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INFERENCES OF GLIA MEDIATED CONTROL IN *CAENORHABDITIS ELEGANS*

by

STEPHANIE BOWLES

Under the Direction of Casonya Johnson, PhD

ABSTRACT

Glia cells are key components of the brain that mediate signaling events between pre- and postsynaptic neurons and that play a vital role in regulating behavior. Depressive disorders are characterized as complex, multifunctional mental disorders that lead to unstable and extreme fluctuation in mood and behavior. Previous studies have shown that loss of glial cells results in emotional and behavioral abnormalities. Here, we exploit the *Caenorhabditis elegans* model, an optimal system in which to study glia-type specific function because the structure and connectivity of the nervous system has been fully described and previous studies have demonstrated that loss of glia is not lethal and does not result in death of the associated neuron. Because of the predetermined cell lineage, *C.elegans* neurons do not require trophic support

from glia. This provides the advantage to separate the supportive role of glia and focus on understanding how glia regulate behavior.

My dissertation research aims to identify the influence of a glia-subtype, the Cephalic sheath glia (CEP_{glia}), and a glia specific basic helix-loop-helix (bHLH) transcription factor, HLH-17, in regulating complex and rhythmic behaviors. Complex behaviors integrate multiple sensory modulatory inputs to orchestrate a specific motor output. Similarly, rhythmic behaviors utilize an intrinsic pacemaker to modulate periodic activation of a stereotyped sequence of behaviors. Work from our lab demonstrates that the unique expression of HLH-17 in an astrocyte-like cephalic sheath (CEP_{glia}) is required to modulate dopamine-dependent behaviors such as swimming, egg laying, and paralysis; and that HLH-17 regulates the expression of genes required for mating and defecation. Results from my research suggest that expression of HLH-17 in the CEP_{glia} may be required for regulating the precision and accuracy of independent motor programs and that CEP_{glia} coordinate multiple motor responses. Findings from my work outline a hypothetical model by which astrocyte-like CEP_{glia} modulate the function of motor neurons, in part, by transmitting signals through interneurons to motor neurons that are required for behavior. Additionally, my dissertation research hints at a mechanism in which glia may exert sexually dimorphic regulation of a rhythmic behavior.

INDEX WORDS: Glia, Cephalic sheath glia, complex behavior, rhythmic behavior, interneuron

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2020

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Office of Graduate Services

College of Arts and Sciences

Georgia State University

May 2020

DEDICATION

I dedicate my doctoral work to my son, my mother, and all my friends and family.

To my son- You helped me discover another level of strength and resilience throughout this journey that I did not know I had. Thank you for bringing so much joy into my life.

To my mother- You stood by me through some of my toughest hurdles and not once did you allow me to give up. You taught me to stand tall with my head high in the midst of every challenge. I am forever grateful for your love and sacrifice.

To my friends and family- Thank you for every encouraging word and act of kindness throughout this journey. Thank you for being my village as I adjusted to becoming a new mom, while working to complete my doctoral work.

I love you all.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Casonya Johnson, for believing in my ability as a scientist. Thank you for exceeding the expectation as a mentor to push me to grow both personally and professionally. I would also like to thank my dissertation committee members Dr. Dan Cox, Dr. Margo Brinton, and Dr. Sarah Pallas. Thank you for your probing questions, feedback, guidance, and support throughout this process.

I would like to thank my lab members and friends that helped me over the years: Chaquettea, Ling, Ke'Ara, Raymarie, Meagan, Thai, Asia, and Han-ting.

Additionally, I am thankful for the financial support provided to me by the Molecular Basis of Disease Program here at Georgia State.

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LIST OF ABBREVIATIONS

CEPglia	Cephalic sheath glia
C. elegans	*Caenorhabditis elegans*
bHLH	basic helix-loop-helix
GABA	Gamma Aminobutyric Acid
DMP	Defecation motor program
EMC	Enteric muscle contraction
pBoc	Posterior body wall contraction
aBoc	Anterior body wall contraction
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
CPG	Central pattern generator
OPC	Oligodendrocyte precursor cell
DVA	Cholinergic interneuron, cell bodies in dorsal rectal ganglion
DVC	Glutamatergic interneuron, cell bodies in dorsal rectal ganglion
DVB	GABAergic motor neuron/interneuron, cell body in dorsal rectal ganglion
AVL	Nerve ring and ventral cord interneuron/GABAergic motor neuron

1 GENERAL INTRODUCTION

1.1 Glia are essential for synaptic maintenance

There are several types of glia that occupy more than half of the human brain. Moreover, these glia are intimately associated with more than 100 trillion synaptic connections (Barres, 2008; Eroglu and Barres, 2010) and are essential for regulating synaptic plasticity (Clarke and Barres, 2013). The first line of evidence to suggest that astrocytes are important for synapse formation was studies using rodent retinal ganglion cells, which demonstrated that in the absence of glia, retinal ganglion cells fail to form excitatory synapses (Pfrieger and Barres, 1997). Additional studies *in vitro* indicated that in the absence of glia, fewer synapses formed in rodent hippocampal (Hughes et al., 2010; Xu et al., 2014), cortical (Diniz et al., 2012), and spinal motor (Ullian et al., 2004) neurons. In mammals, astrocytes regulate synapse formation, function, and plasticity by secreting thrombospondins (Christopherson et al., 2005) and Hevin/SPARC proteins (Kucukdereli et al., 2011), which interact with neurexin and neuroligin adhesion molecules (Clark and Barres, 2013). Astrocytes may secrete glypicans to strengthen and promote functional synapse formation (Allen et al., 2012; Farhy-Tselnicker et al., 2017). Secretion of Hevin/SPARC, thrombospondins, and glypicans from glial cells promotes the expression of AMPA glutamate receptors at postsynaptic terminals (Eroglu, 2009; Allen et al., 2012). Expression of astrocytic $GABA_A$ receptors influences inhibitory synapse formation (Liu et al., 1996; Hughes et al., 2010; Elmariah et al., 2005). Together, these studies demonstrate that glia utilize diverse signaling mechanisms to modulate synaptic activity.

Glia modulate synapse formation by eliminating weak and inappropriate synapses. Synapse elimination is executed by reactive astrocytes and microglia through phagocyte-driven

pathways (Reemst et al., 2016; Allen and Lyons, 2018; Filipello et al., 2018; Vainchtein et al., 2018). Mice retinal ganglion cells (RGCs) that no longer express phagocytic receptors retain an excess of unnecessary synapses (Chung et al., 2013). Microglia express brain-derived neurotrophic factor (BDNF) and cytokines such as interleukin 10 to regulate synapse formation. Oligodendrocyte precursor cells (OPCs), which are undifferentiated oligodendrocyte progenitor cells that have the ability to differentiate into oligodendrocytes or astrocytes (Wu et al., 2003; Marshall et al., 2005), have been implicated in regulating synapse formation. Both hippocampal and cerebellar OPCs express glutamate receptors and voltage-gated Na^+ channels (De Biase et al., 2010), form synapses with glutamatergic neurons (Bergles et al., 2000; Lin and Bergles, 2004), and receive input mediated by glutamatergic and GABAergic signaling (Eroglu and Barres, 2010).

Additional studies in invertebrate and vertebrate models suggest that glia mediated regulation of synapse formation is conserved. In *Drosophila* (Fuentes-Medel et al., 2012) and *Xenopus* (Feng and Ko. 2008), perisynaptic Schwann cells, found in the peripheral nervous system commonly modulate synapses at neuromuscular junctions, in part by secreting TGF-β (Brink et al., 2009; Strauss et al., 2015). In tadpoles, ablating perisynaptic Schwann cells significantly reduced synaptic growth, causing existing synapses to retract from the adjacent postsynaptic muscle (Reddy et al., 2003). In *C. elegans*, the unorthodox, glia-like GLR cells form gap junctions with the GABAergic RME neuron and, through a calcium dependent CDK-5 pathway, modulate axon specification of the neighboring RME neurons (Meng et al., 2016). Together, these studies outline the importance of glia function in the central nervous system.

1.2 Glia are active participants in intercellular signaling

Glial cells are important for brain homeostasis. They regulate the clearance and transport of ions from the extracellular space to the pre-and post-synaptic terminals and express transporters that assist with the uptake of excess neurotransmitters (Figure 1-1), such as glutamate, GABA, serotonin, and glycine (Sykova et al., 1974; Magistretti and Pellerin, 1999; Magistretti, 201, Christensen et al., 2013) in the synaptic cleft. The first line of evidence for active communication between glia and neurons was the discovery that astrocytes release gliotransmitters (Papura et al., 1994). Gliotransmitters are defined as chemical transmitters that are released from glial cells in response to neurotransmitters, chemical transmitters released from neurons (Bezzi and Volterra, 2001; Li et al., 2013). Activation of glutamate receptors in astrocytes triggers an influx of Ca^{2+} that, in turn, initiates the release of glutamate from the glia (Bezzi et al., 1998). Gliotransmitters released from astrocytes include ATP (Caciagli et al., 1988; Maienschein et al., 1999), adenosine (Caciagli et al., 1988), D-serine (Mothet et al., 2005; Henneberger et al., 2010; Martineau et al., 2014), GABA (Gallo et al., 1991), taurine (Pasantes and Schousboe, 1988), TNFα (Beattie et al., 2002), and eicosanoids (Xia and Zhu, 2011) (Figure 1). Glutamate, D-serine, ATP, and GABA released from glia modulate firing frequency and synaptic transmission of neurons (Halassa et al., 2007; Harada et al., 2016).

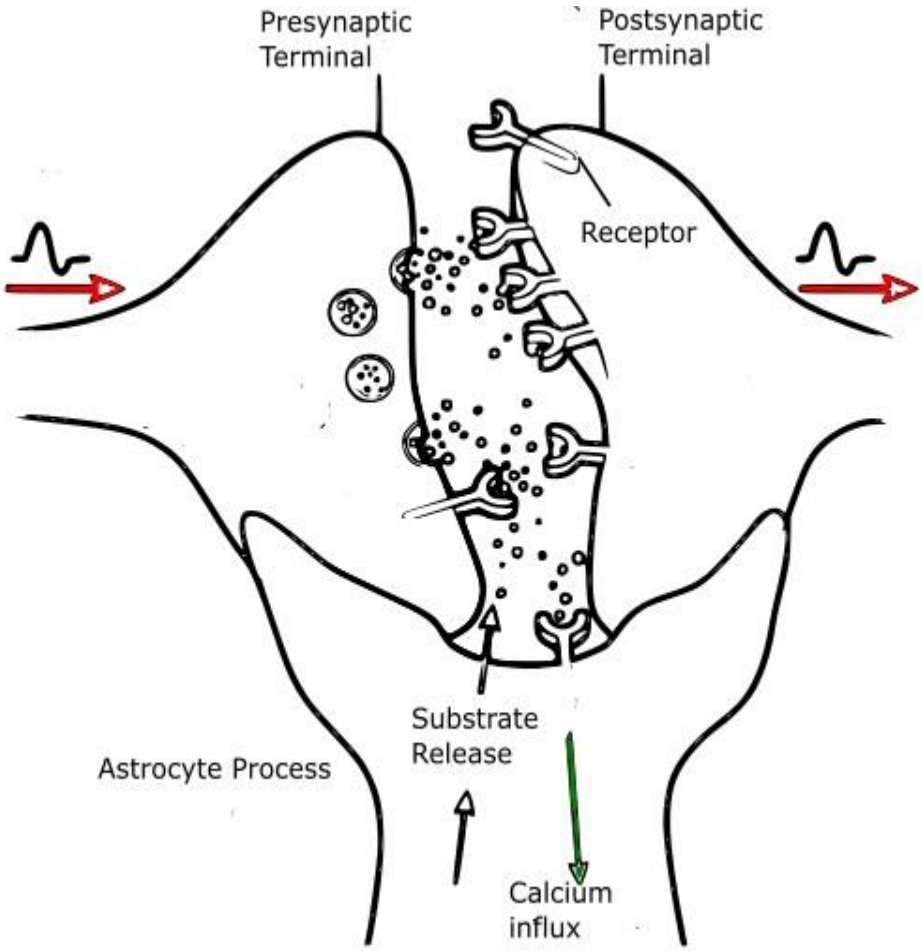


Figure 1-1Astrocytes are active participants in intercellular signaling.

Astrocytes express similar receptors to those expressed by neurons. Expression of these receptors allows astrocytes to respond to neurotransmitters released from the presynaptic neuron, which results in a rise in calcium and the release of substrates known as gliotransmitters. Through this interaction, astrocytes can control synaptic maintenance. Red arrows indicate the flow of signal from the presynaptic neuron to the postsynaptic neuron. Image adapted from Allen and Barres, 2009.

The mechanisms for glio-transmission are divergent and dysfunction of these can lead to abnormal behavior and neurological disorders (Rossi et al, 2011; Verkhratsky et al. 2014). The principle excitatory gliotransmitter in astrocytes, and one of the first to be identified, glutamate, can be released from astrocytes through fusion vesicles (Papura et al., 1994; Montana et al., 2006; Parpura and Zorec, 2010; Harada et al., 2016) or hemichannels (Ye et al., 2003). Astrocytes can respond to signaling molecules through ionotropic receptors (Duan et al., 2003). Ca^{2+}-dependent glutamate release is mediated by the expression of N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins (Montana et al., 2006; Parpura and Zorec, 2010). Glutamate is taken up through vesicular glutamate transporters (VGLUTs).

D-serine, produced from L-serine through the enzyme racemase (de Miranda et al., 2002), is released through fusion vesicles. Martineau et al. (2013) showed that D-serine in cultured cortical astrocytes from newborn rats is stored in synaptic vesicles and secreted from astrocytes in a Ca^{2+}-dependent manner. ATP, which is a gliotransmitter, but not a neurotransmitter, is released through hemichannels, fusion vesicles, and purinergic ion channels (Mothet et al., 2005). When stimulated by glutamate, connexin 43 (Cx43) hemichannels in rat glioma cells and hippocampal astrocyte cells undergo an increase in Ca^{2+} in the glia, which promotes the release of ATP from hippocampal astrocyte slices (Torres et al., 2012). Gliotransmission of ATP stimulates GABAergic interneurons, which promotes GABA release and activates astrocytic $GABA_B$ receptors. Activation of $GABA_B$ receptors in astrocytes successively triggers calcium-mediated ATP release onto neuronal receptors to inhibit synaptic transmission (Boddum et al., 2016; Serrano et al., 2006). Covel and Araque (2018) showed that hippocampal astrocytes can simultaneously release glutamate and ATP into the synaptic cleft, which in turn can induce both inhibitory and stimulatory regulation on the hippocampal synapse activity.

Only within the past decade has GABA been considered a gliotransmitter (Yoon and Lee, 2014). The presence of GABA in glial cells was first noted by Barres et al. (1990) who demonstrated that oligodendrocyte progenitor cells (OPCs), astrocytes, and even differentiated oligodendrocytes express GABA. GABA and the rate limiting enzyme, glutamic acid decarboxylase (GAD) (Buddhala et al., 2009), have been observed in glia processes in rat cerebellum (Martínez-Rodríguez et al., 1993), as well as in astrocytes of the optic nerve in postnatal rats (Ochi et al., 1993). Studies in mouse and fish brain suggest that glia may produce GABA through alternate pathways independent of the biosynthetic enzyme (Seiler and Askar, 1971; Seiler, 1973), GAD, and instead may synthesize GABA through glial monoamine oxidase B (MAOB) (Yoon et al., 2014). In observation of GABA immunoreactivity, studies demonstrate GABA levels are high in the cerebellum compared to the hippocampus (Meur et al., 2012), indicating that GABAergic glia vary depending on the region of the brain (Yoon et al., 2011).

Studies demonstrate that GABA can be released through calcium-activated anion channels (Lee et al., 2010), through volume-regulated anion channels (Pasantes and Schousboe, 1988), or by GABA transporters (Gallo et al., 1991). Persistent release of GABA from astrocytes to neuronal $GABA_A$ receptors was demonstrated in embryonic hippocampal rat cell cultures (Liu et al., 2000), human astrocytes (Lee et al., 2011b), and from glia in the thalamus, olfactory bulb, and cerebellum (Barakat and Bordey, 2002; Kozlov et al. 2006; Lee et al., 2010; Jiménez-González et al., 2011). Oligodendrocyte precursor cells (OPCs) are postsynaptic to GABAergic interneurons in the central nervous system (Orduz et al., 2015; Zonouzi et al., 2015) and signal to the $GABA_A$ receptors expressed in OPCs (Habermacher et al., 2019). Previous work demonstrated that hippocampal astrocytes respond to interneuron signaling through $GABA_B$ receptors (Anderson et al., 2007; Kang et al., 1998; Serrano et al., 2006) and, in turn, astrocytes

release glutamate onto presynaptic terminals, which promotes an increase in inhibitory synaptic transmission into the synaptic cleft (Kang et al., 1998; Losi et al., 2014). Together, these data indicate that glial cells respond to and mediate neuronal activity, which in turn differentially regulates synaptic function, possibly, to mediate temporal activation of neurons in the proper behavioral context.

1.3 Glia influence behavior

Although studies have shown that glia are active participants in regulating neural circuits in the brain, only in the past decade have scientists been able to describe the underlying mechanisms by which glia signaling and regulation of synaptic function influence motor control. Nimmerjahn et al. (2009), reported Ca^{2+} spikes in mice cerebellar Bergmann glia, referred to as radial glia, during locomotor activity and suggest that changes in the flux of Ca^{2+} in these cells were modulated by intracellular glutamatergic transmission. In postnatal mice, Acton and Miles (2015) showed that glia-derived adenosinergic signaling and simultaneous modulation of D_1-like dopamine receptor signaling are important for regulating spinal cord locomotor-related output (Acton et al., 2018). Acton and Miles (2015) demonstrated that stimulation of glia and release of glia-derived adenosine is not required for the excitation of the rhythmic activity modulated by the spinal central pattern generator (CPG), which guides essential locomotor activities such as walking, running, breathing or chewing. Instead glia-derived adenosine signals modulate the activity of inhibitory interneurons within the spinal cord motor circuit to provide a feedback inhibition mechanism that mediates the frequency of locomotor network stimulation. Previous work suggests that astrocytes are essential for regulating breathing under critical conditions such as hypoxia and exercise (Sheikhbahaei et al., 2018) and that astrocytes function through an ATP

response, which in turn initiates glutamate release to modulate the frequency of respiratory output (Huxtable et al., 2010).

Implications of the influence of glia on behavior has been described in studies utilizing glia expressed genes. In mammals, the Clock proteins Clock1, Bamal1, and Per3 have been linked to bipolar disorder. Interestingly, mice that express ClockΔ19 exhibit irregular periodicity in circadian rhythms and hyperactivity, and are used as models of manic behavior in bipolar disorder (Bechtel, 2015). Mice with altered expression of fibroblast growth factor (FGF2) and nerve/glia antigen 2 (NG2) expression in glia display behaviors that mimic those seen in humans with major depressive disorder (MDD), a severe mental disorder that effects mood and behavior, as well as appetite and sleep (Birey et al., 2015). Similar studies in mice have demonstrated that reduced expression of glial glutamate transporter (GLT-1) results in severe major depression (Rajkowska and Miguel-Hidalgo, 2007; Cui et al., 2014; Moraga-Amaro et al., 2014).

Mechanisms underlying control of complex and rhythmic behaviors appear to be directly mediated by proteins with various cellular functions in *Drosophila*. Rhythmic behaviors such as circadian activity coordinate behavior and physiology to allow adaptation to and anticipation of daily environmental changes in light, temperature, and mate availability (Iwasaki and Thomas, 1997; Emery and Freeman, 2007; Peters et al., 2007; Kwan et al., 2008; Haydon, 2011; Zwarts et al., 2015). In *Drosophila*, for example, circadian behavior requires glia-specific expression of Ebony, an N-β-alanyl-biogenic amine synthetase that acts downstream of the regulatory clock proteins, Period (Per) and Timeless (Tim), which are expressed in both glia and neurons. Per and Tim are transcription factors that regulate their own activity as well as the transcription of other Clock genes. Similarly, in *Drosophila*, efficient courtship behavior requires the glial amino acid

transporter protein, genderblind (gb), which modulates neurotransmission and synaptic strength of glutamatergic neurons (Grosjean et al., 2008).

