (Gu and Spitzer, 1995). In *Drosophila*, activity-dependent dendritogenesis is required in the developing
learning and memory circuit (Broadie and Bate, 1993; Doll and Broadie, 2016),
and its disruption causes behavioral deficits in adult animals (Doll and Broadie,
2016). Early Ca\textsuperscript{2+} activity has also been shown to be required to mediate the
selection of the appropriate postsynaptic receptors based on neurotransmitter
specified in the presynaptic neuron (Borodinsky and Spitzer, 2007).

What is the functional importance of early activity in the formation of mature
patterns of activity in neural circuits? The transition from uncoordinated to
coordinated activity could be mediated, in part, by cell-autonomous changes in the
intrinsic firing properties of cells comprising the circuit as they begin to express
different ion channels/channel subtypes, or due to changes in the intracellular
concentration of ions (Moody and Bosma, 2005). The development of electrical
and chemical synaptic structures between cells in the circuit also allows for this
transition (Warp et al., 2012). Apart from changes in activity in motor circuits,
animals also display fictive or spontaneous motor behaviors during embryonic
development raising the question of whether these early behaviors play a role in
circuit development or if they are merely a consequence of the circuits wiring up
(Sanes et al., 2011).

Some of the early experiments looking at the role of early activity and
spontaneous movements in adults were carried out more than a century ago by
raising embryos in anesthetic solutions throughout the period where spontaneous
movements were observed (Harrison, 1904). More recent studies have also
applied anesthetic along with alpha-Bungarotoxin (blocks synaptic transmission)
to embryos to look at behavioral, physiological, and electrophysiological recovery
after the transfer of animals to their normal environment (Haverkamp, 1986; Haverkamp and Oppenheim, 1986). In the cases described above, treated embryos showed full recovery within a short duration following drug (or anesthetic) withdrawal. Studies where more targeted silencing of early activity was performed have yielded interesting results. Blocking early motor circuit CPG rhythms in the chick embryo which is driven by acetylcholine can be blocked in mice lacking choline acetyltransferase (ChAT). In these animals, defects were observed in the fine-tuning of locomotor activity in adult animals (Myers et al., 2005). Surprisingly, other studies targeted genetic manipulations that were expected to silence early activity revealed the existence of robust homeostatic mechanisms by which near normal levels of spontaneous activity are maintained during development (Gonzalez-Islas and Wenner, 2006; Babola et al., 2018).

Recent developments in visualizing circuit activity using live-cell Ca²⁺ imaging and optogenetic techniques to manipulate cell/circuit activity have once again opened the field to systematically address these questions by correlating activity, behavior, and sensory input. In the developing zebrafish spinal column, patterned activity transitions rapidly from sporadic, uncorrelated, long-duration events at 18 hours post fertilization (hpf) to coordinated, fast activity with the stereotypical ipsilateral correlation and contralateral alternation at 20 hpf as a result of the formation of gap junctions and chemical synapses (Warp et al., 2012). Optogenetically suppressing spontaneous activity from 18-19 hpf resulted in an increase in the proportion of cells showing immature patterns of activity at later stage (22 hpf) and a substantial decrease in ipsilateral correlation, suggesting an
important developmental role for early circuit activity. Although this study provides a good example of how to experimentally address the precise role of early activity and synaptic formation in the development of mature patterns of activity, the role of sensory input and how animal behaviors emerge because of maturing activity in the motor circuit could not be addressed. This was in part because the presynaptic Ca$^{2+}$ activity changes were observed in embryos paralyzed with α-bungarotoxin to prevent spontaneous muscle contractions, so the effects of spontaneous muscle contractions on shaping early neural activity could not be addressed.

Developing zebrafish larvae showed three sequential stereotyped motor behaviors during development behaviors (Fig. 1.1): 1) A transient period of alternating, coiling contractions beginning at 17 hpf and showing peak coiling frequency at 19 hpf followed by a gradual decline by 27 hpf. At this stage, animals are unresponsive to touch stimuli; 2) Touch stimulus- induced coiling responses were observed starting at 21 hpf; and 3) Swimming in response to touch stimuli starting at 27 hpf (Saint-Amant and Drapeau, 1998). Figure 1.1 shows a timeline of motor milestones in the developing Zebrafish spinal column.

A fundamental gap in understanding motor circuits is how the three components of circuit development described in Figure 1.1 (Activity, behavior and sensory input) influence each other during development and in adult animals during behaviors. To understand how long-term changes in circuit activity change as animals exhibit adult behaviors and how (and when) sensory input shapes this process, we need a system that allows for us to visualize changes in cell/circuit
activity in freely moving animals in the in developing animals as well as in behaving adults.

**Fig 1.1. Motor circuit development in zebrafish.** Flowchart highlighting changes in circuit activity as described (Warp et al., 2012), behavior pattern development as described (Saint-Amant and Drapeau, 1998), and the effects of hindbrain sensory inputs on driving motor outputs as described (Saint-Amant and Drapeau, 1998) during larval development. Timeline indicates time elapsed during larval development in units of hours post fertilization (hpf).

1.3. Neuromodulation of Motor Circuit Activity and Behavioral Output

Motor outputs in mature circuits are shaped by sensory signals, particularly neuromodulators, that induce or inhibit circuit activity to produce distinct behavior states. Neuromodulators can function as locally released co-transmitters or can
produce their effects on circuits far from their release sites (Cropper et al., 1987; Katz, 1995; Katz and Frost, 1996; Morgan et al., 2000). Such neuromodulators affect neural circuit firing properties by altering neuronal firing properties and synaptic strength (Marder et al., 2005; Marder, 2012). They can act at the level of sensory neurons (Pasztor and Bush, 1987), central circuits (Harris-Warrick and Kravitz, 1984; Hooper and Marder, 1987), as well as in neuromuscular synapses and muscle cells (Schwarz et al., 1980).

Studies in invertebrate motor circuits in crustaceans and mollusks have helped us in understanding the physiological effects of neuromodulators on motor outputs (Marder et al., 2005; Marder and Bucher, 2007; Marder, 2012). The crustacean stomatogastric ganglion (STG) is a Central Pattern Generator (CPG) circuit which generates intrinsic rhythmic activity when the circuit is removed from the animal. It is modulated by projection neurons (in the intact circuit in-vivo), and these same neuromodulators induce the same functional consequences to circuit function when applied exogenously (Marder et al., 2005; Marder and Bucher, 2007; Blitz and Nusbaum, 2011; Marder, 2012). Depending on which neuromodulatory projection neuron is activated (each with its unique complement of co-transmitters), the STG circuit can generate three separate motor rhythms: a fast pyloric rhythm and two slower gastric mill and cardiac sac rhythms, illustrating the power of modulators in increasing the range of motor outputs of an anatomically distinct motor circuit (Blitz and Nusbaum, 2011; Marder, 2012).

A range of chemically-diverse neuromodulators from amino acids (glutamate), biogenic amines (serotonin, noradrenalin, dopamine, and trace
amines), peptides (substance P), endocannabinoids, and nitric oxide signal through G protein coupled receptors to alter the response properties of CPG outputs in the vertebrate spinal cord (Miles and Sillar, 2011). These studies were, however, performed in isolated preparations of the CPG circuits by artificially applying neuromodulators and using electrophysiological approaches to detect changes in cell activity. How these neuromodulators affect intact circuits to regulate behavior in freely moving animals is not clear because functional studies of these signaling pathways are difficult outside of genetically or pharmacologically tractable model systems.

Neuromodulators not only influence circuit activity and motor outputs as described above, they are also critical for the organization of behaviors into discrete states. In their natural environments, animals typically alternate between discrete active and inactive behavior states (Goulding et al., 2008). During the active state, animals engage in locomotor and feeding behaviors, while during the inactive state they engage in behaviors such as rest and sleep (Herbers, 1981; Goulding et al., 2008). Transitioning between active and inactive states is integral to animal fitness and we therefore need to understand the molecular and circuit mechanisms that organize behaviors into discrete bouts and the role of neuromodulators in this process. A study of long-term patterns of mouse behavior in their home cage revealed a similar two-state (active and inactive) organization for behaviors such as locomotion, feeding, and drinking which was perturbed in mutant mice with altered serotonin signaling (Goulding et al., 2008). Although such long-term approaches in mice to identify aberrant behavioral regulation due to
global mutations in neuromodulator signaling can be useful, the underlying changes in circuit activity that cause the changes in behavior and the molecules that mediate these processes are not well understood.

Neurons in a circuit can express combinations of receptors which signal through distinct downstream pathways. An understanding of how such parallel signaling can produce a 'combinatorial' synaptic output is yet to be truly appreciated in the field of neuroscience. Do neurons preferentially express receptors which signal through one or two main G proteins to modulate their excitability? For example, do neurons requiring a tight regulation of cell excitability and rapid transitions between states of low and high activity preferentially use a limited set of G proteins with opposing effects (such as a combination of Gαq and Gαi/o only). Or, do all neurons in a circuit express receptors for most or all G proteins but only employ certain signaling pathways during specific activity states? What is the purpose of having many different serotonin receptors that signal through distinct G proteins? Does this diversity reflect altered receptor expression? Or does this primary difference arise due to differences in ligand affinity, allowing a circuit to respond in different ways to different levels of neurotransmitter, for example by excitation at low levels or inhibition at high levels (or vice versa)? Or, does receptor and G protein diversity reflect receptor function during different circuit activity patterns that underlie unique behaviors? Following are some key experimental steps which can answer these fundamental questions regarding G protein signaling in neural circuits driving behaviors:
1. Identify a genetically tractable neuron/neural circuit that allows for the use of approaches to visualize circuit activity in behaving animals.

2. Identify the signaling molecules (neurotransmitters and neuropeptides), receptors (ionotropic and metabotropic), and their downstream signaling pathways in the circuit. One experimental approach could be to analyze the expression patterns of known neurotransmitters, neuropeptides, and GPCRs using fluorescent reporter constructs. Genetic epistasis experiments with gene mutants can reveal the downstream mediators of neurotransmitter/neuropeptide signaling.

3. Use genetic approaches to manipulate neurotransmitter/peptide signaling in the presynaptic neuron(s) in circuit while simultaneously visualizing changes in the post-synaptic compartment. A similar approach can be used to manipulate key receptors in the postsynapse.

4. Use genetic approaches to abolish signaling through specific alpha subunits (such as Pertussis toxin to block $\text{G}_\text{i/o}$ and Cholera toxin to stimulate $\text{G}_\text{s}$-cAMP signaling) and test the effects on synaptic release and postsynaptic activity.

5. Identify those environmental cues and sensory neurons that release neuropeptides and other hormones and determine how they modulate circuit activity and behavior.

The *C. elegans* genetic model has several advantages which can be leveraged to address some of the fundamental questions posed so far, and also apply the experimental approaches described above. 1) *C. elegans* has exactly 302 neurons...
whose connections have been mapped by electron microscopy (White et al., 1986), 2) *C. elegans* has conserved neurotransmitters, signaling proteins, and synaptic structures, 3) *C. elegans* is optically transparent allowing the recording of Ca\(^{2+}\) activity in circuits and optogenetic approaches to manipulate activity in specific neurons (Collins et al., 2016; Ravi et al., 2018b), 4) *C. elegans* is genetically tractable allowing cell-specific manipulations of neurotransmitter signaling (Ravi et al., 2018a), and 5) *C. elegans* has relatively simple circuits that drive stereotypical behaviors such as locomotion, feeding, defecation, and reproduction. These behaviors provide rich resource to quantify even subtle changes in behavior state that result from specific genetic and activity perturbations.

In the following sections, I will describe a model motor circuit in the nematode model organism *Caenorhabditis elegans* and describe some features of this circuit and the advantages it offers in terms of addressing the fundamental unanswered questions described in this chapter.

### 1.4. Morphology of the *Caenorhabditis elegans* Egg-laying Behavior Circuit

Adult hermaphrodite *C. elegans* are self-fertile, producing self-sperm and oocytes, which fertilize to produce embryos (eggs) which are stored in the reproductive tract (also referred to as ‘uterus’) for a brief time before they are laid. At steady state, wild-type adult hermaphrodites store about 10-15 unlaid eggs in their uterus which are laid when specialized smooth muscles contract to open the
vulva (Chase et al., 2004). The neuromusculature that controls *C. elegans* egg laying is a well-characterized neural circuit (White et al., 1986) that drives a stochastic two-state behavior in adult animals with ~20 minute inactive periods punctuated by ~2 minute active states where ~4-6 eggs are laid (Fig. 1.2) (Waggoner et al., 1998). The underlying signals that drive an animal’s decision to enter or leave the egg-laying active state remain unclear.

**Figure 1.2. Egg laying is a two-state behavior.** (A) Egg-laying active state. Image shows a freely moving worm on a plate with a bacterial (E. coli) food source. Asterisk indicates the beginning of an active state which lasts for ~2 minutes during which eggs (arrowheads, numbered 1-7) are laid at ~20 s intervals. (B) Ethogram of egg-laying patterns in three wild-type animals (1-3) for 2 hours. Vertical dashes indicate single laid eggs. Green and Red brackets indicate one representative ‘active’ and ‘inactive’ behavior state in Animal 1. Figure adapted from Collins and Koelle, 2013.

As shown in Figure 1.3A and 1.3B, the egg-laying circuit comprises two serotonergic Hermaphrodite Specific Neurons (HSNs) (green) which function as
command neurons to activate the circuit (Desai et al., 1988; Waggoner et al., 1998; Emtage et al., 2012). Three locomotion motor neurons (VA7, VB6, and VD7) (grey and light blue), and six cholinergic Ventral C neurons (VC1-6) (dark blue) synapse onto a set of vulval muscles whose rhythmic activity drives either weak twitching or the release of eggs from the uterus in phase with locomotion (White et al., 1986; Collins and Koelle, 2013; Collins et al., 2016). The VC neurons might also serve to regulate locomotion and receive feedback of vulval muscle contractility (Collins et al., 2016). Two pairs of vm1 and vm2 vulval muscles cells control vulval opening (Fig 1.3A and 1.3B). The vm2 cells receive synaptic input from the HSNs and VC neurons and are required to drive egg laying and they also make gap junctions with the vm1 cells (White et al., 1986) (Fig. 1.3C). In addition to indirectly receiving excitatory stimulus via the vm2s upon their activation, the vm1s also receive excitatory synapses from locomotor neurons (White et al., 1986) (Fig. 1.3C).

Uterine muscles (light yellow) wrap around the uterus on the anterior and posterior sides (Fig. 1.3A and 1.3C) and make gap junctions with the vm2 vulval muscles (White et al., 1986) (Fig. 1.3C). Figure 1.3D presents a closer look at the synapses and gap junctions that exist between the HSN (left and right), VCs (1-6), and the vm2 vulval muscles based on the C. elegans wiring diagram (White et al., 1986). Finally, four uv1 neuroendocrine cells (pink) connect the vulval canal to the uterus which holds embryos until they are laid (Newman and Sternberg, 1996; Jose et al., 2007). They are mechanically activated by the passage of eggs through the vulva and release neurotransmitters and peptides which terminate egg laying (discussed in upcoming sections) (Collins et al., 2016).
Figure 1.3. Morphology of the egg-laying behavior circuit. (A) Lateral view of the egg-laying circuit (seen from left) (B) Ventral, vulval side view of the egg-laying circuit (C) Cartoon showing synaptic connections and gap junctions between the cells of the egg-laying circuit. HSNs are green, VC (1-6) are dark blue, VB6 and VA7 are grey, VD7 is light blue, vm2 muscles are red, vm1 muscles are orange, uterine muscles are light yellow, and the uv1 neuroendocrine cells are pink. Arrowheaded lines indicate chemical synapses while bar-headed lines indicate gap junctions. Dashed lines are speculative. (D) Cartoon showing synapses and gap junctions between the HSNs, VCs, and vm2 muscles. ‘+’ and ‘-‘ signs alongside arrowheaded lines indicate whether synapses are excitatory or inhibitory based on our current understanding of the circuit. Figure adapted from White et al. 1986.

1.5. Development of the Egg-laying Circuit

Circuit development occurs during the early-mid L4 larval stages and requires direct interactions of the neurons and muscles with the developing vulval epithelium, but does not appear to require direct interactions between neurons and muscles (Li and Chalfie, 1990; Burdine et al., 1998; Colavita and Tessier-Lavigne, 2003; Shen et al., 2004). In the following section, I will present a brief review of the signaling molecules required for the proper development of each cell and its subsequent assembly into the egg-laying circuit (see also Table 1).

HSN command motor neurons: The command serotonergic HSN motor neurons play a central role in egg laying. Animals in which the HSNs have been ablated have a clear egg-laying defect and accumulate significantly more embryos in the uterus (Trent et al., 1983; Desai et al., 1988). Genetic mutants with defective
HSN neuron development or function can be identified by their sensitivity to serotonin, as their egg-laying defects can be reversed by the application of exogenous serotonin, but not by blockers of serotonin reuptake such as imipramine (Trent et al., 1983; Desai et al., 1988) suggesting HSN is the major site of serotonin release for egg laying. HSNs are generated post-embryonically from the asymmetric divisions of a pair of neuroblasts (Sulston et al., 1983). The precursor cells then migrate from the tail in the anterior direction in response to a posterior, repulsive Wnt signaling gradient toward the developing gonad adjacent to the future location of the vulva (Pan et al., 2006). During the L1 and L2 larval stages, the spherically shaped HSN precursors break symmetry and become ventralized by a process requiring UNC-6 (Netrin) release from the ventral nerve cord (Adler et al., 2006). During the mid-L3 stage (~25 hours after hatching), the HSNs extend several dynamic filopodia, and in between the L3 and L4 larval stages, a single ventral neurite is selected to form the axon (Adler et al., 2006). Netrin signaling not only causes the initial break in HSN symmetry but also maintains and orients the leading edge of the HSN axon via downstream lipid modulators AGE-1 (Phosphoinositide 3-kinase) and actin-regulatory protein MIG-10 (lamellipodin) (Adler et al., 2006). Forward genetic screens have identified key early transcriptional regulators in the birth, migration, and neurotransmitter specification of the HSN neurons (listed in Table 1).

Genetic studies have identified adhesion and cytoskeletal scaffolding molecules that drive HSN presynaptic assembly. The developing HSN axons extend a growth cone ventrally which fasciculates into the ventral nerve cord and
the growth cone migrates into the nerve ring in the head (Garriga et al., 1993a). At the vulva, between the primary and secondary vulval epithelial cells, the HSN axon defasciculates from the nerve cord and subsequently undergoes *en-passant* presynaptic development (Shen and Bargmann, 2003; Shen et al., 2004; Adler et al., 2006). The HSN axon continues to extend anteriorly into the nerve ring (Garriga et al., 1993a). Synaptogenesis in the HSNs relies on signals from specialized vulval epithelial cells (Shen and Bargmann, 2003; Shen et al., 2004; Patel et al., 2006). Forward genetic screens identified mutations that disrupt HSN synapse development. *syg-1* and *syg-2* mutants show a marked reduction in the accumulation of presynaptic proteins such as synaptic vesicle protein synaptobrevin (SNB-1), synaptic vesicle associated protein RAB-3, presynaptic molecules such as ELKS-1, GIT, and SAD-1 (Patel et al., 2006) in addition to reduced morphologically defined active zones at the vulva when examined by serial electron microscopy (Shen et al., 2004). *syg-1* and *syg-2* were found to encode two immunoglobulin superfamily proteins that physically interact to position the HSN presynaptic sites and promote presynaptic development at the vulva (Shen and Bargmann, 2003; Shen et al., 2004). SYG-1 is expressed in the developing HSN process, and SYG-2 is expressed in guidepost cells which are specialized epithelial cells situated near the vulval opening. Transient *syg-2* expression in the primary vulval epithelial cells during L4 defines the location of pre-synaptic sites at the junction between primary and secondary vulval epithelial cells. Mis-expression of SYG-2 in the secondary vulval epithelial cells in null mutants moves those HSN synapses ventrally (Shen et al., 2004), showing SYG-
expression is sufficient to specify HSN postsynaptic targets and drive presynaptic development. Intracellular scaffolding molecules such as SYD-1 and SYD-2 (liprin-α) function downstream of SYG-1 cell-autonomously in the HSNs as master regulators of presynaptic assembly by recruiting a slate of molecules required for presynaptic development such as RAB-3, ELKS-1, and GIT (Patel et al., 2006). Despite such studies defining the role of several molecules in the development of the HSN neurons, key questions remain: 1) Does HSN activity accompany synapse development? and 2) Since egg-laying behavior itself does not occur until much later when animals develop into young adults, is this temporal delay in the onset of behavior signify additional events in the maturation of a functional behavior circuit?

**Cholinergic VC motor neurons:** Like the HSNs, the six VC neurons are generated post-embryonically during the first larval stage along with the other ventral cord motor neurons (Sulston, 1976). VCs undergo synaptic development during L4s, with vulva-proximal VC4-VC5 making synapses onto the vulval muscles (Sulston, 1976). Proper termination of branching of VC synaptic processes is dependent on a neurexin-related transmembrane protein BAM-2 expressed in the vulF epithelial guidepost cells (Colavita and Tessier-Lavigne, 2003). Apart from the synapses made by the VCs onto the vulva muscles, they also extend short, vesicle-filled processes near the vulval hypodermis devoid of synapses, but the function of these neurites is not well understood. It has been proposed that these processes might be activated by mechanosensory input, but
this has not been tested directly (White et al., 1986; Zhang et al., 2008; Collins et al., 2016).

Vulval and uterine muscle cells: The vulval muscle (vm) and uterine muscle (um) cells are derived from the M cell embryonic lineage which gives rise to two, bilaterally symmetric sex myoblast (SM) cells (Sulston and Horvitz 1977). SMs migrate anteriorly from the tail to the future location of the vulva where they undergo a series of divisions to give rise to the egg-laying musculature that is comprised of 16 non-striated muscles: two pairs each of vm2 and vm1 muscles and eight uterine muscle cells. These muscles are organized around the vulva with both anterior/posterior and left/right bilateral symmetry (Figure 1.2). Mutations affecting the M cell lineage, for example in \textit{sem-4}, which encodes a zinc-finger transcription factor, prevent vulval muscle generation (Basson and Horvitz, 1996). Additional mutant screens have identified genes and signaling molecules required for SM migration; in these mutants the vulval muscles are present but are mislocated. Molecular analysis has identified the gonad as an important organizer for vulval muscle development (Li and Chalfie, 1990). EGL-17 (homolog of the Fibroblast Growth Factor) is produced by the gonad and acts via EGL-15 (FGF receptor) in the sex myoblasts to direct their migration (DeVore et al., 1995; Burdine et al., 1998).

Once the SMs arrive at the vulva, they undergo a programmed series of cell divisions that give rise to 16 non-striated muscles with distinct morphologies, cell positions, synaptic connectivity, and function (Hale et al., 2014). Conserved transcription factors play a role in sex muscle fate specification. The T-box
transcription factor MLS-1/TBX-1 is required to specify uterine muscle fate while the knockdown of TALE homeodomain protein UNC-62/MEIS causes all muscle cells to convert to vm1 muscle cells (Kostas and Fire, 2002; Jiang et al., 2009). LIN-12/Notch and its ligand LAG-2/DSL are required for the specification of vm2 cell fate and in their absence a switch from vm2 cell fate to vm1 cell fate is observed (Hale et al., 2014).

The vm1 and vm2 muscles have distinct morphology and synaptic connectivity. vm2 muscles receive direct synaptic input from the HSN and VC motor neurons and regulate the opening of the vulva in conjunction with the vm1 muscles to which they are electrically coupled (White et al., 1986). The vm1 muscles do not receive direct synaptic input from the HSN and VC motor neurons but do receive single synaptic inputs from cholinergic locomotion neurons VA and VB (White et al., 1986). The eight uterine muscles cells ensheath the uterus on the anterior and posterior ends and are electrically coupled to each other and also make electrical synapses with vm2 vulval muscles, but their role in egg laying is not well understood (Schafer, 2006). Laser ablation studies of the SM daughter cells indicate that only loss of the vm2 muscles, but not the vm1 or uterine muscles, grossly affects egg-laying behavior (Michael Stern, personal communication). As the vm2 are the only muscle cells in the circuit that receive significant synaptic input from the serotonergic HSNs, this result suggests that contraction of the vm2 muscles is required for efficient egg release. The vm1 muscle cells may function to coordinate egg-laying behavior with locomotion, while the uterine muscle cells may help the circuit detect the accumulation of unlaid eggs (Collins et al., 2016).
As described in this section (Table 1), despite our understanding of the genes involved in the development of individual cells of the egg laying circuit, we still do not understand when and how the cells in the circuit develop electrical and Ca\textsuperscript{2+} activity. What is the relationship between circuit morphology and Ca\textsuperscript{2+} activity? What is the relationship between Ca\textsuperscript{2+} activity in the circuit and egg-laying behavior? Do all the cells of the circuit begin to show mature patterns of activity as adult animals or do different cells show dynamic changes in activity before behavior? My hypotheses based on these fundamental questions, experimental design, and results will be discussed in upcoming chapters.

1.6. Overview of Expression Patterns of Neurotransmitters, Neuropeptides, Their Receptors, Gap Junctions and Where They Function in the Egg-laying Circuit

In the following section, we will present a brief review of the signaling molecules (neurotransmitters/neuropeptides and their receptors) as well as ion channels that have been shown to control the function of the egg-laying circuit.

**HSN command motor neurons:** The serotonergic HSN neurons are a bilateral pair of neurons which innervate the vulval muscles (White et al., 1986). Genetic loss or laser killing of the HSNs causes a striking egg-laying defect where adult animals continue to make eggs but fail to lay them in timely fashion. HSN-deficient animals accumulate up to 50 eggs in the uterus (Trent et al., 1983). HSNs show Ca\textsuperscript{2+} activity just prior to egg laying (Zhang et al.; Collins et al. 2016), and
sustained optogenetic activation of HSNs induces patterns of egg laying similar to the egg-laying active state (Leifer et al., 2011; Emtage et al., 2012; Collins et al., 2016). Together, these results show the HSNs function as command motor neurons for egg laying. The HSNs are immunoreactive with anti-serotonin antibodies indicating the HSNs are serotonergic (Desai et al., 1988). Egg laying in HSN-deficient animals can also be rescued by treatment with exogenous serotonin but not imipramine, an inhibitor of the serotonin reuptake transporter (Trent et al., 1983; Waggoner et al., 1998).

Serotonin synthesis in *C. elegans* requires the *tph-1* gene which encodes the sole ortholog of Tryptophan Hydroxylase. *tph-1* knockout animals show only a mild egg-laying defect compared to animals without HSNs (Brewer et al., 2019). This result suggests the HSNs promote egg laying by releasing additional neurotransmitters alongside serotonin. Three neuropeptide encoding genes are also expressed in HSNs: *nlp-3, nlp-15* and *flp-19* (Nathoo et al., 2001; Kim and Li, 2004; Brewer et al., 2019). In a screen for neuropeptides that might be released alongside serotonin, NLP-3 was identified as causing a dramatic increase in egg laying when over-expressed (Brewer et al. 2018). While loss of *nlp-3* had only mild egg-laying defects on its own, resembling *tph-1* null mutants, *nlp-3, tph-1* double mutant animals show a strong, synthetic egg-laying defect, comparable to the *egl-1* mutants lacking HSNs (Brewer et al., 2019). These lines of evidence point to a model where serotonin and NLP-3 function in a partially redundant manner to mediate HSN command motor neuron activity for egg laying (Brewer et al., 2019).
The HSNs receive synaptic inputs on their soma from the touch-sensory PLM neurons and a few from the VC neurons and PVN neurons on their neuronal processes near the vulva (White et al., 1986). Activation of the PLM neurons by gentle touch transiently inhibits HSN activity (Zhang et al., 2008). The majority of morphologically identified synapses onto the HSN are found after the long axonal process within the nerve ring (White et al., 1986). Laser ablation of the HSN axon between the vulva and nerve ring, however did not produce an egg-laying defect (Zhang et al., 2008). This result suggests that axotomized HSNs are still active in promoting egg laying and that those axonal HSN synapses in the head are not required for egg laying. If presynaptic inputs from the nerve ring are dispensable, how is HSN activity regulated? The major heterotrimeric G proteins, Gαq and Gαo, signal in HSN to increase and inhibit egg laying, respectively (Ringstad and Horvitz, 2008; Tanis et al., 2008; Koelle, 2016). In the HSN neurons, the G protein coupled receptor EGL-6 signals through the inhibitory Gαo and the inward rectifying K+ channel IRK-1 (homologous to the Kcnj family of inward rectifying K+ channels) to inhibit HSN neurotransmitter release (Ringstad and Horvitz, 2008; Emtage et al., 2012). The EGL-6 receptor is activated by the FLP-17 neuropeptides released by the CO2-sensitive BAG neurons (Ringstad and Horvitz, 2008), allowing worms to acutely inhibit egg laying when environmental CO2 levels become elevated. Another GPCR EGL-47, which is homologous to Drosophila gustatory receptors (Liu et al., 2010) is expressed in the HSNs and is thought to signal via the Gαo pathway to inhibit egg laying (Moresco and Koelle, 2004). Loss-of-function mutations in egl-6 or egl-47 do not cause nearly as strong a hyperactive egg-laying
phenotype as loss of G\(\alpha_o\), suggesting other GPCRs are expressed on the HSNs that negatively regulate its excitability.

Ligands and GPCR receptors that signal in HSN to activate the excitatory G\(\alpha_q\) pathway have not yet been identified, but the G\(\alpha_q\) signaling pathway is more completely understood. Recent work has shown that G\(\alpha_q\) acts in HSN upstream or parallel to CCA-1 (Zang et al., 2017), a T-type voltage-gated Ca\(^{2+}\) channel, and NCA-1 and NCA-2, Na\(^+\) leak channels (Yeh et al., 2008; Topalidou et al., 2017). Because the balance of neuropeptide signaling through receptors and their downstream inhibitory K\(^+\) or excitatory Ca\(^{2+}\) and Na\(^{2+}\) channels have been shown to modulate intrinsic pacemaker activities of other neurons and in cardiac cells of the heart (Hille, 2001), these two antagonistic signaling pathways would allow diverse sensory modulation of HSN neurotransmitter release to ensure egg laying only occurs in favorable environmental conditions.