In *C. elegans*, sleep-like behavior is altered with the loss of CEP_{glia}-mediated inhibitory signaling at tripartite synapses. Glia-like, GLR cells are situated at neuromuscular junctions and assist with motor movement during foraging (Milward et al., 2011; Calhoun et al., 2014; Stout et al., 2014; Pradham et al., 2019). Additionally, loss of glia-derived *hlh-17* expression results in irregular egg laying behavior in hermaphrodites (McMiller and Johnson, 2005; Felton and Johnson, 2011, 2014). Similarly, loss of glia expressed transcription factor PROS-1, the homolog of mammalian Prospero *Prox-1*, alters the function of sensory neurons and influences behavior (Kage-Nakadai et al., 2016). Glia specific expression of SWIP-10, required for swimming behavior in *C. elegans*, modulates neuronal response to dopamine excitability and secretion, in part by modulating transmission of glutamate in the synaptic cleft (Hardaway et al., 2015; Gibson et al., 2018). Additionally, glia-expressed DELM-1 and 2 are required for mechanosensation in nose touch sensitivity and foraging in *C. elegans* (Wang et al., 2008; Han et al., 2013; Stout et al., 2014), a behavior that requires rhythmic dorsal and ventral motion of the animal's nose when in search for food. With the paradigm shift in our understanding of glial function, there is still insufficient data available to fully address the role of glia in integrating complex signaling mechanisms required for balancing complex behavior circuits.

1.4 *Caenorhabditis elegans* are a simple, unique model for probing glial function

C. elegans consist of two naturally occurring sexes, males, and hermaphrodites, each of which have a simple anatomy. Hermaphrodites are females that have a single male characteristic, which is the ability to produce a limited number of sperm for internal self-fertilization, which

allows them to reproduce in the absence of males. However, males, which are less frequent than females or hermaphrodites in the *C. elegans* population can mate with hermaphrodites. In this case, the use of male sperm is preferred and dominates over the hermaphrodite sperm, in turn, increasing the number of progeny produced (Hodgkin et al., 1979; Riddle et al., 1997).

Although *C. elegans* have only 7,000 synaptic connections, these invertebrates are similar to vertebrates in ability to detect and integrate complex information and translate this information into behavioral responses that include escape (Croll, 1975; Chaflie et al., 1985; Akema et al., 2005; Donnelly et al., 2012), mating (Barr and Garcia, 2006), and learning (Ardieal and Rankin, 2010; Sammut et al., 2015). Hermaphrodites have 302 neurons, 132 muscles, and 50 known glial cells. An additional 83 and 23 sex-specific neurons and muscles, respectively, are found in males (White et al, 1986; Lints and Emmons et al., 1999). Morphological and anatomical features of this optically transparent invertebrate model have been mapped out, allowing for simple and comprehensive genetic analysis to study a repertoire of well characterized behaviors. Previous work describes the lineage of neuronal and non-neuronal cells in *C. elegans* (Sulston and Horvitz., 1977; Sulston et al., 1983). Descriptive maps of synaptic connections in the form of electron micrographs (White et al., 1986; Chen et al., 2006) and computer generated connectivity maps (Jarrell et al., 2012; Xu et al., 2013; Cook et al., 2019) explicitly describe the neuronal connectivity of *C. elegans*. Additionally, the chemical connectivity has been described in males and hermaphrodites, outlining the distribution of neurotransmitters such as acetylcholine (Okuda et al., 2000; Garcia et al., 2001; Mullen et al., 2007; LeBouef et al., 2014), dopamine (Zhang et al., 2014; Loer et al., 2015), serotonin (Flames and Hobert, 2009; Pocock and Hobert 2010), GABA (McIntire et al., 1993; Mellem et al., 2002; Mullen et al., 2006), and glutamate (Mano et al., 2007; Ohnishi et al., 2011; Serrano-Saiz et al.,

2013). Moreover, a recent study demonstrated differences in neurotransmitter usage in the male nervous system compared to the hermaphrodite nervous system (Serrano-Saiz et al., 2017). Together, these studies outline the unique characteristics of the *C.elegans* model and the advantages for using *C.elegans* to study glia function.

Work describing the architecture and function of the *C. elegans* neural network demonstrates that glial viability is not required for the survival and reproduction of *C. elegans*. However, studies that have exploited ablation methods, either by laser ablation (Bargmann et al., 1995) or cell-specific expression of cell death proteins (Chelur et al., 2007), have demonstrated that glia are vital components in neural circuit formation and function (Colón-Ramos et al., 2007, 2009).

C. elegans glia are distinguished by morphology, gene expression (Sulston and Brenner, 1974), and the neurons with which they are associated (White et al., 1986). In *C. elegans*, glia grouped with related sensory neurons are called a sensilla. Each sensillium consists of 1-12 sensory organs and two glia, a sheath and socket cell (Bird and Bird, 1991; Doroquez et al., 2014; Singhvi and Shaham, 2019), and are located mainly in the head and the tail of worms. The sheath and socket cells are connected through adherens junctions. However, *C. elegans* also possess glia-like cells called GLR cells, that are not a part of a sensillium. These mesodermally derived GLR cells contact the inner surface of the nerve ring neuropil and form gap junctions with muscle cells in the head. Like GLR cells, CEPglia project posterior processes that contact and wrap around the nerve ring. However, CEPglia are also a part of the cephalic sensillium, consisting of two glia, a socket and a sheath cell (Figure 1-2), which form a channel and wrap around the four dopaminergic cephalic neurons. There are five additional sensilla in the head: anterior derid, amphid, inner labial, outer labial, and posterior derid. The phasmid sensilla is located in the tail of males and hermaphrodites. There are four additional male specific sensilla

located in the tail: hook, ray, spicule, and the post cloacal sensilla (Figure 1-3). The amphid is the largest chemosensory sensilla in *C. elegans*, which include twelve sensory neurons, a socket, and a sheath glial cell (White et al., 1986; 1986; Heiman and Shaham, 2007; Oikonomou and Shaham, 2011). Recent studies have advanced our understanding of mechanisms by which sensory neurons in the amphid sensilla and glia in cephalic sensilla contribute to behavior (Oikonomou and Shaham, 2011).

Studies demonstrate that the CEPglia are considered the morphological and functional homologs of vertebrate astrocytes (Shaham, 2005, 2006, 2015; Yoshimura et al., 2008). Interestingly, Katz et al. (2018) demonstrated that CEP_{glia} form tripartite synapses, similar to the tripartite architecture described with astrocytes and pre- and postsynaptic terminals. Moreover, CEPglia are stimulated at the tripartite synapse and release molecules that promote inhibitory synaptic connections that attenuate sleep behavior (Katz et al., 2018). Additionally, CEP_{glia} secrete guidance molecules that direct the assembly of pre- and postsynaptic terminals in the central neutrophil-rich nerve ring (Colón-Ramos et al., 2007; Shao et al., 2013; Rapti et al., 2017; Dong et al., 2020). Together, the *C. elegans* model provides a powerful tool for investigating glia function in this organism, as well as advancing our understanding of glia function in higher and more complex mammalian systems.

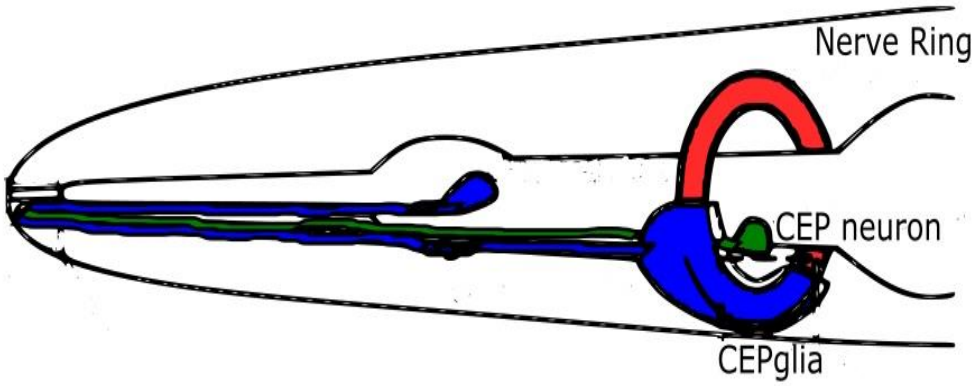


Figure 1-2 The cephalic sensilla.

A simplified schematic of the *C. elegans* cephalic sensilla. One of the four cephalic neurons is depicted in green. The socket and sheath glia are in blue. Projections from the sheath glia wrap around the nerve ring (red). Adapted from Yoshimura et al., 2008.

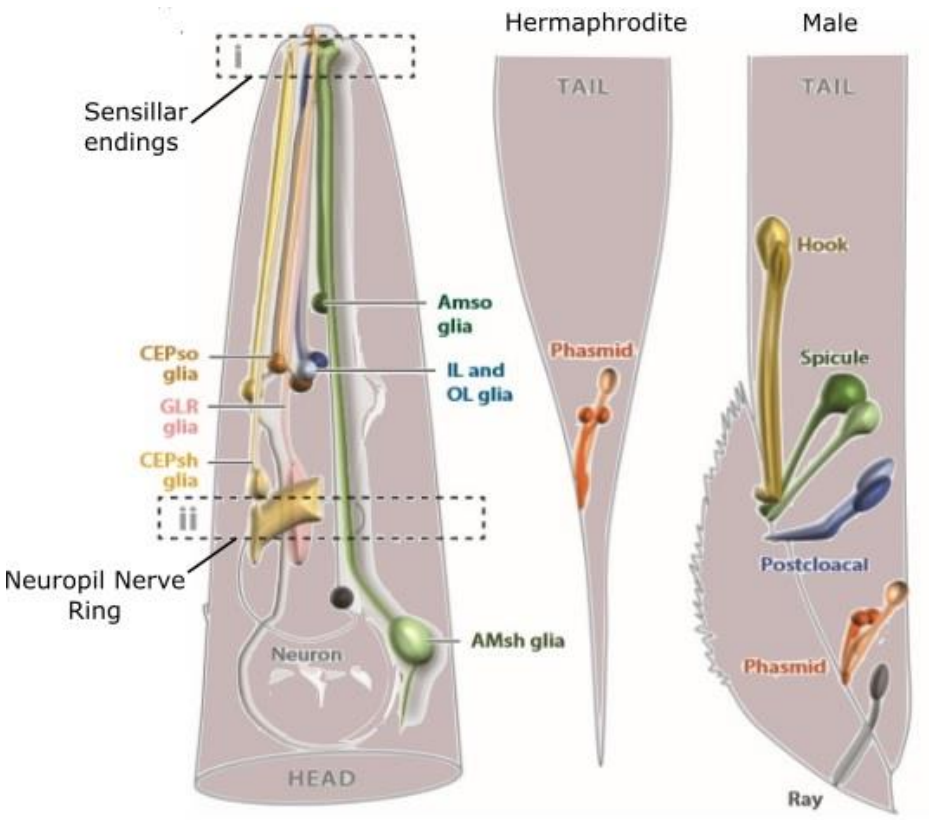


Figure 1-3 The C. elegans glia.

A schematic representation of hermaphrodite and male sensilla in the head (left), hermaphrodite tail (middle), and the male tail (right). Dashed boxes indicate (i) the location of sensillar endings in the lips and (ii) the location of the nerve ring and neuropil glia that wrap around the nerve ring. Adapted from Singhvi and Shaham, 2019.

1.5 HLH-17 is a glia-specific transcription factor that influences behavior

Studies characterizing HLH-17 support a model that suggests CEP_{glia} actively participate in signaling at synaptic junctions to influence behavior. The basic helix-loop-helix transcription factor, HLH-17, an ortholog of the mammalian Olig transcription factor family proteins, is strongly and constitutively expressed in the CEP_{glia} throughout all developmental stages of embryogenesis to adult, and currently is a widely used marker for visualizing CEP_{glia} specific expression and function (McMiller & Johnson, 2005; Yoshimura et al., 2008; Tintori et al., 2016).

It was previously shown that HLH-17 expression is required for modulating dopamine dependent behaviors. Inefficient clearance of dopamine from the synaptic cleft causes nematodes to become paralyzed. Behavioral work by Felton and Johnson (2011) demonstrated that exposure to increased levels of exogenous dopamine paralyzed more than 25% of wild-type animals. However, *dop-3* and *hlh-17* mutant animals were insensitive to the exogenous dopamine. Moreover, loss of the dopamine transporter results in paralysis when worms overexert their body while swimming. This phenotype was also seen in *hlh-17* mutants. These data demonstrated that the transcription factor HLH-17 functions upstream of the dopamine receptors, DOP-1 and DOP-3, and the dopamine transporter, DAT-1, possibly to attenuate the release of dopamine into the synaptic cleft. Gene expression analysis supported this conclusion, demonstrating that HLH-17 regulates the expression of dopamine receptors, DOP-1, DOP-2, and DOP-3 (Felton and Johnson, 2011, 2013).

In mammals, oligodendrocyte precursor cell (OPC) differentiation and myelination of oligodendrocytes is modulated by the level of expression of Olig 1, Olig 2, and Nkx2.2. Moreover, the homeodomain transcription factors Nkx6.1, Nkx2.2, and Pax-6, regulate the

expression of Olig2 in oligodendrocyte differentiation. Similarly, in *C. elegans* glia, Nkx related protein, MLS-2, and Pax-6 related VAB-3, regulate the expression of the Olig related gene HLH-17 during CEPglia development. It was shown that loss of *mls-2* or *vab-3*, hindered CEPglia development, but loss of *hlh-17* did not (Yoshimura et al., 2008). In mammals, Olig2 prevents differentiation of neuronal progenitor cells into neurons, while promoting an oligodendrocyte and astrocyte cell fate. In cell cultures from mouse and rat brains, Olig2 repression led to an increase in the number of astrocytes. HLH-17 shares sequence homology with Olig1 and 2, and is functionally similar to Olig2 (Yoshimura et al., 2008; Felton, 2014). Together, these data suggest that expression of HLH-17 may be involved in cell-type specific regulation of gene expression.

1.6 My dissertation work provides a model for the role of glia in regulating complex and rhythmic behaviors

Gaps persist in our understanding of how intercellular signaling between neurons and glia contribute to behaviors. Moreover, it is not clear how a repertoire of behaviors that are modulated by different signaling mechanisms are integrated in a complex behavioral circuit. The unique overlap in structure and function between mammalian astrocytes and CEP_{glia}, along with the robustness and viability of the *C. elegans* model, provide the opportunity to address these knowledge gaps. The goal of my dissertation work was to uncover mechanisms by which glia modulate complex and rhythmic behaviors. In Chapter 2, I describe the effect of glia loss on complex and rhythmic behaviors. I show that intercellular signaling is differentially regulated by glia, in part, to ensure fluent and accurate execution of behaviors. In Chapter 3, I describe future studies that aim to determine the influence of bidirectional signaling on complex behaviors.

2 INFERENCES OF GLIA MEDIATED CONTROL IN *CAENORHABDITIS ELEGANS*

2.1 Abstract

Astrocytes are essential for modulating synaptic transmission. However, it remains unclear how these glia directly modulate motor activity and influence complex behaviors. Here, we explore the effects of *C. elegans* astrocyte-like cephalic glia (CEP_{glia}) and the glia-specific bHLH transcription factor, HLH-17, on behavior. In *C. elegans*, male mating is a complex behavior that is characterized by accurate execution and coordination of many behaviors to ensure that copulation is achieved. Furthermore, the sex-specific male mating circuitry shares similar components with defecation, which is complex as well as rhythmic, and requires a fixed sequence of behaviors to be activated periodically. We found that loss of CEP_{glia} does not hinder the ability of males to locate the vulva, but instead reduces persistence in execution of behaviors during the copulation stage that ensure breaching of the vulva and sperm transfer. We found that loss of CEP_{glia} results in fewer muscle contractions executed by hermaphrodites in the defecation motor program and hinders males' ability to successfully mate. More importantly, we demonstrate a mechanism for glia-mediated transcriptional regulation of complex and rhythmic behaviors.

2.2 Significance Statement

Mechanisms by which astrocytes, beyond acting as structure supports to neuronal cells, play essential roles in bidirectional signaling with neuronal and non-neuronal cells, has been a burgeoning topic over the last decade. Yet, despite our current understanding of astrocytic signaling and response, it is still unclear how glia regulate complex behaviors. Our work highlights ways in which astrocyte-like CEP_{glia} communicate with interneurons and how that information is translated into a specific behavioral output. More importantly, these proposed CEP_{glia} functions may provide clues for understanding the physiological and pathological relevance for astrocytic responses in mammals.

2.3 Introduction

Globally, approximately 20% of individuals have been diagnosed with mood disorders such as depression, cyclothymia, and bipolar disorder (Sampedro-Piquero & Moreno-Fernandez, 2019; Depression Statistics, 2020). Recently, pathological studies have uncovered astrocytes as an important contributor to neuronal dysfunction (Harada et al., 2016; Konopaske et al., 2008; Moraga-Amaro et al., 2014; Oliveira et al., 2015; Quesseveur et al., 2013; Sanacora & Banasr, 2013; Yamamuro et al., 2015). Patients with depression and major depressive disorder (MDD) have fewer astrocytes in the brain (Rajkowska & Stockmeier, 2013; Sanacora & Banasr, 2013). Moreover, astrocytes present in MDD patients have an altered morphology that is often associated with reduced functional vigor (Wang et al., 2017). Together, these findings illustrate the importance of studying astrocytes and the cellular influence of astrocytic signaling on animal behavior.

To date, scientists have shown that astrocytes not only provide neurotrophic support to their neuronal counterparts, but function to maintain homeostasis in the brain by mediating the levels of ions and neurotransmitters released into the synaptic cleft (Eroglu and Barres, 2010; Li et al., 2013; Mazaud et al., 2019) . Astrocytes modulate synapse formation, acting through the release of gliotransmitters, such as glutamate, ATP, d-serine, and GABA, into the synaptic cleft, and they respond to signals from neighboring cells by expressing functional neurotransmitter receptors (Araque et al., 2014; Araque et al., 2002; Bang et al., 2016; Ishibashi e tal., 2019; Jourdain et al., 2007; Khan et al., 2001; Ota et al., 2013; Schousboe, 2019; Volterra & Meldolesi, 2005). In the past two decades, studies have uncovered potential mechanisms by which astrocytes modulate synaptogenesis through intercellular communication at tripartite synapses (Halassa et al., 2009; Machado-Vieira et al., 2009; Strauss et al., 2015; Swanson et al., 1999) yet only recently have scientists indicated glia as an influential component of the brain that potentially regulates behavior (Christensen et al., 2013; Jackson F.R., 2011; Emery & Freeman, 2007; Jackson et al., 2015; Oliveira et al., 2015; Tso et al., 2017). In view of these findings, glia, like neurons, interact at synapses to maintain structure and function. Yet, how bidirectional communication between glia and neurons is aligned with coordinating complex and rhythmic behavior circuits remains to be thoroughly communicated. Moreover, studies aim to understand mechanisms by which astrocytes regulate diverse signals and how these regulatory mechanisms coordinate behavior in the proper context (Mederos and Perea, 2019).