Additional ion channels have been identified that control the resting excitability of the HSN neurons. TMC-1 mediates a background Na\(^+\) leak conductance and mutations in the \(tmc-1\) gene disrupt normal egg laying (Yue et al., 2018). CLH-3, a member of the class of hyperpolarization activated inwardly-rectifying Cl\(^-\) channels, also regulates HSN neuron excitability (Branicky et al., 2014). How the activity of these ion channels is regulated and the identity of their upstream regulators (if any) is poorly understood. HSNs also express a tyramine-gated chloride channel LGC-55 which is required for full inhibition of egg laying by the uv1 neurotransmitter, tyramine (Pirri et al., 2009; Collins et al., 2016). The HSNs express innexin proteins \(unc-9\), \(inx-4\), \(inx-7\), and \(inx-3\) (Starich et al., 2001;
Altun et al., 2009), and make gap junctions with each other and the VC neurons
(White et al., 1986). Because gap junctions like the UNC-9 partner, UNC-7, have
been suggested to be targets of modulatory signaling (Correa et al., 2015),
changes in gap junction opening or rectification could shape the patterned activity
in the circuit to sustain or terminate the egg-laying active state.

**Vulval Muscles:** The post-synaptic vulval muscles express multiple
serotonin receptors that mediate their response to HSN serotonin input (Carnell et
al., 2005; Dempsey et al., 2005; Hobson et al., 2006; Hapiak et al., 2009). The
SER-1 and SER-7 serotonin receptors are expressed in the vulval muscles and
are required for proper excitation and egg laying. EGL-30 which encodes the Gαq
subunit in C. elegans functions downstream of SER-1 in the vulval muscles leading
to the opening of EGL-19 L-type Ca^{2+} channels (Shyn et al., 2003; Dempsey et al.,
2005) Another receptor, SER-7, a 5-HT7-like receptor, also potentiates the
excitatory effects of serotonin in vulval muscles and *in vitro* evidence suggests
SER-7 signals via Gαs and cAMP (Hobson et al., 2006). Because NLP-3
neuropeptides act in parallel to serotonin to promote egg laying, receptors for NLP-
3 neuropeptides may function similarly in the vulval muscles to enhance their
excitability activity or they may instead act to regulate acetylcholine release from
the presynaptic VA, VB, and VC neurons (Lackner et al., 1999; Nurrish et al.,
1999). The excitatory effects of acetylcholine on vulval muscle activity is likely
mediated by nicotinic acetylcholine receptors (ACHRs) encoded by *unc-29, unc-
38*, and *lev-1* genes (Fleming et al., 1997; Waggoner et al., 2000; Kim et al., 2001).
As shown in Figure 1.2, the vm2 muscles make gap junctions with VC neurons as
well as uterine muscle cells (White et al., 1986). Gap junctions couple the vm1 muscles to the vm2 muscles which receive direct synaptic input. The eight uterine muscles which wrap around the uterus do not receive direct synaptic input and may instead promote vulval muscle activity when they are stretched by the accumulation of eggs in the uterus. Gap junctions may thus modulate vulval muscle excitability through both neuronal and stretch-dependent mechanisms. Whether these two pathways are independent or are regulated by the same set of sensory and neuromodulatory signals remains unclear.

How is vulval muscle excitability regulated? Several potassium channels have been identified that regulate vulval muscle electrical excitability. Most K+ channels in C. elegans were originally defined by dominant, gain-of-function mutations that increase channel opening and result in cell hyperpolarization. Animals carrying these mutant channels inhibit egg-laying behavior, presumably by electrically silencing the vulval muscles. Vulval muscle-expressed channels include egl-36, which encodes shaw voltage gated K+ channel (Elkes et al., 1997; Johnstone et al., 1997), egl-2, encoding an ether-a-go-go homolog (Weinshenker et al., 1999), members of the TWK two-pore domain K+ channels such as sup-9 and egl-23 (Levin and Horvitz, 1993; de la Cruz et al., 2003), and ether-a-go-go related gene homolog unc-103 (Reiner et al., 2006). Loss-of-function mutants in most of these K+ channels show grossly wild-type egg-laying behavior suggesting a large degree of functional redundancy in stabilizing the electrical excitability of the vulval muscles (Schafer, 2006). However null mutations that eliminate unc-103 and egl-36 do cause a hyperactive egg-laying behavior phenotype, suggesting
certain K⁺ channels have a more pronounced role in regulating vulval muscle electrical excitability (Elkes et al., 1997; Johnstone et al., 1997; Reiner et al., 2006; Collins and Koelle, 2013). These K⁺ channels are thought to inhibit excitatory Ca²⁺ channel activity in the muscles. Mutations that impair the functioning of the α1-subunit (contains the ion channel pore and the voltage sensor domain) of the L-type voltage-gated Ca²⁺ channel encoded by egl-19 results in a strong defect in egg laying and also confers resistance to exogenous serotonin, indicating that this channel functions as a downstream regulator of serotonin in the vulval muscles (Lee et al., 1997; Laine et al., 2011). The other two subunits that comprise the voltage-gated Ca²⁺ channel are the α2/δ subunit encoded by unc-36 which modulates the voltage dependence, activation kinetics, and the conductance of calcium currents and the β subunit encoded by ccb-1 which is necessary for viability (Laine et al., 2011). Collectively, the L-type Ca²⁺ channel, EGL-19, mediates action potentials in neurons and vulval muscles.

How is vulval muscle Ca²⁺ activity affected in animals lacking presynaptic HSN activity? Because the HSNs and serotonin promote egg laying, animals lacking HSNs would be predicted to have strongly reduced vulval muscle activity. Surprisingly, HSN-deficient animals have more frequent vulval muscle Ca²⁺ transients during inactive behavior state and also show robust Ca²⁺ activity during the infrequent egg-laying active states (Collins et al., 2016). How are vulval muscles activated in the absence of HSNs? Chemically sterilized wild-type and HSN-deficient mutants showed dramatically reduced vulval muscle Ca²⁺ transient frequency (Collins et al., 2016). These results suggest germ line activity and/or egg
accumulation are required for robust vulval muscle Ca\textsuperscript{2+} activity independent of neurotransmitter release from the HSN command neurons. Animals lacking HSNs continue to make embryos which fill the uterus waiting to be laid. I hypothesize that a mechanical stretch signal reporting this elevation in egg accumulation could be relayed via the eight uterine muscles that line the uterine lumen and form gap junctions to excite the vm2 muscles alongside serotonin and HSN input (White et al., 1986). Therefore, neurotransmitter release from the HSNs initiates vulval muscle activity and the egg-laying active state only when additional signals such as egg accumulation enhance the postsynaptic response of vulval muscles.

**Cholinergic VC motor neurons:** The VC motor neurons express enzymes for acetylcholine (ACh) biosynthesis and release (Duerr et al., 2001; Pereira et al., 2015). The vulva proximal VC4 and VC5 neurons make the most extensive synapses on to the vm2 vulval muscles. The VCs also make synapses with the other VC neurons and the body wall muscles which regulate locomotion (White et al., 1986). The VC neurons express innexin proteins *unc-7* and *unc-9* (Altun et al., 2009), and make gap junctions with each other and the vm2 muscles (Fig. 1.2) (White et al., 1986). Although, ACh receptors agonists receptors strongly stimulate egg laying (Trent et al., 1983; Weinshenker et al., 1995; Waggoner et al., 2000), direct laser ablation of the VCs or mutations that affect VC development and function cause *increased* egg laying. These results suggest that ACh release form the VCs might signal to inhibit egg laying (Bany et al., 2003), perhaps by signaling through GAR-2 receptors expressed on the uv1 neuroendocrine cells (Richard Kopchock and Robert Fernandez, personal communication).
To clarify the precise effects of VC signaling on egg laying, our group employed an optogenetic approach (Collins et al., 2016). Optogenetic activation of the VCs causes hypercontraction of the body and an acute slowing of animal locomotion suggesting VC releases ACh that excites the body wall muscles to control locomotion (Collins et al., 2016). The VCs also express two neuropeptides encoded by \textit{nlp-7} and \textit{flp-11}, which inhibit egg laying when over-expressed (Banerjee et al., 2017). Could the release of these inhibitory peptides explain the why VC ablation causes an egg-laying constitutive phenotype? Since the VCs make synapses (and gap junctions) with the HSNs, do they regulate HSN firing rates? The precise effects of blocking release of all neurotransmitters through transgenic expression of Tetanus toxin may reveal how the VCs function to regulate egg-laying circuit activity and behavior.

\textbf{uv1 neuroendocrine cells:} The uv1 cells synthesize tyramine, a biogenic amine neurotransmitter, which inhibits egg laying. Mutations in \textit{tdc-1} that encodes tyrosine decarboxylase block tyramine biosynthesis and cause hyperactive egg-laying behavior (Alkema et al., 2005; Jose et al., 2007). Tyramine released by the uv1s act at least in part through the LGC-55 \textit{Cl}^- channels on the HSN to inhibit their activity (Collins et al., 2016). The uv1 cells also synthesize two inhibitory neuropeptides encoded by \textit{nlp-7} and \textit{flp-11}, which are co-expressed in the VC neurons and signal to inhibit serotonin release from the HSN neurons (Banerjee et al., 2017). How are the uv1 neuroendocrine cells activated? Our lab has previously shown that the passage of eggs through the vulva physically deforms the uv1 cells to trigger a strong \textit{Ca}$^{2+}$ transient (Collins et al., 2016). Previous work has shown
that the uv1 cells express TRPV channels such as OCR-2 which could function as mechanosensory channels, allowing for the transduction of mechanical strain into a depolarizing stimulus (Jose et al., 2007). However, because *ocr-2(vs29)* dominant-negative mutant still shows uv1 Ca\textsuperscript{2+} transients in response to egg laying (Kevin Collins, unpublished data), other unidentified molecules in the uv1 cells likely mediate their mechanical activation in response to egg passage.
<table>
<thead>
<tr>
<th>Cell</th>
<th>Process affected</th>
<th>Process affected</th>
<th>Gene</th>
<th>Mutant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSN</td>
<td>Development</td>
<td>Birth</td>
<td>\textit{hlh-14} (helix-loop-helix transcription factor)</td>
<td>HSN precursors are not generated</td>
<td>(Frank et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{egl-1 (d)} (upstream activator of apoptotic pathway)</td>
<td>HSNs undergo inappropriate cell death</td>
<td>(Conradt and Horvitz, 1998)</td>
</tr>
<tr>
<td></td>
<td>Migration and differentiation</td>
<td></td>
<td>\textit{egl-5} (Hox gene)</td>
<td>HSN cell death, HSN neuroblasts fail to migrate, no serotonin expression in HSNs</td>
<td>(Chisholm, 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{egl-20} (Wnt)</td>
<td>Abnormal neuroblast migration</td>
<td>(Forrester et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{egl-43 and ham-2} (zinc-finger proteins)</td>
<td>Abnormal HSN migration but not 5-HT expression.</td>
<td>(Garriga et al., 1993b; Baum et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{egl-44 and egl-46} (zinc-finger transcription factors)</td>
<td>Abnormal anterior migration of HSNs, no 5-HT expression, abnormal axonal migration</td>
<td>(Desai et al., 1988)</td>
</tr>
<tr>
<td>Function</td>
<td>Neurotransmitter synthesis</td>
<td>tph-1 nlp-3</td>
<td>Defective egg laying (single mutants- weak phenotype, double mutant-strong phenotype)</td>
<td>Brewer et al., 2019</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------</td>
<td>-------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Cell excitability and neurotransmitter release</td>
<td>goa-1 (Gα_o)</td>
<td></td>
<td>Inhibits synaptic release and egg laying behavior. Null and loss-of-function mutants show hyperactive egg laying.</td>
<td>Trent et al., 1983; Tanis et al., 2008</td>
<td></td>
</tr>
<tr>
<td></td>
<td>egl-10 (RGS protein)</td>
<td></td>
<td>Promotes GTP hydrolysis of Gα_o subunit. Null mutant is egg laying defective</td>
<td>Koelle and Horvitz, 1996</td>
<td></td>
</tr>
</tbody>
</table>

Axon guidance and synaptic development

- **unc-86** (POU transcription factor)
- **unc-6** (Netrin), **unc-40** (Netrin receptor)
- **syg-1, syg-2** (Igg family molecules)
- **syd-1, syd-2** (lprin α), **nab-1** (neurabin)

- No serotonin expression
- No ventral migration of HSN axon
- Abnormal synapse placement
- Abnormal presynaptic development

(Adler et al., 2006)  
(Shen and Bargmann, 2003; Shen et al., 2004)  
(Patel et al., 2006; Chia et al., 2012)  
(Finney and Ruvkun, 1990)
<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Description/Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>egi-30</strong> (Gαo)</td>
<td>Promotes synaptic release and egg laying behavior. Partial loss-of-function mutants are sluggish and egg laying defective</td>
<td>(Lackner et al., 1999; Miller et al., 1999)</td>
</tr>
<tr>
<td><strong>eat-16</strong> (RGS protein)</td>
<td>Promotes GTP hydrolysis of Gαq subunit. Null mutants show hyperactive egg laying.</td>
<td>(Hajdu-Cronin et al., 1999)</td>
</tr>
<tr>
<td><strong>egi-6</strong> (Gαo-coupled GPCR)</td>
<td>Dominant mutation causes egg laying defect. Activated by flp-17 neuropeptides released by BAG. Inhibits HSN activity via IRK-1 K⁺ channel.</td>
<td>(Ringstad and Horvitz, 2008; Emtage et al., 2012)</td>
</tr>
<tr>
<td><strong>egi-47</strong> (Gustatory receptor)</td>
<td>Dominant mutation causes an egg laying defect</td>
<td>(Moresco and Koelle, 2004)</td>
</tr>
<tr>
<td><strong>tmc-1</strong> (transmembrane channel-like protein)</td>
<td>Null mutants are egg laying defective</td>
<td>(Yue et al., 2018)</td>
</tr>
<tr>
<td><strong>cca-1</strong> (T-type Ca²⁺ channel)</td>
<td>Gain-of-function mutant suppresses the egg laying defect in egl-6(dm) mutants</td>
<td>(Zang et al., 2017)</td>
</tr>
<tr>
<td>Vulval muscles</td>
<td>Development</td>
<td>Birth</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Migration and differentiation</td>
<td>egl-15 (FGF receptor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>egl-17 (FGF)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>egl-20 (Wnt)</td>
</tr>
<tr>
<td>Function</td>
<td>Cell excitability</td>
<td>egl-2 (eag K⁺ channel)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>egl-23 (twk K⁺ channel)</td>
</tr>
<tr>
<td>Gene/Protein</td>
<td>Function/Comment</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td><strong>egl-36</strong> (shaw K⁺ channel)</td>
<td>Dominant mutation causes an egg laying defect</td>
<td></td>
</tr>
<tr>
<td><strong>unc-103</strong> (erg K⁺ channel)</td>
<td>Reduced function mutants are egg laying defective</td>
<td></td>
</tr>
<tr>
<td><strong>unc-93, sup-10</strong> (twk accessory proteins)</td>
<td>5-HT resistant. Double mutant ser-1; ser-7 is egg laying defective</td>
<td></td>
</tr>
<tr>
<td><strong>sup-9</strong> (twk K⁺ channel)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>egl-19</strong> (α1 subunit of L-type Ca²⁺ channel)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ser-1</strong> (serotonin receptor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ser-7</strong> (serotonin receptor)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:
- Elkes et al., 1997; Johnstone et al., 1997
- Garcia and Sternberg, 2003; Collins and Koelle, 2013
- Levin and Horvitz, 1992, 1993; de la Cruz et al., 2003
- Lee et al., 1997
- Carnell et al., 2005; Hobson et al., 2006
- de la Cruz et al., 2003
<table>
<thead>
<tr>
<th>VC</th>
<th>Development</th>
<th>Birth</th>
<th><strong>egl-30</strong> (Gαq subunit)</th>
<th>Reduced function mutants are egg laying defective</th>
<th>(Tanis et al., 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-29</strong> (nAChR)</td>
<td>Levamisole resistant</td>
<td>(Fleming et al., 1997; Waggoner et al., 2000; Kim et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-28</strong> (nAChR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>lev-1</strong> (nAChR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>Development</td>
<td>Birth</td>
<td><strong>lin-39</strong> (Antp transcription factor)</td>
<td>Vulvaless. VCs undergo cell death.</td>
<td>(Clark et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>bam-2</strong> (Neurexin-related protein)</td>
<td>Null mutants show abnormal VC branches</td>
<td>(Colavita and Tessier-Lavigne, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-34</strong> (transcription factor)</td>
<td>Null mutants show mildly hyperactive egg laying behavior</td>
<td>(Bany et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-4</strong> (transcription factor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-42</strong> (transcription factor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-76</strong> (PKC binding protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>unc-75</strong> (RNA binding protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Function</td>
<td>Synaptic transmission</td>
<td>cha-1 (choline acetyltransferase)</td>
<td>Null mutants show hyperactive egg laying</td>
<td>(Bany et al., 2003; Zhang et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>-----------------------------------</td>
<td>------------------------------------------</td>
<td>----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>unc-5 (Netrin receptor)</td>
<td></td>
<td>unc-115 (actin binding protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unc-17 (synaptic vesicle acetylcholine transporter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nlp-7</td>
<td></td>
<td>nlp-7;flip-11 double mutants show increased egg laying rate</td>
<td>(Banerjee et al., 2017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flip-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uv1</td>
<td>Development</td>
<td>lin-3 (Epidermal growth factor)</td>
<td>Null mutations result in uv1 cell death</td>
<td>(Chang et al., 1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Birth</td>
<td>let-60 (RAS)</td>
<td></td>
<td>(Han and Sternberg, 1990)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Function</td>
<td>tdc-1 (Tyrosine decarboxylase)</td>
<td>Null mutants show increased egg laying rate</td>
<td>(Alkema et al., 2005; Collins et al., 2016)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neurotransmission</td>
<td></td>
<td></td>
<td>(Banerjee et al., 2017)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>nlp-7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>flip-11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mechanosensation</td>
<td>osm-9, ocr-1, ocr-2 &amp; ocr-4 (TRPV subunit)</td>
<td>Null mutations cause a mild increase in egg laying rate</td>
<td>(Jose et al., 2007)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

Materials and Methods

2.1. Nematode Culture and Developmental Staging

*Caenorhabditis elegans* hermaphrodites were maintained at 20°C on Nematode Growth Medium (NGM) agar plates with *E. coli* OP50 as a source of food as described (Brenner, 1974). Animals were staged and categorized based on the morphology of the vulva as described (Mok et al., 2015). For assays involving young adults, animals were age-matched based on the timing of completion of the L4 larval molt. All assays involving adult animals were performed using age-matched adult hermaphrodites 20-40 hours past the late L4 stage. Table 2 lists all strains used in this study and their genotypes.

**Table 2.** *Strain names and genotypes for all animals used in this study (behavior assays and calcium imaging)*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feature</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LX1832</td>
<td>Strain for transgene production, blue-light insensitive, multi-vulva at 20°C in the absence of <em>lin-15</em> (+) rescue transgene.</td>
<td><em>lite</em>-1*(ce314) lin-15(n765ts) X*</td>
<td>(Gurel et al., 2012)</td>
</tr>
<tr>
<td>N2</td>
<td>Bristol wild-type strain</td>
<td>wild type</td>
<td>(Brenner, 1974)</td>
</tr>
<tr>
<td>LX2004</td>
<td>HSN GCaMP5, mCherry</td>
<td><em>vsIs183 lite-1</em>(ce314) lin-15(n765ts) X</td>
<td>(Collins et al., 2016)</td>
</tr>
<tr>
<td>TV201</td>
<td>HSN presynaptic RAB-3-GFP</td>
<td><em>wyls22 IV</em></td>
<td>(Patel et al., 2006)</td>
</tr>
<tr>
<td>MIA189</td>
<td>mCherry expression under <em>ceh</em>-24 promoter</td>
<td><em>keyEx40; wyls22 IV</em></td>
<td>this study</td>
</tr>
<tr>
<td>Strain</td>
<td>Feature</td>
<td>Genotype</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>LX1918</td>
<td>vulval muscles GCaMP5, mCherry</td>
<td>vsIs164 lite-1(ce314) lin-15(n765ts) X</td>
<td>(Collins et al., 2016)</td>
</tr>
<tr>
<td>AQ570</td>
<td>GFP expression under the ser-4 promoter</td>
<td>jkl570</td>
<td>(Gurel et al., 2012)</td>
</tr>
<tr>
<td>MIA49</td>
<td>HSN, VC, and uv1 cells GCaMP5, mCherry</td>
<td>keyIs11; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA51</td>
<td>Vulval muscles GCaMP, mCherry (under ceh-24 promoter)</td>
<td>keyIs12; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MT1082</td>
<td>No HSNs</td>
<td>egl-1(n487) V</td>
<td>(Trent et al., 1983)</td>
</tr>
<tr>
<td>MT2059</td>
<td>No HSNs</td>
<td>egl-1(n986dm) V</td>
<td>(Conradt and Horvitz, 1998)</td>
</tr>
<tr>
<td>JT47</td>
<td>PLCβ null mutant, Egl, infrequent defecation</td>
<td>egl-8(sa47) V</td>
<td>(Bastiani et al., 2003)</td>
</tr>
<tr>
<td>LX1938</td>
<td>No HSNs, vulval muscles GCaMP5, mCherry</td>
<td>egl-1(n986dm) V; vsIs164 lite-1(ce314) lin-15(n765ts) X</td>
<td>(Collins et al., 2016)</td>
</tr>
<tr>
<td>MIA78</td>
<td>No HSNs, vulval muscles GCaMP5, mCherry</td>
<td>egl-1(n986dm) V; keyIs12; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MT1222</td>
<td>Increased EGL-6 receptor signaling in HSN</td>
<td>egl-6(n5920) X</td>
<td>(Ringstad and Horvitz, 2008)</td>
</tr>
<tr>
<td>MT2258</td>
<td>Increased EGL-47 receptor signaling in HSN</td>
<td>egl-47(n1081) V</td>
<td>(Moresco and Koelle, 2004)</td>
</tr>
<tr>
<td>MT15434</td>
<td>No serotonin</td>
<td>tph-1(mg280) II</td>
<td>(Sze et al., 2000)</td>
</tr>
<tr>
<td>MIA71</td>
<td>vulval muscles expressing Histamine-gated Cl⁻ channels (HisCl)</td>
<td>keyIs19; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA116</td>
<td>HSN expressing Histamine-gated Cl⁻ channels (HisCl)</td>
<td>keyIs21; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA60</td>
<td>All neurons expressing Histamine-gated Cl⁻ channels (HisCl)</td>
<td>keyEx16; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>Strain</td>
<td>Feature</td>
<td>Genotype</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>MIA161</td>
<td>HSN expressing Histamine-gated Cl⁻ channels (HisCl), GCaMP5, mCherry</td>
<td>keyIs21; vsIs186; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA209</td>
<td>HSN Channelrhodopsin-2, GCaMP5, mCherry</td>
<td>wzIs30 IV; vsIs183 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>LX1836</td>
<td>HSN expressing Channelrhodopsin-2</td>
<td>wzIs30 IV; lite-1(ce314) lin-15(n765ts) X</td>
<td>(Collins et al., 2016)</td>
</tr>
<tr>
<td>MIA88</td>
<td>HSN Channelrhodopsin-2, vulval muscle GCaMP5, mCherry (under ceh-24 promoter)</td>
<td>wzIs30 IV; keyIs13; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>LX1932</td>
<td>HSN Channelrhodopsin-2, vulval muscle GCaMP5, mCherry (under unc-103e promoter)</td>
<td>wzIs30 IV; vsIs164 lite-1(ce314) lin-15(n765ts) X</td>
<td>(Collins et al., 2016)</td>
</tr>
<tr>
<td>MIA191</td>
<td>tph-1 null mutant, HSN Channelrhodopsin-2, vulval muscle GCaMP5, mCherry (under unc-103e promoter)</td>
<td>tph-1(mg280) II; wzIs30 IV; vsIs164 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>EU552</td>
<td>Germline defect, sterile when grown at 20-25°C.</td>
<td>glp-1(or178ts) III</td>
<td>(Fujiwara et al., 2016)</td>
</tr>
<tr>
<td>MIA219</td>
<td>Germline defect, HSN GCaMP, mCherry</td>
<td>glp-1(or178ts) III; vsIs183 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA80</td>
<td>Vulval muscles HisCl, HSN GCaMP5, mCherry</td>
<td>keyIs19; vsIs183 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA194</td>
<td>No vm2 vulval muscle arms</td>
<td>lin-12(wy750) III</td>
<td>this study</td>
</tr>
<tr>
<td>MIA196</td>
<td>No vm2 vulval muscle arms, HSN GCaMP, mCherry</td>
<td>lin-12(wy750) III; vsIs183 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA215</td>
<td>Vulval muscles HisCl, VC GCaMP, mCherry</td>
<td>keyIs17; vsIs172; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA217</td>
<td>VC Tetanus Toxin, HSN GCaMP, mCherry</td>
<td>keyI33; vsIs183 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>Strain</td>
<td>Feature</td>
<td>Genotype</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MT2426</td>
<td><em>goa-1(Gα&lt;sub&gt;o&lt;/sub&gt;)</em> reduced-function mutant, hyperactive egg laying</td>
<td><em>goa-1(n1134) I</em></td>
<td>(Segalat et al., 1995)</td>
</tr>
<tr>
<td>DG1856</td>
<td><em>goa-1(Gα&lt;sub&gt;o&lt;/sub&gt;)</em> null mutant, hyperactive egg laying</td>
<td><em>goa-1(sa734) I</em></td>
<td>(Robatzek and Thomas, 2000)</td>
</tr>
<tr>
<td>LX850</td>
<td>HSN and NSM Pertussis Toxin, hyperactive egg laying</td>
<td>vsIs50; lin-15(n765ts)</td>
<td>(Tanis et al., 2008)</td>
</tr>
<tr>
<td>LX849</td>
<td>HSN and NSM activated <em>GOA-1</em>(Q205L), egg laying defective</td>
<td>vsIs49; lin-15(n765ts)</td>
<td>(Tanis et al., 2008)</td>
</tr>
<tr>
<td>MT8504</td>
<td>Increased Gα&lt;sub&gt;o&lt;/sub&gt; signaling due to mutation in RGS protein, EGL-10</td>
<td><em>egl-10(md176)</em></td>
<td>(Koelle and Horvitz, 1996)</td>
</tr>
<tr>
<td>MIA220</td>
<td><em>goa-1(Gα&lt;sub&gt;o&lt;/sub&gt;)</em> reduced-function mutant in lite-1(ce314), lin-15(n765ts) background to facilitate strain construction, hyperactive egg laying</td>
<td><em>goa-1(n1134) I; lite-1(ce314) lin-15(n765ts) X</em></td>
<td>this study</td>
</tr>
<tr>
<td>MIA210</td>
<td><em>goa-1(Gα&lt;sub&gt;o&lt;/sub&gt;)</em> reduced-function mutant, HSN GCaMP, mCherry</td>
<td><em>goa-1(n1134) I; vsIs183 lite-1(ce314) lin-15(n765ts) X</em></td>
<td>this study</td>
</tr>
<tr>
<td>MIA263</td>
<td><em>goa-1(Gα&lt;sub&gt;o&lt;/sub&gt;)</em> null mutant, HSN GCaMP, mCherry</td>
<td><em>goa-1(sa734) I; vsIs183 lite-1(ce314) lin-15(n765ts) X</em></td>
<td>this study</td>
</tr>
<tr>
<td>MIA216</td>
<td>Increased Gα&lt;sub&gt;o&lt;/sub&gt; signaling, HSN GCaMP, mCherry</td>
<td><em>egl-10(md176) V; vsIs183 lite-1(ce314) lin-15(n765ts) X</em></td>
<td>this study</td>
</tr>
<tr>
<td>MIA277</td>
<td>Increased Gα&lt;sub&gt;o&lt;/sub&gt; signaling in HSN, HSN GCaMP, mCherry</td>
<td>vsIs49/+, +/vsIs183 lite-1(ce314) lin-15(n765ts) X (transheterozygote)</td>
<td>this study</td>
</tr>
<tr>
<td>LX2007</td>
<td>HSN GCaMP, mCherry</td>
<td>vsIs186 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA218</td>
<td>HSN and NSM Pertussis Toxin in blue-light insensitive, <em>lin-15</em> multivulva background</td>
<td>vsIs50 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>Strain</td>
<td>Feature</td>
<td>Genotype</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MIA227</td>
<td>HSN and NSM Pertussis Toxin, HSN GCaMP, mCherry</td>
<td>vsIs186; vsIs50 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA214</td>
<td><em>goa-1(Gα) reduced-function mutant</em>, vulval muscles GCaMP, mCherry</td>
<td><em>goa-1(n1134) I</em>; vsIs164 lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>LX1919</td>
<td>Vulval muscles GCaMP, mCherry</td>
<td>vsIs165; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA245</td>
<td><em>goa-1(Gα) null-function mutant</em>, Vulval muscles GCaMP, mCherry</td>
<td>vsIs50 X vsIs165; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA256</td>
<td>Vulval muscles mCherry (<em>ceh-24 promoter</em>)</td>
<td>keyEx45; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA257</td>
<td>Vulval muscles mCherry + Pertussis Toxin (<em>ceh-24 promoter</em>)</td>
<td>keyEx46; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA258</td>
<td>Vulval muscles mCherry + Activated GOA-1(Q205L) (<em>ceh-24 promoter</em>)</td>
<td>keyEx47; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA259</td>
<td>uv1 cells mCherry (<em>tdc-1 promoter</em>)</td>
<td>keyEx48; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA260</td>
<td>uv1 cells mCherry + Pertussis Toxin (<em>tdc-1 promoter</em>)</td>
<td>keyEx49; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA261</td>
<td>uv1 cells mCherry + Activated GOA-1(Q205L) (<em>tdc-1 promoter</em>)</td>
<td>keyEx50; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA262</td>
<td>Vulval muscles mCherry + Tetanus Toxin (<em>ceh-24 promoter</em>)</td>
<td>keyEx51; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA278</td>
<td>HSN/NSM <em>gpb-1</em> and <em>gpc-2</em> overexpression + GFP (<em>tph-1 promoter</em>)</td>
<td>keyEx52; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA279</td>
<td>HSN/NSM GFP (<em>tph-1 promoter</em>)</td>
<td>keyEx53; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
</tbody>
</table>
### Strain and Genotype Details