In this study, we utilize cephalic glia (CEP_{glia}) in *C. elegans,* which are functionally similar to vertebrate glia (Heiman & Shaham, 2007, 2009) and which can regulate synapse formation and function (Bacaj et al., 2008; Colón-Ramos et al., 2007b; Gibson et al., 2018; Hardaway et al., 2015; Meng et al., 2016; Procko et al., 2011; Rapti et al., 2017; Seifert et al.,

2006; Shaham, 2005, 2006; Shao et al., 2013a; Stout et al., 2014; Wallace et al., 2016). *C. elegans* have four main glia types that are distinguished by morphology, gene expression, and the neurons with which they are associated (Procko et al., 2012; Wallace et al., 2016) . Of these glia types, the CEP_{glia} are considered morphological and functional homologs of vertebrate astrocytes (Frakes et al., 2020; Oikonomou & Shaham, 2011). CEP_{glia} are bipolar cells with an anterior process that ensheaths four pairs of dopaminergic neurons, and with a posterior process that ensheaths the central ganglion neuropile, known as the nerve ring (White et al., 1986). Loss of the CEP_{glia} disrupts the organization of the nerve ring and disorients neurons that subsequently fail to innervate postsynaptic targets (Colón-Ramos et al., 2007a; Shao et al., 2013b; Yoshimura et al,, 2008). *C. elegans* have a simple neural anatomy, consisting of 302 neurons and 50 glial cells that, when individually laser- or genetically-ablated, do not cause cell death of the associated neurons (Sulston et al., 1983). Additionally, the neural network is well established, with approximately 7,000 synapses that have been fully described (Cook et al., 2019). Thus, the *C. elegans* model provides an opportunity to explore the requirement of astrocyte function in behavior.

In *C. elegans*, male mating is a complex behavior that is characterized by accurate execution and coordination of many behaviors to ensure copulation (Barr, 2006; Sherlekar & Lints, 2014). Likewise, the defecation motor program (DMP) is a rhythmic and complex behavior in both males and hermaphrodites that requires a sequence of stereotypical behaviors to occur (Branicky & Hekimi, 2006; Thomas, 1990). Previous work describe differences in synapse architecture that are required for proper execution of the DMP in males and hermaphrodites (Reiner & Thomas, 1995). Additionally, the defecation motor program and the male mating circuitry are coupled (LeBoeuf & Garcia, 2017; Nagy et al., 2015). Anatomical features in

hermaphrodites that promote enteric muscle contractions (EMC) during the DMP are rearranged in males to modulate the time in which the copulatory spicules are protracted. Yet, gaps remain in our understanding of how neuronal and non-neuronal cells are stimulated and directed to transmit signals in the proper behavioral context.

Unpublished microarray data from our lab, supported by more recent work (Katz et al., 2018; Katz et al., 2018), suggest that CEP_{glia} are required for synapse maintenance and function prompting us to focus on the complex and rhythmic male mating ritual and DMP. Here we show that CEP_{glia} are needed for proper execution of behaviors in the copulation stage of mating; loss of CEP_{glia} reduces a males' persistence and motivation to surmount the difficulty of transitioning from prodding to spicule protraction and sperm transfer. We show that CEP_{glia} loss causes males to carelessly scan along the body wall at a rapid pace. In turn, males sail off the end the end of the hermaphrodites, losing contact and therefore missing the time in which to execute a ventral turn. In our assessment of independent behaviors in the DMP, we found that loss of CEP_{glia} results in erratic spicule protraction during mating and after enteric muscle contraction and expulsion in the DMP. Our findings suggest that CEP_{glia} stimulate the activity of the GABAergic neurons to promote context-specific execution of behaviors. Together, our work uncovers possible roles for astrocytes in regulating complex and rhythmic behaviors, in part by modulating inhibitory and excitatory signaling at GABAergic synapses.

2.4 Materials and Methods

2.4.1 Strains and maintenance

Strains were acquired from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota (https://cgc.umn.edu). They were maintained at 20°C on solid nematode growth media (NGM) seeded with *E. coli* OP50 (Lewis and Fleming, 1995; Brenner, 1974).Genetically ablated strains were derived from DCR1337 [*nsIs105 (Phlh-17::GFP) I; cima-1(wy84) IV; wyls45(Pttx-3::GFP::rab-3, Punc-122::RFP)X; olaEx805 (Phlh-17::caspase12; Phlh-17::caspase17; Pttx-3::mCherry;* P*glr-3::mcherry* and *Punc-122::GFP)*] (Shao et al, 2013) using traditional crossing techniques, including genotypic/phenotypic confirmation by florescence microscopy and PCR. Strains for this study include: DR466 [*him-5(e1490) V*], OS2649[*hlh-17(ns204) IV*]; CMJ4006 [*hlh-17(ns204) IV; him-5(e1490)*]; CMJ4007 [*nsIs105 (phlh-17::GFP) I; him-5 (e1490) V*]; CMJ4008 [*nsIs105 (phlh-17::GFP) I; hlh-17(ns204) IV; him-5 (e1490) V*]; CMJ4009 [*nsIs105 (phlh-17::GFP) I; him-5 (1490) V; olaEx805 (Phlh-17::caspase12; Phlh-17::caspase17; Pttx-3::mCherry;* P*glr-3::mcherry* and *Punc-122::GFP)*]; CMJ4010 [*nsIs105 (phlh-17::GFP) I; hlh-17(ns204) IV; him-5 (1490) V; olaEx805 (Phlh-17::caspase12; Phlh-17::caspase17; Pttx-3::mCherry;* P*glr-3::mcherry* and *Punc-122::GFP)*]; CB246 [*unc-64(e246)*], COP1997[kNUsI815[pNU1972(F16F9.3p::hlh—17::ttb-2 in ttTi56050]II; unc-119(ed3)II] generated by NemaMetrix, Inc. (https://invivobiosystems.com), CMJ4011 [[*nsIs105 (phlh-17::GFP) I; him-5 (1490) V*]; [kNUsI815[pNU1972(F16F9.3p::hlh—17::ttb-2 in ttTi56050]II; unc-119(ed3)II]], and CMJ4012[[*nsIs105 (phlh-17::GFP) I; hlh-17(ns204) IV; him-5(e1490) V*]; [kNUsI815[pNU1972(F16F9.3p::hlh—17::ttb-2 in ttTi56050]II; unc-119(ed3)II]]. We examined the behaviors of males derived from a *him-5* (e1490) background with intact or ablated (ΔCEP_{glia}) CEP_{glia} and that either expressed or lacked (*hlh-17*)

HLH-17. In our assays, *him*-5 males behaved the same as N2 males (see Figure 2-2); therefore, we hereafter consider the contribution of the *him-5* allele to the described behaviors as negligible, and consider *him-5* males that have intact CEP_{glia} and that produce full-length HLH-17 to be phenotypically WT. Specifically, we refer to the phenotypes of strains as follows: CMJ4007 = WT; CMJ4008 = *hlh-17(ns204)*; CMJ4009 = ΔCEP_{glia}; CMJ4010 = ΔCEP_{glia}; *hlh-17(ns204)*; CMJ4011 = pAmPH::*hlh-17*; and CMJ4012 = *hlh-17(ns204)*; pAmPH::hlh-17.

The following primers were synthesized by a commercial laboratory (www.idtdna.com) and used for genotyping:

hlh-17*:* 5' TCC CTG GGG ACT CTC CTC G 3' and 5' CGA TTT TTG CTG CTA ATG GGC AAC AC 3';

him-5*:* 5' GAC GAT CAC TGT TGA CAA TCA C 3' and 5' GTC CAG AAT TCG TTC TAA TAA CG 3';

cima-1: 5' GAA AAG GAC CAG CCT GTA ATG 3' and 5' GAG ATG TTC TAG AAT TCG CAC C 3'.

2.4.2 Behavioral Assays

2.4.2.1 Mating

One well-fed adult male was added to a seeded, 60 mm NGM agar plate already containing ten partially paralyzed *unc-64(e246)* adult hermaphrodites (Correa *et al*., 2012). Mating events were recorded using the AVI acquisition fast quality mode on a Nikon eclipse 90i

digital microscope. Each assay was limited to a 30-minute duration; failure to respond to the female or complete copulation within this period was considered an unsuccessful mating attempt. The assay started when the male pressed his tail onto the hermaphrodite body wall. The assay stopped after a male transferred sperm, swam away, or when the time exceeded 30 minutes. The mating behaviors were assayed in two stages, response stage and copulation stage. **Response Stage**: In this stage we assessed the male's ability to respond to a hermaphrodite and to initiate a vulva search. 1) Contact: We recorded the total time in which the male tail remained in contact with the hermaphrodite, until locating the vulva. Time was stopped if the male lost contact with the hermaphrodite before locating the vulva. 2) Turning: Males that initiate contact on the dorsal side of the body wall must execute turns to the ventral side, while maintaining contact. To assess turning behavior, we calculated the percentage of completed turns divided by the total number of turn attempts, which included completed and missed turns. The quality of turns included: a *completed* turn, which occurs when the male tail maintains contact or *temporarily* loses contact but quickly regains it while turning to the opposite side of the hermaphrodite; and a *missed* turn, which occurs when the male overshoots the turn and completely loses contact with the hermaphrodite. 3) Detection: To determine whether males were deficient in detecting the vulva, we assayed the number of times a male's tail scanned beyond the vulva without a slight pause or abrupt stop before performing the next behavior. **Copulation stage**: In this stage we assess the male's ability to insert his spicules into the vulva by prodding and to transfer sperm. 1) Prodding: We measured prodding behavior as the accumulated time of each prodding attempt, starting from contacting the vulva until the initiation of sperm transfer, until males gave up and swam away, or until 30 minutes elapsed. 2) Transfer: Copulation was considered successful only after we visually detected protraction of the tail spicules and transfer of sperm into the vulva.

2.4.2.2 Defecation

Defecation behaviors were visualized at 20X magnification using a Nikon eclipse 90i digital microscope. One well-fed adult male or hermaphrodite, grown at 20°C, was placed on a seeded 60 mm NGM plate and allowed to recover for five minutes prior to starting the assay. Time started at the first visually detected posterior body wall contraction and continued for 10 minutes. This period spanned a time that is equivalent to a minimum of 10 cycles of the defecation motor program. One cycle of motor behaviors is initiated by a posterior body wall contraction (pBoc) which is a change in body length at the posterior end of the animal and is visualized as a shrinkage of the tail. Following a pBoc, the anterior body wall muscles contract (aBoc), resulting in a change in body length at the anterior end of the animal and a shrinkage at the head. A similar head movement is depicted during foraging; therefore, aBocs were not assayed. A defecation cycle is complete after enteric muscles contract, which can be visualized as shrinkage at the tip of the tail and quickly followed by expelled gut contents. We assessed periodicity between cycles by calculating the average time between two consecutive pBocs. We scored the average enteric muscle contractions (EMCs) and pBocs as the number of EMCs/10 and the number of pBocs/10, respectively.

2.4.3 Data Analysis

We used Sigma Plot 12.2 to generate standard descriptive statistics for each strain. The Shapio-Wilk test was used to determine whether the sample data set was normally distributed. If so, a standard t-test was used to compare between the two data sets. A p-value < 0.05 was

interpreted as statistically significant. Outliers that were more than two standard deviations plus or minus the mean value were omitted and not included in the statistical analysis.

2.4.4 Analysis of gene expression

For synchronization of development, embryos were collected from healthy gravid, adult populations grown on four 100 mm or two 150 mm 8P-NA22 plates by treatment with sodium hypochlorite (VanDuyn et al., 2010), and then were allowed to hatch over a 17- hour period on unseeded 60 mm NGM plates without tryptone at 20°C (Zhang and Kuhn, 2013). Approximately 10^5 L1 larva were collected and recovered in M9 buffer (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1M $MgSO_4$) as previously described (Nass et al., 2002; Nass and Hamza, 2007; Sun, Ohta et al., 2020). Synchronized L1 larvae were used for L1 cell isolation. Two minutes of SDS-DTT treatment at room temperature (RT) was used to pre-sensitize the worm cuticle. The cuticle was then enzymatically digested with 15 mg/ml pronase for nine minutes and cells were released with mechanical disruption by continuously pipetting as described (Zhang & Kuhn, 2013). Non-cell particles, incompletely lysed worms and intact worms were filtered out through a 10 µm cell strainer, and the isolated cells, which are smaller than 10 µm, were recovered in egg buffer (118mM NaCl, 48 mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 25mM Hepes) (Sangaletti and Bianchi, 2013). The final single cell suspension was used for fluorescence-activated cell sorting (FACs). Forward scatter collection (FSC) was used to detect the size of a cell, while side scatters collection (SSC) was used to detect the complexity of the cell. Combined FSC and SSC was used to asses cell populations. Acquired light scatter data were used to generate scatter plates. To maximize cell purity, we excluded detection of two or more aggregated cells. We added 10 µl 7-

aminoactinomycin D (7-AAD) to the cell suspension to distinguish dead cells (Sun et al., 2020), visualized using the perCP channel, from live GFP^+ cells (Pattanapanyasat et al.,, 1994) that were visualized using the FITC channel. GFP positive, 7AAD negative and GFP negative, 7AAD negative cells were separated by a fusion sorter with a 100 micron aperture to relieve pressure on the cells, and then were collected into 100 µl buffer RA1lysis buffer for RNA isolation (takarabio.com) and 2 µl TCEP (NucleoSpin RNA XS kit, Takara Bio, Cat. No. 740902.10). Total RNA then was isolated from approximately 600 to 1000 CEP_{glia} enriched cells, as previously described (Chao et al., 2019).

cDNA synthesis was performed using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio, Cat. No. 634888). Real-time PCR was performed using the 7500 Fast Real-Time PCR system. Reaction mixtures contained Taqman Universal Master Mix (Applied Biosystems) and the appropriate Taqman Gene Expression Assays (www.thermofisher.com) as per the manufacturer's instructions. The following target specific Taqman Gene Expression Assays were used for real-time PCR:

Gene name	Taqman probe ID
hlh-31	Ce02468489_m1
dgk-1	Ce02491495_g1
dop-3	Ce02496462_m1
pmp-3	Ce02485188_m1
unc-43	Ce02458977_m1
unc-69	Ce02452058_g1
pbo-1	Ce02444102_g1
pkd-2	Ce02468055_g1

lov-1	Ce02437915_g1
sng-1	Ce02497217_g1

2.5 Results

2.5.1 Mating behavior is elusive in males that lack CEP_{glia}

C. elegans males perform a mating ritual that occurs in two stages, each of which consists of a series of key behaviors that must be completed in order to achieve copulation (Figure 2-1). The goal of the first stage, referred to here as the response stage, is to execute the vulva search, during which the male contacts and scans the full length of the hermaphrodite, executes a series of turns, and detects the vulva. The second stage is referred to as the copulation stage, and it involves rhythmic prodding at the vulva, spicule protraction to pry open the vulva, and the transfer of sperm.

As described below, our previous studies suggest that HLH-17 transcriptionally regulates genes that are required for mating in males (Felton, 2014). Because HLH-17 mRNA and protein are predominantly expressed in the CEP_{glia}, we hypothesized that CEP_{glia} are necessary for mating behavior. To examine the role of CEP_{glia} in modulating mating behavior directly, and to determine whether that role is dependent on HLH-17 expression, we examined the behaviors of males derived from a *him-5* (e1490) background. These males either had intact or ablated (ΔCEP_{glia}) CEP_{glia} and either expressed or lacked (*hlh-17*) HLH-17. In our assays, *him*-5 males are phenotypically wild-type (WT) (Figure 2-2); therefore, we refer hereafter to *him-5* males with intact CEP_{glia} and that produce full-length HLH-17 as WT (see Methods and Materials). We found that males lacking CEP_{glia} spend less time in the act of mating (Δ CEP_{glia} = 9.06 +/- 4.71 minutes, n=5) than WT (11.04 +/- 7.38 minutes, n=14) (Figure 2-3a). This generalization

was true whether or not the mating attempt was successful and whether or not the ΔCEP_{glia} males expressed *hlh-17 (*ΔCEP_{glia} ; *hlh-17(ns204)* = 4.84 minutes +/-1.09, n=5). In contrast, *hlh-17(ns204)* males with intact CEP_{glia} took longer to mate (17.31 +/-7.18 minutes, n=14) than WT. Loss of CEP_{glia} correlated with a reduction in the ability of males to complete the mating ritual and subsequently copulate with the hermaphrodite: 40% of ΔCEP_{glia} and Δ*CEP$_{glia}$; hlh-17(ns204)*, versus 67% of WT and 70% of *hlh-17(ns204)* (Figure 2-3b). We found it noteworthy that loss of *hlh-17* caused the total mating time to increase, with a corresponding increase in the percentage of males that were successful in completing the entire mating behavior. Taken together, these data led us to question whether a specific step in mating acts as a bottleneck. If such a bottleneck exists, we would predict that males persisting in overcoming this step would successfully mate, as seen in *hlh-17(ns204)* animals, whereas those that do not persist in overcoming the bottleneck would give up and swim away, as seen in ΔCEP_{glia} animals.

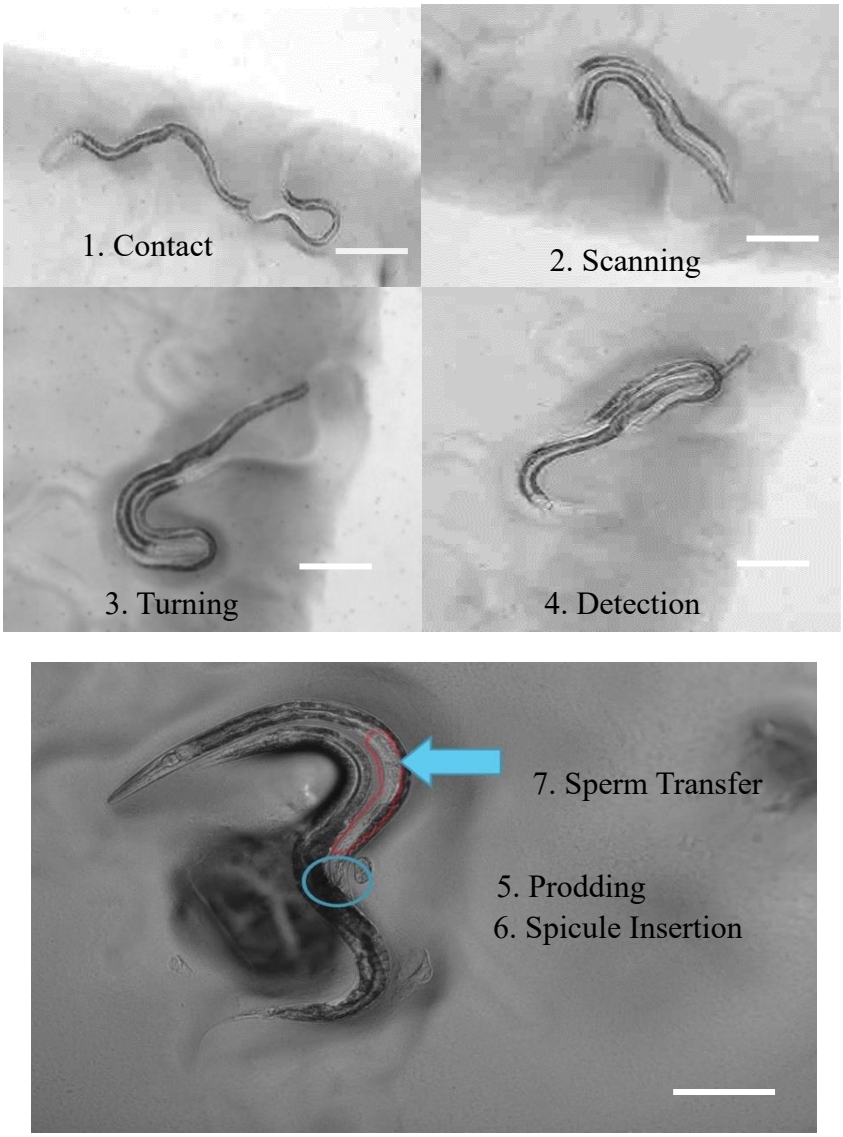


Figure 2-1 Sequence of behaviors executed in the mating ritual. DIC images of a wild type male executing behaviors in the *C.elegans* mating ritual: contact, scanning, turning, vulva detection, prodding, spicule insertion, and sperm transfer. Images were obtained at 20X magnification (scale bar = 100µm). The light blue circle indicates where the male tail is positioned over the vulva during prodding and spicule insertion. The blue arrow indicates where male sperm is stored, and the red outline shows where activated sperm travels upon transfer to the hermaphrodite.

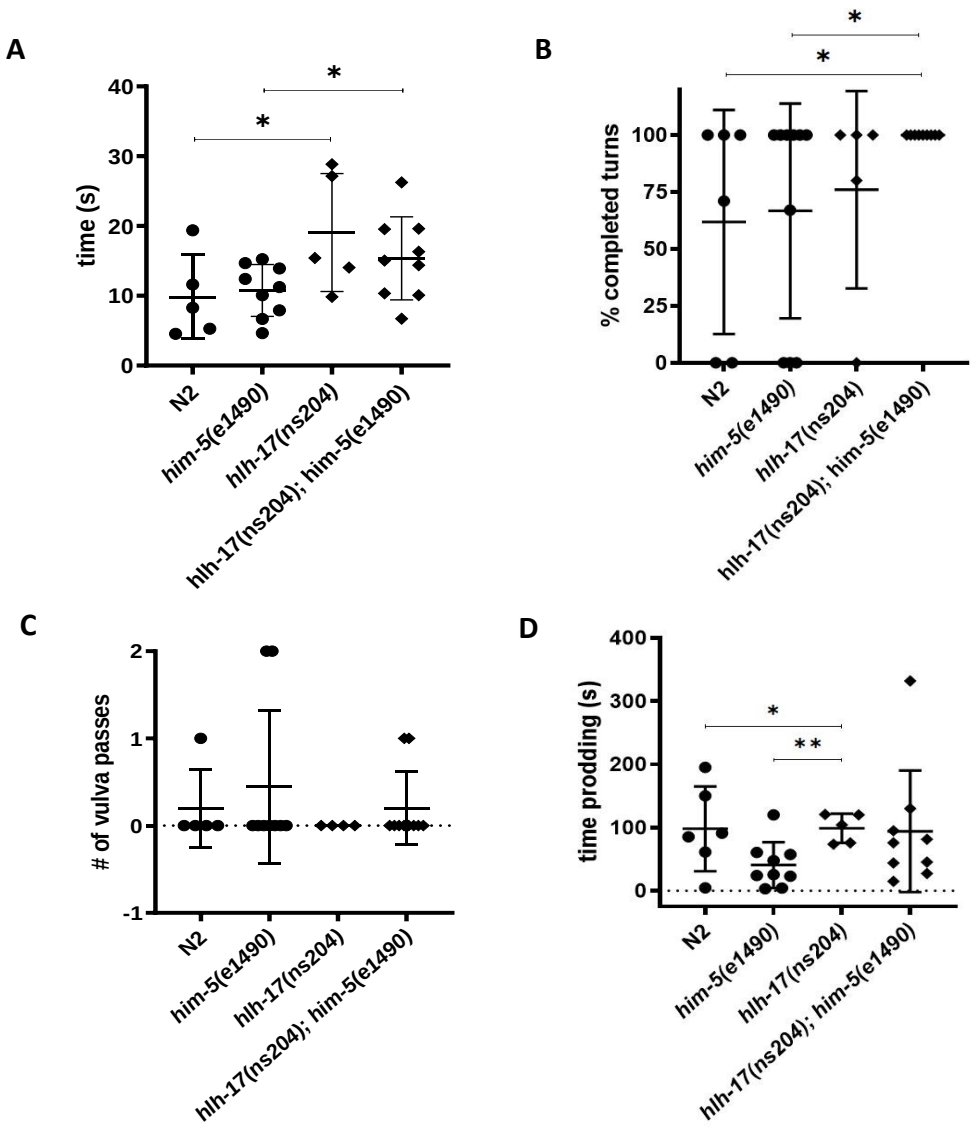


Figure 2-2 The him-5 allele does not influence mating behavior.

Males were assessed for (A) contact: the time in which males contacted and scanned the full length of the hermaphrodite to detecting the vulva; (B) turning: the percentage of dorsal to ventral turns completed; (C) detection: the number of times males scanned and passed the vulva without stopping or pausing; and (D) prodding: the time spent prodding until mating or swimming away after 30 minutes. Lines and bars represent mean and standard deviation. N2 n=5, *hlh-17(ns204)* n=5, *him-5(e1490)* n=9, and *hlh-17(ns204)*; *him-5(e1490)* n=9. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

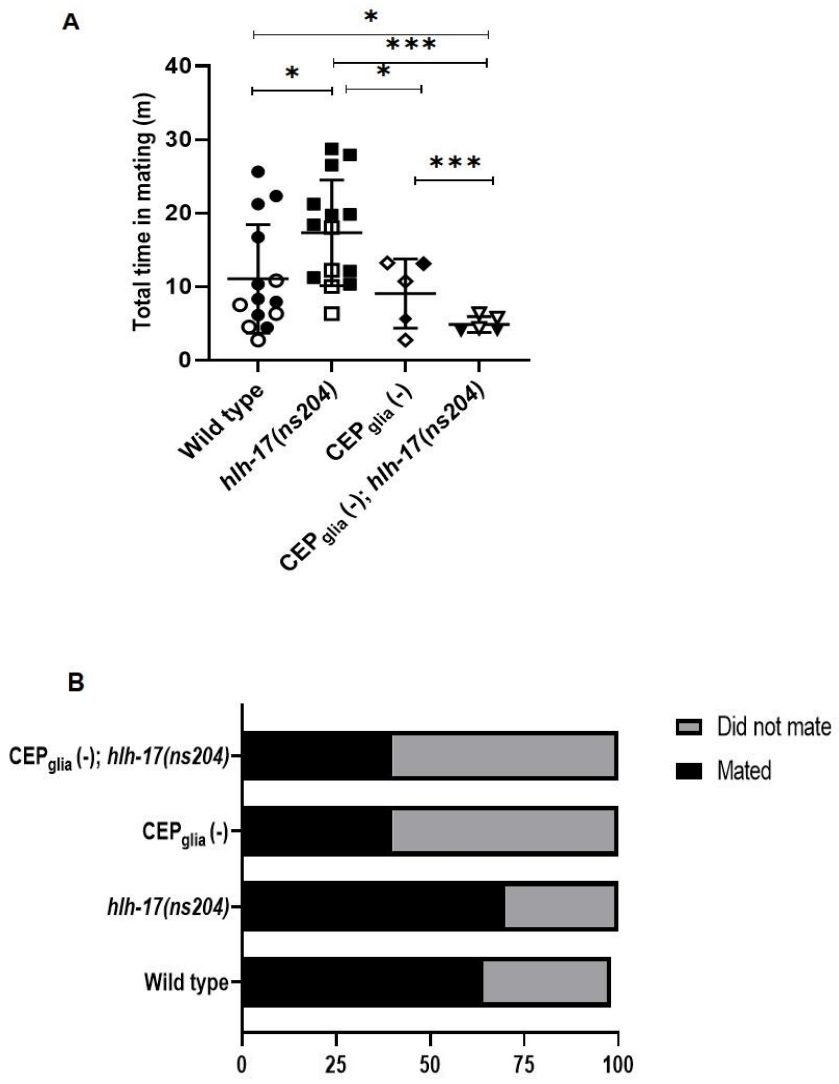


Figure 2-3 Mating behavior is affected by loss of HLH-17.

Males were assessed for (A) the total mating time [response and copulation stage] and (B) the percentage of males that completed both the response and copulation stages. Symbols represent individual males. In A, open symbols represent males that initiated contact but did not copulate. Closed symbols represent males that copulated. In all panels, lines and bars represent mean and standard deviation. WT [nsIs105; *him-5(e1490)*] n=14, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=14 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] n=5 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=5. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

2.5.2 Response stage is not affected by loss of CEP $_{glia}$

To identify the step of the mating process that is potentially acting as a bottleneck in *hlh-17(ns204)* and ΔCEP_{glia} males, we separately analyzed each step of the response and copulatory stages of male mating behavior. Locating the vulva during the response stage likely requires the polycystin transmembrane receptor LOV-1 and the polycycstin-2 TRP channel, PKD-2 (reviwed by Barr, 2006), given that *lov-1* and *pkd-2* males have trouble locating the vulva (Penden and Barr, 2005). Our previous, unpublished data suggest that *lov-1* and *pkd-2* mRNA levels are decreased in *hlh-17(ns204)* mutants. In this study, we compared the levels of *lov-1* and *pkd-2* mRNA in fluorescently sorted CEP_{glia} from WT and *hlh-17(ns204)* males. We found that expression of *lov-1* and *pkd-2* was higher (6.9 fold and 8.5 old, respectively) in CEP_{glia} from *hlh-17(ns204)* males than from WT males, suggesting that HLH-17 may function as a transcriptional repressor by forming heterodimers with LOV-1 or PKD-2 modulate detection and the transition to behaviors in the copulation stage. However, analysis of relative expression with CEP_{glia} cells using fluorescent activated cell sorting suggest that HLH-17 expression in the CEP_{glia} may be required for promoting the expression of LOV-1 and PKD-2 (Figure 2-4). Based on these data, we expected the vulva detection step to be the bottleneck that affected mating time and mating ability in *hlh-17(ns204)* males. We predicted that CEP_{glia} may have a more global affect and therefore be required to modulate many behaviors in the mating ritual. We expected that the ΔCEP_{glia} males experience difficulty getting to the vulva detection step or getting past this step, in order to successfully mate.

To test this prediction, we examined male performance during two behaviors within the response stage: locating the vulva and turning ability. In the response stage, males initiate the

vulva search by using their tails to press against and scan along the body of the hermaphrodite. Males turn from the dorsal body wall of the hermaphrodite by executing sharp turns that propel them towards the ventral body wall, which allows them to continuously scan towards the vulva. Once the vulva is detected, males pause slightly before moving to the copulation stage. As shown in Figure 2-5a, we found that the time to complete this process, from initial contact to locating the vulva, was not significantly different in WT (16.32 +/- 12.10s, n=14) and *hlh-17(ns204)* (16.86 +/- 9.10 s, n = 14, p-value = 0.447) males. However, this time was shorter in ΔCEP_{glia} males, whether they expressed (9.94 +/- 5.43s, n=5 p-value = 0.160) or lacked HLH-17 (9.08 +/- 1.08s, n=4, p-value = 0.130).

Loer and Kenyon (1993) demonstrated that *cat-1* and *cat-4* animals, which lack dopamine signaling due to mutations in the genes required for dopamine synthesis, execute "sloppy" or "missed" turns, resulting in loss of contact with the hermaphrodite and failure to copulate. Additionally, approximately 30% of animals with mutations in the *flp-10* gene, which encodes an FMRFamide-like neuropeptide, exhibit a repetitive turning behavior, causing males to prematurely initiate sharp ventral turns, stop before reaching the ventral side of the hermaphrodite, and then repeat the turn-stop behavior approximately four more times before finally transitioning to the next step (Liu et al., 2007; Peymen et al., 2014). Our previous data (unpublished work; Felton & Johnson, 2014) suggest that HLH-17 differentially regulates *cat-1, cat-4*, and *flp-10*, and we previously demonstrated that *hlh-17(ns204)* animals experience trouble executing dopamine-dependent behaviors due to abnormal dopamine signaling (Felton & Johnson, 2011, 2014). Additionally, the CEP_{glia} ensheath the dopaminergic cephalic (CEP) neurons and male specific cephalic CEM neurons (Sulston et al., 1975; White et al., 1986). CEP_{glia} project posterior processes to innervate the "synapse-rich" central neuropile known as the

nerve-ring (White et al., 1986). Based on these observations, we hypothesized that *hlh-17(ns204)* and ΔCEP_{glia} males would phenocopy *flp-10* males and, as a result, these males would execute and increased number of ventral turns. We further predicted that this would lead to heightened motor neuron and muscle activity, thereby diminishing the persistence to copulate.

To assess turning behavior, we calculated the percentage of completed turns. We found that both WT and *hlh-17(ns204)* males were proficient in executing turns; that is, 100% of the animals that we tested completed 100 % of the turns they attempted (Figure 2-5c). In contrast, only 60% ΔCEP_{glia} and 17% ΔCEP_{glia}; *hlh-17(ns204)* of the males completed 75.2% and 78.2% of the turns, respectively (Figure 2-5c-d). Additionally, although loss of CEP_{glia} resulted in at least one vulva pass for 40% of both ΔCEP_{glia} and ΔCEP_{glia}; *hlh-17(ns204)* males (Figure 2-5b), the time required to locate the vulva was not significantly different from that of WT. However, loss of CEP_{glia} resulted in failure to maintain contact with hermaphrodites when executing turns. These data suggest that ΔCEP_{glia} males phenocopy *fllp-10* males, as predicted; however, *hlh-17(ns204)* males do not.

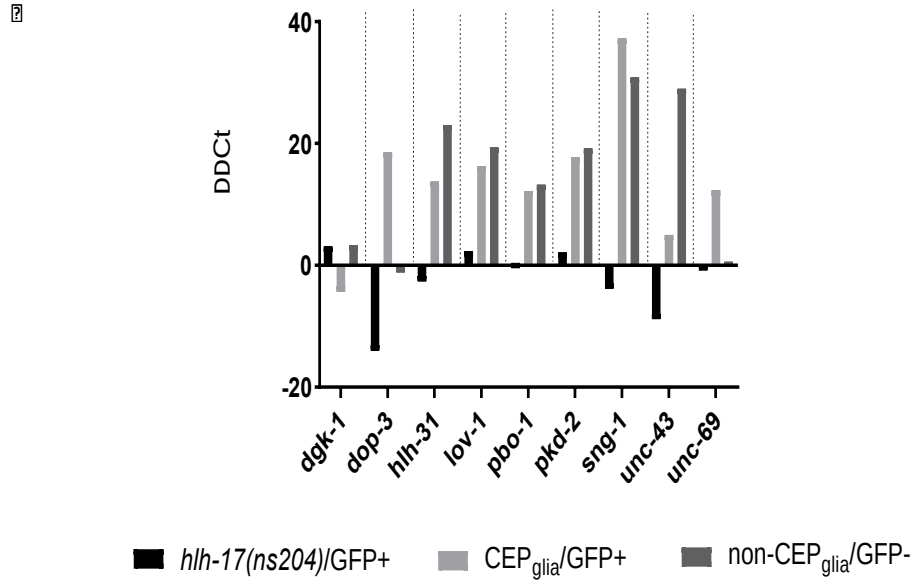


Figure 2-4 HLH-17 expression in CEP_{glia} regulates genes required for behavior.

The relative change in expression levels of mRNA sorted non-fluorescent cells or fluorescent CEP_{glia}. mRNA was extracted from CEP_{glia} cells from *hlh-17(ns204)* wildtype hermaphrodites with intact CEP_{glia} and expressing full-length HLH-17. We analyzed the relative change in expression for complex and rhythmic behavior genes.

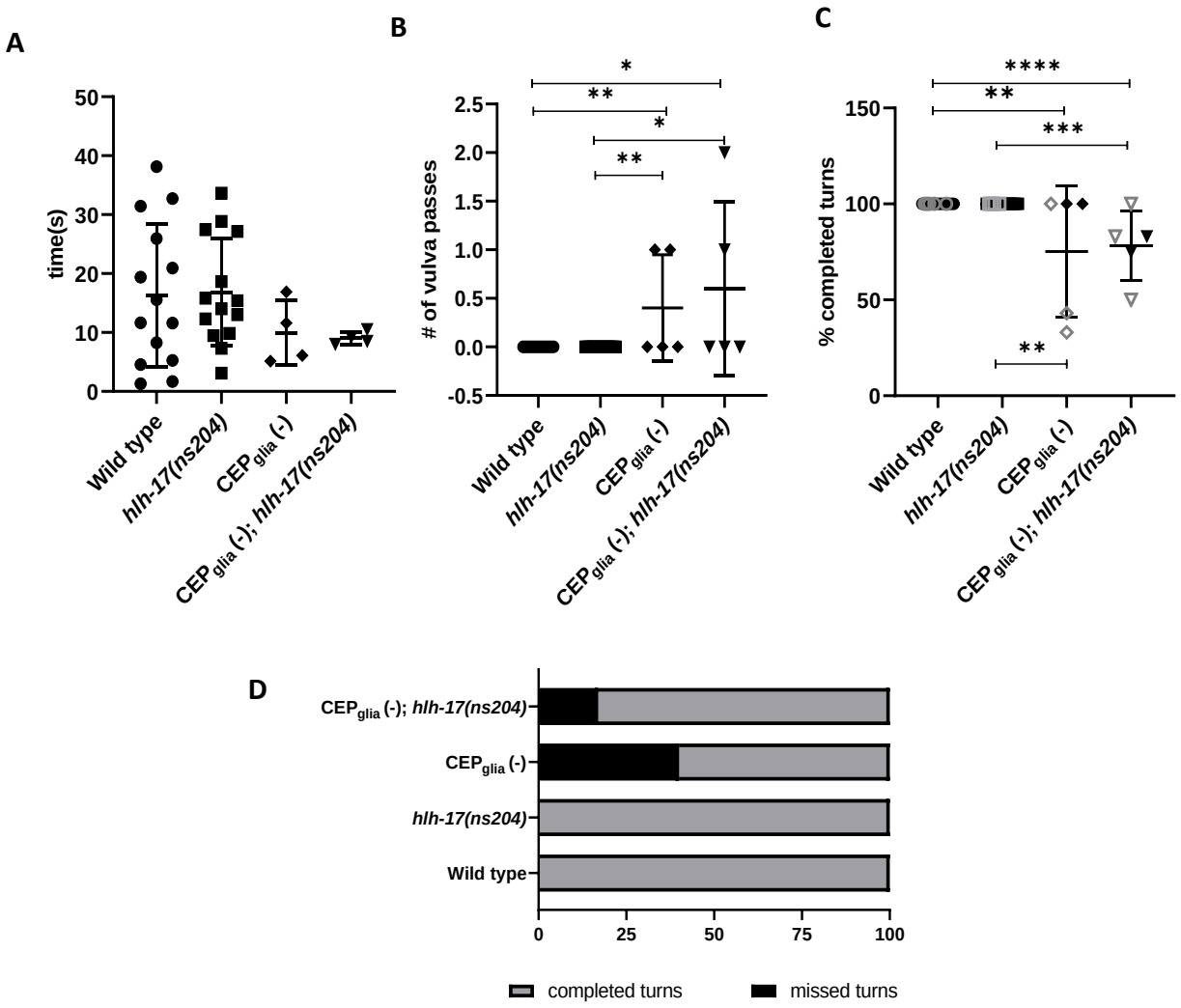


Figure 2-5 Response stage is not affected by loss of HLH-17.

Males were assessed for (A) Contact: the time from which males contacted and scanned the full length of the hermaphrodite to detecting the vulva; (B) Detection: the number of times males scanned and passed the vulva without stopping or pausing; (C) Turning: the percentage of dorsal to ventral turns completed by the male divided by total turn attempted; and (D) Completed versus missed turns. Types of turns are defined in the materials and methods section. Symbols represent individual males in each assay. Open symbols in C represent males that initated contact but did not mate. Closed sysmbols represent males that mated. Lines and bars represent mean and standard deviation. WT [nsIs105; *him-5(e1490)*] n=13, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=11 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] n=5 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=5. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

2.5.3 CEP_{glia} promote prodding persistence

The second stage of mating behavior, the copulation stage, consists of prodding, spicule insertion, and sperm transfer (Figure 2-1). As shown in Figure 2-3, our data when examining mating as a single comprehensive behavior showed that although a high percentage of WT and CEP_{glia} males completed the copulation stage to mate successfully, these males took longer than ΔCEP_{glia} or ΔCEP_{glia}; *hlh-17(ns204)* males to do so. We questioned whether the differences in time could be attributed to differences in prodding behavior. To address this question, we determined the percentage of males to exhibit prodding behavior after locating the vulva and then measured total time that those males spent prodding. We found that 93% of WT (n=14), 100% of *hlh-17(ns204)* (n=15), 80% of ΔCEP_{glia} (n=5) and 60% ΔCEP_{glia}; *hlh-17(ns204)* (n=5) males exhibited prodding behavior after locating the vulva (Figure 2-6a). WT males spent 155.17 +/-168.16 s (n=14) in the act of prodding. Similarly, ΔCEP_{glia} males spent 198.26 +/- 203.14 s (n=5, p-value=0.427) and *hlh-17(ns204)* spent 266.30 +/-223.19 s (n=15, p-value= 0.1340) prodding. Interestingly, ΔCEP_{glia}; *hlh-17(ns204)* males spent significantly less time prodding (9 +/- 12.31 s, n=5, p-value=0.031) before mating or swimming away (Figure 2-6b). We found that, although not significantly different from WT, more than half of *hlh*-17*(ns204)* males spent longer than 10 minutes in the prodding behavior before eventually completing the copulation stage to successfully mate (Figure 2-7a). Additionally, ΔCEP_{glia} males that transitioned to the prodding step spent a longer time prodding than WT males. While increased prodding time correlated with increased mating success for *hlh-17(ns204)* males, fewer ΔCEP_{glia} and ΔCEP_{glia}; *hlh-17(ns204)* mated even after prodding for as long as 10 minutes (Figure 2-7a-b).