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feature</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIA280</td>
<td>HSN/NSM photoactivable cAMP protein bPAC (tph-1 promoter)</td>
<td>keyEx54; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>MIA281</td>
<td>Cholinergic neurons photoactivable cAMP protein bPAC (unc-17 promoter)</td>
<td>keyEx55; lite-1(ce314) lin-15(n765ts) X</td>
<td>this study</td>
</tr>
<tr>
<td>KG421</td>
<td>GSA-1(Gαs) gain-of-function mutant. Hyperactive locomotion</td>
<td>gsa-1(ce81) I</td>
<td>(Schade et al., 2005)</td>
</tr>
<tr>
<td>KG518</td>
<td>ACY-1 (Adenylate Cyclase) gain-of-function mutant. Hyperactive locomotion</td>
<td>acy-1(ce2) III</td>
<td>(Schade et al., 2005)</td>
</tr>
<tr>
<td>KG532</td>
<td>KIN-2 (Protein Kinase A inhibitory regulatory subunit) loss-of-function mutant. Hypersensitive to stimuli</td>
<td>kin-2(ce179) X</td>
<td>(Schade et al., 2005)</td>
</tr>
<tr>
<td>KG744</td>
<td>PDE-4 (cAMP phosphodiesterase) loss-of-function mutant, Hyperactive locomotion</td>
<td>pde-4(ce268) II</td>
<td>(Charlie et al., 2006a)</td>
</tr>
<tr>
<td>MIA282</td>
<td>PDE-2 (cGMP phosphodiesterase) loss-of-function mutant, Increased cGMP signaling</td>
<td>pde-2(qj6)</td>
<td>(Fujiwara et al., 2015)</td>
</tr>
<tr>
<td>MIA283</td>
<td>cGMP-dependent Protein Kinase and cGMP phosphodiesterase loss-of-function double mutant. Defective egg laying</td>
<td>pde-2(qj6), egl-4(ky185)</td>
<td>(Fujiwara et al., 2015)</td>
</tr>
</tbody>
</table>

---

### 2.2. Plasmid and Strain Construction

Calcium reporter transgenes

**Vulval Muscle Ca²⁺**: To visualize vulval muscle Ca²⁺ activity in adult animals, I used LX1918 vsIs164 [unc-103e::GCaMP5::unc-54 3'UTR + unc-103e::mCherry::unc-54 3'UTR + lin-15(+)] lite-1(ce314) lin-15(n765ts) X strain as
described (Collins et al., 2016). In this strain, GCaMP5G (Akerboom et al., 2013) and mCherry are expressed from the unc-103e promoter (Collins and Koelle, 2013). The unc-103e promoter is only weakly expressed in vulval muscles during the L4 stages. To visualize vulval muscle activity during development, I generated new transgenes that express GCaMP5G and mCherry from the ceh-24 promoter (Harfe and Fire, 1998). Briefly, a ~2.8 kb DNA fragment upstream of the ceh-24 start site was amplified from genomic DNA by PCR using the following oligonucleotides: 5'-GCG GCA TGC AAC GAG CCA TCC TAT ATC GGT GGT CCT CCG-3' and 5'-CAT CCC GGG TTC CAA GGC AGA GAG CTG CTG-3'. This DNA fragment was ligated into pKMC257 (mCherry) and pKMC274 (GCaMP5G) from which the unc-103e promoter sequences were excised to generate pBR3 and pBR4, respectively. pBR3 (20 ng/µl) and pBR4 (80 ng/µl) were injected into LX1832 lite-1(ce314) lin-15(n765ts) X along with the pLl5EK rescue plasmid (50 ng/µl) (Clark et al., 1994). The extrachromosomal transgene produced was integrated by irradiation with UV light after treatment with trimethylpsoralen (UV/TMP) creating two independent transgenes keyIs12 and keyIs13, which were then backcrossed to LX1832 parental line six times to generate the strains MIA51 and MIA53. Strain MIA51 keyls12 [ceh-24::GCaMP5::unc-54 3'UTR + ceh-24::mCherry::unc-54 3'UTR + lin-15(+)] IV; lite-1(ce314) lin-15 (n765ts) X was subsequently used for Ca²⁺ imaging. I noted repulsion between keyls12 and wzIs30 IV, a transgene that expresses Channelrhodopsin-2::YFP in HSN from the egl-6 promoter (Emtage et al., 2012), suggesting both were linked to chromosome IV. As a result, I crossed MIA53 keyIs13 [ceh-24::GCaMP5::unc-54 3'UTR + ceh-
24::mCherry::unc-54 3'UTR + lin-15(+)]; lite-1(ce314) lin-15(n765ts) X with LX1836 wzls30 IV; lite-1(ce314) lin-15(n765ts) X, generating MIA88 which was used to activate HSN neurons and record vulval muscle Ca$^{2+}$ in L4 animals. In the case of young adults (3.5 & 6.5h post molt) and 24h old adults, strain LX1932 wzls30 IV; vsls164 lite-1(ce314) lin-15(n765ts) X was used as described (Collins et al., 2016).

To visualize vulval muscle activity in goa-1(n1134) animals, I crossed LX1918 vsls164 [unc-103e::GCaMP5::unc-54 3'UTR + unc-103e::mCherry::unc-54 3'UTR + lin-15(+)] lite-1(ce314) lin-15(n765ts) X strain with the MT2426 goa-1(n1134) I strain to generate MIA214 goa-1(n1134) I; vsls164 lite-1(ce314) lin-15(n765ts) X strain. To visualize vulval muscle activity in transgenic animals expressing the catalytic subunit of Pertussis Toxin in the HSN neurons, strain MIA218 vsls50 lite-1(ce314) lin-15(n765ts) X was crossed with LX1919 vsls165; lite-1(ce314) lin-15(n765ts) X strain to generate MIA245 vsls165; vsls50 lite-1(ce314) lin-15(n765ts) X.

**HSN Ca$^{2+}$:** To visualize HSN Ca$^{2+}$ activity in both L4 and adult animals, I used the LX2004 vsls183 [nlp-3::GCaMP5::nlp-3 3'UTR + nlp-3::mCherry::nlp-3 3'UTR + lin-15(+)] lite-1(ce314) lin-15(n765ts) X strain expressing GCaMP5G and mCherry from the nlp-3 promoter as previously described (Collins et al., 2016). In order to visualize HSN Ca$^{2+}$ activity in lin-12(wy750) mutant animals lacking post-synaptic vm2 vulval muscle arms, I crossed MIA194 lin-12(wy750) III with LX2004 vsls183 lite-1(ce314) lin-15(n765ts) X to generate MIA196 lin-12(wy750) III; vsls183 X lite-1(ce314) lin-15 (n765ts) X. In order to visualize HSN Ca$^{2+}$ activity in glp-1(or178ts) mutant animals, we crossed EU552 glp-1(or178ts) III with LX2004 vsls183 lite-
(ce314) lin-15(n765ts) X to generate MIA219 glp-1(or178ts) III; vsIs183 lite-1(ce314) lin-15(n765ts) X. In order to visualize HSN Ca\(^{2+}\) activity in \textit{goa-1(n1134)} mutant animals, I crossed MT2426 \textit{goa-1(n1134)} I with LX2004 vsIs183 lite-1(ce314) lin-15(n765ts) X to generate MIA210 \textit{goa-1(n1143)} I; vsIs183 X lite-1(ce314) lin-15 (n765ts) X. I noticed a repulsion between alleles vsIs50, the transgene that expresses the catalytic subunit of Pertussis Toxin from the \textit{tph-1} promoter, and vsIs183 suggesting both were linked to the X chromosome. I crossed LX850 vsIs50 \textit{lin-15(n765ts)} X strain with LX1832 lite-1(ce314) \textit{lin-15(n765ts)} X to generate the strain MIA218 vsIs50 \textit{lite-1(ce314) lin-15(n765ts)} X.

In order to visualize HSN Ca\(^{2+}\) activity in transgenic animals expressing Pertussis Toxin, I crossed MIA218 with LX2007 vsIs186; lite-1(ce314) lin-15(n765ts) X to generate MIA227 vsIs186; vsIs50 lite-1(ce314) \textit{lin-15(n765ts)} X. To visualize HSN Ca\(^{2+}\) activity in \textit{egl-10(md176)} mutant animals, I crossed MT8504 \textit{egl-10(md176)} V with LX2004 vsIs183 lite-1(ce314) \textit{lin-15(n765ts)} X to generate MIA216 \textit{egl-10(md176)} V; vsIs183 lite-1(ce314) \textit{lin-15(n765ts)} X. In order to visualize HSN Ca\(^{2+}\) activity in transgenic animals expressing a constitutively active mutant \textit{GOA-1^{Q205L}} protein which increases G\(\alpha_c\) signaling in the HSN neurons, I crossed the LX849 vsIs49; \textit{lin-15(n765ts)} X strain with LX2004 vsIs183 lite-1(ce314) \textit{lin-15(n765ts)} X. I noted a repulsion between the vsIs183 and vsIs49 transgenes, thus not allowing for two copies of both transgenes to co-exist. One clone in which all adult animals showed the egg laying defective phenotype of the LX849 parent strain but in which the \textit{vsIs183} allele showed heterozygous pattern of expression was selected for Ca\(^{2+}\) imaging and was designated the strain name MIA277.
visualize HSN Ca\(^{2+}\) activity in transgenic animals expressing Tetanus Toxin in the VC neurons, I crossed the MIA144 \textit{keyls33; lite-1(ce314) lin-15(n765ts)} X strain with LX2004 \textit{vsls183 lite-1(ce314) lin-15(n765ts)} X to generate MIA217 \textit{keyls33; lite-1(ce314) lin-15(n765ts)} X.

**VC Ca\(^{2+}\):** To visualize VC Ca\(^{2+}\) activity in adult animals, I used the LX1960 \textit{vsls172 [VC::GCaMP5 + VC::mCherry + lin-15(+)]} \textit{lite-1(ce314) lin-15(n765ts)} X strain expressing GCaMP5G and mCherry in the VC neurons as previously described (Collins et al., 2016). To test the effect of silencing vulval muscles on VC Ca\(^{2+}\) activity, I crossed MIA70 \textit{keyls17; lite-1(ce314) lin-15(n765ts)} X strain with LX1960 \textit{vsls172; lite-1(ce314) lin-15(n765ts)} X to generate MIA215 \textit{keyls17; vsls172; lite-1(ce314) lin-15(n765ts)} X.

Histamine gated chloride channel (HisCl) expressing transgenes

**Vulval muscle HisCl:** To produce a vulval muscle-specific HisCl transgene, coding sequences for mCherry in pBR3 were replaced with that for HisCl. First, an Eagl restriction site (3’ of the mCherry encoding sequence) was changed to a Notl site using Quickchange site-directed mutagenesis to generate pBR5. The \(~1.2\) kB DNA fragment encoding the HisCl channel was amplified from pNP403 (Pokala et al., 2014) using the following oligonucleotides: 5’- GCG GCT AGC GTA GAA AAA ATG CAA AGC CCA ACT AGC AAA TTG G-3’ and 5’-GTG GCG GCC GCT TAT CAT AGG AAC GTT GTC-3’, cut with Nhcl/Notl, and ligated into pBR5 to generate pBR7. pBR7 \((80\) ng/\(\mu l\)) was injected into LX1832 along with pLI5EK \((50\) ng/\(\mu l\)). One line bearing an extrachromosomal transgene was integrated with UV/TMP,
and six independent integrants (keyls14 to keyls19) were recovered. Four of these were then backcrossed to the LX1832 parental line six times to generate strains MIA68, MIA69, MIA70, and MIA71. All four strains were used for behavioral assays in adult animals to test the effect of vulval muscle silencing on egg laying (Fig. 3.6B). MIA71 keyls19 [ceh-24::HisCl::unc-54 3'UTR + lin-15(+); lite-1(ce314) lin-15(n765ts) X strain was used to study the effect of acute silencing of early activity on egg-laying behavior (Fig. 3.6C). To visualize HSN Ca²⁺ activity after vulval muscle silencing, I crossed MIA71 with LX2004 to generate strain MIA80 keyls19; vsls183 lite-1(ce314) lin-15(n765ts) X. To visualize VC Ca²⁺ activity after vulval muscle silencing, I crossed MIA70 keyls17 [ceh-24::HisCl::unc-54 3'UTR + lin-15(+); lite-1(ce314) lin-15(n765ts) X; lite-1(ce314) lin-15(n765ts) X with the LX1960 vsls172; lite-1(ce314) lin-15(n765ts) X strain to generate MIA215 keyls17; vsls172; lite-1(ce314) lin-15(n765ts) X animals.

HSN HisCl: The ~1.2 kB DNA fragment encoding the HisCl channel was amplified from pNP403 using the following oligonucleotides: 5'- GCG GCT AGC GTA GAA AAA ATG CAA AGC CCA ACT AGC AAA TTG G -3' and 5'- GCG GAG CTC TTA TCA TAG GAA CGT TGT CCA ATA GAC AAT A- 3'. The amplicon was digested with Nhel/Sacl and ligated into similarly cut pSF169 (pegl-6::mCre (Flavell et al., 2013)) to generate pBR10. To follow expression in HSN, mCherry was amplified using the following oligonucleotides: 5'- GCG GCT AGC GTA GAA AAA ATG GTC TCA TAG GAA CGT TGT CCA ATA GAC AAT A- 3'. The amplicon was digested with Nhel/Sacl and ligated into pSF169 to generate pBR12. pBR10 (HisCl; 5 ng/µl) and pBR12 (mCherry; 10 ng/µl) were
injected into LX1832 \textit{lite-1}(ce314) \textit{lin-15}(n765ts) along with pLI5EK (50 ng/µl). The extrachromosomal transgene produced was integrated with UV/TMP, creating three independent integrants (\textit{keyIs20}, \textit{keyIs21}, and \textit{keyIs22}). The resulting animals were backcrossed to the LX1832 parental line six times to generate strains MIA115, MIA116, and MIA117. The MIA116 strain had a low incidence of HSN developmental defects and was used subsequently for behavioral assays.

\textbf{All neuron HisCl:} pNP403 was injected into LX1832 \textit{lite-1}(ce314) \textit{lin-15}(n765ts) animals at 50 ng/µl along with pLI5EK (50 ng/µl) to produce strain MIA60 carrying extrachromosomal transgene \textit{keyEx16}[\textit{tag-168::HisCl::SL2::GFP + lin15(+)}]. Non-Muv, \textit{lin-15(+)} animals with strong GFP expression in the HSNs and other neurons were selected prior to behavioral silencing assays. All animals showed histamine-dependent paralysis that recovered after washout.

Transgenic reporters of circuit development and morphology

\textbf{Vulval muscle morphology:} To visualize vulval muscle development at the L4 stages, I injected pBR3 [\textit{pceh-24::mCherry}] (80 ng/µl) along with a co-injection marker pCFJ90 (10 ng/µl) (Collins and Koelle, 2013) into TV201 \textit{wyls22}[\textit{punc-86::GFP::RAB-3 + podr-2::dsRed}] (Patel et al., 2006) to generate an extrachromosomal transgene, \textit{keyEx42}. To visualize adult vulval muscle morphology, I used the LX1918 \textit{vsls164}[\textit{unc-103e::GCaMP5::unc-54 3'UTR + unc-103e::mCherry::unc-54 3'UTR + lin-15(+)} \textit{lite-1}(ce314) \textit{lin-15}(n765ts) X strain (Collins et al., 2016). To visualize the expression of the \textit{ser-4} gene, I used the strain AQ570 [\textit{ijls570}] (Tsalik and Hobert, 2003; Gurel et al., 2012).
**HSN morphology:** I used the LX2004 strain expressing mCherry from the *nlp-3* promoter to visualize HSN morphology at L4 stages as well as in adults. To visualize GFP::RAB-3 synaptic localization in HSNs during development, the *wyls22* transgene was used (Patel et al., 2006).

**Whole circuit morphology (HSN, VC and uv1 cells):** A ~3.2 kB DNA fragment upstream of the *ida-1* start site (Cai et al., 2004) was cloned using the following oligonucleotides: 5’-GCGGCATGCCCTGCCTGTGCCAACTTACCT-3’ and 5’-CATCCC GGGCGGGATGACACAGAGATGCGG-3’. The DNA fragment was digested with Sphl/Xmal and ligated into pKMC257 and pKMC274 to generate plasmids pBR1 and pBR2. pBR1 (20 ng/µl) and pBR2 (80 ng/µl) were co-injected into LX1832 along with pLI5EK (50 ng/µl). The extrachromosomal transgene produced was integrated with UV/TMP creating four independent integrants *keyls8* to *keyls11*, which were then backcrossed to LX1832 parental line six times. MIA49 *keyls11* [ida-1::GCaMP5::unc-54 3’UTR + ida-1::mCherry::unc-54 3’UTR + lin-15(+); lite-1(ce314) lin-15 (n765ts) X] was used subsequently to visualize whole-circuit morphology.

Transgenic strains to manipulate Gαo signaling in the HSN neurons, vulval muscles, and uv1 neuroendocrine cells

**HSN neurons:** To produce a HSN (and NSM) specific GPB-1 expressing construct, the *gpb-1* cDNA fragment was amplified from pDEST-*gpb-1* (Yamada et al., 2009) using the following forward and reverse oligonucleotides: 5’-GAGGCTAGCGTAGAAAAATGAGCGAACTTGACCAACTTCGA-3’ and 5’-
GCGGGTACCTCATTAATTCCAGATCTTGAGGAACGAG-3’. The ~1 kb DNA fragment was digested with NheI/KpnI and ligated into pJT40A (Tanis et al., 2008) to generate pBR30. To produce a HSN (and NSM) specific GPC-2 expressing construct, the gpc-2 cDNA fragment was amplified from worm genomic DNA using the following forward and reverse oligonucleotides: 5’-GAGGCTAGCGTAGAAAAAATGGATAAATCTGACATGCAACGA-3’ and 5’-GCGGGTACCTTAGAGCATGCTGCACTTGCT-3’. The DNA ~250 bp DNA fragment was digested with NheI/KpnI and ligated into pJT40A to generate pBR31.

To overexpress the βγ G protein subunits in the HSN neurons, I injected pBR30 (50ng/µl), pBR31 (50ng/µl), and pJM60 [ptph-1::GFP] (80 ng/µl) (Moresco and Koelle, 2004) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA278 [keyEx52; lite-1(ce314) lin-15(n765ts)], was kept. To generate a control strain for comparison in the egg-laying assays, I injected pJM66 [ptph-1::empty] (100 ng/µl) (Tanis et al., 2008) and pJM60 (80 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA279 [keyEx53; lite-1(ce314) lin-15(n765ts)], was kept. To activate cAMP synthesis in HSNs, the optogenetically activated Beggiatoa-photoactivated adenylyl cyclase (bPAC) (Steuer Costa et al., 2017) was used. To generate a HSN-specific bPAC transgene, the coding sequence for bPAC-YFP was amplified from pWSC22 plasmid (Steuer Costa et al., 2017) using the oligonucleotides: 5’-
GAGGCTAGCGTAGAAAAATGAAGCGGCTGGTGTACATCAG-3’ and 5’-GCGGGTACCTCATTACTTGACTAGCTCGTCCATGCC-3’. The ~1.8 kb DNA fragment was digested with NheI/KpnI and ligated into pJT40A to generate pBR28.

I then injected pBR28 (30 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA280 [keyEx54; lite-1(ce314) lin-15(n765ts)], was kept. In order to generate the control transgenic strain for cAMP activation using bPAC, I injected pWSC22 (5 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA281 [keyEx55; lite-1(ce314) lin-15(n765ts)], was kept.

**Vulval muscles:** To produce vulval muscle-specific Pertussis Toxin transgene, the coding sequences for mCherry in pBR3 were replaced with Pertussis Toxin. The Pertussis toxin coding sequence was obtained from pJT40A (ptph-1::Pertussis Toxin (Tanis et al., 2008)) by digesting with Nhel/KpnI and ligating into pBR3 to generate pB20. To abolish Gαo signaling in vulval muscles, I injected pBR20 [pceh-24::Pertussis Toxin] (10 ng/µl) and pBR3 [pceh-24::mCherry] (10 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA257 [keyEx46; lite-1(ce314) lin-15(n765ts)], was kept. To produce vulval muscle-specific GOA-1(Q205L), the coding sequence of GOA-1(Q205L) was obtained from pKMC268 by digesting with
NheI/NcoI and ligated into pBR3 to generate pBR21. To increase Gαo signaling in vulval muscles, I injected pBR21 [pceh-24::GOA-1Q205L] (10 ng/µl) and pBR3 [pceh-24::mCherry] (10 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pL15EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavior assays. One representative transgenic strain, MIA258 [keyEx47; lite-1(ce314) lin-15(n765ts)], was kept. To generate a control strain for comparison in the egg-laying assays, I injected pBR3 [pceh-24::mCherry] (20 ng/µl) alone into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pL15EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA256 [keyEx45; lite-1(ce314) lin-15(n765ts)], was kept. To produce a vulval muscle-specific Tetanus toxin transgene, the Tetanus toxin coding sequence was amplified from pAJ49 (pocr-2::Tetanus toxin) using the following oligonucleotides: 5’-GAGGCTAGCTAGAAAAATGCCGATCACCATCAACAACTTC-3’ and 5’-GCGCAGGCGGCCGCTCAAGCGGTACGGTTGTACAGGTT-3’. The DNA fragment was digested with NheI/NotI and ligated into pBR6 to generate pBR27. To block any possible neurotransmitter release from the vulval muscles, I injected pBR27 (10 ng/µl) and pBR3 (10 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pL15EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavior assays. One representative transgenic strain, MIA262 [keyEx51; lite-1(ce314) lin-15(n765ts)], was kept.

**uv1 neuroendocrine cells:** To generate a uv1 cell-specific Pertussis toxin transgene, pBR20 (pceh-24::Pertussis toxin) was digested with NheI/NcoI and the
coding sequence of Pertussis toxin was then ligated into pAB5 (ptdc-1::mCherry) to generate pBR25. To abolish Gαo signaling in uv1 neuroendocrine cells, I injected pBR25 [ptdc-1::Pertussis Toxin] (10 ng/µl) and pAB5 [ptdc-1::mCherry] (5 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA260 [keyEx49; lite-1(ce314) lin-15(n765ts)], was kept. To generate a uv1 cell-specific GOA-1(Q205L) transgene, pKMC268 (punc-103e::GOA-1(Q205L)) was digested with NheI/Ncol and the coding sequence of GOA-1(Q205L) was then ligated into pBR25 to generate pBR26. To increase Gαo signaling in uv1 cells, I injected pBR26 [ptdc-1::GOA-1Q205L] (10 ng/µl) and pAB5 [ptdc-1::mCherry] (5 ng/µl) into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One transgenic strain MIA261 [keyEx50; lite-1(ce314) lin-15(n765ts)] was kept. To generate a control strain for comparison in our egg laying assays, I injected pAB5 [ptdc-1::mCherry] (15 ng/µl) alone into the LX1832 lite-1(ce314) lin-15(n765ts) animals along with pLI5EK (50 ng/µl) to generate five extrachromosomal transgenes which were used for behavioral assays. One representative transgenic strain, MIA259 [keyEx48; lite-1(ce314) lin-15(n765ts)], was kept.

2.3. Fluorescence Imaging

3D confocal microscopy: To visualize the morphological development of the egg-laying system, L4s and age-matched adults were immobilized using 10 mM
muscimol on 4% agarose pads and covered with #1 or #1.5 coverslips. Two-channel confocal Z-stacks (along with a bright-field channel) using a pinhole opening of 1 Airy Unit (0.921 μm thick optical sections, 16-bit images) were obtained with an inverted Leica TCS SP5 confocal microscope with a 63X Water Apochromat objective (1.2NA). GFP and mCherry fluorescence was excited using a 488 nm and 561 nm laser lines, respectively. Images were analyzed in Volocity 6.3.1 (Perkin Elmer) and FIJI (Schindelin et al., 2012).

**Ratiometric Ca\(^{2+}\) Imaging:** Ratiometric Ca\(^{2+}\) recordings were performed on freely behaving animals mounted between a glass coverslip and chunk of NGM agar as previously described (Collins and Koelle, 2013; Li et al., 2013; Collins et al., 2016; Ravi et al., 2018b). Recordings were collected on an inverted Leica TCS SP5 confocal microscope using the 8 kHz resonant scanner at ~20 fps at 256x256 pixel resolution, 12-bit depth and ≥2X digital zoom using a 20x Apochromat objective (0.7 NA) with the pinhole opened to ~20 μm. GCaMP5G and mCherry fluorescence was excited using a 488 nm and 561 nm laser lines, respectively. L4 animals at the relevant stages of vulval development were identified based on vulval morphology (Mok et al., 2015). Adult recordings were performed 24 hours after the late L4 stage. Young adults (3.5–6.5 h) were staged after cuticle shedding at the L4 to adult molt. After staging, animals were allowed to adapt for ~30 min before imaging. During imaging, the stage and focus were adjusted manually to keep the relevant cell/pre-synapse in view and in focus.

Ratiometric analysis (GCaMP5:mCherry) for all Ca\(^{2+}\) recordings was performed after background subtraction using Volocity 6.3.1 as described (Collins
et al., 2016; Ravi et al., 2018a). The egg-laying active state was operationally defined as the period one minute prior to the first egg-laying event and ending one minute after the last (in the case of a typical active phase where 3-4 eggs are laid in quick succession). However, in cases where two egg-laying events were apart by >60 s, peaks were considered to be in separate active phases and transients between these were considered to be from the inactive state.

**Ratiometric Ca\(^{2+}\) comparisons with different reporters and developmental stages:** To facilitate comparisons of ΔR/R between different reporters at different developmental stages, particularly during periods of elevated Ca\(^{2+}\) activity, HSN recordings in which baseline GCaMP5/mCherry fluorescence ratio values that were between 0.2-0.3 were selected for the analysis, while vulval muscle recordings with GCaMP5/mCherry ratio values between 0.1-0.2 were chosen (≥80% of recordings). Because HSN Ca\(^{2+}\) transient amplitude did not change significantly across developmental stages or in mutant or drug-treatment backgrounds, our analyses focused on HSN Ca\(^{2+}\) transient frequency. To test whether vulval muscle Ca\(^{2+}\) transient amplitudes recorded using different transgenes were suitable for quantitative comparisons, we measured the average GCaMP5:mCherry fluorescence ratio from two 15 by 15 µm regions of interest (ROI) from the anterior and posterior vulval muscles under identical imaging conditions (data not shown). The ROIs were positioned so as to ensure maximal coverage of the muscle cell area. We found that resting GCaMP5:mCherry ratios (±95% confidence intervals) bearing either the *ceh-24* (*keyIs12*) or *unc-103e* (*vsls164*) vulval muscle Ca\(^{2+}\) reporter transgenes were not statistically different at
the developmental stages under comparison in Fig. 3H (L4.7-8 (ceh-24): 1.055 ±0.027; L4.9 (ceh-24): 1.055 ±0.061; Adult (unc-103e): 1.15 ±0.064; n≥10 animals measured per developmental stage). The coordination of vulval muscle contraction was determined as described (Li et al., 2013).

**ERG expression analysis:** To measure ERG (unc-103e) expression in the vulval muscles during development in staged LX1918 L4.7-8 and L4.9 larvae and 24-hour adults, I used identical imaging conditions to measure mCherry fluorescence through a 20x Plan Apochromat objective (0.8NA) using a Zeiss Axio Observer microscope onto a Hamamatsu ORCA Flash 4.0 V2 sCMOS sensor after excitation with a 590 nm LED (Zeiss Colibri.2). After import into Volocity, two 15 x 15 µm ROIs were placed on the anterior and posterior vulval muscles, and the mCherry fluorescence of the two objects was averaged. A control ROI placed outside of the animal was used for background subtraction.

### 2.4. Behavior Assays and Microscopy

**Optogenetics and Defecation Behavior Assays:** ChR2 expressing strains were maintained on OP50 with or without all-trans retinal (ATR) (0.4 mM). ChR2 was activated during Ca²⁺ imaging experiments with the same, continuous laser light used to excite GCaMP5 fluorescence. Intervals between expulsion (Exp) steps of the defecation motor program were determined as described from brightfield and HSN Ca²⁺ recordings (Thomas, 1990). To test whether optogenetic activation of the HSNs affected defecation behavior on plates, a OTPG_4 TTL Pulse Generator (Doric Optics) was used to trigger image capture (Grasshopper 3, 4.1 Megapixel,
USB3 CMOS camera, Point Grey Research) and shutter opening on a EL6000 metal halide light source generating 8-16 mW/cm² of ~470±20nm blue light via a EGFP filter set mounted on a Leica M165FC stereomicroscope. Late L4 and adult LX1836 transgenic animals were maintained on OP50 seeded with or without all-trans retinal (ATR) (0.4 mM). Animals were illuminated with continuous blue light for a duration of 2 minutes, and video recordings were analyzed to identify the timing of defecation events within the duration of blue light activation. In the experiments looking at the correlation between HSN Ca²⁺ and defecation events, HSN Ca²⁺ ratiometric recordings were obtained for L4 animals using a Leica TCS SP5 confocal microscope as described in the previous section and in (Ravi et al., 2018a) and for adult animals, I performed Ca²⁺ imaging using a 20x Plan Apochromat objective (0.8NA) using a Zeiss Axio Observer microscope onto a Hamamatsu ORCA Flash 4.0 V2 sCMOS sensor and pulsed LED illumination. In both cases, a bright-field recording obtained simultaneously was used to identify the timing of defecation events and the Ca²⁺ recordings were analyzed to identify HSN Ca²⁺ peak timepoints.

**Acute silencing experiments using HisCl:** For acute silencing assays, NGM plates containing 10 mM histamine were prepared and used as described (Pokala et al., 2014). For adult behavioral assays, HisCl expressing strains were staged as late L4s with histamine treatment and behavior assays performed 24 hours later. For L4 activity silencing, L4.7 animals were placed on NGM plates with or without 10 mM histamine and were monitored to note when the animals complete the L4
molt. Each animal was then transferred to a new seeded plate (lacking histamine), and the time for each animal to lay its first egg was recorded.

**Animal sterilization:** Animals were sterilized using Floxuridine (FUDR) as follows. 100 µl of 10 mg/ml FUDR was applied to OP50 seeded NGM plates. Late L4 animals were then staged onto the FUDR plates and the treated adults were imaged 24 hours later. MIA219 *glp-1(or178ts) III; vsIs183 lite-1(ce314) lin-15(n765ts) X* animals were sterilized during embryogenesis as described (Fujiwara et al., 2016). L1-L2 animals were shifted to 25 °C and returned to 15 °C after 24 hours. Late L4 animals were then staged and grown at 15°C and imaged 24 hours later.

**Egg laying assays:** Unlaid eggs were quantitated as described (Chase et al., 2004). Staged adults were obtained by picking late L4 animals and culturing them for 40 hr at 20°.

**Long-term recording of egg-laying behavior:** To record long-term egg laying behavior on plates, a Leica M165FC stereomicroscope was used along with an image capture setup (Grasshopper 3, 4.1 Megapixel, USB3 CMOS camera, Point Grey Research). Late L4 were staged and assayed 24 hours later. NGM plates having a thin lawn of OP50 bacterial food was used to facilitate the visualization of egg-laying events. Recordings were obtained at 4 or 5 frames/second. Wildtype strain and the egg laying hyperactive strains (MT2426 and LX850) were recorded for 3 hours. The egg laying defective strains MT8504 and LX849 were recorded for 8-10 hours as the intervals between egg laying events are very long in these strains.
2.5. Experimental Design and Statistical Analysis

Sample sizes for behavioral assays followed previous studies (Chase et al., 2004; Collins and Koelle, 2013; Collins et al., 2016). No explicit power analysis was performed before the study. Statistical analysis was performed using Prism 6 (GraphPad). Ca$^{2+}$ transient peak amplitudes, widths, and inter-transient intervals were pooled from multiple animals (typically ~10 animals per genotype/condition per experiment). No animals or data were excluded except as indicated above to facilitate comparisons of Ca$^{2+}$ transient amplitudes between different development stages and reporters. Individual $p$ values are indicated in each Figure legend, and all tests were corrected for multiple comparisons (Bonferroni for ANOVA; Dunn for Kruskal-Wallis).
Chapter 3

Development of Mature Patterns of Activity in the C. elegans Egg-laying Behavior Circuit

3.1. Rationale and Introduction to Experiments

Developing neural circuits in the cortex, hippocampus, cerebellum, retina, and spinal cord show spontaneous neural activity (Wong et al., 1995; Garaschuk et al., 1998; Garaschuk et al., 2000; Watt et al., 2009; Warp et al., 2012). In contrast, mature neural circuits show coordinated patterns of activity required to drive efficient behaviors. Activity-dependent mechanisms have been shown to play key roles during development in vertebrate neural circuits (Gu et al., 1994; Gu and Spitzer, 1995; Jarecki and Keshishian, 1995; Borodinsky et al., 2004; Hanson et al., 2008), but the complexity of such circuits poses limitations in terms of understanding how developmental events, neurotransmitter specification, and sensory signals act together to promote the transition from immature to mature patterns of circuit activity. Genetically tractable invertebrate model organisms, such as the nematode Caenorhabditis elegans, have simple neural circuits and are amenable to powerful experimental approaches allowing us to investigate how activity in neural circuits is shaped during development.

1 Results in Chapter 3 were published as part of the following manuscript: Bhavya Ravi, Jessica Garcia, and Kevin M. Collins (2018) "Homeostatic feedback modulates the development of two-state patterned activity in a model serotonin motor circuit in Caenorhabditis elegans." Journal of Neuroscience; 38(28):6283-6298. PMID: 29891728.
The C. elegans egg-laying behavior circuit is a well-characterized neural circuit to understand the role of early circuit activity in synaptic development and also its relation to behavior development. The key questions that I address in Chapter 3 are:

1. How do mature activity patterns required to drive robust behaviors in the C. elegans egg-laying circuit develop?
2. What (if any) role does circuit activity during development play in the development of the cells of the egg-laying circuit and egg-laying behavior in adult animals?

3.2. Results

Asynchronous presynaptic and postsynaptic development in the C. elegans egg-laying behavior circuit

The function of cell activity in the adult egg-laying behavior circuit and how developmental mutations impact circuit activity and adult behavior have been previously described (Collins and Koelle, 2013; Li et al., 2013; Collins et al., 2016). Because development of the cells in the circuit is known to be complete by the end of the fourth larval (L4) stage (Li and Chalfie, 1990), I wanted to determine the relationship between circuit development and the emergence of cell activity as the animals mature from juveniles into egg-laying adults. I exploited the stereotyped morphology of the developing primary and secondary vulval epithelial cells in the fourth (final) larval stage to define discrete half-hour stages of development until
the L4-adult molt (Fig. 3.1A-F) as described (Mok et al., 2015). I observed NLP-3 neuropeptide promoter expression in HSNs of late L4 animals (Fig. 3.1G-I), showing that L4.7-8 HSNs have specified a transmitter phenotype. Consistent with L4.7-8 HSN being functional, the presynaptic marker GFP::RAB-3 expressed from the unc-86 promoter showed clear punctate localization in HSN at synaptic sites at these stages (Fig. 3.1J-L), confirming previous observations with light microscopy and serial electron microscopy reconstruction that HSN development is complete by L4.7-8 (Shen and Bargmann, 2003; Shen et al., 2004; Adler et al., 2006; Patel et al., 2006).

Unlike HSNs, I found the post-synaptic vulval muscles completed their morphological development during the L4.9 stage, just prior to the L4 molt. I expressed mCherry in the vulval muscles from the ceh-24 promoter (Harfe and Fire, 1998) and found that the vm1 and vm2 vulval muscles were still developing at the L4.7-8 stage (Fig. 3.1M). After lumen collapse at the L4.9 stage, the tips of the vm1 muscles extended ventrally to the lips of the vulva, and the anterior and posterior vm2 muscle arms extended laterally along the junction between the primary and secondary vulval epithelial cells (Fig. 3.1N), making contact with each other at the HSN (and VC) synaptic release sites that continues in adults (Fig. 3.1O). Previous work has shown that mutations that disrupt LIN-12/Notch signaling perturb development of the vm2 muscle arms in late L4 animals (Li et al., 2013), a time when vm2 muscle arm extension was observed.
**Fig. 3.1. Morphological development of the C. elegans egg-laying circuit.** (A-F) Representative images of vulval morphology at late L4 stages- (A) L4.7, (B) L4.7-8, (C) L4.8, (D) L4.9, (E) Molt and (F) Young adult. (G-I) Morphology of HSN labeled with mCherry (top) and the vulva (bottom) in L4.7-8 (G) and L4.9 (H) larval stages and in adults (I). (J-L) Morphology of HSN synapses labeled with GFP::RAB-3 (top) and the vulva (bottom) in L4.7-8 (J) and L4.9 (K) larval stages and in adults (L). (M-O) Morphology of vm1 and vm2 vulval muscles labeled with mCherry (top) and the vulva (bottom) in L4.7-8 (M) and L4.9 (N) larval stages and in adults (O). (P-R) Developmental expression of ser-4 from a GFP transcriptional reporter (top) at the L4.7-8 (P) and L4.9 (Q) larval stages and in adults (R) and the
vulva (bottom). (S-U) Morphology of HSN, VC4, VC5, and the uv1 neuroendocrine cells labeled with mCherry (top) and the vulva (bottom) in L4.7-8 (S) and L4.9 (T) larval stages and in adults (U) visualized using the ida-1 promoter. Arrowheads in all images indicate the location of presynaptic boutons or postsynaptic vm2 muscle arms. Scale bar is 10 µm; anterior is at left and ventral is at bottom unless indicated otherwise. Asterisks indicate the position of the developing or completed vulval opening. Vertical half-brackets indicate the approximate position of primary (1°) vulval epithelial cells, and horizontal bracket indicates progress of vulval lumen collapse at each larval stage.

Vulval muscles express multiple serotonin receptors that mediate their response to HSN input (Carnell et al., 2005; Dempsey et al., 2005; Hobson et al., 2006; Hapiak et al., 2009). In order to look at the developmental expression pattern of one such serotonin receptor, I examined a transgenic reporter line expressing GFP under the ser-4b gene promoter (Tsalik and Hobert, 2003; Gurel et al., 2012). As shown in Fig. 3.1P and 3.1Q, I observed strong GFP expression in VulF and VulE primary and VulD secondary epithelial cells. The ser-4b promoter also drove weak GFP expression in the vm2 muscles in L4.7-9, and this was elevated in adults (Fig. 3.1P-R). Serial EM reconstruction has shown that HSN makes transient synapses onto the vulval epithelial cells in developing L4 animals, and the expression of a serotonin receptor in these cells and the vm2 muscles during this period suggests they have specified a receptor phenotype (Shen et al., 2004). Lastly, I wanted to determine whether the VC motor neurons and uv1 neuroendocrine cells had completed their development in late L4 animals. To simultaneously visualize HSN, VC, and the uv1 neuroendocrine cells, I expressed
mCherry from the *ida-1* promoter, a gene expressed in a subset of peptidergic cells, including those in the egg-laying circuit (Cai et al., 2004). I observed mCherry expression in all three cell types in L4.7-8 animals, consistent with their development of a peptidergic phenotype in late L4 animals (Fig. 3.1S-U). As expected, HSN and VC presynaptic termini assembled at the junction between the primary and secondary vulval epithelial cells in L4.7-8. The uv1 cells were positioned laterally to the HSN/VC synaptic regions and extended dorsal processes around the primary vulval epithelial cells (Fig. 3.1S-U). These results indicate that the morphological development and peptidergic expression phenotype of the HSN, VC, and uv1 cells is largely complete by L4.7-8 stage. In contrast, vulval muscle morphological development is completed in the L4.9 stage when the vm2 muscle arms reach each other and the HSN and the VC presynaptic boutons and begin to express the serotonin receptor SER-4b.

**HSNs switch from tonic activity in juveniles to burst firing in egg-laying adults**

I next wanted to determine if the HSNs show activity as they develop and how that activity compares to that seen in egg-laying adults. To follow HSN activity, I expressed the Ca²⁺ reporter GCaMP5 along with mCherry in HSN using the *nlp-3* promoter and performed ratiometric Ca²⁺ imaging in freely behaving animals as previously described (Collins et al., 2016). Starting at the L4.7-8 larval stage, I observed rhythmic Ca²⁺ activity in both HSN presynaptic termini and in the soma.
During the L4.9 larval stage, when animals exhibited behavioral features of the developmentally timed L4 quiescence (Raizen et al., 2008), rhythmic Ca$^{2+}$ activity in the HSNs slowed (Fig. 3.2B; Movie 1). The tonic HSN activity I observed in juveniles (Fig. 3.2B; Movie 2) differed from the alternating, two-state pattern previously seen in adult animals where periods of infrequent activity are interrupted by bouts of HSN burst firing that drive the egg-laying active state (Collins et al., 2016).

I quantitated changes in HSN Ca$^{2+}$ transient peak amplitude and frequency during the different developmental stages and behavior states. I found no significant differences in HSN Ca$^{2+}$ transient amplitude (Fig. 3.2C), but I did observe significant changes in frequency. The median inter-transient interval in L4.7-8 animals was ~34 s, and this interval increased to ~60 s as animals reached the L4.9 stage (Fig. 3.2D). The reduction of HSN transient frequency seen in L4.9 animals resembled the egg-laying inactive state. However, none of the developmental stages recapitulated the ‘burst’ Ca$^{2+}$ activity with <20 s inter-transient intervals seen during the egg-laying active state (Fig. 3.2D). Together, these results indicate that the HSNs show tonic Ca$^{2+}$ activity after their morphological development is complete. HSN activity then switches into distinct inactive and active states as animals become egg-laying adults.
Fig. 3.2. HSN neurons show tonic Ca$^{2+}$ activity during the late L4 stage and burst firing during the egg-laying active state. (A) Representative images of the intensity-modulated GCaMP5:mCherry fluorescence ratio during HSN Ca$^{2+}$ transients in L4.7-8 and L4.9 larval stages, and in adults. White arrowheads show Ca$^{2+}$ activity localized to the anterior and posterior presynaptic boutons. Scale bar is 10 µm; anterior is at left, ventral is at bottom. See also Movies 1 and 2. (B) Representative GCaMP5:mCherry ratio traces ($\Delta R/R$) of HSN Ca$^{2+}$ activity in L4.7-8 (top), L4.9 (middle), and in adult animals (bottom). Adults show distinct active (yellow) and inactive (grey) egg-laying behavior states. Black arrowheads indicate egg-laying events. (C) Cumulative distributions of HSN Ca$^{2+}$ peak amplitudes in L4.7-8 (filled black circles), L4.9 (open black circles), and adults (filled green circles). n.s. indicates $p>0.0809$ (one-way ANOVA). (D) Cumulative distribution plots of instantaneous HSN Ca$^{2+}$ transient frequencies (and inter-transient intervals) from L4.7-8 (filled black circles) and L4.9 (open black circles) animals,
and from adult egg-laying inactive (filled green circles) and active (green open circles) states. Asterisks (*) indicate p<0.0001; pound sign (#) indicates p=0.0283; n.s. indicates p=0.1831 (Kruskal-Wallis test). N≥10 animals for each developmental stage. Number of HSN Ca^{2+} peak inter-transient intervals (peak frequencies) values used in the cumulative distribution plots for the developmental stages: L4.7-8 (n=159), L4.9 (n=162), Adult inactive (n=66), and Adult active (n=92). Number of HSN Ca^{2+} peak amplitudes used in cumulative distribution plots for the developmental stages: L4.7-8 (n=171), L4.9 (n=121), and Adult (n=148).

**Role of HSN L4 Ca^{2+} activity in locomotor activity and the Defecation Motor Program (DMP)**

The onset of Ca^{2+} activity in the HSN neurons during the late L4 stage coincided with changes in animal locomotion, pharyngeal pumping, and defecation behaviors that accompany the L4 lethargus (Raizen et al., 2008). Previous published work has shown that there is an increase in animal locomotion in adult animals around egg-laying active states driven by serotonin signaling from HSN onto AVF (Hardaker et al., 2001). Loss of HSN neurons or serotonin signaling from HSN reduces reversals and increases forward locomotion and exploratory behavior (Flavell et al., 2013). To understand if the tonic HSN activity seen in juveniles was associated with locomotor arousal, I analyzed movement in L4.9 animals ten seconds before and after each HSN Ca^{2+} transient. As shown in Figure 3.3 About one third of L4.9 HSN transients failed to show any movement before or after the transient (35±7%), and the remaining HSN transients were about evenly split between those which showed movements before (30±7%), after (15±7%), or
before and after (20±7%) the transient (n=156 transients). These results show that although HSN Ca\textsuperscript{2+} transients can occur around locomotion events, there does not appear to be a causal relationship between HSN activity and movement in juvenile animals. Analysis of vulval muscle Ca\textsuperscript{2+} transients in L4.9 animals showed a significantly different pattern of activity (Fig. 3.3). As with HSN, I observed vulval muscle Ca\textsuperscript{2+} transients in otherwise non-moving animals, but these comprised about 17±4% of L4.9 Ca\textsuperscript{2+} transients analyzed (e.g. half the level seen for HSN). The largest fraction of vulval muscle Ca\textsuperscript{2+} transients were accompanied by locomotion both before and after the Ca\textsuperscript{2+} transient, with smaller proportions occurring only before or after.

**Fig. 3.3. HSN Ca\textsuperscript{2+} activity during the late L4 stage is not correlated with animal locomotor state.** Bars indicate the percentage of HSN and vulval muscle Ca\textsuperscript{2+} transients at the L4.9 larval stage with no movement at all (either before or after a Ca\textsuperscript{2+} transient) (white); movement before the observed transient (light grey); movement after the transient (dark grey); and movement both before and after each transient (black). Total transients from N≥10 L4 animals were used for analysis: HSN (n=156) and vulval muscles (n=291) (indicated above the bars).
I anticipate these differences in HSN activity during locomotion are related to developmental changes in HSN serotonin levels. Adult HSNs show increased GFP expression from a \textit{tph-1} transcriptional reporter (data not shown) and have elevated serotonin levels measured by immunostaining (Desai et al., 1988).

The \~50 s rhythm of HSN activity in L4.9 animals resembles the defecation rhythm, prompting us to investigate whether there is a relationship between HSN activity and the defecation motor program (DMP). I found that defecation intervals in L4.7-8 and adult animals were significantly longer when they were accompanied by one or more HSN Ca$^{2+}$ transients (Fig. 3.4 A and B). HSNs make and receive synapses from the excitatory GABAergic AVL motoneuron that regulates defecation, and serotonin and G$_{\alpha_\text{lo}}$ signaling have previously been shown to inhibit defecation behavior (White et al., 1986; Segalat et al., 1995). However, I found that optogenetic activation of the HSN neurons did not affect the defecation rhythm in L4 or adult animals (Fig. 3.4 C). Two independent mutants lacking HSNs showed a significant decrease in DMP frequency (Fig. 3.4 D), although this defecation phenotype was not observed in \textit{egl-47(dm)} animals which also reduce HSN neurotransmitter release (Moresco and Koelle, 2004). The egg-laying and defecation circuits both drive expulsion behaviors and are regulated by a common set of signaling molecules (Reiner and Thomas, 1995), but a role for HSN in coordinating these behaviors will require further study.
Fig. 3.4: HSN regulates the defecation motor program. (A) Representative HSN Ca\(^{2+}\) traces at the L4.7-8 larval stage (top) and adults (bottom). Vertical lines indicate the expulsion step of the defecation motor program (DMP); arrowheads indicate adult egg-laying events. (B) Cumulative distribution plots showing DMP intervals with no HSN Ca\(^{2+}\) transient (black) versus those with one or more HSN Ca\(^{2+}\) transients (green) in L4.7-8 (closed circles) and adult (open circles). Pound indicates p=0.0058; asterisk indicates p<0.0001 (Kruskal-Wallis test with Dunn’s correction for multiple comparisons). (C) Scatter plots showing the consequences of HSN optogenetic activation on the DMP Expulsion step frequency. L4.7-8 and adult animals expressing Channelrhodopsin-2 in HSN neurons were grown in the absence (-, grey) or presence (+, blue) of all-trans retinal (ATR), illuminated with blue light for two minutes, and the timing of DMP events was used to calculate an instantaneous DMP frequency. Error bars show 95% confidence intervals for the mean; n.s. indicates p=0.0645 (L4.7-8) or p=0.1866, (adult) (Student’s t test). (D) Scatter plots showing DMP frequencies (min\(^{-1}\)) in wild-type (grey), egl-1(n487dm)
and egl-1(n986dm) (red), gain-of-function egl-47(n1082dm) (pink), and PLCβ null egl-8(sa47) (brown) mutant adults. Error bars indicate the 95% confidence interval for the mean. Asterisk indicates $p<0.0001$; n.s. indicates $p=0.5208$ (One-way ANOVA with Bonferroni’s correction for multiple comparisons). Data from $N\geq10$ animals were used for both developmental stages. Total transients used for analysis in (B): L4.7-8, no HSN Ca$^{2+}$ ($n=33$); L4.7-8, w/ HSN Ca$^{2+}$ ($n=62$); Adult, no HSN Ca$^{2+}$ ($n=39$); Adult, w/ HSN Ca$^{2+}$ ($n=72$). Total DMP interval (frequency) values used for analysis in (C): L4.7-8 no ATR ($n=43$); L4.7-8 plus ATR ($n=40$); Adult no ATR ($n=67$); Adult plus ATR ($n=55$). Total intervals used for analysis in (D): 10 per strain.

**Vulval muscle Ca$^{2+}$ transients increase in strength and frequency during development**

I next wanted to determine if the HSN activity I observe in late L4 animals drives early vulval muscle activity. I used the *ceh-24* promoter to drive expression of GCaMP5 and mCherry in the vulval muscles of L4 animals. I detected Ca$^{2+}$ transients at the L4.7-8 larval stage in the still-developing vulval muscles, and these transients continued and increased in frequency as the muscles completed their development at the L4.9 stage (Fig. 3.5 A-C, 3.5F and 3.5G; Movies 3-5). The median interval between vulval muscle Ca$^{2+}$ transients was $\sim32$ s in L4.7-8 animals which dropped to 18 s in L4.9 animals. L4 vulval muscle activity differs from that observed previously in egg-laying adults (Fig. 3.5D and 3.5E; Movie 6). The frequency of vulval muscle Ca$^{2+}$ transients increased significantly in animals during the egg-laying active state with median intervals dropping to $\sim7$ s phased with each body bend (Fig. 3.5G), as previously described (Collins and Koelle, 2013; Collins
et al., 2016). I found that vulval muscle Ca$^{2+}$ transients become stronger after development. While Ca$^{2+}$ transient amplitudes in the L4.7-8 and L4.9 stages were not significantly different, inactive phase Ca$^{2+}$ transients of adults were stronger than those observed in L4 animals (Fig. 3.5H). In adult animals, strong Ca$^{2+}$ transients were observed during the egg-laying active states, with the strongest Ca$^{2+}$ transients driving the complete and simultaneous contraction of anterior and posterior vulval muscles to allow egg release (Fig. 3.5E and 3.5H).

I was surprised that vulval muscle transient frequencies decreased in adults as circuit activity bifurcated into distinct inactive and active egg-laying behavior states. I quantified periods of increased activity by measuring time spent with vulval muscle Ca$^{2+}$ transient intervals less than one minute. I found that vulval muscle activity increased as L4.7-8 animals developed into L4.9 animals but then dropped significantly in egg-laying adults. L4.7-8 animals on average spent ~50% of their time in periods of increased vulval muscle activity, and this increased to 85% as animals entered the L4.9 stage (Fig. 3.5I). In contrast, adult animals spent only about ~33% of their time in periods with elevated vulval muscle activity (Fig. 3.5I) about half of which were coincident with the ~3 minute egg-laying active states that occur about every 20 minutes (Waggoner et al., 1998). What depresses vulval muscle activity in adult animals? Our group has previously shown that the loss of unc-103, which encodes Ether-a-Go-Go Related Gene (ERG) K$^+$ channel, results in increased vulval muscle excitability and egg-laying behavior (Collins and Koelle, 2013). Using an mCherry transcriptional reporter transgene, I found that unc-103e
F. 

L4.7-8 transition

G. Cumulative distribution of vulval muscle Ca\(^{2+}\) transient frequency (mHz)

H. Cumulative distribution of vulval muscle Ca\(^{2+}\) transient peak amplitude (\(\Delta R/R\))

I. Total time spent with VM intervals 500 s (%)

J. Coordinated VM transients (%)

K. P\(_{pc\rightarrow1054\rightarrowERG5\rightarrowmCherry}\) vulval muscle expression

L. P\(_{pc\rightarrow1054\rightarrowERG5\rightarrowmCherry}\) vulval muscle expression (AU)
**Fig. 3.5. Development of coordinated vulval muscle Ca\(^{2+}\) transients in the L4.9 stage does not require presynaptic HSN input.** (A-E) Representative images of the intensity-modulated GCaMP5:mCherry fluorescence ratio during vulval muscle Ca\(^{2+}\) transients at the L4.7-8 (A), L4.9 larval stages (B,C), and during the adult active state (D,E). White arrowheads show localization of Ca\(^{2+}\) transients. Scale bars are 10 µm; anterior at left, ventral at bottom. See also Movies 3-6. (F) Representative GCaMP5:mCherry (ΔR/R) ratio traces of vulval muscle Ca\(^{2+}\) activity at L4.7-8 (top), L4.9 (middle), and in adult animals (bottom) during an inactive (grey) and active (yellow) egg-laying state. Uncoordinated transients are indicated by blue circles (°), coordinated transients by orange carets (^), egg-laying events by black arrowheads. (G and H) Cumulative distribution plots of instantaneous vulval muscle Ca\(^{2+}\) transient peak frequencies (G) and amplitudes (H) at L4.7-8 (pink), L4.9 (blue), and in the egg-laying inactive (green) and active state (orange) of adults. Asterisks indicate p<0.0001; n.s. indicates p>0.9999 (Kruskal-Wallis test). (I) Scatterplots show time spent by 9-10 animals with frequent Ca\(^{2+}\) transients (inter-transient intervals ≤60 s) at L4.7-8 (pink), L4.9 (blue), and in adults (gray). Error bars show 95% confidence interval for the mean. Asterisks indicates p≤0.0002 (one-way ANOVA). (J) Scatterplots show percent synchronous anterior and posterior vulval muscle Ca\(^{2+}\) transients in each individual at L4.7-8 (pink), L4.9 (blue), and in adult egg-laying inactive (green) and active states (orange) in wildtype (top) and egl-1(n986dm) animals (red) lacking HSNs (bottom). Error bars show 95% confidence intervals for the mean from ≥5 animals. Asterisks indicate p≤0.0022; n.s. indicates p≥0.1653 (one-way ANOVA). (K) Representative images of mCherry fluorescence in the vulval muscles from a unc-103e (ERG) transcriptional reporter in an L4.7-8, L4.9, and adult animal. White arrowheads show anterior (left) and posterior (right) vulval muscle cells; scale bar is 10 µm. (L) Scatterplots show mCherry fluorescence from the unc-103e promoter in ten animals. Error bars show 95% confidence interval for the mean; ‘#’ indicates p=0.0288 and asterisk indicates p≤0.0001 (one-way ANOVA). Number of vulval muscle Ca\(^{2+}\) inter-transient intervals (peak frequency) values used in the cumulative distribution plots for the developmental stages: L4.7-8 (n=186), L4.9
(n=328), Adult inactive (n=132), and Adult active (n=169). Number of vulval muscle Ca^{2+} peak amplitude used in cumulative distribution plots for the developmental stages: L4.7-8 (n=186), L4.9 (n=324), Adult inactive (n=155), and Adult active (n=149). Total percentage values used for analysis in (I): 10 for each developmental stage. Percentage of coordinated transients from N≥5 animals for each developmental stage (both wildtype and animals lacking HSNs) were used for analysis in (J). Data from 10 animals for each developmental stage were used for analysis in (L).

expression in vulval muscles is low in L4 animals and increases >15-fold as animals mature into egg-laying adults (Fig. 3.5K and 3.5L). These results are consistent with our previous functional results that show that ERG depresses vulval muscle electrical excitability in adults to promote distinct inactive and active egg-laying behavior states (Collins and Koelle, 2013).

Development of coordinated vulval muscle activity for egg laying

Egg release through the vulva requires the synchronous contraction of the anterior (A) and posterior (P) vulval muscles (Fig. 3.5E). Previous work has shown that loss of Notch signaling blocks postsynaptic vm2 muscle arm development in L4 animals resulting in asynchronous vulval muscle contractility and defects in egg-release in adults (Li et al., 2013). Because of the vulval slit, the lateral vm2 muscle arms that develop between L4.7-8 and L4.9 form the only sites of potential contact between the anterior and posterior vulval muscles (Fig. 3.1M and 3.1N). To determine the relationship between vulval muscle morphology and activity, I
examined the spatial distribution of vulval muscle Ca\textsuperscript{2+} during identified transients. I found that only 5\% of vulval muscle Ca\textsuperscript{2+} transients were coordinated in the L4.7-8 stage (Fig. 3.5A; Movie 3), with nearly all transients occurring in either the anterior or posterior muscles (Fig. 3.5F and 3.5J). The degree of vulval muscle coordination increased significantly to ~28\% of transients during L4.9 (Fig. 3.5J; compare Movies 4 and 5) a time when vm1 and vm2 muscles, as well as vm2 muscle arms, complete their development (compare Fig. 3.1M and 3.1N). This level of coordinated muscle activity was not significantly different to that found in adult animals during the egg-laying inactive state (Fig. 3.5J; compare Fig. 3.5C and 3.5D). During the egg-laying active state ~60\% of vulval muscle transients were found to be coordinated, with Ca\textsuperscript{2+} transients occurring synchronously in the anterior and posterior muscles (Movie 6).

To test whether HSN activity was required for the development of coordinated muscle activity, I analyzed muscle activity in animals missing the HSNs. Surprisingly, I observed that vulval muscles develop wild-type levels of coordinated activity even without HSN input (Fig. 3.5J). I have previously shown that vulval muscle activity in adults is phased with locomotion (Collins et al., 2016), possibly via rhythmic acetylcholine release from the VA7 and VB6 motor neurons that synapse onto the vm1 muscles (White et al., 1986). Vulval muscle activity in L4.9 animals accompanied ongoing locomotion as well. I analyzed recordings from L4.9 animals for movement ten seconds before and after each vulval muscle Ca\textsuperscript{2+} transient. A clear majority of transients (62±5\%) were accompanied by movements occurring both before and after vulval muscle activity, with a smaller fraction of
transients occurring just before or just after movement (11±4% and 10±4%, respectively; n=291 transients). Movement was not strictly required for vulval muscle activity, as Ca$^{2+}$ transients were still observed in non-moving animals (17±4%) (Fig. 3.3). My results show that coordinated vulval muscle activity in L4.9 stage is independent of HSN input and may instead be driven by input from the locomotion motor neurons into vm1 and through the lateral vm2 muscle contact along the vulval slit.