Taken together, these data show that loss of CEP_{glia} hindered the ability of males to complete ventral turns. Additionally, *hlh-17(ns204)*, ΔCEP_{glia}, and ΔCEP_{glia}; *hlh-17(ns204)* had difficulty prodding, as suggested by the increased time spent in prodding behavior. Moreover, fewer ΔCEP_{glia} , and ΔCEP_{glia}; *hlh-17(ns204)* males executed prodding, however the males that prodded were able to complete spicule insertion and sperm transfer within 20 seconds (Figure 2-6c).

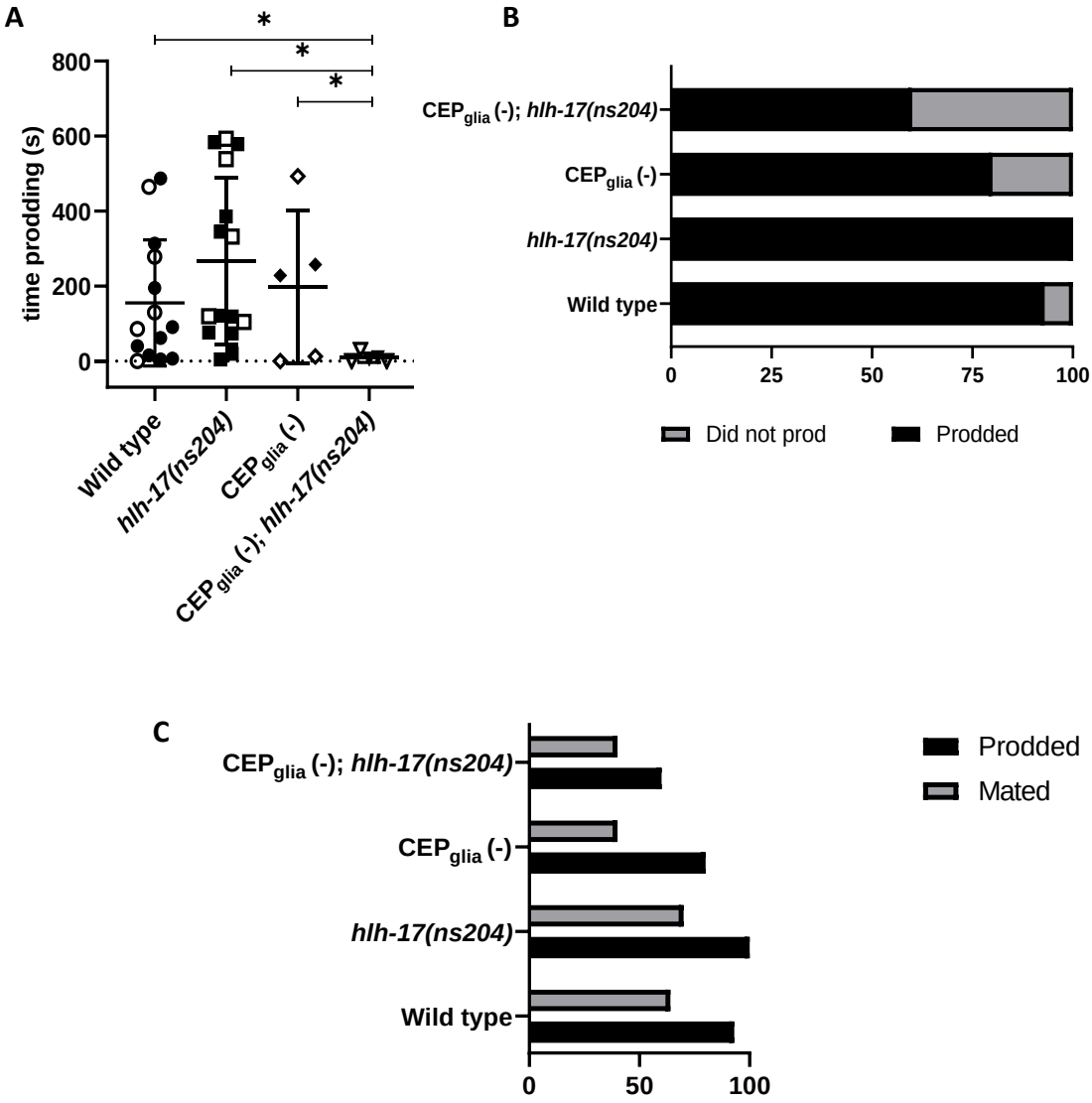


Figure 2-6 ΔCEP_{glia} males rarely executed prodding behavior.

Males were assessed for (A) Prodding: the time spent prodding until mating or swimming away after 30 minutes; (B) males that prodded versus males that did not prod and (C) The percent of males that prodded compared to the percent that mated. Intervals of prodding behavior were added together for the total prodding time. Open symbols in A represent males that initiated contact but did not copulate. Closed sysmbols represent males that copulated. Lines and bars represent mean and standard deviation. WT [nsIs105; *him-5(e1490)*] n=15, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=14 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] n=5 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=5. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

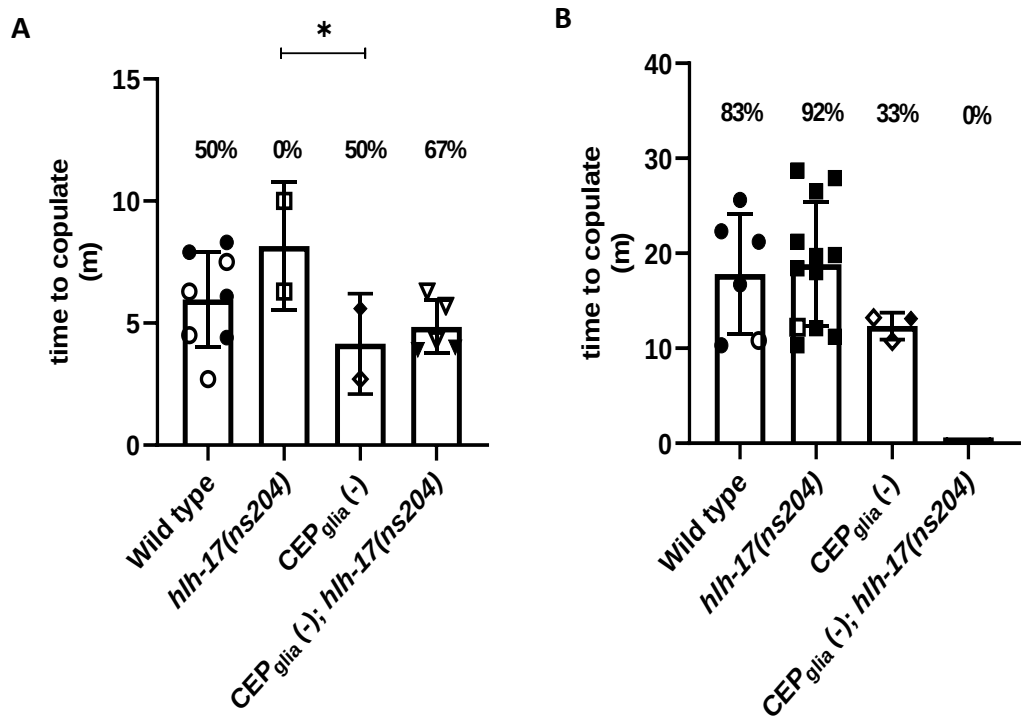


Figure 2-7 Fewer ΔCEP$_{glia}$ males mate as prodding time increases.
Quantification of the total time in mating [response and copulation stage] (A) for ten minutes or less and (B) more than ten minutes. Percentages show the number of males that mated. Percentage = number that copulated/ total number assayed * 100. Open symbols represent males that initiated contact but did not copulate. Closed symbols represent males that copulated. Lines and bars represent mean and standard deviation. WT [nsIs105; *him-5(e1490)*] n=15, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=14 , ΔCEP$_{glia}$ [nsIs105; *him-5(e1490)*; ola*Ex805*] n=5 , and ΔCEP$_{glia}$; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=5. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

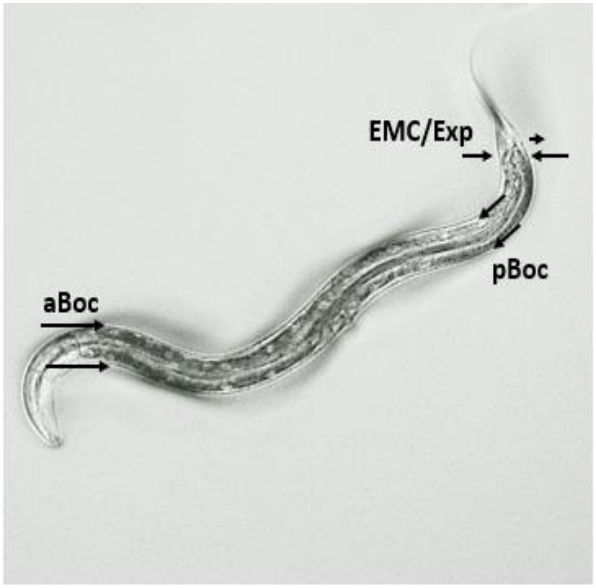
Our mating data suggests that males experience difficulty executing essential behaviors in the mating ritual with loss of CEP_{glia}. However, male mating behavior has only been described in the context of the male tail neural network. To understand how CEP_{glia}, which are located in the head, communicate with the tail neural network to modulate behavior, we examined the effect of CEP_{glia} loss on the defecation motor program (DMP). In both males and hermaphrodites, the DMP is initiated by sequential contractions of the posterior body wall, anterior body wall, and enteric muscles, which ultimately results in the expulsion of pressurized waste in the gut (Branicky & Hekimi, 2006; Thomas, 1990) (Figure 2-8). The neuronal connections that drives this program are sexually dimorphic. In hermaphrodites, gap junctions electrically couple the anal depressor to the intestinal muscle and the sphincter, all of which are stimulated by enteric muscle contractions. In males, however, the anal depressor and the sphincter are not in direct communication, relying instead on intermediary interactions with the male specific protractor muscle (Nagy et al., 2015; Reiner and Thomas, 1995). The anal depressor is connected through gap junctions to the copulation sex muscle- the gubernacular erector muscle- to promote spicule insertion (LeBoeuf & Garcia, 2017). Interestingly, the gubernacular erector muscles functionally links the DMP to mating behavior because they reorient the protracted spicule muscles posteriorly to allow for sperm transfer during mating (Liu et al., 2011). Similarly, the GABAergic DVB and AVL neurons are sexually dimorphic. DVB, located in the tail, forms a chemical synapse with the anal depressor, which in hermaphrodites is required for enteric muscle contractions in the DMP. However, in males, DVB does not synapse with the anal depressor, instead it forms a chemical synapse with the protractor muscle that is coupled to the anal depressor. In males, the anal depressor no longer functions to regulate the DMP but instead is required for spicule insertion during mating (LeBoeuf & Garcia, 2017; Hart

& Hobert, 2018) (Figure 2-9). The GABAergic AVL neuron, located in the head, synapses with DVB and the enteric intestinal muscle to regulate enteric muscle contractions in hermaphrodites. In males, AVL no longer forms a chemical synapse with the intestinal muscle, however the synapse between AVL and DVB is maintained (LeBouf & Garcia, 2017).

We questioned whether loss of CEP_{glia}, located in the head, affects the activity of DVB, the processes of which do not extend to the nerve ring in the head and the cell body that resides in the tail. We predicted that if CEP_{glia} are required for modulating the activity of DVB, which synapses with the protractor muscle and regulates the activity of the male spicules, then loss of CEP_{glia} may result in abnormal spicule protraction. Therefore, we examined the effect of CEP_{glia} and HLH-17 on spicule protraction. During mating, we found that while WT males protracted their spicules only at the spicule insertion step of the copulation stage, 13% of *hlh-17(ns204)* (n=15), 60% of ΔCEP_{glia} (n=5) and 67% of ΔCEP_{glia}; *hlh-17(ns204)* (n=6) males prematurely protracted their spicule in the response stage of mating, well before locating the vulva (Figure 2-10). Similarly, 38% ΔCEP_{glia} (n=8) and ΔCEP_{glia};*hlh-17(ns204)* (n=8) males protracted and retracted their spicule at least once per DMP cycle whereas WT males (n=8) and *hlh-17(ns204)* males (n=8) did not. Together, these data suggest that CEP_{glia} are essential for reducing unorthodox spicule protraction, supporting a model in which CEP_{glia} in the head can influence tail-mediated behaviors.

Although the DVB neuron no longer synapses with the enteric muscles in males, the sex muscles that regulate spicule protraction are coupled to the sphincter and intestinal enteric muscles, which are both required for enteric muscle contractions during the defecation motor program. Therefore, we expected that the rate of enteric muscle contractions (EMC) would be

inhibited by loss of CEP_{glia}. Instead, we found that the rates of EMCs were similar in WT (0.69 +/- 0.09 EMC/minutes, n=7) and ΔCEP_{glia} (0.63 +/- 0.18 EMC/minutes, n=8, p-value= 0.2124) males, but the rates were significantly slower in *hlh-17(ns204)* males (0.36 +/- 0.09 EMC/minutes, n=8, p-value= <0.0001) and in ΔCEP_{glia}; *hlh-17(ns204)* males (0.21 +/- 0.09 EMC/minutes, n=7, p-value= <0.0001) (Figure 2-11b). Our results were similar when we examined posterior muscle contractions, which follow enteric muscle contractions in response to intestinal signaling (Figure 2-11a). The rate of pBoCs in WT (0.71 +/- 0.07 pBoc/minute, n=7) and ΔCEPglia males (0.77 +/- 0.08 pBoc/minute, n=7, p= 0.0827) did not differ significantly but the rates were significantly slower in *hlh-17(ns204)* (0.51 +/- 0.10 pBoc/minute, n=8, p=0.0003) and ΔCEP_{glia}; *hlh-17(ns204)* (0.54 +/- 0.11 pBoc/minute, n=8, p-value=<0.0001) males. Taken together, these data suggest that loss of *hlh-17*, but not loss of CEP_{glia}, regulates the defecation motor program in males.



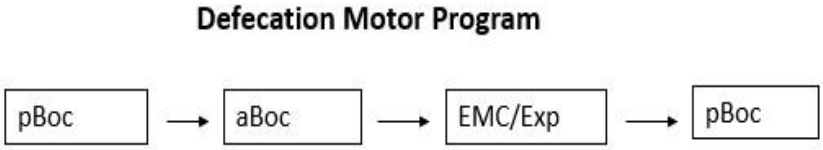


Figure 2-8 Sequence of behaviors executed in the defecation motor program.

Image of an adult hermaphrodite is shown. Arrows indicate site and direction of muscle contraction for steps in the defecation motor program. The defecation motor program is initiated by posterior body wall muscle contractions (pBocs), followed by anterior body wall muscle contractions (aBocs), and finally enteric muscle contractions (EMCs) that are followed by expulsion (Exp) of waste from the gut. Image was taken at 20X magnification (no scale bar 100µm). Photo modified from Branicky and Hekimi, 2006.

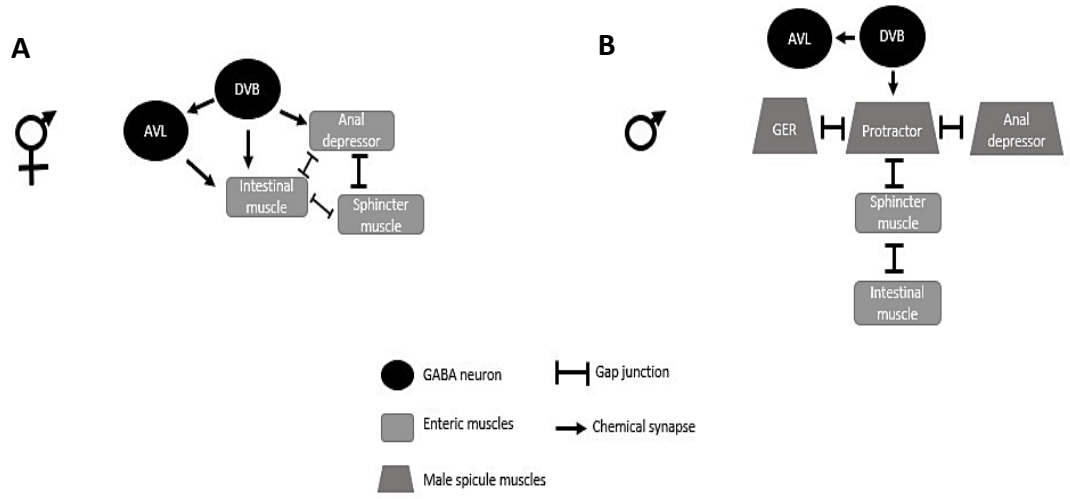


Figure 2-9 Defecation and male mating circuitry are coupled in males. (A) Connectivity of the defecation circuitry in hermaphrodites. (B) Connectivity of the defecation and male mating circuitry in males. ger, gubernacular erector muscle. Adapted from LeBoeuf and Garcia, 2017; Hart and Hobert, 2018.

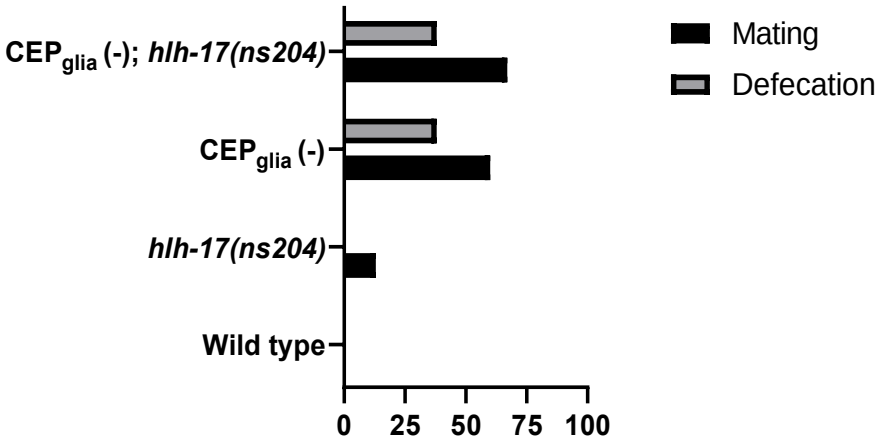


Figure 2-10 Percent premature spicule protractions.

The percentage of males that executed premature spicule protraction equals the number of males that prematurely protracted their spicule / the total number of males assessed *100. Premature spicule protraction during mating occurred before prodding and occurred after expulsion during the defecation motor program. WT [nsIs105; *him-5(e1490)*] n=15 (mating) n=8 (DMP), *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=15 (mating) n=8 (DMP) , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] n=5 (mating) n=8 (DMP) , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=6 (mating) n=8 (DMP).