**Early neuronal and vulval muscle activity is not required for the onset of adult egg-laying behavior**

Activity in developing circuits has previously been shown to contribute to mature patterns of activity that drive behavior. Is the early activity I observe in HSN and vulval muscles required for the proper onset of egg-laying behavior in adults? To test this, I first set out to determine when adults initiate egg laying. I found wild-type animals laid their first egg at about ~6-7 hours after the L4-adult molt (Fig. 3.6A) after accumulating ~8-10 eggs in the uterus, a time when VC and uv1 Ca$^{2+}$ activity is first observed (data not shown). Animals without HSNs laid their first egg much later, ~18 hours post molt (Fig. 3.6A). Gain-of-function receptor mutations in EGL-6, a neuropeptide receptor coupled to $G_{\alpha_o}$ (Ringstad and Horvitz, 2008), or EGL-47, a putative gustatory receptor block neurotransmitter release from HSN (Moresco and Koelle, 2004) and delay egg release until ~15-17 hours after the L4 molt, resembling animals without HSNs (Fig. 3.6A). Surprisingly, tryptophan
hydroxylase (*tph-1*) knockout animals that are unable to synthesize serotonin showed only a small albeit significant delay in egg release compared to wild type (~7-8 hours post L4 molt), suggesting that HSN promotes egg laying via release of neurotransmitters other than serotonin.

**Fig. 3.6. Early HSN and vulval muscle activity is not required for the onset of egg-laying behavior.** (A) Scatter plots of the first egg-laying event in wild-type (grey), HSN-deficient gain-of-function egl-1(n986dm) (red open circles), serotonin-deficient *tph-1*(mg280) null mutant (green triangles), gain-of-function egl-6(n592dm) mutant (purple squares), and gain-of-function egl-47(n1082dm) mutant (pink open squares) mutant animals. Error bars show 95% confidence intervals for the mean from ≥19 animals. Asterisks indicate *p*≤0.0016 (One-way ANOVA). (B) Scatter plots showing eggs laid by three 24-hour adult animals in two hours before (filled circles) and in two hours after incubation on plates with 10 mM histamine (open circles). Transgenic animals expressing HisCl in vulval muscles (orange), HSN neurons (green), all neurons (blue) were compared with the non-transgenic wild-type (grey). Error bars indicate 95% confidence intervals for the mean from ≥17 paired replicates. Asterisks indicate *p*<0.0001; n.s. indicate *p*=0.5224 (paired Student’s t test). (C) Top, transgenic L4.7 animals expressing HisCl channels were incubated on NGM plates with or without 10 mM histamine until the L4-Adult molt. Animals were then moved to plates lacking histamine and allowed to recover and lay eggs. Bottom, scatter plots show the timing of the first egg-laying event with (open circles) and without (filled circles) histamine. Error bars indicate 95%
To silence HSN and vulval muscle activity acutely and reversibly, I expressed *Drosophila* Histamine-gated chloride channels (HisCl) using cell-specific promoters and tested how histamine affected egg-laying behavior (Pokala et al., 2014). Egg laying was unaffected by exogenous histamine in non-transgenic animals but was potently inhibited when HisCl channels were transgenically expressed in the HSNs, the vulval muscles, or in the entire nervous system (Fig. 3.6B). Silencing these cells in late L4 animals for the entire period where I observe early activity caused no significant changes in the onset of adult egg laying after histamine washout in molted adults (Fig. 3.6C). I also observed no change in the steady-state number of unlaid eggs in the uterus after developmental silencing of L4 animals with histamine (data not shown). To ensure that the opening of HisCl channels at the juvenile stages does not produce a net depolarization resulting from Cl⁻ efflux, I constructed a strain that allows for monitoring HSN Ca²⁺ in transgenic animals expressing HisCl in the HSNs (see Table 2). My preliminary observations in a few animals (data not shown) indicated that HSN Ca²⁺ transients were reduced in L4 animals. Additionally, in strains expressing HisCl in all neurons, exposure to histamine rendered juvenile animals completely paralyzed for up to several hours (similar to that seen in adults) indicating a robust and strong hyperpolarizing effect of Cl⁻ in motor neurons. Overall, my results suggest that
presynaptic and postsynaptic activity in the developing circuit is not required for circuit development or behavior.

3.3. Major Findings and Discussion

I used a combination of molecular genetic, optogenetic, and ratiometric Ca\(^{2+}\) imaging approaches to determine how coordinated activity develops in the *C. elegans* egg-laying behavior circuit. Figure 3.7 summarizes the timeline of key developmental events in the egg-laying circuit (HSN, VC, vulval muscles, and uv1 cells) from birth of the individual cells/precursors to their final differentiated state in adults. I find the pre-synaptic HSNs, VCs, and uv1 neuroendocrine cells complete morphological development in the early-mid L4 stages, while the vulval muscles finish developing at the late L4 stages. Like HSNs, the vulval muscles show Ca\(^{2+}\) activity in the L4.7-8 stage. Coordinated vulval muscle Ca\(^{2+}\) transients are not observed until the L4.9 stage, a time when the anterior and posterior vm2 muscle arms complete a Notch signaling-dependent lateral extension around the primary vulval epithelial cells (Li et al., 2013). I did not observe Ca\(^{2+}\) transients in the VC neurons and uv1 cells except in egg-laying adults (data not shown) suggesting activity in these cells does not contribute to circuit development. Hence, similar to circuit morphology, the onset of activity in the cells of the circuit occurred at different developmental stages and continued to be shaped after morphological development. In adults, the juvenile HSN and vulval muscle activity disappears, leading to the establishment of characteristic ‘inactive’ states in which adult
animals spend ~85% of their time. Inactive state activity closely resembles that seen in sterilized animals that do not accumulate any eggs (Collins et al., 2016).

Changes in gene expression likely contribute to the changes in circuit activity patterns I observe between L4s and adults. Since mutants lacking serotonin have little effect on the timing of the first egg-laying event, I anticipate other neurotransmitters, like NLP-3 neuropeptides, are released from the HSNs promote egg laying in young adults (Brewer et al., 2019). Previous work has found that serotonin expression is low in L4 and increases as animals increase egg laying (Tanis et al., 2008). KCC-2 and ABTS-1, two Cl⁻ extruders required for inhibitory neurotransmission, also show a developmental increase in HSN expression from L4 to adult (Tanis et al., 2009; Bellemer et al., 2011) which may be associated with the disappearance of spontaneous rhythmic activity in the HSNs after the late L4.

**Fig. 3.7. Timeline of key developmental events and the onset of Ca²⁺ activity in the C. elegans egg-laying circuit.** Refer text for details.

<table>
<thead>
<tr>
<th>Time</th>
<th>HSN</th>
<th>Vulval muscles</th>
<th>VC</th>
<th>uv1</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1 hour</td>
<td>Embryo</td>
<td>Birth &amp; anterior migration</td>
<td>Birth of progenitor sex myoblasts</td>
<td>Birth</td>
</tr>
<tr>
<td>Hatching</td>
<td>L1</td>
<td>Neurite outgrowth or growth</td>
<td>Anterior migration of sex myoblasts</td>
<td>Axon outgrowth</td>
</tr>
<tr>
<td>Molt</td>
<td>L2</td>
<td>Ventral leading edge forms</td>
<td>Mitotic division of sex myoblasts</td>
<td>VC branching</td>
</tr>
<tr>
<td>Molt</td>
<td>L3</td>
<td>NLP-3 expression</td>
<td>Maturation of sex myoblasts into vulval muscles</td>
<td>KCC-2 &amp; ABTS-1</td>
</tr>
<tr>
<td>Molt</td>
<td>L4</td>
<td>Growth cone extension</td>
<td>Cell positioning &amp; attachment</td>
<td>Birth; uv1 cell fate specification</td>
</tr>
<tr>
<td>Molt</td>
<td>adult</td>
<td>Presynaptic development</td>
<td>serotonin in vm2 muscles (weak)</td>
<td>Ca²⁺ activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IDA-1 peptideergic marker expression</td>
<td>Expression of ERG (weak)</td>
<td>Methyltransferase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-HT expression (weak)</td>
<td>Coordination of Ca²⁺ activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca²⁺ activity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:
- Brewer et al., 2019
- Collins et al., 2016
- Tanis et al., 2008
- Tanis et al., 2009
- Bellemer et al., 2011
stages. At the same time, we find that inhibitory ERG K⁺ channel expression becomes upregulated in the vulval muscles of young adults. Studies in vertebrate models have shown that mechanical stretch can increase the transcription of receptors that enhance muscle contraction during parturition (Terzidou et al., 2005; Shynlova et al., 2007). I speculate that similar mechanisms may operate in the C. elegans reproductive system to drive expression of receptors and channels that modulate vulval muscle excitability to synaptic input during development.

The adult animals, HSNs show dramatic changes in Ca²⁺ transient frequency between the inactive and active states. The major G proteins, Gαq and Gαo, signal in HSN to increase and inhibit egg laying, respectively (Ringstad and Horvitz, 2008; Tanis et al., 2008). These effects are in part by their opposing effects on serotonin biosynthesis in the HSN neurons (Tanis et al., 2008). G protein signaling in HSN may also modulate an intrinsic pacemaker activity, similar to that seen in other central pattern generator circuits and in the cardiac pacemaker (Hille, 2001). Gαo signaling in HSN activates inhibitory IRK K⁺ channels (Emtage et al., 2012), and recent work has identified the T-type Ca²⁺ channel, CCA-1, and the Na⁺ leak channels, NCA-1 and NCA-2, as possible targets of excitatory Gαq signaling (Yeh et al., 2008; Topalidou et al., 2012; Zang et al., 2017). How the balance between signaling through the inhibitory Gαo and excitatory Gαq pathway is regulated during development and which receptors in the HSN signal through the two signaling pathways is not clear.
The early vulval muscle Ca\textsuperscript{2+} activity I observe could result from spontaneous activity or by neuronal input. Spontaneous Ca\textsuperscript{2+} transients promote the maturation of activity in many other cells (Moody and Bosma, 2005). I observed no change in behavioral onset or egg-laying rate in animals in which neuron or vulval muscle activity was silenced in the L4 stage. While this may result from incomplete silencing using the HisCl based approach, previous results in other circuits indicate synapse development does not require Ca\textsuperscript{2+}-dependent excitatory transmission (Verhage et al., 2000; Lu et al., 2013; Sando et al., 2017). In future experiments, alternative approaches to silence vulval muscles could be tested such as the light-activated chloride channel Halorhodopsin which would allow for a pulsed stimulation to prevent sensitization due to long-term acute silencing approaches like the HisCl method. Alternative approaches would include cell-specific transgenic approaches to express gain-of-function K\textsuperscript{+} channels that would hyperpolarize the cell and prevent electrical excitation. While neurotransmitter activation of G protein signaling could still drive early Ca\textsuperscript{2+} activity in the absence of electrical activity, this would still require Ca\textsuperscript{2+}-dependent vesicle fusion. The persistence of vulval muscle activity in animals that lack HSNs entirely and its recovery after acute neural silencing suggests the activity I observe arises from a shared mechanism that is separable from those processes of synapse development and/or recovers quickly after histamine washout.

The functions of early HSN activity during the late L4 stage also poses some interesting questions for further study. The activity I observe began during the L4.7-8 substage, well after the molecular components of the HSN presynaptic terminal
were assembled (Shen and Bargmann, 2003; Shen et al., 2004; Patel et al., 2006) and well before the extension of the vulval muscle arms had been completed. Does early HSN activity play a role in directing the growth of the muscle arms during the late L4 stage? Is activity required for the maintenance of the HSN presynaptic termini once they have assembled? Although I did not observe a delay in egg laying in worms in which early HSN activity was silenced, it does not rule out the possibility that this activity may be required for some aspect of neuromuscular synapse development/maintenance. In other systems where early activity has been shown to play key roles in synaptic development such as the vertebrate auditory system, mutations that profoundly affect the activity of cells from which early activity originates have surprisingly near normal levels of activity. This highlights the importance of homeostatic plasticity mechanisms which can alter other factors influencing cell excitability such as resting potential in order to maintain some amount of activity in the developing circuit which may be sufficient for proper synapse and circuit development (Babola et al., 2018). Whether such mechanisms play a role in the egg laying circuit remain to be studied. In the next chapter, I will discuss a key question about how the egg laying circuit is activated in adult animals during behaviors.
Chapter 4

Mature Patterns of Activity are Driven by Sensory Signals That Detect the Accumulation of Unlaid Eggs in the Uterus

4.1. Rationale and Introduction to Experiments

In Chapter 3, I described how mature patterns of cell and circuit activity develop in the egg-laying circuit. What activates the cells of the circuit during egg laying in adult animals? After the completion of the L4 molt, young adult animals begin to accumulate unlaid eggs in the uterus and lay their first egg at ~6-7 hours after the L4-Adult molt. At this stage, animals have around ~8-10 unlaid eggs in the reproductive tract. The first unlaid egg can be seen in the uterus at ~3-4 hours but is not laid immediately. Injection of fluid into the worm gonad triggers egg-laying events suggesting a role for mechanical stretch in the induction of activity in the egg-laying circuit. Molecular changes during development such as the neurotransmitter specification or the expression of receptors and ion channels can also contribute to changes in cell/circuit activity. My results presented in the previous chapter point to an upregulation in the expression of a post-synaptic receptor for serotonin, SER-4, in the vulval muscles. Previous work also points to

---

2 Results in Chapter 4 were included as part of the following manuscripts:
an upregulation of serotonin neurotransmitter in the HSNs in young adults (Desai et al., 1988).

I proposed two hypotheses for the emergence of mature patterns of activity in the egg-laying circuit:

1. Mature patterns of cell and circuit activity emerge with circuit maturity and age, and is coincident with the accumulation of unlaid eggs in the uterus
2. Mature patterns of activity are driven by sensory signals that detect the accumulation of eggs

I tested these hypotheses by looking at how muscle excitability to presynaptic HSN input changes as juvenile animals become egg laying adults using optogenetics and calcium imaging. I then used pharmacological and reversible silencing approaches to understand how the accumulation of unlaid eggs in the uterus affects circuit activity and HSN burst firing. My results show that the accumulation of eggs in the adult uterus renders the muscles sensitive to HSN input. Sterilization or acute electrical silencing of the vulval muscles inhibits presynaptic HSN activity, and reversal of muscle silencing triggers a homeostatic increase in HSN activity and egg release that maintains ~12-15 eggs in the uterus. Feedback of egg accumulation depends upon the vulval muscle postsynaptic terminus, suggesting a retrograde signal sustains HSN synaptic activity and egg release. Thus, the circuit, while not seeming to require early activity, does depend on modulation by sensory feedback to drive activity and the two-state egg-laying behavior in adults.
4.2. Results

Vulval muscle responsiveness to HSN activity increases as maturing animals accumulate unlaid eggs

Optogenetic activation of the HSNs in adult animals is sufficient to induce egg-laying circuit activity and behavior (Emtage et al., 2012; Collins et al., 2016). Despite the fact that both the HSNs and vulval muscles show activity in L4.9 animals, I have previously shown that egg laying does not begin until 6-7 hours later when the animals have accumulated ~8-10 unlaid eggs in the uterus (Fig. 3.6A). In order to dissect the relationship between developmental time, egg production, and circuit functionality, I tested when the vulval muscles develop sensitivity to HSN input. I optogenetically activated the HSNs using Channelrhodopsin-2 (ChR2) while simultaneously recording Ca\(^{2+}\) activity in the vulval muscles at 3 stages: in L4.9 juveniles and in 3.5 hour and 6.5-hour old adults. L4.9 animals have no eggs in the uterus, 3.5-hour adults contained 0-1 unlaid eggs, while 6.5-hour old adults had accumulated ~8-10 eggs. Stimulating HSNs in L4.9 juveniles or in 3.5-hour adults failed to induce detectable changes in vulval muscle Ca\(^{2+}\) activity (Fig. 4.1A, 4.1B, 4.1F). In contrast, optogenetic activation of HSNs in 6.5-hour adults significantly increased vulval muscle Ca\(^{2+}\) activity and triggered egg laying (Fig. 4.1C and 4.1F). L4.9 juveniles or 3.5-hour adults with 0-1 eggs in the uterus had a mean transient frequency of ≤100 mHz, similar to the inactive state vulval muscle Ca\(^{2+}\) response seen in 6.5-hour adult animals with ~8 unlaid eggs grown in the absence of ATR, a cofactor necessary for ChR2 activation. The vulval muscle Ca\(^{2+}\) response to HSN input was increased
to ~170 mHz in 6.5-hour adults that had accumulated ~8 unlaid eggs (Fig. 4.1G). Surprisingly, vulval muscles in serotonin-deficient mutants responded normally to HSN activation at 6.5 hours (Fig. 4.1D and 4.1E), a finding consistent with the normal onset of egg laying in these mutants (Fig. 3.7A). A recent study from our lab has shown that the ~4 minute continuous blue light stimulation used to activate the HSNs is effective in inducing strong vulval muscle Ca\(^ {2+} \) and egg-laying events throughout the stimulation period, indicating the effectiveness of this approach. Additionally, to confirm that ChR2 activation in the HSNs is able to activate HSN Ca\(^ {2+} \) robustly across developmental stages, I crossed the wzIs30 transgene expressing ChR2 in the HSNs into a strain expressing GCaMP5 and mCherry in the HSNs. My preliminary observations in a few animals at each developmental stage show that the HSNs could be activated in response to continuous blue light during a ~2 minute period of excitation, as described previously (Chapter 2 and data not shown). Together, these results show that despite having significant Ca\(^ {2+} \) activity in juveniles, the adult vulval muscles only develop a robust response to HSN input ~6 hours after the molt, a time when fertilized embryos are being deposited in the uterus to be laid.
Fig. 4.1. Vulval muscle responsiveness to HSN input correlates with egg accumulation. (A-D) Representative traces of vulval muscle Ca\(^{2+}\) activity in L4.9 juveniles (A, blue), 3.5-hour adults (B, orange), 6.5-hour wild-type adults (C, black), and 6.5-hour serotonin-deficient tph-1(mg280) mutant adults (D, green) with and without optogenetic activation of HSN. Animals were grown in the presence (plus ATR, top) or absence (no ATR, bottom) of all-trans retinal (see cartoon schematic). Continuous 489 nm laser light was used to simultaneously stimulate HSN ChR2 activity and excite GCaMP5 fluorescence for the entire recording. Arrowheads indicate egg-laying events. Blue bars under the Ca\(^{2+}\) traces indicate the period of continuous blue light exposure. (E) Cumulative distribution plots of instantaneous peak frequencies (and inter-transient intervals) of vulval muscle Ca\(^{2+}\) activity in 6.5-hour adult wild-type (black filled circles, no ATR; black open circles, plus ATR) and tph-1(mg280) null mutant animals (green filled circles, no ATR; green open circles, plus ATR). Asterisks indicate p<0.0001; n.s. indicates p≥0.2863 (Kruskal-Wallis test). (F) Cumulative distribution plots of instantaneous peak frequencies
(and inter-transient intervals) of vulval muscle Ca\(^{2+}\) activity in L4.9 juveniles (blue filled squares, no ATR; blue open squares, plus ATR), 3.5-hour old adults (orange filled circles, no ATR; orange open circles, plus ATR), and 6.5-hour old adults (black filled circles, no ATR; black open circles, plus ATR). Asterisk indicates \(p<0.0001\); n.s. indicates \(p\geq0.3836\) (Kruskal-Wallis test). (G) Plot shows the average number of unlaid eggs present in the uterus and the average vulval muscle Ca\(^{2+}\) transient peak frequency, ±95% confidence intervals. Total vulval muscle Ca\(^{2+}\) inter-transient intervals (and instantaneous peak frequencies) used for analysis in (F) for each group were as follows: L4.9, no ATR \((n=83)\); L4.9, plus ATR \((n=103)\); 3.5 h Adult, no ATR \((n=90)\); 3.5 h Adult, plus ATR \((n=140)\); 6.5 h, no ATR \((n=185)\); 6.5 h, plus ATR \((n=204)\). Total instantaneous peak frequencies (and inter-transient intervals) of vulval muscle Ca\(^{2+}\) used for analysis in (E) for each group were as follows: wildtype 6.5 h, no ATR \((n=50)\); 6.5 h, plus ATR \((n=231)\); no serotonin 6.5 h, no ATR \((n=82)\); no serotonin 6.5 h, plus ATR \((n=189)\). \(N\geq10\) animals for each stage (both no ATR and plus ATR groups).

I next examined whether this change in vulval response in older adults was caused by ongoing developmental events or was instead a consequence of egg accumulation. We previously demonstrated that adults sterilized with FUDR, a chemical blocker of germline cell division and egg production (Mitchell et al., 1979), showed inactive state levels of vulval muscle activity (Collins et al., 2016). I found that vulval muscles in FUDR-treated animals 24 hours after the molt were also significantly less responsive to HSN optogenetic stimulation (Fig. 4.2A and 4.2B). The residual vulval muscle response in FUDR-treated animals is likely caused by incomplete sterilization when FUDR is added to L4.9 animals. I interpret these results as indicating that animal age or circuit maturity are not sufficient for the onset of the egg-laying active state.
Fig. 4.2. **Sterilization decreases vulval muscle responsiveness to optogenetic HSN activation.** (A) Traces of HSN-induced vulval muscle Ca\(^{2+}\) activity in untreated (top, black) and FUDR-treated 24-hour adult animals (bottom, red). Arrowheads indicate egg-laying events. (B) Cumulative distribution plots of instantaneous peak frequencies (and inter-transient intervals) of vulval muscle Ca\(^{2+}\) activity after optogenetic activation of HSNs in untreated animals grown with ATR (+ATR, open black circles), FUDR-treated animals with ATR (+ATR, open red circles), and in untreated animals without ATR (no ATR, closed black circles). Asterisks indicate p<0.0001 (Kruskal-Wallis test with Dunn’s correction for multiple comparisons). Total vulval muscle Ca\(^{2+}\) inter-transient intervals (and instantaneous peak frequencies) used for analysis in (B) for each group were as follows: 24 h Adult, no ATR (n=57), 24 h Adult, plus ATR (n=304), and 24 h Adult, plus ATR plus FUDR (n=170). N≥10 animals for each treatment.

A retrograde signal of egg accumulation and vulval muscle activity drives presynaptic HSN activity

HSN activity can be inhibited by external sensory signals and feedback of egg release (Ringstad and Horvitz, 2008; Emtage et al., 2012; Collins et al., 2016; Banerjee et al., 2017), but the factors that promote HSN activity are not clear. I
tested whether egg accumulation promotes circuit activity through the presynaptic HSNs, the postsynaptic vulval muscles, or both. I found that HSN Ca\textsuperscript{2+} activity, particularly the burst firing activity associated with the active state, was dramatically reduced in FUDR-treated animals (Fig. 4.3A). Although I did observe single HSN Ca\textsuperscript{2+} transients after FUDR treatment, the intervals in between were prolonged, often minutes apart (Fig. 4.3C). I quantified the total time spent by animals with HSN Ca\textsuperscript{2+} transient intervals <30 s apart as a measure of HSN burst-firing seen in the active state. I found that while untreated animals spent ~13% of their time with the HSNs showing high-frequency activity, such bursts were eliminated in FUDR-treated animals (Fig. 4.3D). I confirmed the FUDR results using a conditional glp-1(or178ts) Notch receptor mutant that causes germline loss and sterility when shifted to 25°C during the L1 stage (Fujiwara et al., 2016) (Fig. 4.3B). I observed a dramatic reduction in HSN Ca\textsuperscript{2+} transient frequency in sterile glp-1(or178ts) adults, phenocopying the results seen with FUDR (Fig. 4.3B and 4.3C). While glp-1(or178ts) fertile animals (raised at 15°C) animals spent a typical 13% of their time with the HSNs showing high frequency activity, such bursts were eliminated in sterile glp-1(or178ts) adults (Fig. 4.3D). These results show that feedback of germline activity, egg production, and/or egg accumulation modulates the frequency of HSN activity.

I then performed a reciprocal experiment to test how an over-accumulation of unlaid eggs would affect presynaptic HSN activity. We have previously shown that passage of eggs through the vulva mechanically activates the uv1 neuroendocrine cells which release tyramine and neuropeptides that inhibit HSN
activity and egg laying (Collins et al., 2016; Banerjee et al., 2017). I hypothesized that prevention of egg release would block inhibitory uv1 feedback and increase HSN activity. I expressed HisCl channels in the vulval muscles and recorded HSN Ca\(^{2+}\) activity after silencing with exogenous histamine. Surprisingly, I found that acute silencing of vulval muscles significantly reduced presynaptic HSN Ca\(^{2+}\) activity, resembling the effects of animal sterilization (Fig. 4.4A and 4.4B). While untreated animals spent \(~16\%\) of recording time with high frequency HSN activity, this was reduced to \(~2\%\) of the total recording time in histamine-treated animals (Fig. 4.4C). These results indicate that postsynaptic vulval muscle activity is required for the burst firing in the presynaptic HSN neurons that accompanies the egg-laying active state.

I next looked at how HSN Ca\(^{2+}\) activity recovers when histamine inhibition of the vulval muscles and egg laying is reversed. As shown in Fig. 4.4A, adult animals were treated with or without histamine for 3-4 hours and then moved to plates without histamine for a 20-30 minutes recovery period. Presynaptic HSN Ca\(^{2+}\) activity was then recorded as the animals resumed egg-laying behavior. The HSNs showed a rapid and dramatic recovery of Ca\(^{2+}\) activity after histamine washout resulting in a prolonged active state with increased HSN Ca\(^{2+}\) transient frequency and numerous egg-laying events (Fig. 4.4A and 4.4B). Washout animals spent \(~40\%\) of their recorded time with elevated HSN activity compared to \(15\%\) of the total recorded time in untreated controls (Fig. 4.4C). During this recovery period, I observed increased vulval muscle twitching contractions in the bright field.
A

HSN Ca\(^{2+}\), plus FUDR

B

HSN Ca\(^{2+}\), glp-1\((or178ts)\)

25 °C

15 °C

sterile

assay as 24 h adult

fertile

25 °C birth L1

20 s

sterile

egg-laying events

fertile

C

Cumulative Distribution (%)

Inter-transient interval (s)

HSN Ca\(^{2+}\) peak frequency (mHz)

D

Total time spent with HSN intervals <30 s (%)

wild type

glp-1\((or178ts)\)
Fig. 4.3. Germ line activity is required for HSN burst firing and the active state. (A) Representative HSN Ca$^{2+}$ traces in untreated (top) and FUDR-treated (bottom) adult animals. (B) Representative HSN Ca$^{2+}$ traces in adult glp-1(or178ts) sterilized animals (L1s shifted to 25°C for 24h and raised to adults at 15°C) (top) and glp-1(or178ts) fertile animals (raised at 15°C) (bottom). Arrowheads indicate egg laying events. (C) Cumulative distribution plots of instantaneous HSN Ca$^{2+}$ transient peak frequencies (and inter-transient intervals) of adult HSN Ca$^{2+}$ activity. Asterisks indicate $p<0.0001$ (Kruskal-Wallis test). (D) Scatterplots show total time spent by each individual with HSN transients ≤30s apart in untreated (black filled circles) and FUDR-treated (black open circles) wild type animals or fertile (red filled circles) or sterile (red open circles) glp-1(or178ts) mutant animals. Asterisks indicate $p≤0.0001$ (one-way ANOVA); error bars indicate 95% confidence intervals for the mean. N≥10 animals for each group in (C) and (D). Total HSN Ca$^{2+}$ inter-transient intervals (and instantaneous peak frequencies) used for analysis in (C) for each group were as follows: wildtype, no FUDR (n=105); wildtype, plus FUDR (n=25); glp-1(or178ts), fertile (n=253); glp-1(or178ts), sterile (n=64).

channel, indicating that muscle activity was restored (data not shown). These results are consistent with a model whereby accumulation of unlaid eggs promotes vulval muscle activity which drives a homeostatic increase in presynaptic HSN activity and burst-firing that sustains egg laying.
Fig. 4.4. Vulval muscle activity and egg accumulation promote HSN burst firing. (A) 24-hour old adult animals expressing HisCl in the postsynaptic vulval muscles (vm) and GCaMP5/mCherry in the presynaptic HSNs were placed onto NGM plates with (red, bottom) or without histamine (black, top) for 3-4 hours to induce muscle silencing and cessation of egg laying. Animals were then moved to plates without histamine and allowed to recover for 30 minutes before HSN Ca\textsuperscript{2+} imaging. HSN Ca\textsuperscript{2+} imaging was also performed on adults not removed from histamine (red, middle). Arrowheads indicate egg laying events. (B) Cumulative distribution plots of instantaneous HSN Ca\textsuperscript{2+} transient peak frequencies (and inter-transient intervals) on histamine (open red circles), and after histamine washout (filled red circles) compared with untreated controls (filled black circles). Asterisks indicate p<0.0001 (Kruskal-Wallis test). (C) Scatter plots show fraction of time spent by each individual with frequent HSN Ca\textsuperscript{2+} transients characteristic of the egg-laying active state (<30 s) in untreated controls (black circles), on histamine (red open circles), and after histamine washout (red circles). Error bars indicate 95% confidence intervals for the mean; asterisks indicate p≤0.0061 (one-way ANOVA). Data from N≥10 animals were used for all treatments indicated in (B) and (C). Total HSN Ca\textsuperscript{2+} and inter-transient intervals (and instantaneous peak frequencies) used for analysis in (B) for each treatment were as follows: no Histamine (n=295), plus Histamine (n=76), and plus Histamine, after washout (n=403).
HSN synapses are formed exclusively on the lateral vm2 muscle arms that provide sites of contact between the anterior and posterior vulval muscles (White et al., 1986; Feinberg et al., 2008; Collins and Koelle, 2013). Hypomorphic Notch signaling mutants fail to develop vm2 muscle arms, and are egg-laying defective, but have normal pre-synaptic HSN and VC development (Sundaram and Greenwald, 1993; Li et al., 2013). To determine if retrograde signaling from the vulval muscles to the HSNs occurs through the vm2 muscle arms, I recorded HSN Ca\(^{2+}\) activity in \textit{lin-12(wy750)} Notch receptor mutant animals lacking vm2 muscle arms (Fig. 4.5A and 4.5B). I found that HSN Ca\(^{2+}\) transient frequency was strongly reduced in the \textit{lin-12(wy750)} mutants compared to wild-type control animals (Fig. 4.5C and 4.5D). HSN Ca\(^{2+}\) transients still occurred in \textit{lin-12(wy750)} mutants but burst-firing was eliminated. Wild-type animals spent ~13% of their time with HSN transients <30 s apart, while this was reduced to zero in the \textit{lin-12(wy750)} mutant (Fig. 4.5E), resembling activity seen in sterilized or vulval muscle-silenced animals.
Fig. 4.5. The vm2 muscle arms are required for vulval muscle feedback to HSN and burst firing. (A-B) Cartoon of egg-laying circuit structure (ventral view) in wild-type (A) and lin-12(wy750) hypomorphic loss-of-function mutant (B) animals missing lateral vm2 muscle arms (arrowheads). (C) Representative traces show HSN Ca²⁺ activity in wild-type (black) and lin-12(wy750) mutant animals (red). Arrowheads indicate egg-laying events. (D) Cumulative distribution plots of instantaneous Ca²⁺ transient peak frequencies (and inter-transient intervals) in wild-type (black circles) and lin-12(wy750) mutants (red circles). Asterisks indicate p<0.0001 (Mann-Whitney test). (E) Scatter plots show fraction of time spent by each individual with frequent HSN Ca²⁺ transients characteristic of the egg-laying active state (<30 s) in wild-type (filled black circles) and lin-12(wy750) mutant animals (open red circles). Error bars indicate 95% confidence intervals for the
mean. Asterisk indicates $p=0.0011$ (Student’s t test). Data from $N \geq 10$ animals were used for both groups in (D) and (E). Total HSN $Ca^{2+}$ inter-transient intervals (and instantaneous peak frequencies) used for analysis in (D) for each group were as follows: wildtype ($n=155$) and lin-12(wy750) mutant ($n=100$).