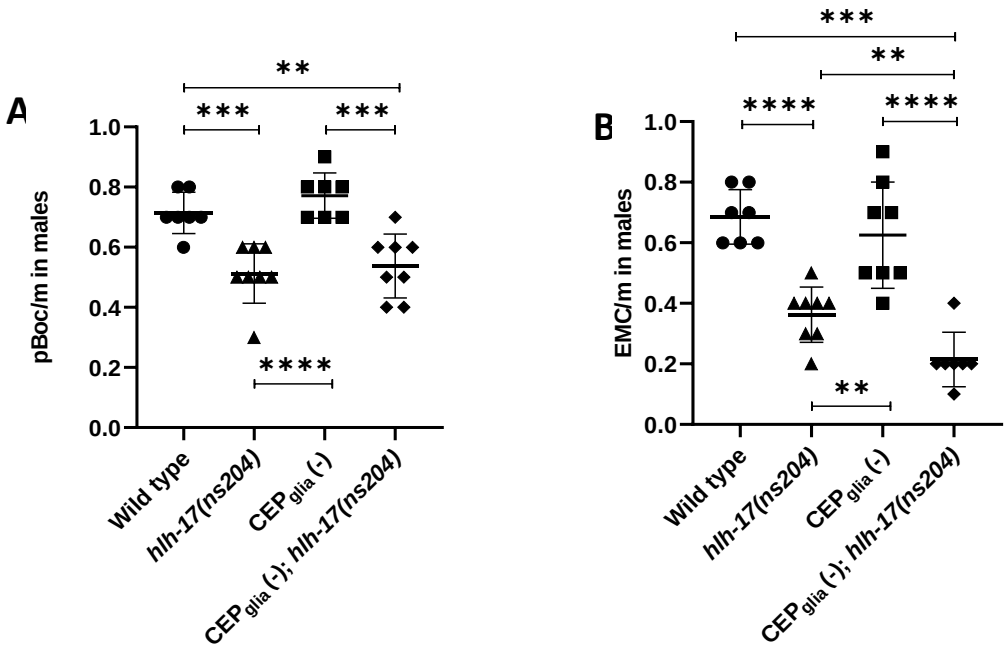


Figure 2-11 Steps in the defecation motor program are not affected in ΔCEP_{glia} males.
The rate of (A) posterior body muscle contractions and (B) enteric muscle contractions was quantified in males. WT [nsIs105; *him-5(e1490)*] pBoc, n=7; EMC, n=7, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] pBoc, n=8; EMC, n=8 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] pBoc, n=7; EMC, n=8 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] pBoc, n=8; EMC, n=7. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

2.5.5 Loss of CEP_{glia} and HLH-17 reduces pBocs and EMCs in the DMP of hermaphrodites

In males, we find that CEP_{glia} are required for behaviors in mating but not in the defecation motor program. Recent studies have shown that, while the sequence of the DMP is unchanged in males and hermaphrodites, the architectures of the DMP neurons and musculature differ in the two sexes (Reiner and Thomas 1995; Nagy et al., 2015; LeBoeuf and Garcia, 2017). Our mating data and the aberrant spicule protraction phenotype of ΔCEP_{glia} males suggest that the CEP_{glia} mitigate these effects via the DVB neuron. Therefore, it is possible that the CEP_{glia} would have a sexually dimorphic effect on defecation. To test this possibility, we examined pBocs in hermaphrodites. As shown in Figure 2-12a, we found that the rates of pBocs in *hlh-17(ns204)* (0.88 +/- 0.21, n=30 pBoc/minute, p-value= <0.0001), ΔCEP_{glia} (0.97 +/- 0.22 pBoc/minute, n=19, p-value= 0.0003), and ΔCEP_{glia}; *hlh-17(ns204)* (0.87 +/- 0.21 pBoc/minute, n=20, p-value= <0.0001) hermaphrodites were significantly slower than those in WT (1.17 +/- 0.15 pBoc/minute (n=28) hermaphrodites. These data suggest that loss of HLH-17 has similar effects in both hermaphrodites and males, but loss of CEP_{glia} affects hermaphrodites differently than it affects males, by significantly reducing posterior contractions.

Previous data suggest that posterior contractions are initiated through signals secreted from the intestine, which functions like a central pacemaker that modulates the periodic activation of DMPs. Therefore, we questioned whether fewer pBocs in ΔCEP_{glia} animals could be attributed to changes in intestinal signaling by examining periodicity of the DMP, which is measured as the time between two consecutive pBocs (Liu & Thomas, 1994; Thomas, 1990). We expected that the decrease in pBocs in ΔCEP_{glia} hermaphrodites would correlate with a change in

the periodicity of the defecation cycle. Instead, we found that periodicity was unaffected in *hlh-17(ns204)*, ΔCEP_{glia}, and ΔCEP_{glia}; *hlh-17(ns204)* hermaphrodites (Figures 2-13), suggesting that although loss of CEP_{glia} reduces pBocs/minutes, signaling from the intestine is not altered. Nevertheless, these results are in line with previous studies suggesting that cycle length is uncoupled to the DMP (Teramoto & Iwasaki, 2006; Thomas, 1990).

Our data in males suggest that CEP_{glia} influence the activity of the protractor and anal depressor muscles, which regulate spicule insertion during mating. In hermaphrodites, the protractor muscle does not develop; instead, the anal depressor forms gap junctions with the sphincter and intestinal enteric muscles and is for enteric muscle contractions. Therefore, we questioned whether, unlike in males, loss of CEP_{glia} in hermaphrodites would affect enteric muscle contractions. To address this question, we measured the rate of EMCs in hermaphrodites. We found that the rates of enteric muscle contractions were significantly lower in *hlh-17 (ns204)* (0.81 +/- 0.08 EMC/minutes, n=12, p-value= 0.0024) and ΔCEP_{glia} (0.81 +/- 0.23 EMC/minutes, n=13, p-value= 0.0097) hermaphrodites than they were in ΔCEP_{glia}; *hlh-17(ns204)* (0.89 +/- 0.20 EMC/minutes, n=10, p-value= 0.0559) and WT (1.03 +/- 0.23 EMC/minute, n=14) hermaphrodites (Figure 2-12b).

Together, our data show that in hermaphrodites, loss of either HLH-17 or CEP_{glia} affects posterior and enteric muscle contractions; however, the DMP cycle length is not affected. How is it that ΔCEP_{glia} and *hlh-17 (ns204)* hermaphrodites execute fewer contractions than WT when the overall cycle length remains the same? We wondered if hermaphrodites that have difficulty contracting their enteric muscles and expelling waste execute fewer contractions as a result, thereby prolonging the time before the subsequent pBoc is initiated. Therefore, we measured the

inter-cycle length, defined here as the time between an enteric muscle contraction at the end of one cycle and the posterior muscle contraction (pBoc) at the beginning of the subsequent cycle (Figure 2-12c). We found that the inter-cycle lengths for WT (38.17 +/- 4.23 seconds, n=4) and *hlh-17(ns204)*(37.58 +/- 1.10 seconds, n=4, p-value= 0.3985) hermaphrodites were not significantly different from each other. In contrast, the inter-cycle length in ΔCEP_{glia} (44.69 +/- 4.27 seconds, n=4) and ΔCEP_{glia}; *hlh-17(ns204)* (57.26 +/- 8.65 seconds, n=5) hermaphrodites was significantly longer than that of WT hermaphrodites. These data show that while the DMP cycle length is unaffected with loss of HLH-17 and CEP_{glia}, enteric muscle contractions are affected in animals that lack CEP_{glia}, and therefore the inability to execute enteric muscle contractions causes hermaphrodites take longer to initiate a subsequent pBoc.

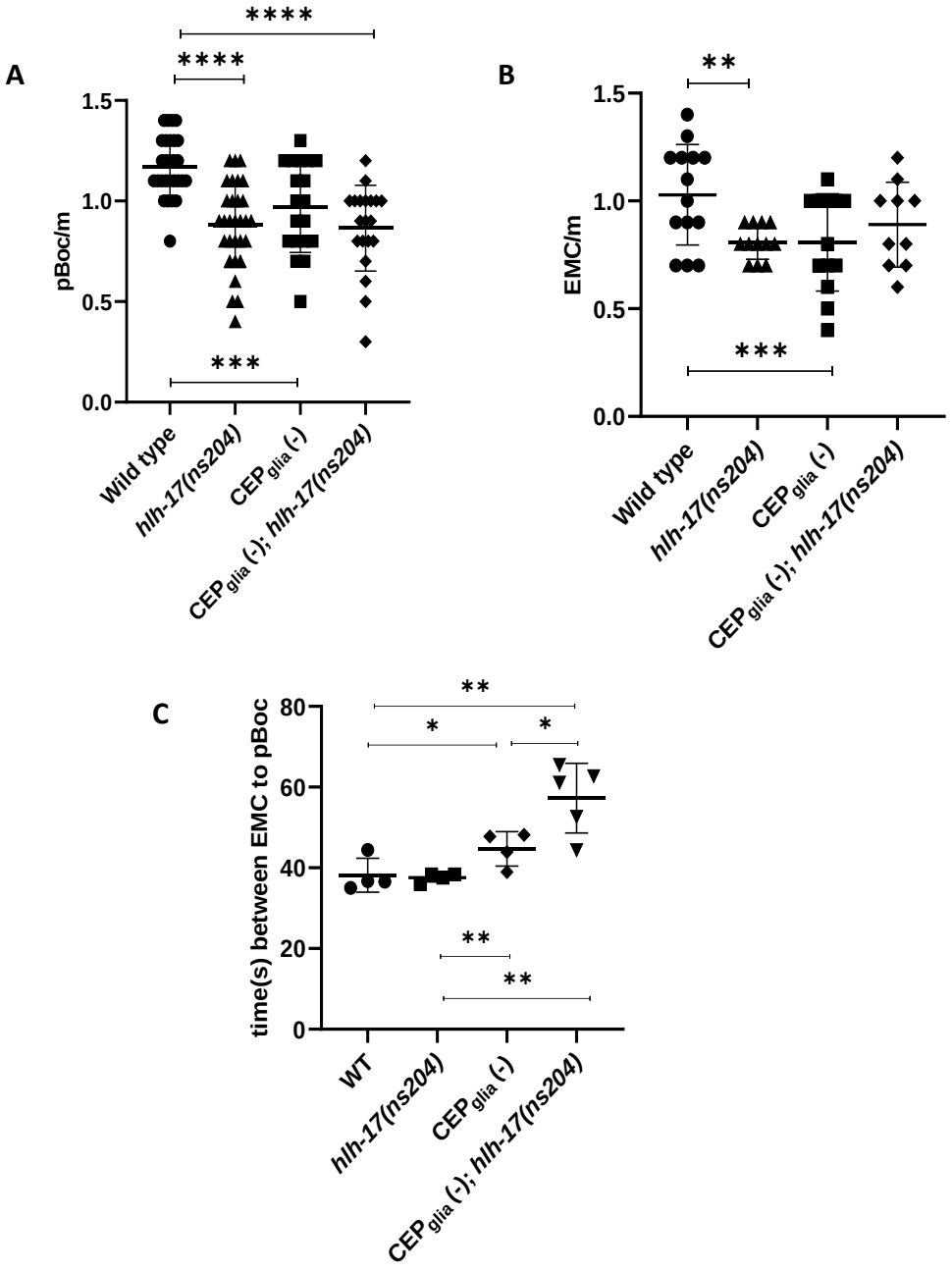


Figure 2-12 Steps in the defecation motor program were affected in ΔCEP_{glia} hermaphrodites.

The rate of (A) posterior body muscle contractions and (B) enteric muscle contractions was quantified in hermaphrodites. (C) Quantification of the time between enteric muscle contractions (EMC) and posterior body contractions (pBoc). Int= Inter-cycle length. WT [nsIs105; *him-5(e1490)*] pBoc n=28 ; EMC, n= 14; Int, n=7, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] pBoc, n= 30; EMC, n= 12; Int, n=8 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] pBoc, n=19 ; EMC, n=13; Int, n=7 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] pBoc, n= 20, EMC, n=10, Int, n=8. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

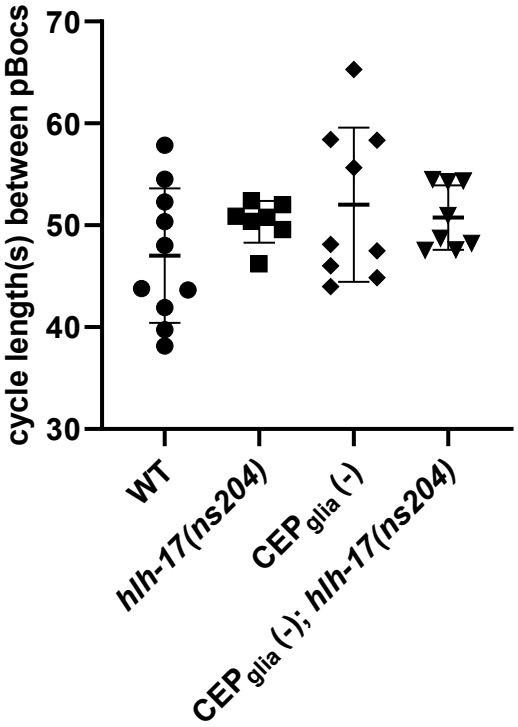


Figure 2-13 Periodicity was not affected by loss of CEP_{glia}.

Cycle length was calculated as the time between two consecutive posterior body contractions (pBoc). WT [nsIs105; *him-5(e1490)*] n=10, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] n=7 , ΔCEP_{glia} [nsIs105; *him-5(e1490)*; ola*Ex805*] n=9 , and ΔCEP_{glia}; *hlh-17(ns204)* [nsIs105; *hlh-17(ns204)*; *him-5(e1490)*; ola*Ex805*] n=8.

2.5.6 Ectopic Expression of HLH-17

Previous work shows that HLH-17 is constitutively expressed in the CEP_{glia} throughout all developmental stages from embryogenesis to adult (McMiller& Johnson, 2005; Yoshimura et al., 2008; Tintori et al., 2016). Additionally, *hlh-17* expression is detected in presumptive CEP_{glia} cells in the tail (Appendix A1-3) and very faintly in the inner and outer labial glia that are closely associated with the CEP_{glia} (www.wormatlas.org). Sporadic expression of HLH-17 using a myristolated GFP marker has been reported in commissures (Yoshimura et al., 2008), a region that modulates the laterality of axons in response to guidance cues such as netrins. Previous work shows that the netrin protein, UNC-6, is exclusively expressed in ventral CEPglia, guides the assembly of the neural network in the nerve ring, and modulates synaptogenesis (Colon-Ramos et al., 2007), raising the possibility that HLH-17 may assist in synapse maintenance and response to signaling molecules (Appendix B)..

Currently, activity of the *hlh-17* promoter is the only cell-specific marker for CEP_{glia}, and here we used that promoter to drive expression of apoptotic caspases so that we could genetically ablate the CEP_{glia}. This consistent and persistent expression of *hlh-17* in the CEP_{glia} led us to question whether expression of *hlh-17* is critical to the unique function of the $CEP_{glia,}$ or whether ectopic HLH-17 activity in glia cells that do not normally express it was sufficient to rescue the pBoc and EMC phenotype in *hlh-17(ns204)* hermaphrodites. To test our question, we ectopically expressed *hlh-17* in glia cells of the amphid sensilla. These glia are similar to the CEP_{glia} in that their cell bodies are located in the head, and that they send a single process to the nose tip; they, and the 12 neuron pairs that they ensheath, form the major chemosensory organs of *C. elegans* (Bacaj et al., 2008; Wang et al, 2008, 2012; Han et al., 2013; Stout et al., 2014). We

hypothesized that HLH-17 function is unique to CEP_{glia} and that ectopic expression in amphid glia is not sufficient to rescue the *hlh-17(ns204)* pBoc and EMC phenotype in hermaphrodites.

As shown in Figure 2-14a, WT hermaphrodites executed 1.17 +/- 0.15 pBocs/minute, (n=28), while *hlh-17(ns204)* animals executed significantly fewer (0.88 +/- 0.21 pBoc/minute, n=30, p-value <0.0001). Ectopic expression of *hlh-17* in the amphid glia of *hlh-17(ns204)* hermaphrodites rescued the *hlh-17(ns204)* phenotype so that the rate of pBocs was not significantly different from that in WT hermaphrodites (1.15 +/- 0.17 pBoc/minute, n=15, p-value= 0.311). Interestingly, ectopic expression of *hlh-17* in the amphid glia of otherwise WT hermaphrodites (pAmPH::hlh-17, 1.23 +/- 0.07 pBoc/minute, n=12, p-value= 0.0) had no effect on pBocs. As shown in Figure 2-14b, WT hermaphrodites (1.03 +/- 0.23 EMC/minute, n=14) executed significantly more EMCs than *hlh-17(ns204)* hermaphrodites (0.81 +/- 0.08 EMC/minute, n=12, p-value= 0.0024). Ectopic expression of *hlh-17* in the amphid glia of *hlh-17(ns204)* hermaphrodites did not rescue the *hlh-17(ns204)* phenotype (0.59 +/- 0.21 EMC/minute, n=13, p-value= <0.0001). Ectopic expression of *hlh-17* in the amphid glia of otherwise WT hermaphrodites (pAmPH::hlh-17) had no effect on pBocs (1.23 +/- 0.07 pBoc/minute, n=12, p-value= 0.0) or on EMCs (1.11 +/-0.17 EMC/minute, n=13, p-value= 0.1615). Together, these data show that *hlh-17* expression in CEP_{glia} hermaphrodites is not required for posterior muscle contractions because *hlh-17* expression in other glial cells is sufficient for promoting posterior muscle contractions. In contrast, *hlh-17* expression from CEP_{glia} in hermaphrodites is necessary to influences enteric muscle contractions and expression in other glia is not sufficient to rescue *hlh-17(ns204)* EMC phenotype.

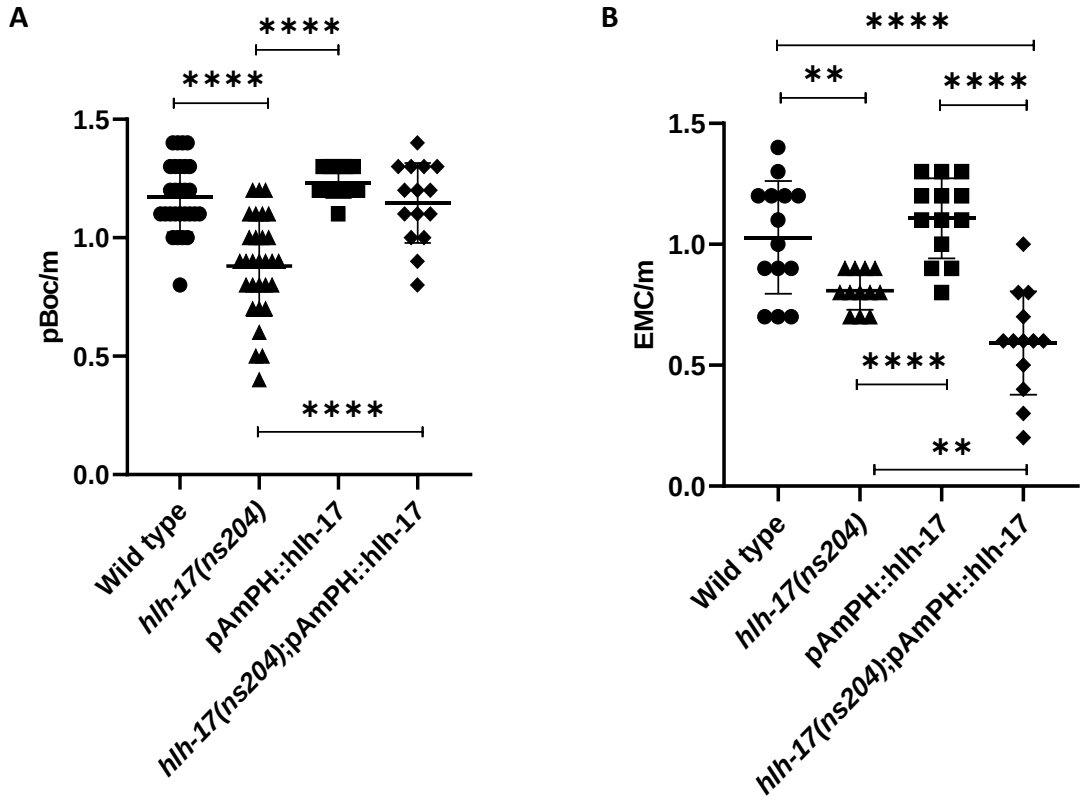


Figure 2-14 Ectopic expression of HLH-17 in hermaphrodites.