Together, these results suggest that muscle activity feeds back through the vm2 muscle arms onto the pre-synaptic HSN neurons to promote additional $Ca^{2+}$ transients that drive burst firing and sustain the egg-laying active state.

**The VC neurons are activated during periods of increased vulval muscle activity, but inhibits burst firing in the HSN neurons**

As previously described, the role of the cholinergic VC motor neurons in the egg-laying circuit has been difficult to dissect. The VC neurons make synapses onto both the vm2 vulval muscles and the body wall muscles (Refer Figure 1.3). VC $Ca^{2+}$ activity peaks at the moment of vulval muscle contraction, but optogenetic activation of the VCs fails to elicit egg-laying events and instead slows locomotion. Moreover, VC- and acetylcholine-defective mutants show increased egg laying (Bany et al., 2003; Ringstad and Horvitz, 2008), suggesting a loss of inhibitory feedback. Interestingly, the VC motor neurons share key functional features of sensory neurons and interneurons which modulate CPG rhythms in other circuits. VC extends non-synaptic processes along the vulval hypodermis which could be mechanically activated by vulval muscle contraction (White et al., 1986; Colavita and Tessier-Lavigne, 2003). Thus, the VCs, instead of releasing acetylcholine at
the vm2 synapse to drive vulval muscle contraction, may function in part as baroreceptors to slow locomotion during vulval opening and egg release. An alternate hypothesis is that the VCs are activated during periods of increased vulval muscle twitching and in turn promote feed-forward HSN Ca$^{2+}$ activity that drives burst-firing pattern that sustains the active state. To test this, I expressed HisCl channels in the vulval muscles and recorded VC Ca$^{2+}$ activity after silencing with exogenous histamine. Surprisingly, I found that acute silencing of vulval muscles significantly reduced presynaptic VC Ca$^{2+}$ activity, resembling the effects observed on the HSN neurons after vulval muscle silencing (Fig. 4.4A). This silencing could be in part due to gap junctions between the VC neurons and vulval muscles (White et al., 1986). While untreated animals show high frequency VC activity during the egg-laying active state, I never observed Ca$^{2+}$ activity in the VC neurons in histamine-treated animals (Fig. 4.7A and 4.7B). These results indicate that postsynaptic vulval muscle activity is required for the activity in the presynaptic VC neurons that accompanies the egg-laying active state. Further, they show that the VCs, like the HSNs, fail to maintain their activity when the postsynaptic vulval muscles targets are silenced. That is, the HSN and VCs do not appear to enter independent active states, releasing neurotransmitter onto muscles even when
that is ineffective at exciting the muscles. Instead, activity in each cell in the circuit is unexpectedly influenced by ongoing post-synaptic activity.

**Fig. 4.6. VC neurons show a homeostatic increase in Ca\(^{2+}\) activity with increased egg accumulation** (A) 24-hour old adult animals expressing HisCl in the postsynaptic vulval muscles and GCaMP5/mCherry in the presynaptic VCs were placed onto NGM plates with (orange, bottom) or without histamine (blue, top) for 3-4 hours to induce muscle silencing and cessation of egg laying. Animals were then moved to plates without histamine and allowed to recover for 30 minutes before VC Ca\(^{2+}\) imaging (bottom). VC Ca\(^{2+}\) imaging was also performed on animals placed on plates with histamine (orange, middle). Arrowheads indicate egg laying events. (B) Scatter plots show VC Ca\(^{2+}\) peaks per minute measurements for each individual in untreated controls (blue circles), on histamine (orange open circles), and after histamine washout (orange circles). Error bars indicate 95% confidence intervals for the mean for 10 animals; asterisks indicate \(p\leq0.0001\) (one-way ANOVA with Bonferroni correction for multiple comparisons). Data from \(N\geq10\) animals were used for the three treatment groups in (A) and (B). Total peak values of VC Ca\(^{2+}\) used for analysis in (B) for each treatment group were as follows: no
Histamine \((n=11)\), plus Histamine \((n=13)\), and plus Histamine, after washout \((n=12)\).

I next looked at how VC Ca\(^{2+}\) activity recovers when histamine inhibition of the vulval muscles and egg laying is reversed. As shown in Fig. 4.6A, adult animals were treated with or without histamine for 3-4 hours and then moved to plates without histamine for a 20-30 minutes recovery period. Presynaptic VC Ca\(^{2+}\) activity was then recorded as the animals resumed egg-laying behavior. The VCs showed a rapid and dramatic recovery of Ca\(^{2+}\) activity after histamine washout resulting in a prolonged active state with increased VC Ca\(^{2+}\) transient frequency and numerous egg-laying events (Fig. 4.6A and 4.6B). These results are consistent with a model whereby increased vulval muscle activity during the active state of egg laying drives VC Ca\(^{2+}\) activity, and that rebounds of vulval contractility after histamine-washout cause a corresponding increase in VC Ca\(^{2+}\) activity.

What is the function of the vulval muscle induced VC neuron activity? Does it drive feed-back inhibition of HSN activity as proposed (Bany et al., 2003; Ringstad and Horvitz, 2008), or does it instead drive a feed-forward increase in HSN activity and burst firing? To test this, I expressed Tetanus Toxin in the VC neurons to block synaptic release and recorded HSN Ca\(^{2+}\) transient frequency. I observed a marked increase in HSN Ca\(^{2+}\) transient frequency and burst firing in animals where VC synaptic transmission was blocked compared to non-transgenic control animals (Fig. 4.8A and 4.8B). While wild-type animals spent about \(~11\%\) of their time showing high frequency activity in the HSN neurons, transgenic
animals expressing Tetanus Toxin in the VC neurons spent about ~21% showing high frequency burst firing Ca$^{2+}$ transients in the HSN neurons, a significant increase (Fig. 4.8C). These results support the hypothesis that neurotransmitter release from the VCs inhibits HSN Ca$^{2+}$ activity, consistent with previous genetic data (Bany et al., 2003; Ringstad and Horvitz, 2008).

**Fig. 4.7. VC neurotransmission inhibits HSN burst firing** (A) Traces show HSN Ca$^{2+}$ activity in wild-type (black) and transgenic animals expressing Tetanus toxin (TeTX) in the VC neurons (red). Arrowheads indicate egg-laying events. (B) Cumulative distribution plot of instantaneous Ca$^{2+}$ transient peak frequencies (and inter-transient intervals) in non-transgenic wild-type control animals (black circles) and in transgenic animals expressing TeTX in the VC neurons (red open circles).
Asterisks indicate $p<0.0001$ (Mann Whitney test). (C) Scatter plots show mean fraction of time spent by each animal with frequent HSN $\text{Ca}^{2+}$ transients characteristic of the egg-laying active state ($<30$ s) in wild-type (black circles) and VC::TeTX animals (red open circles). Error bars indicate 95% confidence intervals for the mean for $\geq10$ animals; asterisk indicates $p<0.0001$ (Student’s t test). Data from $N\geq10$ animals were used for both groups in (B) and (C). Total HSN $\text{Ca}^{2+}$ inter-transient intervals (and instantaneous peak frequencies) values used for analysis in (B) for each group were as follows: wildtype ($n=154$) and VC::Tetanus toxin transgenic animals ($n=282$).

It had been proposed that acetylcholine released from the VCs act via inhibitory G$\alpha$-coupled receptor GAR-2 in the HSNs to provide inhibitory feedback (Bany et al., 2003). However, recent more detailed analysis of the expression patterns of several G-protein coupled receptors in the egg-laying circuit has failed to show that GAR-2 is expressed in the HSN neurons. Other muscarinic receptors of acetylcholine, namely GAR-1 and GAR-3, were also found to not be expressed in the HSNs (Personal communication from Dr. Michael Koelle at Yale University). Apart from acetylcholine, the VCs also express two peptides which produce an inhibitory effect on egg-laying behavior, NLP-7 and FLP-11 (Banerjee et al., 2017). It is therefore possible that the expression of Tetanus Toxin in the VCs negatively affects the release of these two peptides, thereby increasing the duration of HSN active phase activity.
4.3. Major Findings and Discussion

Figure 4.8 shows a working model for how postsynaptic muscle activity could promote burst firing in the presynaptic HSNs. I propose that changes in the accumulation of unlaid eggs depress or excite the uterine muscle cells which then cross-excite the vm2 vulval muscles via gap-junctions. Stretch-activation of the uterine muscles would serve as a coincidence detector, increasing vulval muscle sensitivity to serotonin and other neurotransmitters released from HSN only when sufficient eggs are in the uterus. This enhanced vm2 electrical excitability allows for rhythmic acetylcholine input from the VA/VB locomotion motor neurons to drive vulval muscle twitching contractions with each body bend. Coordinated Ca\(^{2+}\) activity in the anterior and posterior vulval muscles allows the vm2 muscle arms to restimulate the HSNs and prolong the egg-laying active state for as long as eggs are still present in the uterus. VC activity is coincident with strong vulval muscle contractions, while uv1 activity follows passage of eggs through the vulva (Collins et al., 2016). Once sufficient eggs have been laid, excitatory feedback into the vulval muscles and back to the HSNs is reduced, increasing the probability that inhibitory acetylcholine, tyramine, and neuropeptides released from VC and uv1 will block subsequent HSN Ca\(^{2+}\) transients, returning the circuit to the inactive state.
My results point to a retrograde signaling mechanism in the egg laying circuit which scales presynaptic HSN Ca^{2+} activity in response to the accumulation of eggs (sensory signal) and the levels of postsynaptic vulval muscle activity. In my study, I have not identified specific mechanosensory molecules in the uterine...
muscles or other cells in the egg-laying circuit which transduce the stretch signal resulting from egg accumulation. The post-synaptic vm2 muscle arms play a key functional role in retrograde signaling and in coordinating muscle activity during egg-laying behavior. Because of the intervening vulval slit through which eggs are laid, the vm2 muscle arms are the only sites of contact between the anterior and posterior muscles. Coordinated muscle Ca\(^{2+}\) transients appear during the L4.9 larval stage after vm2 muscle arm development. After development, the vm2 muscle arms may be electrically coupled at their points of contact, allowing for the immediate spread of electrical activity and/or Ca\(^{2+}\) signals between the anterior and posterior muscles. Mutants missing the vm2 muscle arms do not show regenerative HSN Ca\(^{2+}\) activity, resembling the consequences of vulval muscle electrical silencing (Li et al., 2013). Alternatively, neuromodulator signals like serotonin may ensure the anterior and poster muscles are being activated in a coordinated manner that allows sufficient vulval opening for egg laying. The vm2 muscle arms form the sites of synaptic input from HSN and VC. We have previously shown that the ERG K\(^+\) channel and SER-1 serotonin receptor localize to the vm2 muscle arm region (Collins and Koelle, 2013; Li et al., 2013). Both ERG and SER-1 have C-terminal PDZ interaction motifs, and SER-1 has been shown to interact with the large PDZ scaffold protein MPZ-1 that may drive the local organization of these and other molecules to the vm2 muscle arms (Xiao et al., 2006). Innexin gap junction proteins which are potential targets of G protein signaling (Correa et al., 2015) may also play a role in driving the development of coordinated vulval muscle contractility and HSN ‘burst’ activity in the circuit during
egg laying. I will speculate on possible molecules in *C. elegans* which might function as retrograde signaling messenger in Chapter 6.

Stretch-mediated feedback is essential in circuits that control autonomic functions (Dethier and Gelperin, 1967; Gelperin, 1971; Spencer et al., 2002), the rhythmic uterine activity during parturition (Ferguson’s reflex) (Ferguson, 1941), and in circuits which generate rhythmic motor outputs (Grillner, 2003; Marder et al., 2005; Blitz and Nusbaum, 2011). Stretch can provide either positive or negative feedback to downstream reflex and homeostatic circuits. For example, specialized mechanosensory neurons activated by gastric stretch induce satiety by providing negative feedback to neural circuits reducing food consumption when the stomach is full (Dethier and Gelperin, 1967; Zagorodnyuk et al., 2001). In guinea pigs, stretch-sensitive interneurons provide ascending excitatory and descending inhibitory inputs to generate peristaltic neural reflexes in the distal colon (Spencer and Smith, 2004). Mechanical stretch (from egg accumulation) or artificially induced distension of the reproductive tract in female flies induces an attraction to acetic acid so that eggs can be laid in optimal environments (Gou et al., 2014). In the cases described above, how stretch sensory inputs modulate the activity of neural circuits and synaptic transmission is not always clear.

The *C. elegans* egg-laying homeostat is regulated by egg accumulation which sustains rhythmic activity in a motor neuron for muscle contraction and egg release. This phenomenon is observed in the case of other stretch-activated neural circuits. In the case of the Ferguson’s parturition reflex, initial stretch-induced myogenic contractions engage the neuroendocrine feed-forward loop, similar to
our results showing that vulval muscle activity promotes a feed-forward increase in HSN activity. Does mechanosensory stretch also play a role in the feedback inhibition of *C. elegans* egg-laying? While the release of eggs and loss of uterine stretch should reduce excitatory drive into the vulval muscles and HSN, mechanical feedback activity of the VC motor neurons and the uv1 neuroendocrine cells may provide additional negative feedback required to exit the active state completely. VC Ca\(^{2+}\) activity is coincident with egg release and the results discussed in this chapter bolster our hypothesis that VC neurotransmission inhibits HSN Ca\(^{2+}\) activity. The uv1 cells are mechanically deformed and activated by egg release, and tyramine and inhibitory neuropeptides released from uv1 inhibit HSN activity (Collins et al., 2016; Banerjee et al., 2017). Both feed-forward and feedback systems operating in parallel likely contribute to changes in circuit activity that allow distinct patterns of behavior, for example whether multiple eggs are laid within long active states rather than single eggs being laid as soon as they are deposited into the uterus. Further studies of the *C. elegans* egg-laying homeostat described here should allow the dissection of conserved molecular, cellular, and synaptic mechanisms that drive stretch-dependent feedback. Because dysregulation of similar stretch-dependent circuits likely contributes to pervasive human disorders including obesity and urinary incontinence, a basic understanding of the molecular signaling mechanisms would help inform the generation and improvement of treatments.
Chapter 5

Major G-protein, GOA-1 (G\textsubscript{\alpha\text{o}}), Regulates Neuronal Excitability of the Serotonergic HSN Neuron in Caenorhabditis elegans\textsuperscript{3}

5.1. Rationale and Introduction to Experiments

In Chapters 3 and 4, I examined how mature pattern of cell and circuit activity develop in the egg-laying circuit and how egg accumulation (not circuit age) is sufficient to activate the circuit for behavior. Because eggs are only laid in favorable environmental conditions (Dong et al., 2000), how do positive and negative external sensory signals affect egg-laying circuit activity to influence where and when animals lay eggs? Heterotrimeric G proteins, comprising the \(\alpha, \beta\) and \(\gamma\) subunits are key transducers of various signals in the nervous system and have been shown to both stimulate and inhibit egg-laying circuit activity and behavior. Of the four major members of alpha subunit family, Pertussis toxin sensitive subunit, G\(\alpha_{\text{o}}\), is the most abundantly expressed alpha protein in both the mammalian central and peripheral nervous system, comprising about 1% of membrane proteins in the brain (Sternweis and Robishaw, 1984). Animals missing inhibitory G\(\alpha_{\text{o}}\) signaling have dramatically increased neural excitability and behavioral defects (Jiang et al., 1998; Koelle, 2016). G\(\alpha_{\text{o}}\) knockout mice show hyperactive motor behaviors and neurological defects such as tremors, seizures,

\textsuperscript{3} Results in Chapter 5 will be included as part of the following manuscript: Bhavya Ravi, Jian Zhao, Lijun Kang, and Kevin M. Collins "Major G-protein G\(\alpha_{\text{o}}\) regulates neuronal excitability of the serotonergic HSN neuron in Caenorhabditis elegans." (In Preparation)
sensory deficits, and hyperalgesia (Jiang et al., 1998; Tanaka et al., 1999; Luo et al., 2002). De novo mutations in GNOA-1, which encodes the Gαo subunit, have been implicated in epileptic encephalopathies, a group of neurological disorders that are characterized by progressive neurodevelopmental and motor impairments which are most likely caused by increased epileptic activity (Nakamura et al., 2013). A wide array of ligands bind to and activate Gαo coupled receptors including the neurotransmitters acetylcholine, GABA, serotonin and dopamine, as well as neuropeptides (Jiang and Bajpayee, 2009). In the mammalian nervous system, Gαo signaling regulates a range of functions including neurite extension, sensory perception, and the regulation of neuronal excitability (Nakata and Kozasa, 2005; Nakamura et al., 2013; Pal Choudhuri et al., 2016). Knockout of the Gαo in the C. elegans, which shares more than 80% sequence identity with its corresponding mammalian protein, leads to hyperactive locomotion and egg-laying behaviors (Koelle, 2016), phenotypes that closely mirror the consequences of too much Gαq signaling (Brundage et al., 1996; Hajdu-Cronin et al., 1999). Pioneering genetic studies in C. elegans indicate that Gαo signaling functions to antagonize signaling by the other major G protein Gαq, whose effectors, the PIP2-specific Phospholipase, PLCβ, and the RhoGTPase Exchange Factor, Trio, signal to promote lipid, Ca^{2+}, and Rho signaling. Because direct effectors of Gαo remain unidentified, how Gαo-coupled receptors signal to antagonize Gαq in vivo to inhibit neuronal excitability and control behavior remains largely unknown.

The C. elegans model system is ideally suited to investigate the effects of Gαo signaling on neuronal activity, as well as to identify its upstream and
downstream effectors. The Gαo protein is expressed in all the cells in this circuit (Tanis et al., 2008). Previous work has shown that Gαo functions in the presynaptic HSNs to regulate synaptic transmission in the C. elegans egg-laying behavior circuit (Koelle, 2016). Animals with reduced Gαo signaling show hyperactive locomotion and egg-laying while mutants with too much Gαo signaling are sluggish and egg-laying defective (Koelle, 2016). Gαq acts in exact opposition to Gαo signaling thereby promoting serotonin release from the HSNs (Tanis et al., 2008), but receptors which act upstream of Gαq in HSN and which ligands activate their signaling have not yet been identified.

Previous studies have shown that Gαo signaling acts downstream of sensory cues released during aversive conditions to inhibit HSN activity and egg release. Neuropeptides released by the CO2 sensing BAG neurons signal through Gαo coupled EGL-6 receptors in the HSNs to activate inward rectifying K+ channels including IRK-1 to diminish cell electrical excitability (Ringstad and Horvitz, 2008; Emtage et al., 2012). HSN-specific expression of Pertussis Toxin, which ADP-ribosylates and inhibits Gαo signaling, causes strong hyperactive egg laying behavior phenotypes, similar to goa-1 null mutants (Tanis et al., 2008). Animals lacking egl-6 have normal egg-laying behavior supporting a model where other inhibitory signals act in parallel to activate Gαo and inhibitory ion channels like IRK-1 to depress HSN electrical excitability to inhibit serotonin release and egg laying. Alternatively, loss of Gαo in the cholinergic locomotor neurons has been shown to increase the localization of synaptic vesicle priming protein UNC-13S::GFP to presynaptic termini (Nurrish et al., 1999). However, in the HSN neurons, no
changes in overall synaptic volume or increase in the localization of synaptic UNC-13S::GFP have been observed in Gαo loss-of-function mutants (Tanis et al., 2008). These mutants did show an increase in the expression of a tph-1 reporter transgene suggesting a role for Gαo signaling in regulating long-term changes in serotonin biosynthesis (Tanis et al., 2008), perhaps explaining how loss of Gαo increases egg laying. Despite clear egg-laying phenotypes in mutants with altered Gαo signaling, genetic studies have not yet identified direct effectors for Gαo that would help clarify whether Gαo principally inhibits HSN activity through ion channels, altered DAG levels and synaptic vesicle fusion, through transcriptional changes that altered serotonin levels, or through all or additional mechanisms (Koelle, 2016). In this Chapter, I aimed to test directly how Gαo signaling regulates HSN and circuit Ca2+ activity to maintain the inactive behavior state, even under conditions that normally favor egg laying.

In this part of my study I tested the following hypothesis about the role of Gαo signaling on egg-laying circuit activity and behavior:

1. **Mutants with altered Gαo signaling show changes in the durations of active and inactive egg-laying behavior states.** I tested this hypothesis by recording long-term egg-laying behavior in wildtype animals and mutant and transgenic animals with altered Gαo signaling in the egg laying circuit.

2. **Mutations which reduce inhibitory Gαo signaling will show increased HSN and vulval muscle excitability and Ca2+ activity.** Mutations which increase inhibitory Gαo signaling will show decreased HSN and vulval muscle excitability and Ca2+ activity. I tested this hypothesis by crossing
reporters of Ca^{2+} activity with mutants and transgenic animals with altered G\(\alpha_0\) signaling and visualizing circuit activity.

3. **G\(\alpha_0\) has additional signaling functions in the vulval muscles and uv1 neuroendocrine cells of the egg-laying circuit.** I tested this hypothesis by cell-specifically perturbing G\(\alpha_0\) signaling in vulval muscles and uv1 cells using transgenic approaches.

5.2. Results

**Reduced inhibitory G\(\alpha_0\) signaling leads to premature egg laying and decreases the duration of egg-laying inactive states**

As mentioned above, inhibiting or increasing G\(\alpha_0\) signaling has opposite effects on steady-state measures of egg accumulation as a readout of behavior. Whether these manipulations caused a change in the duration of the active state (e.g. how frequently eggs are laid within an active state), the inactive state (how frequently animals enter an egg-laying active state), or both, remains unclear. To address this gap in our understanding of how G\(\alpha_0\) signaling contributes to the pattern of circuit activity that underlies two-state behaviors, I wanted to determine how genetic manipulations that increased and decreased G\(\alpha_0\) signaling affected the onset of egg laying as well as the temporal pattern of egg laying within adult active states.

My data show that G\(\alpha_0\) signaling regulates the onset of egg-laying in young adult animals. I first performed a ‘time to first egg’ assay (described in Chapter 3)
in wild-type animals and in mutants with too much or too little Gαo signaling. As previously described, wild-type animals laid their first egg ~6-7 hours after becoming adults. Animals bearing goa-1 loss-of-function (and reduced-function) mutations laid their eggs much earlier, 3-4 hours after becoming adults (Fig. 5.1A). Both goa-1 mutants, sa734, which results in an early stop during translation (hence is predicted to be a molecular null), and n1134, a hypomorphic mutant that eliminates the initiating ATG, producing a gene product predicted to encode an in-frame protein lacking the N-terminal myristoylation and palmitoylation sequence (Segalat et al., 1995; Robatzek and Thomas, 2000), preventing its proper trafficking to the cell membrane, showed a similar precocious onset in egg laying (Fig. 5.1A). This same phenotype was observed in transgenic animals expressing Pertussis Toxin cell-specifically in HSNs (Tanis et al., 2008), suggesting the egg-laying phenotype is caused by a specific loss of inhibitory Gαo signaling that results in potentiated serotonin and neuropeptide release from HSN (Fig. 5.1A). To test the effects of increased Gαo signaling, I analyzed the behavior of egl-10(md176) null mutants which lack the major RGS protein that terminates Gαo signaling by promoting Gαo GTP hydrolysis (Koelle and Horvitz, 1996). egl-10(md176) mutants showed a strong and significant delay in the onset of egg laying, ~15 hours after reaching adulthood (Fig. 5.1A), similar to animals that lack the HSN motor neurons entirely (Ravi et al., 2018a). Animals expressing the constitutively active Gαo (Q205L) mutant specifically in the HSNs showed a similar delay in the onset of egg laying, consistent with Gαo signaling acting to inhibit neurotransmitter release from the HSNs.
To understand how changes in $\Gamma_{\alpha_o}$ signaling disrupt the two-state pattern of egg-laying behavior, I made long-term recordings of animal behavior. Intervals between egg-laying events were operationally classified into two categories: intra-cluster intervals and inter-cluster intervals as described (Waggoner et al., 1998; Banerjee et al., 2017; Zang et al., 2017; Chew et al., 2018). Intra-cluster intervals (< 4 minutes) are intervals between consecutive egg laying events within a single active state which typically lasts for ~2 minutes. Inter-cluster intervals (> 4 minutes) are the intervals between distinct active states. Inter-cluster intervals provide us with a measure of how frequently egg laying occurs (Waggoner et al., 1998). Wild-type animals displayed a two-state pattern of egg laying as described (Waggoner et al., 1998), with multiple egg-laying events clustered within brief, ~2 minute active states about every 20-30 minutes (Fig. 5.1B and Table 3). Animals with reduced inhibitory $\Gamma_{\alpha_o}$ signaling entered active states 2- or 3-fold more frequently, often laying single eggs during active states separated by only ~12-13 minutes (Fig. 5.1B and Table 3). The pattern of egg laying events in animals expressing Pertussis Toxin in the HSN neurons was indistinguishable from the $goa-1(n1134)$ hyperactive egg laying mutant, indicating that $\Gamma_{\alpha_o}$ signaling is required in HSN for proper inhibition of synaptic transmission (Fig. 5.1A, 5.1B, 5.1C, and Table 3). The average intra-cluster intervals of the hyperactive $goa-1(n1134)$ and transgenic animals expressing pertussis toxin in the HSN neurons were almost doubled in comparison to wildtype animals (Fig. 5.1C and Table 3). In contrast, $egl-10(md176)$ mutant animals and animals expressing $\Gamma_{\alpha_o}(Q205L)$ mutant specifically in the HSNs had very infrequent egg laying, lengthening the inactive
period to 258 and 66 min, respectively. Interestingly, animals with too much Gαo signaling still laid eggs in clusters of multiple eggs (Table 3). I performed a linear regression analysis on the intervals between egg laying events in the different Gαo signaling mutants (Fig. 5.1D), grouping intervals into intra-clusters intervals (<4 minutes apart) and inter-cluster intervals (greater than >4 minutes). As shown in Table 3, the Gαo mutant and transgenic animals show a specific differences in the inter-cluster interval compared to wild-type control animals with little difference in the pattern of egg laying within an active state. These results show that Gαo signaling does not simply reduce circuit activity to the point where only single eggs are laid. Instead, Gαo specifically acts to determine how frequently animals enter into the egg-laying active state. In animals with reduced Gαo signaling, the animals lay eggs soon after they are deposited (Fig. 5.1B).
Fig. 5.1. $\alpha_\text{G}$ signaling maintains the inactive egg-laying behavior state. (A) Scatter plots of the first egg-laying event in wild-type (black), null goa-1(sa734) mutants (blue), hypomorphic loss-of-function (lof) goa-1(n1134) mutants (red), egl-10(md176) null mutants (orange), and transgenic animals expressing Pertussis Toxin (grey) and GOA-1$^{Q205L}$ in the HSNs (green). Error bars show 95% confidence intervals for the mean from $\geq$10 animals. Asterisks indicate $p \leq 0.0001$ (One-way ANOVA with Bonferroni correction for multiple comparisons) (B) Representative plots showing temporal pattern of egg laying for three hours in wildtype animals and in $\alpha_\text{G}$ signaling mutants (Refer (A) for color scheme). Vertical lines indicate single egg-laying events. (C) and (D) Cumulative distributions of intra-cluster and
inter-cluster intervals (min) in wild-type (black), goa-1(n1134) mutant animals (red), egl-10(md176) (orange) mutant animals, HSN::Pertussis toxin (grey) and HSN::GOA-1Q205L (green) transgenic animals. Inter-cluster intervals are <4 minutes while intra-cluster intervals are >4 minutes duration. Asterisks indicate p<0.0001 (Kruskal-Wallis test with Dunn’s correction for multiple comparisons). Refer Table 3 for details. N≥10 animals were used for analysis for each strain in (A). Total intra-cluster intervals (intervals < 4 minutes) used for analysis in (C) for each strain were as follows: wildtype (n=75), hypomorphic goa-1(n1134) mutants (n=36), HSN::Pertussis toxin transgenic animals (n=24), egl-10(md176) null mutants (n=161), and HSN::GOA-1Q205L transgenic animals (n=75). Total inter-cluster intervals (intervals > 4 minutes) used for analysis in (D) for each strain were as follows: wildtype (n=33), hypomorphic goa-1(n1134) mutants (n=88), HSN::Pertussis toxin transgenic animals (n=79), egl-10(md176) null mutants (n=147), and HSN::GOA-1Q205L transgenic animals (n=56).
Table 3. Intra-cluster and inter-cluster intervals in wildtype animals and mutants with altered Gαₒ signaling. Asterisks indicate significant differences compared to Wildtype (p<0.0001, Kruskal-Wallis test with Dunn’s correction for multiple comparisons). ‘ǂ’ indicate significant differences compared to Wildtype (p<0.0001, One-way ANOVA). ## indicate that this result was previously reported (Tanis et al., 2008).

Gαₒ signaling inhibits HSN neuron Ca²⁺ activity and burst firing

To understand how Gαₒ signaling regulates HSN activity, I performed ratiometric Ca²⁺ imaging in our panel of Gαₒ signaling mutants. Animals bearing the goa-1(n1134) hypomorphic or goa-1(sa734) null mutations that strongly reduce Gαₒ signaling and promote egg laying showed a clear change in HSN Ca²⁺ activity from burst to tonic firing (Fig. 5.2A). This led to a reduction in the number of high-frequency bursts observed during egg-laying active states (Fig. 5.2B). The
increase in the 'burst' firing duration of the *goa-1(n1134)* mutants was not statistically significant (Fig. 5.2C), despite the strong hyperactive egg-laying behavior of this mutant. I did however observe a significant quantitative increase in 'burst' firing duration in the *goa-1(sa734)* null mutants compared to both wildtype and *goa-1(n1134)* mutant animals (Fig. 5.2C). This result suggests that the *goa-1(n1134)* hypomorphic mutant, despite having a strong hyperactive egg-laying phenotype, has residual Gαo signaling activity despite lacking a proper membrane anchor sequence. This result also suggests that insignificant or merely modest changes in HSN Ca2+ activity are sufficient to induce dramatic changes in egg-laying behavior perhaps a result of long-term changes in serotonin biosynthesis (Tanis et al., 2008).

I next tested how increased inhibitory Gαo signaling affects HSN activity. *egl-10(md176)* mutants or transgenic animals expressing the activated GOA-1(Q205L) in the HSN neurons both show a significant and dramatic reduction in the frequency of HSN Ca2+ transients, with single HSN Ca2+ transients occuring several minutes apart (Fig. 5.2A and 5.2B). The rare egg-laying events seen in animals with increased Gαo signaling were were mostly associated with single HSN Ca2+ transients, not multi-transient bursts (Fig. 5.2C). In one *egl-10(md176)* animal, we observed one egg-laying event that was not accompanied by an HSN Ca2+ transient, suggesting Gαo signaling may depress HSN activity sufficiently to effectively silence the HSNs, with egg laying becoming HSN independent. Consistent with this complete silencing of HSNs, *egl-10(md176)* and *egl-1(n986dm)* mutants that lack HSNs show similar defects in the timing of first egg
laid (Ravi et al., 2018a) and are similarly resistant to imipramine, a serotonin reuptake transporter inhibitor (Trent et al., 1983). Alternatively (or additionally) because our imaging conditions only allow us to visualize a single HSN, $G_{\alpha_o}$ signaling may also function to depress coordinated activity between the contralateral HSNs, despite the neurons forming gap-junctions in the head.

To determine how disruption of inhibitory $G_{\alpha_o}$ signaling specifically in HSN affects activity, I recorded HSN Ca$^{2+}$ transients in transgenic animals expressing Pertussis Toxin in the HSNs. $G_{\alpha_o}$ silenced HSNs showed a dramatic increase in the frequency of HSN Ca$^{2+}$ activity, leading to a nearly constitutive ‘burst’ firing activity not seen in hypomorphic $goa-1(n1134)$ mutant animals but closely resembling that of $goa-1(sa734)$ null mutants (Fig. 5.2D). While control animals spent ~4% of the total recorded time showing high frequency ‘burst’ Ca$^{2+}$ activity in the HSN neurons, Pertussis Toxin expressing animals spent ~42% of their time showing ‘burst’ Ca$^{2+}$ activity. These results suggest that unidentified neurotransmitters and/or neuropeptides signal act under ‘optimal’ steady-state conditions to activate HSN receptors and $G_{\alpha_o}$, reducing cell excitability such that the observed two-state pattern of HSN activity and egg-laying behavior is maintained. Importantly, these results show that changes in $G_{\alpha_o}$ signaling directly regulate HSN excitability as measured by Ca$^{2+}$ activity. $G_{\alpha_o}$ does not merely antagonize $G_{\alpha_q}$ to promote indirect changes in presynaptic UNC-13 localization (Nurrish et al., 1999) and/or long-term changes in serotonin biosynthesis (Tanis et al. 2008).
**Fig. 5.2. G\(\alpha\)o signaling inhibits HSN neuron Ca\(^{2+}\) activity and burst firing.** (A) Traces show HSN Ca\(^{2+}\) activity in wild-type (green), hypomorphic loss-of-function goa-1(n1134) mutants (blue), null goa-1(sa734) mutants (dark blue), egl-10(md176) null (orange) mutants, and transgenic animals expressing the activated GOA-1(Q205L) (purple) and Pertussis Toxin (blue, bottom) in the HSN neurons. Arrowheads indicate egg-laying events. (B) Cumulative distribution plots of instantaneous Ca\(^{2+}\) transient peak frequencies in wild-type (green circles), goa-1(n1134) (light blue circles), goa-1(sa734) (dark blue circles), egl-10(md176) (orange circles) mutant animals, and transgenic animals expressing the activated GOA-1(Q205L) in the HSN neurons. Asterisks indicate \(p<0.0001\) (Kruskal-Wallis test with Bonferroni’s test for multiple comparisons). (C) Scatter plots show fraction of time spent by each individual with frequent HSN Ca\(^{2+}\) transients characteristic of the egg-laying active state (<30 s) in wild-type (green circles). Error bars indicate
95% confidence intervals for the mean. Asterisk indicates \( p<0.0001 \) (One-way ANOVA, Dunn’s correction for multiple comparisons). (D) Traces show HSN \( \text{Ca}^{2+} \) activity in wild-type (green) and animals expressing Pertussis toxin (PTX) in the HSN neurons (blue). Arrowheads indicate egg-laying events. (F) Cumulative distribution plots of instantaneous \( \text{Ca}^{2+} \) transient peak frequencies in wild-type (green circles) and in transgenic animals expressing PTX in the HSN neurons (blue circles). Asterisks indicate \( p<0.0001 \) (Mann Whitney test). (C) Scatter plots show fraction of time spent by each individual with frequent HSN \( \text{Ca}^{2+} \) transients characteristic of the egg-laying active state (<30 s) in wild-type (green circles) and HSN::PTX animals (blue open circles). Error bars indicate 95% confidence intervals for the mean. Asterisk indicates \( p<0.0001 \) (Student’s t test). Data from \( N\geq10 \) animals were used for each strain for analysis. Total HSN \( \text{Ca}^{2+} \) inter-transient intervals (and instantaneous peak frequencies) used for analysis in (B) for each group were as follows: wildtype (n=160), goa-1(n1134) hypomorphic mutant (n=236), goa-1(sa734) null mutant (n=410), egl-10(md176) null mutant (n=7), HSN::GOA-1Q205L transgenic animals (n=19). Total instantaneous peak frequencies (and inter-transient intervals) values of HSN \( \text{Ca}^{2+} \) used for analysis in (E) for each group were as follows: wildtype (n=74) and HSN::Pertussis toxin transgenic animals (n=425).

**Post-synaptic activity in the vulval muscles is dramatically increased in mutants with reduced G\( \alpha_o \) signaling**

In the previous section, I discussed how the loss of inhibitory G\( \alpha_o \) signaling increases HSN \( \text{Ca}^{2+} \) activity and leads to a transition from two-state activity to tonic firing in the HSN neurons. To test how increased HSN \( \text{Ca}^{2+} \) activity due to decreased G\( \alpha_o \) signaling affects the postsynaptic vulval muscles, I recorded \( \text{Ca}^{2+} \) activity in the vulval muscles in goa-1(n1134) mutants and in transgenic animals
expressing Pertussis Toxin in the HSN neurons. As shown in Figure 5.3A, 5.3B, and 5.3C, inactive state vulval muscle Ca^{2+} twitching activity is slightly increased in goa-1(n1134) mutants and is dramatically increased in transgenic animals expressing HSN-expressed Pertussis toxin transgenic animals. In contrast, egg-laying active state Ca^{2+} activity was not significantly different from that seen in wildtype control animals (Fig. 5.3B and 5.3C). These results suggest that the increase in tonic HSN activity that accompanies loss of inhibitory Gα_o signaling potentiates vulval muscle excitability such that the muscles respond to rhythmic ACh input with each body bend, a feature rarely observed in wild type animals with infrequent HSN Ca^{2+} transients.

**Fig. 5.3 Gα_o signaling in HSN reduces vulval muscle excitability.** (A) Representative GCaMP5:mCherry (ΔR/R) ratio traces of vulval muscle Ca^{2+} activity in LX1918 wild-type strain (top, black), hypomorphic loss-of-function goa-1(n1134) mutant animals (top, red), LX1919 wild-type strain (bottom, black), and
animals expressing Pertussis Toxin in the HSN neurons (bottom, red). (B) Cumulative distribution plots of instantaneous vulval muscle Ca\textsuperscript{2+} transient peak frequencies in wild-type (LX1918 strain) and goa-1(n1134) mutant animals in the egg-laying inactive (black and red open triangles) and active state (green and blue closed circles) of adults. (B) Cumulative distribution plots of instantaneous vulval muscle Ca\textsuperscript{2+} transient peak frequencies in wild-type (LX1919 strain) and strains expressing Pertussis Toxin in the HSN neurons in the egg-laying inactive (black and red open triangles) and active state (black and red closed circles) of adults. Asterisks indicate p<0.0001 (Kruskal-Wallis test). Data from N≥10 animals were used for each strain for analysis. Total of vulval muscle Ca\textsuperscript{2+} inter-transient intervals (and instantaneous peak frequencies) used for analysis in (B) and (C) for each group were as follows: LX1918 wildtype inactive (n=217), LX1918 wildtype active (n=94), goa-1(sa734) null mutant inactive (n=491), goa-1(n1134) null mutant active (n=165), LX1919 wildtype inactive (n=232), LX1919 wildtype active (n=105), HSN::Pertussis toxin inactive (n=877), and HSN::Pertussis toxin active (n=155).

\textbf{Gα\textsubscript{o} signals in other cells of the egg-laying circuit where it regulates behavior}

My data presented in the previous sections and other studies show that the HSNs are the principal cells in the circuit in which Gα\textsubscript{o} signals to regulate serotonin release and egg laying. Gα\textsubscript{o} is also expressed in all neurons in \textit{C. elegans} and in all the cells of the egg laying system, raising questions as to what Gα\textsubscript{o} is doing in those cells to regulate egg-laying behavior (Tanis et al., 2008). Transgenic expression of the activated GOA-1(Q205L) in the HSNs, but not the VCs or vulval muscles, rescued the hyperactive egg behavior of the goa-1(n1134) mutants (Tanis et al., 2008). Expressing Pertussis Toxin in the uv1 neuroendocrine cells
under the ocr-2 promoter (which also expresses in the a subset of sensory neurons and the uterine seam cell) causes an increased egg-laying rate (Jose et al., 2007), but whether this effect was due to expression in the uv1 cells alone has not been determined. Previous work failing to identify a function for Gαo in the vulval muscles used a modified Nde-box element from the ceh-24 promoter that drove transgene expression only in the vm1 muscle cells that do not directly mediate egg-laying. In order to test if Gαo plays a role in the entire set of vulval muscles and the uv1 neuroendocrine cells, I used more specific promoters to abolish and increase Gαo signaling by transgenically expressing Pertussis toxin and the activated GOA-1(Q205L). I used the full ceh-24 gene promoter as described to drive expression in the vulval muscles (Harfe and Fire, 1998; Ravi et al., 2018a), and I used the tdc-1 gene promoter and ocr-2 3’ untranslated region which drives expression in the uv1 cells but not sensory neurons in the head or the utse cells (Alkema et al., 2005).

I tested how changes in inhibitory Gαo signaling in specific cells of the circuit affected egg-laying behavior. Under steady-state conditions, wild-type animals typically accumulate 12-15 eggs in the uterus. As shown in Figure 5.4A, goa-1(n1134) Gαo mutant animals are strongly hyperactive for egg laying and accumulate only 1-2 eggs in the uterus. Conversely, egl-10(md176) mutations missing the Gαo-specific RGS protein or transgenic animals expressing a Gαo(Q205L) constitutive activated mutant in the HSNs accumulate up to ~30-50 eggs. Expression of Pertussis toxin in the vulval muscles did not produce any significant change on the steady-state number of eggs present in the uterus compared to
control animals expressing only mCherry (Fig. 5.4A). However, expression of the activated GOA-1(Q205L) in the vulval muscles did cause a mild egg-laying defect, accumulating 24.06±2.0 eggs compared to control transgenic animals expressing mCherry (13.2±0.7 eggs; Fig. 5.4A). This egg-laying defect was much weaker than in egl-10(md176) mutants HSN-specific GOA-1(Q205L) transgenic animals. Collectively, these results show that while Gαo does not appear to signal to inhibit vulval muscle excitability under state-state conditions, activated Gαo can signal in these cells to induce a mild but significant inhibition of cell activity and egg-laying behavior.

The uv1 cells synthesize and release neurotransmitter tyramine and neuropeptides encoded by genes nlp-7 and flp-11 which inhibit egg laying (Alkema et al., 2005; Collins et al., 2016; Banerjee et al., 2017). Blocking neurotransmitter release from the uv1 cells by transgenically expressing Tetanus Toxin (a protease that cleaves synaptobrevin) resulted in a hyperactive egg laying phenotype, supporting a role for neurotransmitter and peptides release by the uv1 signaling to inhibit egg laying (Jose et al., 2007). In the same study the authors found that a GOA-1::GFP fusion reporter is expressed in the uv1 cells in addition to all egg laying neurons and vulval muscles (Jose et al., 2007). Does Gαo regulate neurotransmitter release from uv1? Based on the function of Gαo signaling in inhibiting neurotransmitter release in neurons, I would expect that inhibition of Gαo would enhance the release of inhibitory tyramine and neuropeptides from uv1, reducing egg laying, and increasing egg accumulation. Surprisingly, previous work has shown that transgenic expression of Pertussis toxin in uv1 cells caused a
hyperactive phenotype, similar to the blocking of neurotransmitter release by Tetanus toxin (Jose et al., 2007). A caveat of these experiments was that the expression of Tetanus Toxin and Pertussis Toxin was driven by the ocr-2 gene promoter which in addition to the uv1 cells is also expressed in the utse (uterine-seam) associated cells and head sensory neurons which may additionally regulate egg laying behavior.

I used the tdc-1 promoter to test whether loss of Gαo in uv1 showed a similar activation of egg laying. As shown in Figure 5.4B, I observed a quantitatively significant decrease in egg accumulation (10.9±1.5) compared to control, mCherry-expressing transgenic animals (15.3±1.2). This hyperactive phenotype was not nearly as strong as published data using the ocr-2 promoter (Jose et al., 2007), suggesting that Gαo may signal in sensory neurons and/or utse to inhibit egg laying. I also tested how elevated Gαo signaling in uv1 affects egg laying. I used the tdc-1 promoter to transgenically express the activated GOA-1(Q205L) mutant in uv1 cells, but I found no quantitative differences egg accumulation (15.5±2.1 eggs; Fig. 5.4B). Gαo has been proposed to signal in parallel to heteromeric TRPV channels (which may be directly mechanically gated) to regulate uv1 transmitter release, but the relationship between uv1 mechanical activation, TRPV channel activity, and Gαo signaling is yet to be determined (Jose et al., 2007). Genetic epistasis experiments using the strongly hyperactive ocr-2(vs29) TRPV channel mutant and transgenes described here could help determine whether the Gαo and TRPV act in the same or parallel pathways (Huang and Sternberg, 2006).
Fig. 5.4. $G_{\alpha_o}$ signaling in the vulval muscles and uv1 neuroendocrine cells also regulates egg laying (A) Scatter plots show average number of eggs retained by wild-type animals (grey closed circles), hypomorphic loss-of-function goa-1 mutants (green closed circles), egl-10(md176) null mutants (orange closed circles), and transgenic animals in which Pertussis Toxin (blue open circles) and GOA-1$^{Q205L}$ (grey open circles) was expressed in the vulval muscles (red open circles) under the ceh-24 gene promoter. (B) Scatter plots show average number
of eggs retained by wild-type animals (grey closed circles), goa-1 mutants (green closed circles), egl-10 mutants (orange closed circles), and transgenic animals in which Pertussis Toxin (blue open circles) and GOA-1Q205L (grey open circles) was expressed in the uv1 neuroendocrine cells (red open circles) under the tdc-1 gene promoter. Five extrachromosomal arrays were generated for each transgene and 10 animals from each extrachromosomal array were used (n=50). Error bars indicate 95% confidence intervals for the mean. Asterisk indicates p<0.0001 (One-Way ANOVA). Total animals for each strain in (A) and (B) were as follows: wildtype (n=30), goa-1(n1134) hypomorphic mutant (n=30), egl-10(md176) null mutants (n=30), HSN::GOA-1Q205L (n=30), transgenic animals expressing mCherry (n=50), mCherry plus Pertussis toxin (n=50), and mCherry plus GOA-1Q205L in the vulval muscles, and transgenic animals expressing mCherry (n=50), mCherry plus Pertussis toxin (n=50), and mCherry plus GOA-1Q205L (n=49) in the uv1 cells.

5.3. Major Findings and Discussion

Using a combination behavior assays and Ca2+ imaging, I found that the major G protein, Goa, regulates the duration of the inactive behavior states as well as activity in the egg-laying behavior circuit. Mutations that reduce Goa signaling in the whole animal and cell-specifically in the HSN neurons resulted in a shortening of the duration of the inactive behavior state and more frequent egg laying. In these animals, I observed that eggs were not laid in the clusters typically seen for wild-type animals (3-4 eggs per active state). Instead, egg-laying active states in hyperactive animals were typically of a single egg. Conversely, mutations that increased Goa signaling caused infrequent egg laying with longer inactive behavior states. Ca2+ imaging of HSNs in the hyperactive animals revealed strong
burst-firing both in and out of the egg-laying active state. This suggests that loss of G\(_{\alpha_0}\) signaling in HSN induces egg-laying as soon as they are deposited next to the vulva.

What accounts for the observed difference in the HSN Ca\(^{2+}\) activity levels between the goa-1 null mutants and transgenic animals expressing Pertussis Toxin in the HSNs? The mutations in goa-1 reduces G\(_{\alpha_0}\) signaling in the entire animal, and G\(_{\alpha_0}\) is expressed in all neurons and many muscles in C. elegans. This causes increased neurotransmitter release from motor neurons including the HSNs as well as cholinergic neurons including VA/VB/VC that synapse onto the vulval muscles to regulate egg laying (Nurrish et al., 1999; Tanis et al., 2008). Interestingly, mutations which disrupt the development of the VC neurons or their acetylcholine release show increased egg laying (Bany et al., 2003; Ringstad and Horvitz, 2008). In addition, my findings in Chapter 4 indicate that loss of neurotransmitter release from the VCs also increases HSN activity. The VC neurons express NLP-7 and FLP-11 neuropeptides which signal to inhibit HSN activity and egg laying (Banerjee et al., 2017). Increased neurotransmitter and inhibitory peptide release from inhibitory neurons like the VCs might cause result in enhanced inhibitory signaling onto HSN that does not reach a similar level of activity as seen in Pertussis toxin-silenced HSNs and the goa-1(sa734) G\(_{\alpha_0}\) null mutant. Also, goa-1(n1134) mutant only reduces G\(_{\alpha_0}\) protein localization to the membrane, leaving other G\(_{\alpha_0}\) functions intact (Solis et al., 2017). Pertussis Toxin catalyzes the ADP-ribosylation of G\(_{\alpha_0}\), eliminating its proper function only in HSN. Thus, Pertussis Toxin is predicted to eliminate inhibitory G\(_{\alpha_0}\) signaling completely,
explaining the stronger HSN activity phenotype that mimics the \textit{goa-1(sa734)} $\alpha_o$ null mutant. \textit{goa-1(n1134)} and animals expressing Pertussis Toxin in the HSN neurons have identical behavior despite differences in circuit activity. A possible explanation for this could be that animals reach a behavioral steady state based on the number of eggs stored in the uterus. In order to test if the circuit itself is more ‘effective’ in null mutants or transgenic animals, we would need to use a reversible vulval muscle silencing approach (Chapter 4) to test which strains lay their accumulated eggs the fastest.

Although GOA-1 appears to be the sole ortholog of $\alpha_o$ in \textit{C. elegans}, two distantly related $\alpha_o$ homologs, GPA-16 and GPA-7, show some functional redundancy with GOA-1 and are co-expressed with GOA-1 in some neurons (mostly sensory neurons) (Jansen et al., 1999). However, these proteins do not seem to contain residues which might be substrates for Pertussis Toxin (Jansen et al., 1999), and the effects of altering their function on egg laying is not clear. Is the stronger effect of Pertussis toxin (which also enhances cAMP synthesis via the $\alpha_s$ pathway) also a consequence of increased cAMP synthesis? Mutations that increase $\alpha_s$ signaling in \textit{C. elegans} leads to smoothly hyperactive sinusoidal locomotion, similar to that seen in $\alpha_o$ mutants (Mendel et al., 1995; Segalat et al., 1995; Schade et al., 2005). This effect is due to the increase in acetylcholine release from cholinergic motor neurons (Schade et al., 2005). In the ALA neurons, downstream activation of PKA by $\alpha_s$ signaling can also induce dense-core vesicle release (Zhou et al., 2007). However, $\alpha_s$ and adenylate cyclase gain-of-function mutants do not show the strong hyperactive egg-laying behavior phenotypes seen
after loss of $\mathrm{G} \alpha_\text{o}$. Future experiments to how mutations that increase $\mathrm{G} \alpha_s$ signaling and the cell-specific expression of Cholera Toxin (which activates $\mathrm{G} \alpha_s$ signaling) affect egg-laying behavior should reveal whether $\mathrm{G} \alpha_\text{o}$ antagonizes $\mathrm{G} \alpha_s$ signaling in addition to its role in inhibited $\mathrm{G} \alpha_q$.

A critical finding from this study is that $\mathrm{G} \alpha_\text{o}$ depresses HSN excitability and egg-laying behavior under steady-state conditions, even in what were thought to be optimal laboratory conditions strongly favorable for egg laying. Previous work has focused mainly on the role of $\mathrm{G} \alpha_\text{o}$ in inhibiting egg laying in the context of inhibitory peptides released under specific, noxious conditions such as increase $\mathrm{CO}_2$. The dramatic increase in burst firing in animals where $\mathrm{G} \alpha_\text{o}$ signaling is abolished in the HSNs suggests that unidentified neurotransmitters and/or neuropeptides activate $\mathrm{G} \alpha_\text{o}$-coupled receptors which signals to reduce HSN excitability to maintain the inactive egg-laying behavior state. Therefore, $\mathrm{G} \alpha_\text{o}$ does not merely antagonize $\mathrm{G} \alpha_q$ to promote indirect changes in presynaptic UNC-13 localization (Nurrish et al., 1999) and/or long-term changes in serotonin biosynthesis (Tanis et al. 2008). Which ligands act via $\mathrm{G} \alpha_\text{o}$ to promote the inactive state? In the HSN neurons, EGL-6 and the gustatory EGL-47 receptor are thought to activate $\mathrm{G} \alpha_\text{o}$ (Moresco and Koelle, 2004; Ringstad and Horvitz, 2008; Emtage et al., 2012). While the ligand for the EGL-6 receptor are the peptides encoded by the $\text{flp-17}$ gene which are released by $\mathrm{CO}_2$-sensing BAG neurons, the ligands for EGL-47 are not clear. Animals lacking $\text{egl-6}$ and $\text{egl-47}$ have grossly wild-type egg-laying behavior supporting a model where convergent inhibitory $\mathrm{G}$ protein coupled receptors (GPCR) signal through $\mathrm{G} \alpha_\text{o}$ to depress HSN activity to maintain the egg-
laying inactive state. Identification of all receptors expressed in the HSNs and the ligands they bind to will be the next important step in understanding how signaling through $\mathrm{G}_\alpha_0$ reduces HSN excitability and neurotransmitter release. This inhibition is overcome when the stretch homeostat (described in Chapter 4) activates the HSNs in a retrograde manner by increasing vulval muscle excitability and Ca$^{2+}$ activity. Other excitatory signals that act through the downstream $\mathrm{G}_\alpha_q$ pathway likely promote HSN excitability, but the receptors and ligands which signal through this pathway are yet to be determined.

Are the inhibitory effects of $\mathrm{G}_\alpha_0$ also mediated in part by the inhibition of the synthesis of cyclic nucleotides such as cAMP and cGMP? cAMP activates protein kinase A (PKA) which phosphorylates unknown downstream effectors which augment neurotransmitter release (Koelle, 2016). Mutations that increase $\mathrm{G}_\alpha_s$ signaling in C. elegans cause smoothly sinusoidal hyperactive locomotion distinct from the deep body bends induced by the loss of $\mathrm{G}_\alpha_0$ (Schade et al., 2005; Charlie et al., 2006b). The effects of increased $\mathrm{G}_\alpha_s$ signaling on the egg-laying circuit and any possible suppression of this pathway by the $\mathrm{G}_\alpha_0$ signaling is unclear. Interestingly, preliminary data shown in Figure 5.5 shows that animals carrying dominant, gain-of-function mutations in $\textit{gsa-1(ce81)}$ which increase $\mathrm{G}_\alpha_s$ signaling, animals bearing a partial loss-of-function mutation in $\textit{pde-4(ce268)}$, reducing the activity of a cAMP-specific phosphodiesterase, animals carrying a dominant gain-of-function mutation in $\textit{acy-1(ce2)}$ conferring increased adenyl cyclase activity, $\textit{kin-2(ce179)}$ loss-of-function mutant animals lacking the PKA regulatory subunit, and $\textit{pde-2(qj6)}$ null mutants which lack the enzyme PDE-2 which hydrolyzes cyclic
nucleotides all showed a slight hyperactive egg-laying phenotype. Future work will be necessary to test whether the reduced egg accumulation is caused by an increase in the frequency if egg laying or egg production. If it is the case that these mutants are indeed hyperactive, I will test if increased Ga₉ signaling in the HSN neurons can suppress this effect. Another future goal of this study will be to test the effects of global increases in cAMP synthesis on egg-laying circuit activity in addition to behavior.

**Fig. 5.5. Increased Ga₉ signaling causes hyperactive egg laying.** Scatter plots show average number of eggs retained by wild-type animals (grey circles), hypomorphic loss-of-function goa-1(n1134) mutants (green circles), egl-10(md176) null mutants (orange circles), and in mutant animals with increased cAMP signaling: dominant gain-of-function gsa-1(ce81) mutants (brown open circles), partial loss-of-function pde-4(ce268) mutants (blue open circles), dominant gain-of-function acy-1(ce2) mutants (purple open circles), loss-of-function kin-2(ce319) mutants (green open circles), and pde-2(qj6) null mutant animals (pink open circles). N≥40 for wildtype, egl-10(md176) mutants, and the
Ga\textsubscript{s} mutants. N=15 for goa-1(n1134) mutant animals. Error bars indicate 95% confidence intervals for the mean. Asterisk indicates p<0.0001 (One-Way ANOVA). Total animals for each strain were as follows: wildtype (n=60), goa-1(n1134) hypomorphic mutant (n=15), egl-10(md176) null mutants (n=30), gsa-1(ce81) (n=40), pde-4(ce268) (n=40), acy-1(ce2) (n=40), kin-2(ce319) (n=40), and pde-2(qj6) (n=40).
Chapter 6

Perspectives and Future Directions

6.1. Role of Early Activity in the Egg-laying Circuit

The development of neural circuits represents a highly dynamic period where cell fate and neurotransmitter specification occur coincident with morphological changes, synaptic development, and the maturation of cell activity patterns prior to the onset of behaviors. The ways in which cells in circuits communicate with and signal to each other also change during development. Internal and external sensory signals are integrated at appropriate timepoints to ensure that mature patterns of activity drive behaviors in the appropriate context. In Chapter 3, I examined the timing of key molecular changes and how they correlate with changes in Ca\textsuperscript{2+} activity in individual cells of the egg-laying circuit (Fig. 3.7).