The rate of (A) posterior body muscle contractions and (B) enteric muscle contractions was quantified in hermaphrodites that ectopically expressed *hlh-17*. WT [nsIs105; *him-5(e1490)*] pBoc, n=28; EMC, n= 14, *hlh-17(ns204)* [nsIs105; *hlh-17(ns204); him-5(e1490)*$^{-}$] pBoc, n=30; EMC, n=12 , pAmPH pBoc, n=12; EMC, n= 13 , and *hlh-17(ns204)*; pAmPH pBoc, n=15; EMC, n= 13. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

2.6 Discussion

In this study, we show that loss of CEP_{glia} has a sexually dimorphic effect on the ability of *C. elegans* males and hermaphrodites to execute complex and rhythmic behaviors. Complex behaviors integrate multiple modulatory sensory inputs from independent motor programs to orchestrate specific motor outputs. In *C. elegans*, mating behavior in males and the defecation motor program (DMP) in both sexes are complex behaviors, and DMP is a rhythmic behavior that utilizes an intrinsic pacemaker to modulate periodic activation of a stereotyped sequence of movements. Interestingly, mating and DMP share similarities in anatomical features and circuitry, as well as neurotransmitter identity, making these behaviors ideal for probing glia function.

Here, we propose a model in which CEP_{glia} regulate these two behaviors by signaling through the DVC interneuron to regulate activity of the DVB neuron. In males, as shown in Figure 2-15b, DVB regulates spicule protraction during mating by synapsing with the protractor muscle, which is electrically coupled to the anal depressor. The protractor muscle is coupled to the sphincter muscle, and through it, to the intestinal muscle, which regulates enteric muscle contraction during defecation. The DVB neuron switches between excitatory and inhibitory signaling to fine-tune execution of spicule protraction. Based on our data, we propose that the CEP_{glia} modulate protractor muscle contraction by signaling through the DVC interneuron to mediate neurotransmission from the DVB neuron and to regulate synapse formation with the protractor muscle. The CEP_{glia} can then stimulate the protractor and anal depressor muscles simultaneously to regulate spicule protraction during mating. it is possible that, by regulating synapse formation and neurotransmission of the DVB neuron, CEP_{glia} signaling ensures that the

DVB neuron synapses with the protractor muscle in males and modulates the time during which the protractor muscle is stimulated, thereby promoting the release of inhibitory signal from the DVB neuron to ensure that spicule protraction occurs in the proper behavioral context. As shown in Figure 2-15a, in hermaphrodites, DVB synapses with the anal depressor and intestinal muscles, both of which are electrically coupled to the sphincter muscle to regulate enteric muscle contractions during defecation. In hermaphrodites, the DVB neuron transmits excitatory GABA to stimulate contraction of the anal depressor and intestinal muscles. In contrast to males, our model predicts that CEP_{glia} signaling through DVC interneuron in hermaphrodites regulates the dyadic synapse formation between the anal depressor and intestinal muscles and promotes the excitation and contraction of these muscles. By regulating synapse formation and neurotransmission of the DVB neuron in hermaphrodites, CEP_{glia} signaling may act as a guide to ensure that the DVB neuron synapses with the intestinal and anal depressor muscles and promotes the transmission of excitatory signals from the DVB neuron to modulate the frequency of muscle contractions.

Loss of CEP_{glia} results in the abnormal execution of behaviors, resulting in an overall reduction in the percentage of males that complete that mating behavior. These data indicate that CEP_{glia} not only influence individual motor behaviors but coordinate the temporal activity of motor neuron signaling. This finding is supported by previous work in hippocampal astrocytes, showing that glia can transmit glutamate and adenosine to either potentiate or depress activity-dependent neurotransmitter release, implicating astrocytes in the fine-tuning of neuronal responses by differentially regulating synaptic transmission (Covelo and Araque, 2018).

Our results show that *hlh-17* expression in CEP_{glia} may have a less global, yet important role in modulating and integrating neurotransmitter signaling that contributes to the mechanism

by which CEP_{glia} fine-tune the fluent coordination of complex behaviors. We found that *hlh-17* expression in CEP_{glia} did not significantly affect male mating behavior. However, *hlh-17(ns204)* males were impaired in the act of prodding and experienced difficulty transitioning to spicule insertion. These males occasionally executed premature spicule protraction. Our finding here that HLH-17, previously shown to be required for normal dopaminergic behaviors (Felton and Johnson, 2011, 2014), influences male mating behavior by modulating neurotransmitter signaling, aligning with previous work demonstrating that prodding and spicule insertion are dopamine dependent behaviors and that dopamine signaling fine-tunes and reduces ineffective and repetitive movements during mating (Correa et al., 2012). Similarly, studies in zebrafish suggest that inhibiting dopamine reduces persistence in swimming behavior (Tran et al., 2015), and that noradrenergic neurons respond to feedback from failed swim attempts by progressively activating radial astrocyte glia (Mu et al., 2019). In turn, the radial astrocyte glia are able to sense ineffective behavior and in turn initiate changes in the behavioral state, which suppresses swimming behavior (Mu et al., 2019).

In *C. elegans* males, precision and accuracy during the mating ritual ensure robust mating success and can assist in reducing repetitive behaviors that are ineffectively executed. The quality of the attempts during each individual behavior of the mating ritual promotes fluent coordination of subsequent behaviors, ensuring that the ultimate goal of spicule insertion and sperm transfer is achieved. After the vulva is detected, the post cloacal neurons assist in ensuring that the male tail is precisely positioned over the vulva (Liu and Sternberg, 1995; Loer et al., 1999) and then initiates insertion behavior by releasing acetylcholine onto the protractor muscle, which is then directly activated by the cholinergic SPC neuron (Garcia et al., 2001). Periodic contraction of the spicule muscles during prodding behaviors is directed by the post cloacal

sensilla, and stimulation by the cholinergic SPC neuron promotes prolonged contraction of the protractor muscle to ensure spicule insertion and sperm transfer (Garcia et al., 2001; Liu et al., 2007). Our model predicts that CEP_{glia} signaling assists in reducing prolonged contraction, which can be detrimental to males, by temporally mediating release of inhibitory GABA to ensure accuracy in the timing of spicule protraction. Hart and Hobert (2018) recently suggested that the DVB neuron, which synapses with the SPC neuron and the protractor muscle, assists in regulating the activation of the protractor muscle. The branching and signaling from the DVB neuron promotes synaptic connectivity between the DVB neuron and the protractor muscle and regulates the excitatory signals released from the SPC neuron by releasing inhibitory GABA into the neuromuscular synapse. In support of our model, CEP_{glia} signaling through the DVC interneuron eliminates unorthodox spicule protraction. By regulating synapse formation with the protractor muscle, CEP_{glia} may ensure that the DVB neuron synapses with the protractor muscle to inhibit over stimulation by the SPC neuron.

Hart and Hobert (2018) mention that expression of the neuroligin protein NLG-1 in the postsynaptic protractor and anal depressor muscles and expression of the neurexin protein NRX-1 specifically in the DVB neuron regulates neurite outgrowth. Therefore, the cell autonomous expression of NRX-1 in the DVB neuron may be required for guiding synaptic connectivity with the DVC interneuron. Südhof (2008) suggests that complementary expression of neuroligins and neurexins modulates synaptic transmission. Additionally, Mariotti et al. (2018) show that astrocyte signaling depresses in parvalbumin synapses but potentiates in response to somatostatin interneurons. Previous works demonstrate that hippocampal astrocytes respond to interneuron signaling through $GABA_B$ receptors (Andersson et al., 2007; Kang et al., 1998; Serrano et al., 2006) and in turn respond with release of glutamate onto presynaptic terminals, which promotes

an increase in inhibitory synaptic transmission into the synaptic cleft (Kang et al., 1998; Losi et al., 2014). These data demonstrate the importance of intercellular signaling between glia and interneurons and supports our model, which emphasizes that CEP_{glia}-DVC interaction is required for differentially regulating DVB neuron activity.

CEP_{glia} signaling may promote synapse formation with the DVC and in turn regulate the expression of neuroligin proteins in the DVC neuron to promote synapsing with DVB (White et al., 1986; Hart and Hober, 2018). Cholinergic interneurons are tonically active and can receive input from both glutamatergic and GABAergic neurons (Wilson and Goldberg, 2006) to promote a feed forward control system (Bennett and Wilson, 1989; Chang and Kitai, 1992; DiFiglia, 1987; DiFiglia and Carey, 1986; Nanda et al., 2009; Sullivan et al., 2008; Szydlowski et al., 2013). Therefore, CEP_{glia}-DVC interaction may be required for promoting feedforward inhibition of the cholinergic signaling from the SPC neuron to modulate protractor muscle activity. Together these data predict that modulation of DVB is complex and requires bidirectional signaling that modulates functional synapse formation with neurons and muscles.

Here, we have provided the first behavioral evidence that the invertebrate CEP_{glia} are sexually dimorphic in regulating behaviors. Loss of CEP_{glia} resulted in premature spicule protraction during mating but had no effect on enteric muscle contractions of the defecation motor program (DMP). In males, CEP_{glia} signaling is required to negatively regulate muscle contractions. However, in hermaphrodites, loss of CEP_{glia} altered DMP behaviors, suggesting that CEP_{glia} signaling promotes excitation of the muscles to initiate contraction. This finding is supported by previous work in mammalian glia demonstrating that glia response mechanisms to signaling molecules differ in males and females. In rats, for example, microglia inflammatory signaling response to lipopolysaccharide and estradiol treatment differs in males and females

(Loram et al., 2012). Additionally, adult female rats have a higher number of activated microglia and pro-inflammatory cytokines than males, which is thought to contribute to how mood disorders are mediated in the male and female brain (Schwartz et al., 2012; Lenz and McCarthy, 2015). Previous studies show that hippocampal astrocytes differentially respond to estradiol (Wise et al., 2009). The male astrocyte response decreased the expression of pro-apoptotic factors but in females the expression of pro-apoptotic genes was not altered. Apoptotic cell death was decreased in female astrocytes (Frago et al., 2017), however. Our work suggests that, in contrast to males, the CEP_{glia}-DVC interaction in hermaphrodites positively regulates behaviors.

During defecation, the GABAergic DVB neuron releases GABA to the enteric muscles to promote contractions. McIntire et al., (1993b) demonstrated that GABA released from the DVB neuron is excitatory GABA (Schuske and Jorgensen, 2004; Jobson et al., 2015). Although it remains to be determined whether GABAergic neurotransmission stimulates the enteric muscle cells, studies have proposed an alternate pathway that includes activation of the enteric muscles through FMRFamide neuropeptide signaling from the DVB neuron (Thomas 1990; Schinkmann and Li, 1992). Pereira et al. (2015) showed that the sex-shared AIM interneuron utilizes glutamate in hermaphrodites and acetylcholine in males. Similarly, the PVN interneuron that is glutamatergic in males acquires a cholinergic indentity in hermaphrodites (Landis and Keefe, 1983; Pereira et al., 2015; Serrano-Saiz et al., 2017). Moreover, previous work demonstrate that astrocytes have the ability to alter neurotransmitter effects (Perea et al., 2016). Supporting our model, CEP_{glia} differentially regulate neutransmission of the sexually dimorphic DVB neuron in males and hermaphrodites, in part to assist in coordinating the complex signaling mechanisms required for directing specific motor outputs.

In both males and hermaphrodites, loss of HLH-17 significantly altered the rate at which posterior and enteric muscle contraction were executed. The isomorphic role of HLH-17 in defecation may be explained by a difference in the transcriptional signature of male and hermaphrodite CEP_{glia}, which would have to be further explored. Guneykaya et al. (2018) showed that genes are differentially expressed and regulated in hippocampal and cortex microglia from males versus females. Additionally, previous studies show that expression of receptors are differently regulated in hippocampal astrocytes from males and females (Tetel and Pfaff, 2010; Frago et al., 2017). Moreover, our ectopic expression results suggest that *hlh-17* expression in other glia is sufficient for regulating behavior. Previous work demonstrates that the expression of certain transcription factors in glia can alter morphological plasticity (Procko et al., 2011, 2012) and may contribute to changes in glia function. Additionally, recent work in *Drosophila* shows the importance of *mir-1* expression in glia for regulating behavior (Mazaud et al., 2019). Future studies may identify the influence of *hlh-17* expression on the transcriptional network of glia and further outline the mechanisms by which HLH-17 regulates behavior in males and hermaphrodites.

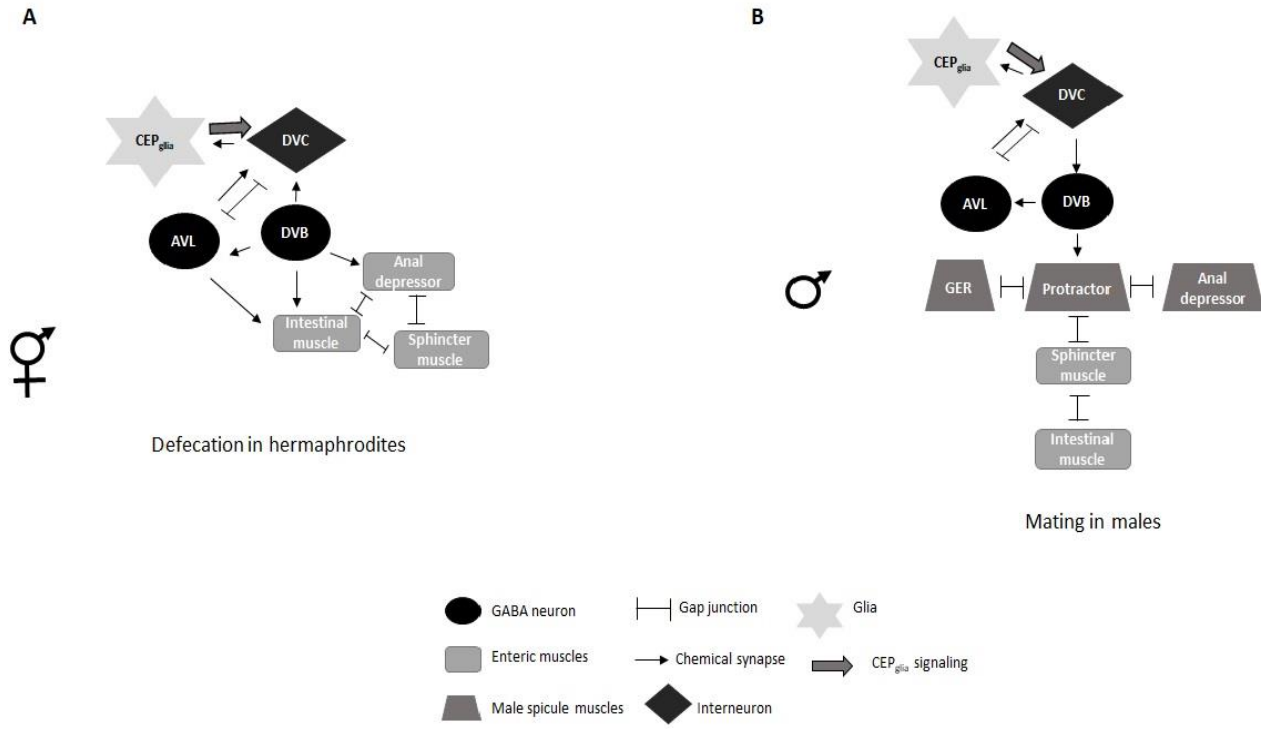


Figure 2-15 CEP_{glia} are sexually dimorphic in regulating behavior.

Proposed mechanisms by which CEP_{glia} transmit signaling molecules to direct behavior in (A) hermaphrodites and (B) males.

3 FUTURE WORK

3.1 CEP_{glia} signaling is required for DVB function

3.1.1 General Background and Significance

Not only have recent studies recognized glia as essential components and active participants in the brain (Losi et al., 2014; Meyer and Kasper, 2017; Dzyubenko et al., 2016), but many reports demonstrate the importance of glia-neuron communication in regulating normal behavior responses (Colón-Ramos et al., 2007; Shao et al., 2013; Mederos and Perea, 2019). However, the mechanisms by which the uptake and release of gliotransmitters such as ATP/adenosine, GABA, and glutamate in modulating the balance between excitatory and inhibitory synapses has yet to be elucidated. Only recently have scientists explored the significance of glial modulation on plasticity and function of neural circuits (Lim et al., 2013; Lee et al., 2017; Tan et al., 2017) and how loss of glial modulation influences changes in behavioral states (Chen et al., 2014; Mu et al., 2019). Mechawar and Savitz (2016) demonstrated that mood disorder and bipolar disorder patients have a reduced number of astrocytes and functional changes in GABAergic interneurons. Previous work in ALS mouse models demonstrate that loss of inhibitory signaling from GABAergic interneurons (Zhang et al., 2016) and astrocytes (Pardo et al., 2006) results in hyperexcitability. The onset of hyperexcitability, which is a drastic increase in response to stimuli common in Amyotrophic Lateral Sclerosis (ALS) patients (Bae et al., 2013), is due to dysfunctional signaling from GABAergic interneurons, as a result of glia loss.

The results from this study are exciting because they demonstrate for the first time the mechanisms by which glia-neuron interaction modulate both excitatory and inhibitory synapses to influence the execution of multiple behaviors. In Chapter 2, we proposed a model by which CEP_{glia} differentially regulates synapse formation and synaptic transmission of the GABAergic DVB neuron by signaling through the DVC interneuron. Results from our study demonstrated that CEP_{glia} influence the activity of the DVB neuron. However, the cell body of the DVB neuron is located in the tail of males and hermaphrodites and extends processes that stop halfway along the body axis (Garcia and Collins, 2019; Ravi et al., 2019). Therefore, it was unclear how CEP_{glia}, which reside in the head and extend processes anteriorly towards the mouth of *C.elegans*, would transmit signals to the tail. Previous work showed that the cell body of the DVB neuron bundled with the DVA and DVC interneuron in the tail to form the dorso-rectal ganglion (White et al., 1986; Li et al., 2006). While the DVB processes extend mid-way along the body, DVA and DVC send their processes to the nerve ring in the head. In analyzing previously published electron micrographs (White et al., 1986) and recent computer-based studies that provided synapse lists from reconstructed *C.elegans* neural networks (Bhatla, 2009; Jarell et al., 2012, Xu et al., 2013; Sammut et al., 2015), we found that the glutamatergic DVC interneuron forms chemical synapses with both the DVB neuron and CEP_{glia}. Previous work suggests that stimulation of the transient receptor potential (TRP) channel, expressed throughout the DVC axon, inhibits body wall muscle contractions by negatively regulating downstream command interneurons and motor neurons (Li et al., 2006). To determine whether intercellular signaling between the CEP_{glia} and the DVC interneuron is required for regulating behavior, future studies would test CEP_{glia} response to DVC signaling and the effect of CEP_{glia} signaling on behavior.

3.1.2 CEP_{glia} are required for DVB function

Based on our model, DVB neurons switch between releasing GABA or neuropeptides onto the enteric muscles (anal depressor, intestinal, and sphincter muscles) to increase enteric muscle contraction and expulsion during the defecation motor program in hermaphrodites. DVB releases GABA to reduce contraction of the spicule protractor muscle in the wrong behavioral context. To determine whether CEP_{glia} signaling is required for DVB function, we planned to assess whether loss of CEP_{glia} signaling would result in dysregulation of the release of GABA. We predicted that loss of CEP_{glia} would result in overstimulation of the enteric muscles. Although our model predicts that CEP_{glia} function to promote excitation of the enteric muscles, GABA or neuropeptide release is temporally modulated by CEP_{glia}. Our data suggests that the time between an enteric muscle contraction and a posterior muscle contraction is increased with the loss of CEP_{glia} and results in fewer posterior contractions, therefore we rationalized that loss of CEP_{glia} signaling would reduce posterior contractions as well.

We hypothesized that CEP_{glia} signaling not only regulates DVB neuron synaptic plasticity but also assists in directing synapse formation with the anal depressor and intestinal muscles in hermaphrodites and the spicule protractor muscles in males. Therefore, by inhibiting CEP_{glia} signaling we propose that DVB may also form inappropriate and ectopic synapses. In males, loss of CEP_{glia} signaling would result in unorthodox spicule protraction due to overstimulation of the protractor muscle by the cholinergic SPC neuron and reduced inhibitory signaling from the DVB neuron to the protractor muscle. Alternatively, if CEP_{glia} signaling had no effect on DVB function then loss of CEP_{glia} may have altered neurite outgrowth from the DVC neuron, eliminating synapse formation with the DVB neuron. This result would suggest that CEP_{glia} is

required for synapse formation with the DVC interneuron and that the DVC interneuron is necessary and sufficient to regulate DVB neuron activity for defecation and mating behavior.

To inhibit signaling we planned to hyperpolarize the CEP_{glia} by directing the expression of an inwardly rectifying potassium (Kir) channel, (Emtage et al., 2012) IRK-1 in the CEP_{glia}. Kir channels allow a small amount of potassium (K^+) to flow out of the cell but a large amount of potassium to move into the cell. The large inflow of potassium ions distorts the electrochemical equilibrium resulting in a negative membrane potential. This change in the membrane potential causes the cell to become hyperpolarized in which the cell can no longer fire action potentials or release signaling molecules (Franc, 2014). To inhibit CEP_{glia} from responding and releasing signals to the DVC interneuron, we would use the *hlh-17* promoter to drive the expression of IRK-1 in the CEP_{glia}. Because CEP_{glia} act asguideposts and modulate synaptogenesis, we predicted that loss of CEP_{glia} signaling early in development may alter patterning and organization of cells during embryogenesis (Sulston et al., 1977, 1983). To temporally control the expression of IRK-1 in CEP_{glia}, we would utilize the light sensitive ion channel, Channelrhodopsin-2. The *hlh-17* promoter and IRK-1 would be cloned into a Channelrhodopsin-2 (ChR2) expression vector and co-injected with a fluorescent marker (*myo*-2p::mCherry) to assess expressivity, as previously described by Ardiel and Rankin, 2015. Mating behavior in males and the defecation motor program in males and hermaphrodites would be assessed using a real-time computer vision worm tracking system, previously used to quantify behaviors in *C. elegans* (Swierczek et al., 2011).

3.1.3 CEP_{glia} are stimulated by the cholinergic DVC interneuron

Previous work demonstrated that DVC forms a chemical synapse with CEP_{glia}, however it is unclear whether these cells implement bidirectional signaling to modulate behavior. In the

previous section, I describe methods to determine whether CEP_{glia} signaling is required for regulating behavior. However, it is unclear how the CEP_{glia} are stimulated. Since DVC is presynaptic to the CEP_{glia}, we predict that signaling from the DVC interneuron promotes the release of modulatory signals from the CEP_{glia} to regulate behavior. To determine whether the CEP_{glia} mediated response requires stimulation from the DVC interneuron, we planned to artificially express the biosynthetic glutamine synthetase enzyme, *gln-3*, which is also found in our microarray analysis (Felton, 2014). We predicted that by driving the artificial expression of *gln-3* under the direction of the *hlh-17* promoter, we could mimic DVC signaling to CEP_{glia}. We predict that CEP_{glia} respond to glutamatergic signaling from the DVC by releasing gliotransmitters such as glutamate, GABA, or adenosine to modulate behavior (Acton and Miles, 2017). Release of these gliotransmitters in turn directs the activity of the DVB neuron.

Recently, Oh et al. (2019) developed a GAL4-UAS temperature-sensitive system in *C. elegans* that utilized the *ceh-63* promoter to drive expression of GFP in DVC neurons. Future work would have utilized this robust system to drive the expression of previously characterized *cha-1* mutants in the DVC interneuron to alter biosynthesis of acetylcholine (Rand 1989). To assess CEP_{glia} activity and response to the cholinergic signaling, we would perform calcium imaging using a microfluidic device, a method previously described in *C.elegans* (Chronis et al., 2007; Katz et al., 2018). Together, results from this work would demonstrate that signaling from the DVC interneuron prompts CEP_{glia} function and that bidirectional signaling between DVC and CEP_{glia} is required for regulating behavior.

Figure 3-1 The DVC interneuron sends its axon to the nerve ring.

A schematic showing that the cell body for the DVC interneuron resides in the tail and sends processes to the head of the nematode at the nerve ring. Adapted from Li et al., 2006.

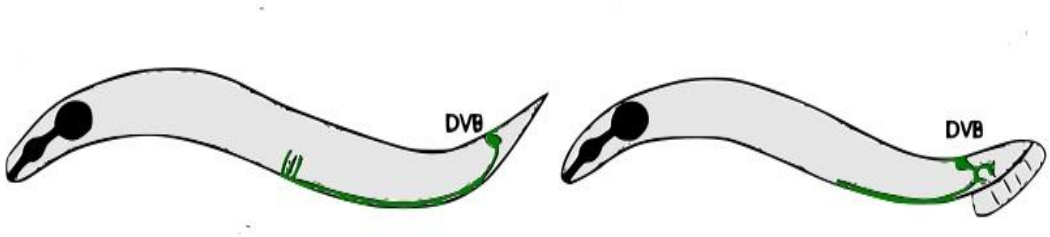


Figure 3-2 The DVB neuron does not extend to the nerve ring.

A schematic of the DVB neuron. The cell body for the DVB neuron resides in the tail and sends processes along the body wall that stop at the mid region of males (left) and hermaphrodites (right). Adapted from Hart and Hobert, 2018.

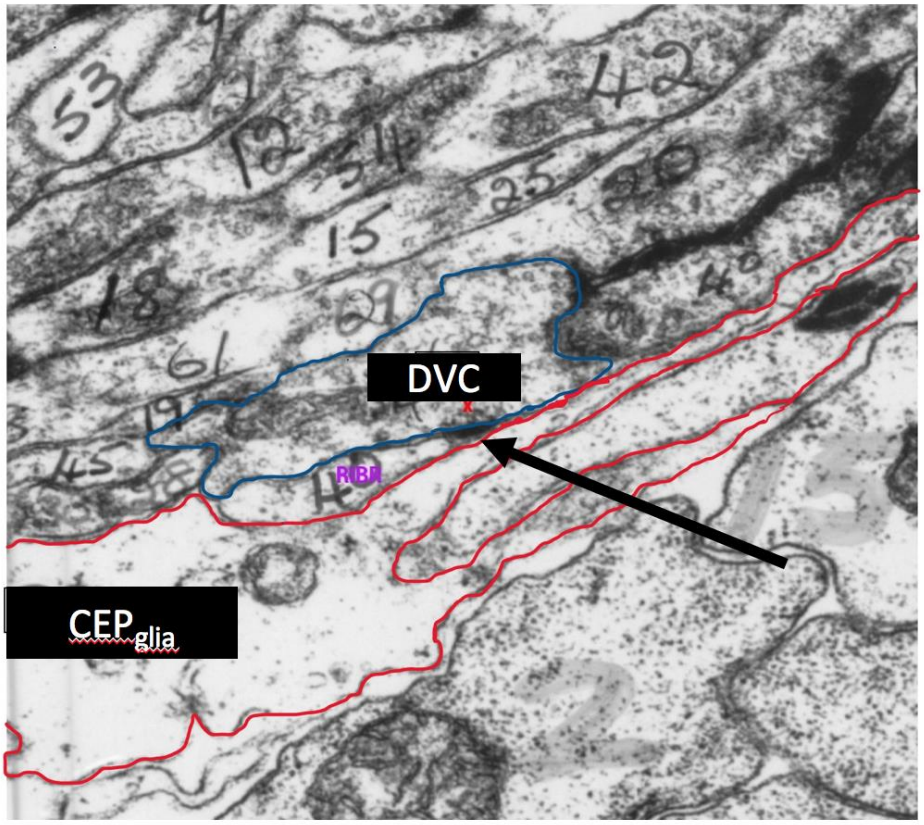


Figure 3-3 CEP_{glia} form a chemical synapse with the DVC interneuron.
Micrograph of a CEP_{glia} (red) and its presynaptic partner, DVC (blue). Black arrow indicates the chemical synapse. Adapted from White et al., 1986.

4 CONCLUSION

Multiple recent studies suggest glia to be a vital component in mediating behavior (Ongur and Drevets, 1998; Uranova et al., 2004; Cao et al, 2013; Cui et al., 2014; Moraga-Amaro et al., 2014). A key question that has yet to be thoroughly addressed is how intercellular signaling pathways are interconnected and aligned with behavior. My doctoral work identifies mechanisms by which glia regulate complex behaviors and infers functional homology to mammalian astrocytes in regulating behavior. Additionally, I demonstrate that CEP_{glia} and CEP_{glia}-expressed HLH-17 together regulate the synergy between behaviors in the complex male mating ritual. Moreover, although expression of HLH-17 is not necessary for the completion of these behaviors, HLH-17 expression does assist in fine-tuning to minimize redundancy and promote coordination and control. Lastly, future studies are outlined that may contribute to emerging discoveries about the role of interneuron-astrocyte interaction and intercellular signaling (Pereas et al., 2016; Mederos and Perea, 2019).

4.1 Glia regulate sexually dimorphic behaviors

The regulation of sexually dimorphic behaviors such as mating, courtship, and aggression has largely been described in the context of neural control (Kelley, 1988; Xu et al., 2012; Manoli et al., 2013). Only recently have studies demonstrated the importance of glia in regulating sexually dimorphic behaviors. Yet, the link between sexual dimorphism in behavior and glia function has mostly been implied by sexual dimorphism in astrocyte number in regions of the brain which would affect the significance and impact of a glial response. Here, we provide the first line of behavioral evidence demonstrating that the astrocyte-like CEP_{glia} communicate with neurons to regulate sex-specific behaviors. In a recent review, Wickens et al. (2018), emphasizes the importance of regulating the neurotransmitter, glutamate. Glutamatergic signaling from glia

plays a role in psychiatric disorders, such as schizophrenia and major depressive disorder, which exhibit sex bias (Noble, 2005; Markham, 2012). Frankurt et al. (1984) demonstrated that in rodents, males and females have a high concentration of glutamate in different region of the brain. Moreover, sex differences are apparent in synaptic glutamate signaling, in that females, opposed to males, commonly respond to glutamate signaling through AMPR receptors, which assist in modulating cell excitability (Monofort et al., 2014). Additionally, NMDA receptor antagonists increased the concentration of dopamine in male rats but decreased dopamine in females in the ventral tegmental area and the prefrontal cortex (Locklear et al., 2016). In our model, we describe the astrocyte-like CEP_{glia} as an essential component in mediating diverse neurotransmitter signals in males and hermaphrodites. We show that CEP_{glia} signaling promotes excitatory neurotransmission in hermaphrodites to modulate the defecation motor program while promoting inhibitory neurotransmission in males to diminish redundancy and inaccuracy in executing sex specific behaviors. In all, my dissertation work demonstrates the importance glia function and establishes the foundation for future studies in uncovering sex-specific roles of glia.

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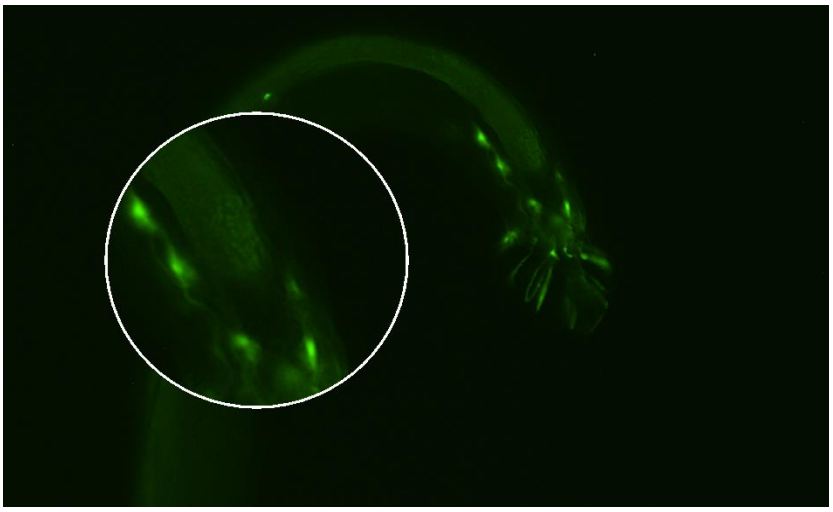
APPENDICES

Appendix A Image analysis HLH-17 expression in the male tail

Appendix A.1 Ray structural glia in ram-5::gfp males



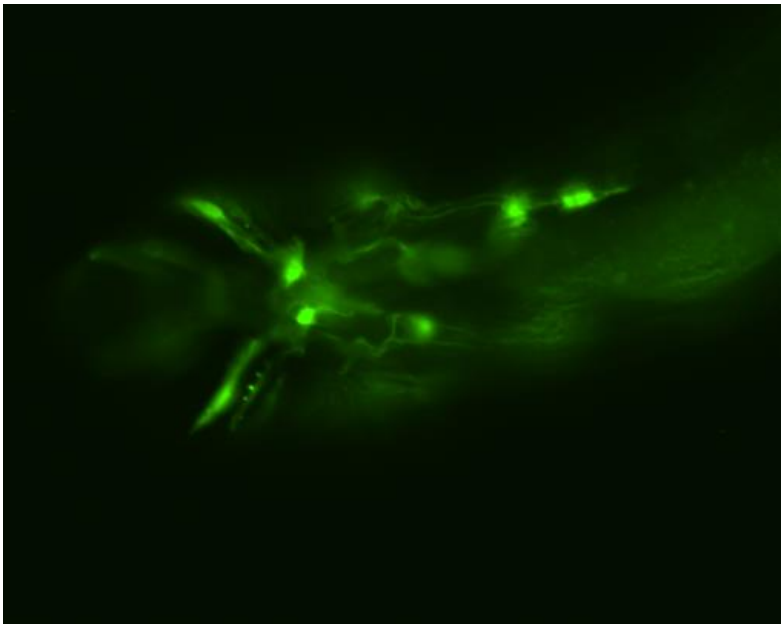


Image of wild-type tail ray sensilla in adult males. GFP fluorescence indicates ray structural glia in *ram-5*::gfp males. Images were obtained at 20X magnification (scale bar = 100µm).

Appendix A.2 HLH-17 localizes to neuronal support cells required for mating

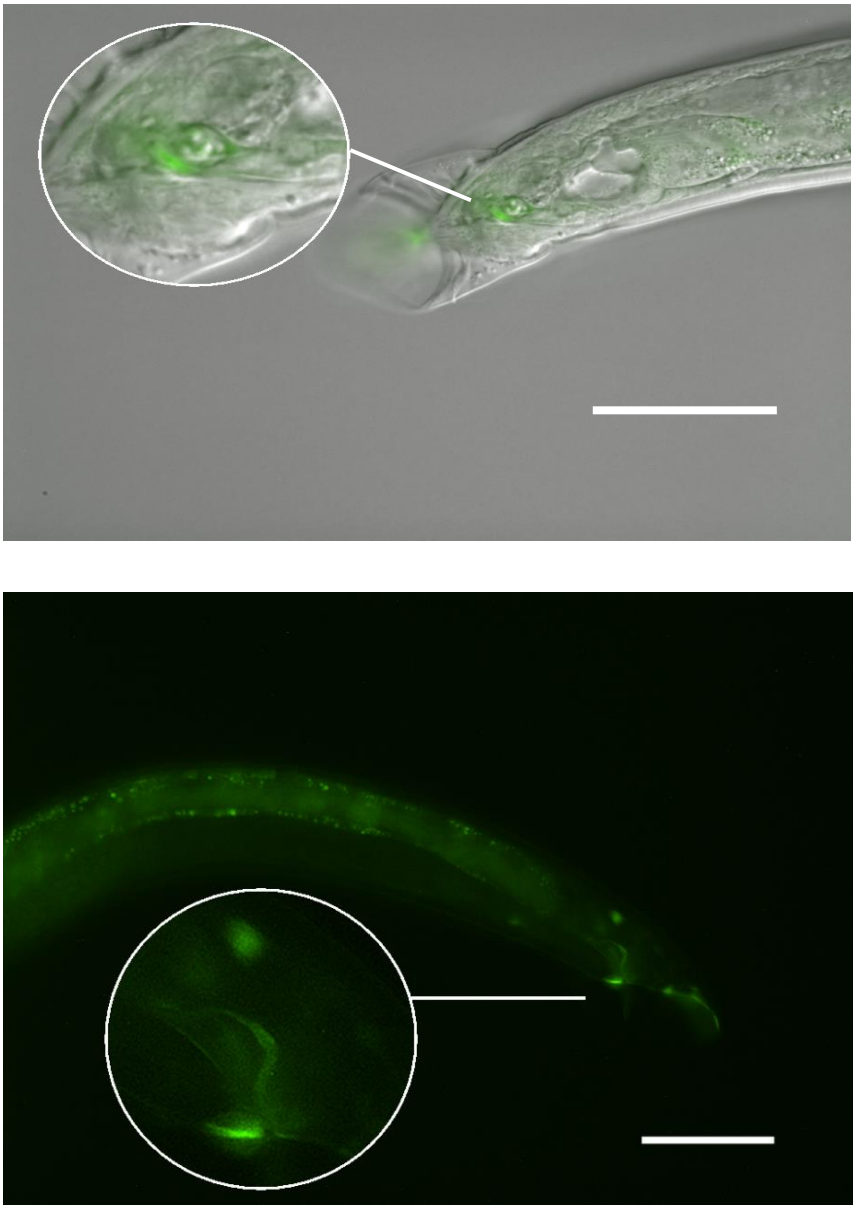
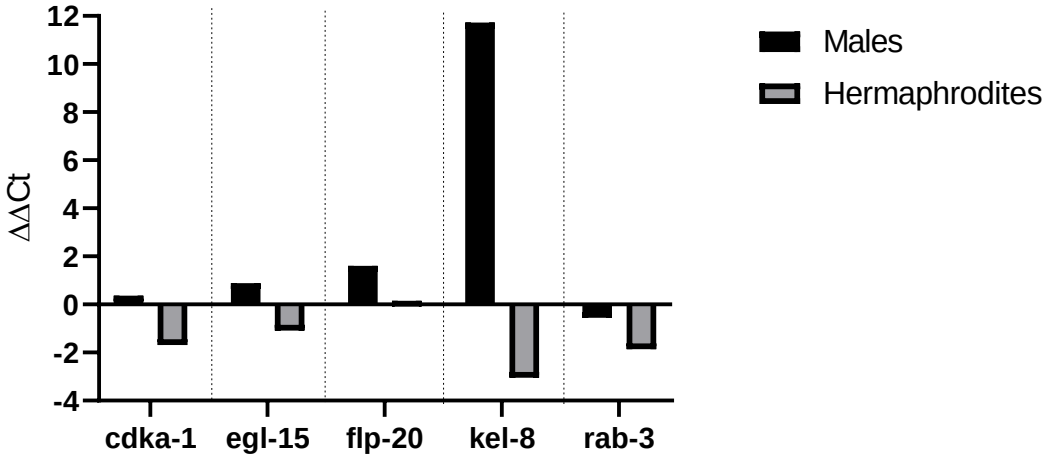


Image of wild-type male tail sensilla. GFP fluorescence indicates expression of HLH-17 in tail of p*hlh-17*; HLH-17::GFP males. Images were obtained at 20X magnification (scale bar = 100µm).

Appendix B HLH-17 differentially regulates genes required for synaptic transmission in males and hermaphrodites



RNA extraction from whole worm and expression analysis. For three biological replicates, a total of 300 males and hermaphrodites were collected separately for WT and *hlh-17(ns204).*