The HSN neurons show Ca\textsuperscript{2+} activity 1-2 hours after synaptic development is complete (early-mid L4) at a stage when they robustly express the neuropeptide NLP-3, but only weakly express neurotransmitter serotonin. The vulval muscles show activity during the late L4 stages as they continue to undergo morphological development and complete the postsynaptic vm2 muscle arm extension prior to the L4-Adult molt. Vulval muscle Ca\textsuperscript{2+} transients in juveniles do not correlate with HSN Ca\textsuperscript{2+} transients showing that activity in the pre and postsynaptic cells can occur independently during development. The development of postsynaptic muscle activity has not received much attention in previous studies looking at
motor circuit development. Given that early movements (fictive behaviors) shown by animals are usually myogenic in nature (Crisp et al., 2008), it is important to understand if and how myogenic events influence circuit development. In both the HSNs and vulval muscles, spontaneous activity seen during juvenile stages disappears and gives rise to a two-state activity, likely driven by changes in the expression of ion channels that control cell excitability. The VCs and uv1 cells show activity during egg laying but not during the L4 stage, indicating that activity in these cells is dependent on the induction of the active behavior state. Hence, Ca\textsuperscript{2+} activity in the cells of the *C. elegans* egg-laying circuit develops asynchronously.

An important question that needs to be addressed is the how early activity in the HSN neurons and vulval muscles is driven in juveniles. Since I observe no correlation between presynaptic HSN Ca\textsuperscript{2+} and postsynaptic vulval muscle Ca\textsuperscript{2+} activity at the L4, it is possible that early muscle activity is driven by synaptic input from the cholinergic neurons (VA7, VB6, VD7) neurons but when these synapses form and become functions is not clear. Another way to test if early vulval muscle activity is neurogenic would be use Histamine-gated chloride channels to silence all neuronal activity during L4s and look at how vulval muscle Ca\textsuperscript{2+} activity is affected. L4 Ca\textsuperscript{2+} activity in both the HSNs and vulval muscles could be driven by permissive signals from the germline and one could test this hypothesis by testing whether FUDR treatment or the *glp-1* germline mutation affects the rhythmic activity seen in L4. It is possible that early activity is an intrinsic property of cells at this stage perhaps due to their moderately depolarized membrane potentials. Or it
could be driven by the release of Ca\(^{2+}\) from intracellular stores through the activation of IP\(_3\) receptors as a consequence of Ga\(_q\) signaling.

In adults, the HSN neurons drive an increase in locomotion during the active state by influencing the AVF interneuron (White et al., 1986; Hardaker et al., 2001) and an increase in HSN Ca\(^{2+}\) causes a reduction in defecation events (Fig. 3.4). In juveniles, although we did not observe a correlation between HSN Ca\(^{2+}\) activity and locomotion, we did find an inverse correlation between increased HSN Ca\(^{2+}\) and defecation events. Since I was unable to observe any effect of optogenetically activating HSNs on the defecation motor program, further experiments will need to be performed looking at mutations that affect the HSN neurons and defecation behavior to test how these two expulsive behaviors influence each other. Optogenetically driving activity in the excitatory GABAergic AVL motoneuron which drives defecation and also makes (and receives) synapses with the HSN neurons could answer how the defecation motor program affects egg laying and vice versa. Neuromodulatory and sensory cues have been shown to ‘reconfigure’ neural circuits such that neurons that usually drive one motor rhythm can drive a completely new motor rhythm by the effects of neuromodulators in altering relative synaptic strengths (Marder, 2012). Future experiments looking at how the ability of the HSNs to drive changes in animal locomotion, arousal, and egg laying behaviors is correlated with synaptic development and sensory signals will help us understand if the HSNs function as serotonergic arousal neurons capable of regulating behaviors in addition to egg laying.
Silencing early activity in the HSN and vulval muscles using the HisCl approach did not reveal a significant delay in the onset of egg laying or the rate of egg-laying behavior. This could either be due to insufficient silencing by the HisCl channels because they become sensitized and inactivated after prolonged exposure to histamine. In order to circumvent sensitization, other approaches to silencing such as the light-activated hyperpolarizing channel Halorhodopsin and a pulsed blue light stimulation could be used as an alternative as described (Warp et al., 2012). An important factor to consider when using chloride channels to silence cells during development is the switch in the direction of Cl⁻ ions from cells which occurs during development. In mammals, the upregulation of potassium chloride cotransporter (KCC) establishes Cl⁻ gradients necessary for GABA-gated Cl⁻ channels to hyperpolarize neurons in adult circuits (Hubner et al., 2001). In the HSN neurons, the C. elegans KCC-2 is upregulated during synaptic development to establish Cl⁻ gradients to mediate the inhibitory effects of inhibitory neurotransmitters coupled to hyperpolarizing Cl⁻ channels (Tanis et al., 2009). Therefore, expressing channels that provide Cl⁻ influx during development may not be the best approach to silence early activity in neural circuits. The ectopic expression of dominant mutant K⁺ channels which lead to hyperpolarization is another approach (Paradis et al., 2001), but a disadvantage of this method is that it does not allow for temporally restricted silencing during critical activity windows.

My findings could alternatively support the existence of homeostatic mechanisms which regulate spontaneous activity in the developing circuit similar to a recent study in the auditory system (Babola et al., 2018). The bursts of
spontaneous activity observed in the Spiral Ganglion neurons (SGN) are driven by glutamate release from the cochlear hair cells, and mutations that affect glutamate release result in deafness in mice and progressive hearing loss in humans (Ruel et al., 2008; Seal et al., 2008). Remarkably, in Vglut3−/− KO mice spontaneous bursts of activity in isofrequency zones is preserved at early stages because of a homeostatic increase in the excitability of the SGNs (Babola et al., 2018). Similar mechanisms might play a role in the egg-laying circuit which lead to the maintenance and quick recovery of circuit activity during and after periods of acute silencing.

6.2. The Egg-laying Circuit Functions as a Stretch Homeostat

In Chapter 4, I describe how the adult egg-laying circuit is activated by the accumulation of unlaid eggs in the uterus. The circuit having completed its development during L4 remains in a non-functional state until it receives feedback of egg accumulation. My results support the hypothesis that circuit activity is driven by sensory signals that detect egg accumulation and is not merely coincident with circuit age and maturity. The accumulation of eggs in young adult animals renders the vulval muscles responsive to presynaptic HSN input. Stretch stimuli have been shown to induce transcriptional changes in other cells (Mitchell et al., 2004; Terzidou et al., 2005), but it remains to be studied if mechanical stretch due to the accumulation of unlaid eggs lead to transcriptional changes in the egg-laying musculature. Egg accumulation promotes and homeostatically scales levels of
HSN $\text{Ca}^{2+}$ activity and burst firing. This happens via the regulation of vulval muscle excitability suggesting a retrograde signaling mechanisms which operates at the synapse. To further support this hypothesis, we see the postsynaptic vulval muscle arms regulate HSN burst firing such that mutants lacking muscle arms show reduced HSN $\text{Ca}^{2+}$ and no burst firing.

How similar or different is the egg-laying circuit in comparison with other stretch sensory neural circuits? Mechanosensory stretch is an important source of sensory feedback, necessary to initiate and/or modulate activity in neural circuits regulating important physiological functions such as the maintenance of blood pressure, micturition reflex, parturition reflex, and peristalsis in the gut. The neuroendocrine reflex required during parturition, also called the Ferguson reflex, is one of the best understood examples of positive feedback in animal physiology. During labor, fetal distension is detected by specialized mechanosensory neurons, which carry sensory information to the hypothalamus (Ferguson, 1941; Debackere, 1961; Johnson, 2013). This leads to oxytocin release from the pituitary promoting a feed-forward increase in uterine contraction and parturition. What is not well understood is how the mechanosensory neurons are activated, their effects on downstream circuits in the brain, and how these neurons are silenced following parturition. The egg-laying circuit provides a model to understand the how stretch initiates, sustains, and the terminates of activity in the circuit.

In flies, distension of the internal reproductive tract induces a behavioral preference for acetic acid seen when female flies are actively laying eggs (Gou et al., 2014). Specialized Piezo channel-expressing neurons whose dendrites tile the
surface of the reproductive tract sense uterine stretch induced by egg accumulation in the tract. They project to sensory regions in the brain to mediate behavioral changes but the mechanisms by which this occurs is unclear. The HSN neurons possibly function like Piezo neurons described above via the synapses they make in the nerve ring. They could promote and sustain forward locomotion bouts to help the animal navigate towards favorable environments during egg laying by signaling to locomotor interneurons such as the AVF and other sensory neurons in the head. In the egg-laying circuit, although we have the tools to understand effects on circuit activity, what remains to be looked at is the expression patterns of mechanosensory channels/molecules in the cells of the circuit, particularly in the uterine and vulval muscles.

Many mechanosensory circuits possess specialized classes of sensory neurons responsible for detecting stretch and activating downstream motor circuits. In the gut, specialized stretch-sensitive interneurons provide ascending excitatory and descending inhibitory inputs to downstream motor neurons which generate peristaltic neural reflexes (Spencer and Smith, 2004). Does the egg-laying circuit possess such specialized mechanosensory cells? The VC motor neurons share key functional features of sensory neurons and interneurons which modulate CPG rhythms in other circuits. VC extends several non-synaptic processes along the vulval hypodermis which could be mechanically activated by vulval muscle contraction (White et al., 1986; Colavita and Tessier-Lavigne, 2003). Recent studies in our lab have demonstrated that optogenetic activation of vulval muscle Ca^{2+} activity and contractions can stimulate the VC neurons (unpublished).
The VC neurons make synapses onto both the vm2 vulval muscles and the body wall muscles. VC Ca\(^{2+}\) activity peaks at the moment of vulval muscle contraction, but optogenetic activation of the VCs fails to elicit egg laying events and instead slows locomotion. Moreover, VC- and acetylcholine-defective mutants show increased egg laying (Bany et al., 2003; Ringstad and Horvitz, 2008), suggesting a loss of inhibitory feedback. Thus, the VCs, instead of releasing acetylcholine at the vm2 synapse to drive vulval muscle contraction and egg laying itself, may function in part as baroreceptors to slow locomotion during egg release (Collins et al., 2016).

In our model for the activation of the egg-laying circuit in Chapter 4 (Fig. 4.6), I proposed a key role for the uterine muscles in transducing the stretch stimulus. Excitation in these muscles is relayed to the vm2 vulval muscles via gap junctions to increase vm2 excitability. Visualizing uterine muscle Ca\(^{2+}\) activity during the active state and in response to increased reproductive stretch could clarify their role as critical stretch sensors in the egg-laying circuit. In addition to the VC neurons, the uv1 neuroendocrine cells are mechanically deformed and activated by the passage of eggs through the vulva and feedback inhibit egg laying (Collins et al., 2016). Unpublished data from the lab indicate that egg release is not required for uv1 mechanical activity, occurring during strong bouts of vulval muscle contractility and during male spicule insertion during copulation. Subunits of the TRPV channels are expressed in the uv1 cells and are thought to transduce mechanosensory stimuli (Jose et al., 2007), but this remains to be formally tested. Hence, multiple cells of the egg-laying circuit such as the VC neurons, uv1 cells,
and the muscle cells (uterine and vulval muscles) may respond to different kinds of mechanosensory/stretch stimuli using very different mechanisms so as to initiate, sustain, and inhibit circuit activity during behaviors.

6.3. Candidate Retrograde Signaling Molecules in C. elegans

In Chapter 1, I reviewed briefly some of the known retrograde trans-cellular signaling molecules that function in neural circuits in a range of organisms. A key finding in Chapter 4 was that the degree of uterine stretch scales presynaptic HSN Ca\(^{2+}\) activity in a retrograde manner that depends on postsynaptic vulval muscle activity. The reduced frequency of Ca\(^{2+}\) activity and the absence of high frequency burst firing in the HSN neurons in animals lacking postsynaptic vm2 muscle arms suggests a retrograde signal from the vulval muscles to the HSNs maintains the active state. What are some possible candidate molecules and/or signaling systems which could be functioning in this circuit?

C. elegans provides an excellent model system to genetically dissect retrograde signaling pathways (Doi and Iwasaki, 2002). Mutations that affect presynaptic development and the clustering of acetylcholine receptors induce abnormal sprouting in the SAB cholinergic motor neurons indicating that reciprocal communication between neurons and muscles plays a role during development in C. elegans (Zhao and Nonet, 2000). A novel protein, AEX-1, which is homologous to presynaptic protein UNC-13 and contains a Ca\(^{2+}\) binding domain was found to regulate retrograde signaling at neuromuscular junction in C. elegans (Doi and
Iwasaki, 2002). Mutations in \textit{aex-1} cause altered defecation rhythms and abnormal presynaptic UNC-13 localization at neuromuscular junctions, in addition to producing a mild egg-laying defect. Defects in defecation behavior and synaptic clustering defects were rescued by \textit{aex-1} expression in the postsynaptic intestinal muscles but not in neurons. AEX-1 was found to be a component in the exocytic secretory pathway in postsynaptic muscles to release factors that promote presynaptic activity and transmitter release in a G\textsubscript{q}-dependent manner.

Defecation and egg laying are regulated by common signaling proteins (Reiner and Thomas, 1995). Could AEX-1 function as a retrograde signal in the context of egg laying? GFP expression under a truncated promoter for the \textit{aex-1} gene failed to show expression in the vulval or uterine muscles but whether the full-length promoter drives expression in the vulval muscles needs to be determined. I hypothesize that if the retrograde signal in the egg-laying circuit is a molecule that is exocytosed, the transgenic expression of Tetanus Toxin, which cleaves the vesicle associated membrane protein synaptobrevin, should inhibit vesicle release and affect egg laying (Link et al., 1992). To my surprise, the transgenic expression of Tetanus Toxin in the vulval muscles did not produce any effect on egg laying (Fig. 6.1) indicating that another mechanism of signaling system regulates retrograde signaling in the egg-laying circuit.
**Fig. 6.1. Effect of blocking synaptic transmission in vulval muscles.** Plots show number of unlaid eggs stored in utero in transgenic animals expressing only mCherry (grey circles) or Tentanus toxin along with mCherry (orange circles) in the vulval muscles using the ceh-24 gene promoter. Error bars indicate means with 95% confidence intervals. $P=0.2197$ (Student’s t test). Number of animals used for analysis for the two groups are as follows: ceh-24::mCherry ($N=49$) and ceh-24::mCherry plus Tetanus toxin ($N=40$).

In *C. elegans*, a micro-RNA miR1 has been shown to function in body wall muscles to inhibit presynaptic acetylcholine release from cholinergic motor neurons by regulating levels of muscle-specific transcription factor MEF-2 which controls a retrograde signal (Simon et al., 2008). Trans-synaptic Neurexin-neuroligin interaction at the neuromuscular junction has also been shown to repress ACh release by inhibiting the function of presynaptic UNC-2/CaV2 channels (Tong et al., 2017). TGFβ is part of a large family of secreted peptide growth factors in metazoans that includes the Bone Morphogenetic Proteins...
(BMPs) in vertebrates (Shi and Massague, 2003). Signaling by the TGFβ family is required during neural development and proper functioning of the nervous system throughout life (Meyers and Kessler, 2017). In C. elegans, TGFβ signaling regulates sensory signaling, dauer formation, cell migration, body size regulation (Patterson and Padgett, 2000; Gumienny and Savage-Dunn, 2013). Mutations the TGFβ signaling components also produce egg-laying defects (Trent et al., 1983; Schafer, 2006). It is however not clear if TGFβ signaling acts directly on the cells of the egg-laying circuit, in sensory cells which signal to modulate egg-laying circuit activity, or instead function in additional developmental processes necessary for the proper maturation of fertile females. Identification of the cells required for release of TGFβ and the sites of expression of TGFβ receptors should inform whether TGFβ acts in function or development.

Lipid messengers such as endocannabinoids regulate presynaptic release during certain forms of long-term depression (LTD) (Kano, 2014). In C. elegans, cannabinoids activate monoaminergic signaling to modulate behaviors such as locomotion and nociception (Oakes et al., 2017). Cannabinoid receptor agonist 2-arachidonoylglycerol (2-AG) causes a global increase in serotonin levels (Oakes et al., 2017). Future experiments examining the role of 2-AG in promoting HSN activity and their expression patterns could clarify their role as retrograde synaptic messengers in the egg-laying circuit. There are no reports describing if C. elegans utilizes gaseous retrograde messengers such as nitric oxide (NO) or carbon monoxide (CO). Overall, the genetically tractable egg-laying neural circuit would serve as an excellent model to facilitate the discovery of novel retrograde synaptic
regulators. Forward genetic screen screens can also be used to identify genes required for burst-firing in HSN during sustained periods of egg-laying behavior, for example after optogenetic activation of egg laying. Examining how HSN activity is affected due to mutations or knockdown of known genes required for lipid signaling, trans-synaptic communication, and the trafficking of post-synaptic components to the vulval muscle arms could similarly unveil novel retrograde synaptic regulators.

6.4. Gαo Signaling in Regulating Neural Excitability in Circuits

Gαo is the most abundant G protein in the mammalian brain but its functional importance in the CNS is not well defined. Gαo is highly conserved across species and the C. elegans orthologue is >80% identical to the mammalian ortholog (Koelle, 2016). Interestingly, behavioral abnormalities associated with mutants lacking Gαo bear remarkable similarities. Mice lacking Gαo have reported several neurological deficits, including tremors, seizures, hyperactive behaviors, hyperalgesia, and a repetitive turning behavior (Jiang et al., 1998). In humans, de novo mutations in GNOA-1, which encodes the Gαo subunit, have been implicated in epileptic encephalopathies, a group of neurological disorders that are characterized by progressive neurodevelopmental and motor impairments which are most likely caused by increased epileptic activity (Nakamura et al., 2013). The C. elegans ortholog of Gαo (GOA-1) is expressed in all the neurons and some muscle cells, including all the cells of the egg-laying behavior circuit (Koelle, 2016).
In Chapter 5, I looked at the role of Gα<sub>o</sub> in regulating cell and circuit excitability in the context of egg-laying behavior.

A key finding in Chapter 5 was that Gα<sub>o</sub> signaling modulates HSN neuronal excitability and neurotransmitter release. Previous studies have determined how G proteins regulate synaptic transmission and localization of presynaptic UNC-13 in cholinergic neurons assayed using aldicarb assays, imaging, and electrophysiological approaches (Miller et al., 1996; Miller et al., 1999; Nurrish et al., 1999; Miller and Rand, 2000; Hu et al., 2015). Similar increases in UNC-13S localization was not observed in the HSN neurons where it was clear that Gα<sub>o</sub> was functioning cell-autonomously (Tanis et al., 2008). In the HSNs, Gα<sub>o</sub> signaling can also have long term effects on serotonin biosynthesis and overall serotonin levels (Tanis et al., 2008). My work shows that Gα<sub>o</sub> plays a major role in suppressing HSN Ca<sup>2+</sup> to maintain the inactive state. I hypothesized two possible ways in which HSN excitability is suppressed in the HSN neurons by Gα<sub>o</sub> signaling: 1) Molecules signaling via Gα<sub>o</sub> maintain the HSN in a hyperpolarized state by influencing the activity of resting channels such as activating inward rectifying K<sup>+</sup> channels (such as IRK-1) or inhibiting Na<sup>+</sup> leak channels (such as TMC-1) and 2) Gα<sub>o</sub> signaling acts to antagonize the excitatory Gα<sub>q</sub> pathway which signals to promote HSN excitability and neurotransmitter release (Miller et al., 1996; Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999; Miller et al., 2000; Tanis et al., 2008).

Through a collaboration with the Lijun Kang lab in China, we were able to show that resting potentials were indeed altered in Gα<sub>o</sub> mutants (data not shown). Animals expressing Pertussis Toxin in the HSN showed significantly more
depolarized membrane potentials compared to wildtype control animals. In contrast, *egl-10* null mutants with increased \( \text{G}_\alpha_0 \) signaling showed significantly hyperpolarized resting membrane potentials. Although this supports our first hypothesis, it is still unclear how \( \text{G}_\alpha_0 \) signaling promotes HSN hyperpolarization under steady state. The activation of IRK-1, one of three inward rectifying \( K^+ \) channels of the Kcnj family, was found to inhibit HSN excitability downstream of the FLP-17 neuropeptides, the EGL-6 GPCR, and \( \text{G}_\alpha_0 \) (Ringstad and Horvitz, 2008; Emtage et al., 2012). *irk-2* and *irk-3* mutations, which encode additional IRK \( K^+ \) channel orthologs, did not appreciably cause an additional increase in HSN excitability (Emtage et al., 2012). However, this does not rule out the possibility that IRK-2 and IRK-3 might mediate the effects of yet undetermined \( \text{G}_\alpha_0 \)-coupled GPCRs, although that would suggest the receptors, their G proteins, and effectors act in distinct pools with distinct channel coupling. Other possible channels that might mediate the effects of \( \text{G}_\alpha_0 \) on resting membrane potential are the NCA-1 \( Na^+ \) leak channels (and the UNC-70 and UNC-80 NALCN proteins that regulate their function), other \( K^+ \) channels including ERG which is also expressed in HSN, or the hyperpolarization-activated CLH-3 \( Cl^- \) channel (Branicky et al., 2014).

Genetic epistasis experiments looking at which combinations of null mutants for the abovementioned resting channels can suppress the effect of increased \( \text{G}_\alpha_0 \) signaling can clarify the identity and specificity of \( \text{G}_\alpha_0 \) signaling through distinct effectors in the HSNs.

Does the suppression of HSN activity occur predominantly by the modulation of resting channels by \( \text{G}_\alpha_0 \) signaling as described above, is it possible
that this could be an indirect effect of the repression of the $\text{G}_\alpha_q$ signaling pathway by $\text{G}_\alpha_o$? One prediction would be that ligands that signal through $\text{G}_\alpha_o$ (but not $\text{G}_\alpha_q$) are produced at all times to maintain the inactive behavior state resulting in the inhibition of $\text{G}_\alpha_q$ through the recruitment of its RGS protein, EAT-16 (Hajdu-Cronin et al., 1999) thereby overpowering any signal that might promote HSN from reaching its depolarizing threshold. R7 RGS proteins like EGL-10 and EAT-16 have a unique structure that suggests the RGS proteins themselves may be a direct effector of $\text{G}_\alpha_q$ and $\text{G}_\alpha_o$, respectively (Cheever et al., 2008). RGS proteins have a $\gamma$-like domain (GGL) that interacts with a variant $\text{G}_\beta$ protein called GPB-2, that could form an unconventional heterotrimer with a specific $\text{G}_\alpha$ protein. Based on the opposite behavior phenotypes of each RGS protein and its G protein target, it is reasonable to propose that one effector for activated $\text{G}_\alpha_o$ could be a heterodimer of GPB-2 and EAT-16, forming an active heterotrimeric RGS effector complex which would function to inhibit $\text{G}_\alpha_q$ signaling. A similar complex with activated $\text{G}_\alpha_q$, GPB-2, and EGL-10 would then function to inhibit $\text{G}_\alpha_o$ signaling. In this way, the clear antagonism would result from the activation of the reciprocal RGS protein to term off the opposing pathway. Active state induction may involve the combined action of two or more elements: First, the release of hormones or neurotransmitters that activate HSN receptors that couple to $\text{G}_\alpha_q$ signal through lipid, $\text{Ca}^{2+}$, and Rho effectors that bring the HSN resting potential closer to threshold. Second, release of serotonin by HSN enhances vulval muscle excitability, which, in the presence of eggs in the uterus, triggers a rhythmic $\text{Ca}^{2+}$ transient and vulval muscle contractility that promotes the release of retrograde
signals that maintain HSN excitability and the egg-laying active state. At present, it’s not clear if the default state is ‘ON’ which is then inhibited tonically by Gαo only when unfavorable environmental conditions are removed. Alternatively, the default state could be ‘OFF’ with the circuit only activated when there is circuit feedback of eggs in the uterus. Integrating these findings with results from another ongoing study in our lab looking at the effects of the Gαq pathway on the HSNs could provide more evidence to help identify the signals other than egg accumulation that promote the circuit into its ‘ON’ state. Activation of the corresponding RGS protein and termination of the opposite G protein pathway would help stabilize one circuit outcome (e.g. either ‘ON’ or ‘OFF’), leading to the clear ~2 minute active and inactive behavior states observed in wild-type animals.

In light of the ideas presented above, determining the expression patterns of all GPCRs expressed in the egg-laying circuit and the G proteins through which they exert their effects could clarify how combinatorial parallel signaling defines the ‘activity state’ in of the circuit. Efforts towards this end are currently underway by expressing GPCR-GFP fusion constructs and creating detailed maps of GPCR (for neurotransmitters and neuropeptides) expression patterns in the cells of the egg-laying circuit (Michael Koelle, Yale University). Once we determine that a GPCR is expressed in the egg-laying circuit, how do we determine its function within the circuit? It has become clear that gene knockouts for GPCR genes have rarely been isolated through genetic screens. Most receptors identified genetically in C. elegans have been through gain-of-function mutations that hyperactivate the receptor; gene knockouts for those GPCRs, including egl-6 and egl-47, do not
grossly affect egg-laying behavior to the degree as the loss of the relevant G protein target. This suggests that cells like HSN express several GPCRs, and that cell excitability is gated by the integration of numerous inputs that signal through a common G protein. One way to circumvent this problem caused by an absence of clear phenotypes for the knockout mutant would be to cell-specifically overexpress candidate GPCR genes, mimicking the effects of a dominant mutation and then screen for induced egg-laying phenotypes. There are ~22 neurotransmitter and ~128 neuropeptide GPCR genes encoded in *C. elegans* (Koelle, 2016) making receptor overexpression a more effective approach than overexpressing specific neurotransmitters and peptides. Nevertheless, overexpressing neuropeptides such as NLP-3 and NLP-7 which produced clear egg-laying phenotypes (Banerjee et al., 2017; Brewer et al., 2019). Screens for receptor mutations that reduce these behavior defects caused by ligand over-expression may reveal how these molecules regulate egg-laying circuit activity. Confirming the interaction between ligand and receptors will require high-throughput *in-vitro* binding assays (Bender et al., 2002; Frooninckx et al., 2012; Caers et al., 2014).

Finally, a key motivation for this body of work has been to address a fundamental gap in our current understanding of how the egg-laying active state is induced. In Chapter 4, I discussed the role of the stretch homeostat and a vulval-muscle derived retrograde signal in inducing burst firing in the HSN neurons and the active behavior state. The homeostat works to increase HSN Ca$^{2+}$ activity, but in mutants with reduced HSN excitability such as *egl-6(dm)* and *egl-47(dm)*, the homeostat is unable to produce burst firing (Zang et al., 2017) suggesting that the
homeostat works in conjunction with other necessary sensory signals (likely coupled to $G\alpha_q$) to induce HSN burst firing. In fact, animals expressing Pertussis Toxin in the HSNs store only 1-2 eggs in the uterus at steady state and the HSNs show constitutive burst firing. Such a strong reduction in egg accumulation typically causes a dramatic inhibition of both HSN and vulval muscle $Ca^{2+}$ activity (Fig. 4.3). Loss of inhibitory $G\alpha_o$ signaling also causes animals to predominantly lay single eggs and rarely show clustered double or triple egg laying events. An important area of work is to determine how the circuit maintains is activity in hyperactive mutants even when there are few eggs in the uterus.

Major G protein, $G\alpha_o$, functions as a fast ON-OFF molecular switch owing to its rapid GDP to GTP exchange rate (necessary to activate signaling) because its rate of dissociation with GDP occurs about 3 times faster than $G\alpha_i$ (Jiang and Bajpayee, 2009). Additionally, the intrinsic GTPase activity (which determines signaling duration) of $G\alpha_o$ is about 3-7 times higher than that of $G\alpha_i$ (Jiang and Bajpayee, 2009). Taken together, these features may point to its unique role as a highly effective molecular signaling transducer to control rapid behavior transitions. As opposed to its PTX-sensitive sister protein $G\alpha_i$ which negatively regulates the $G\alpha_s$ pathway and cAMP synthesis, $G\alpha_o$ might be a highly specialized ancient protein that performs a broader role in inhibition of circuit activity in the regulation of behaviors which rely on tight control of activity levels in neural circuits.
Overall, in this dissertation, I have used a model motor circuit from the nematode worm to understand how mature patterns of activity develop that drive behaviors. My study is the first to describe in detail the relationship between morphological development, activity development, and behavior development in freely behaving animals. Previous work has shown that the individual cells of the egg-laying behavior circuit develop asynchronously and independent of each other. My work has shown that mature patterns of cell activity in the circuit also develop asynchronously. The HSNs and vulval muscles are the first cells to show activity in juveniles while the VC neurons and uv1 cells show activity only in egg-laying adults. I also observed that activity in the circuit during the first/early egg-laying events resembled activity observed in 24-30 hour old adult animals (data not shown). Thus, neural activity that drives behaviors in animals are robust and observed prior to the onset of behaviors.

In this study, I have also demonstrated how the presence of eggs is required for the activation of the circuit and for adult patterns of circuit activity. Other motor systems involving CPG circuits continue to maintain intrinsic patterns of activity even after sensory inputs are removed. But this does not seem to be the case in the egg-laying motor circuit, suggesting that different motor circuits can show variable degrees of dependence on sensory inputs to maintain robust levels of activity. Lastly, the mechanisms mediating the establishment, maintenance, and transitioning between opposing behavior states, particularly arousal states, is critical in neuroscience. Opposing behavioral states have been shown to be regulated by distinct neuromodulator systems which act through separate
downstream circuits to promote a given behavior state (Flavell et al., 2013). My study shows that opposing neuromodulators (along with other sensory or homeostatic signals) could also act at the level of a single command neuron whose differential activity can then initiate particular behavior states. My study has also established that the highly abundant major G protein, Gαo, is a key molecular mediator of behavioral switches by regulating the excitability and neurotransmitter release from command neurons and I speculate that it may have similar functions in vertebrates by controlling activity in neuronal populations (cholinergic or serotoninergic projections neurons for example) that influence global activity states in the brain.
References


Charlie NK, Schade MA, Thomure AM, Miller KG (2006a) Presynaptic UNC-31 (CAPS) is required to activate the G alpha(s) pathway of the Caenorhabditis elegans synaptic signaling network. Genetics 172:943-961.


