The Neural Circuits and Synaptic Mechanisms Underlying Motor Initiation in *C. elegans*

by

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DEDICATION

This thesis is dedicated to my family, who provided me with continual support and unconditional love. To my mother, Lori J. Piggott, whose strength in times of hardship is unprecedented, and who maintains a childlike excitement about life. To my father, Thomas D. Piggott, who often sacrifices all of himself to ensure my family's well-being and has supported me in all endeavors scholastically as well as athletically. To my sister, Kimberly S. Piggott, who has become both my best friend and continual cheerleader. To my brother William T. Piggott, whose support and advice have been instrumental along the way. Without all of you, surely I would never have reached such great heights.

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LIST OF ABBREVIATIONS

AGE-1	AGEing alteration 1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ATP	Adenosine Tri-Phosphate
AVR-14	Avermectin Resistant 14
CINs	Commissural interneurons
CPG	Central Pattern Generator
DA	Dorsal A-type motor neuron
DAF-2	abnormal DAuer Formation
DB	Dorsal B-type motor neuron
DD	Dorsal D-type motor neuron
DEGT-1	DEGeneration of Touch neurons 1
DRG	Dorsal Root Ganglia
EAT-4	EATing abnormal
EGL-3	EGg Laying defective 3
EM	Electron Microscope
ENaC	Epithelial sodium (Na+) Channel
EPSP	Excitatory Post Synaptic Potential
GABA	ɣ-aminobutyric acid
GLR-1	Glutamate Like Receptor 1

GRK-2	G-protein-coupled Receptor Kinase 2
HD	Huntington's Disease
Ia-INs	Ia Interneurons
INs	Interneurons
INS-1	INSulin related 1
IPSP	Inhibitory Postysynaptic Potential
LGC-55	Ligand Gated ion Channel 55
MEC	MEChanosensory abnormal
MLR	Mesencephalic Locomotor Region
MN	Motor Neuron
MRI	Magnetic Resonance Imaging
NGM	Normal Growth Media
NMDA	N-Methyl-D-aspartic acid or N-Methyl-D-aspartate
NMR-1	N-Methyl-D-aspartic acid or N-Methyl-D-aspartate class glutamate receptor 1
OC	Obsessive Compulsion
OCD	Obsessive Compulsion Disorder
OCR-2	Osm-9 and Capsaicin receptor-Related
ODR-3	ODoRant response abnormal
OSM-10	OSMolarity avoidance abnormal 10
OSM-9	OSMolarity avoidance abnormal 9
PD	Parkinson's Disease
PUFA	Poly Unsaturated Fatty Acid
RCs	Renshaw Cells
TRP-4	Transient Receptor Potential 4
TRPV	Transient Receptor Potential channel, Vanilloid subfamily

TS	Tourette's Syndrome
UNC-8	UNCoordinated 8
VA	Ventral A-type motor neuron
VB	Ventral B-type motor neuron
VD	Ventral D-type motor neuron

ABSTRACT

Understanding the neural circuits and genes that underlie behavior is a fundamental question in the field of neuroscience. While behaviors diverge across species, recent structural analysis of neural anatomy suggests that many patterns of neural connectivity are conserved across species. These neural motifs can be thought of as building blocks that may be increased or reconfigured to generate nervous system complexity. It can be difficult to define and characterize properties of neural circuits in complex systems, such as the human brain, which possesses an estimated 100 billion neurons and 3 trillion synapses. In contrast, with only 302 neurons and 7,000 synapses, the genetic model, *Caenorhabditis elegans,* has become an attractive system to dissect how neural circuits and genes generate behavior. *Caenorhabditis elegans* exhibits a number of complex behaviors, all of which involve basic locomotion. During locomotion, worms initiate backward movement to change direction spontaneously or in response to sensory cues; however, the underlying neural circuits are not well defined. We applied a multidisciplinary approach to map neural circuits in freely behaving worms by integrating functional imaging, optogenetic interrogation, genetic manipulation, laser ablation, and electrophysiology. Using this approach,

we discovered that the long standing model for backward movement in *C. elegans* required substantial revision. Previously, it was thought that a set of command interneurons acting as a stimulatory circuit were required to drive backward movement. We discovered that although important for execution and coordination, backward movement persisted in the absence of the command interneurons. Importantly, we identified a new disinhibitory circuit that acts in parallel to the stimulatory circuit to promote initiation of backward movement and that circuitry dynamics is differentially regulated by sensory cues. Both circuits require glutamatergic transmission but depend on distinct glutamate receptors. This dual mode of motor initiation control is found in mammals, suggesting that distantly related organisms with anatomically distinct nervous systems may adopt similar strategies for motor control. Additionally, our studies illustrate how a multidisciplinary approach facilitates dissection of circuit and synaptic mechanisms underlying behavior in a genetic model organism.

CHAPTER I:

INTRODUCTION

INTRODUCTION

The mammalian brain has evolved over millions of years. Compared to simpler invertebrates, mammals exhibit larger brain size, behavioral complexity and flexibility in environmental interactions [1]. While behaviors diverge across species, recent structural analysis of neural anatomy suggests that many patterns of neural connectivity are conserved across species [2]. These neural motifs can be thought of as building blocks that may be increased or reconfigured to generate nervous system complexity. At the core, elemental features of nervous system architecture and function may be conserved throughout evolution as they are conducive to information processing.

Brain disorders such as schizophrenia, autism, depression, and Parkinson's, among others, result from aberrant function of neural circuits [3]. These disruptions can be manifested during development, due to deficits of structural and functional integration or as a result of brain lesions [4]. As diverse disruptions in circuit function can generate similar clinical symptoms, a major challenge going forward is to define specific mechanisms that underlie a particular disease. Many diseases of the nervous system are heritable,

suggesting that they result from specific genetic abnormalities [4]. Therefore, defining the genetic specificity of particular brain disorders could lead to the development of better, tailored therapies. As neural motifs and genes underlying neurotransmission are conserved throughout species, a more complete understanding of neural circuit function could benefit from investigating neural networks in models with simpler nervous system structure [1, 5]. Deriving key principles of how these neural circuits function and the mechanism of their dysfunction holds promise for revealing elemental features of nervous system dynamics that may be extended to higher ordered species.

NEURAL CONTROL OF LOCOMOTION

Behavior is generated from neural circuits. Locomotion, in particular, represents a major feature of behavior across the animal kingdom. It is characterized by rhythmic patterns of body movement. In many cases this underlying rhythmicity is achieved through alternating patterns of muscle contraction. For example, during walking, knee extensor muscles will contract while flexor muscles are relaxed and *vice versa* [6]. Rhythmic locomotor patterns are stereotyped-repetitive motions that can be generated automatically by lower levels of the nervous system independent of input from higher brain centers [7]. Two fundamental questions underlie how the nervous system controls locomotion [6]. How are rhythmic motor patterns generated by the nervous system? How are locomotor patterns adjusted by sensory stimuli? This section will address these questions, highlighting landmark studies that form our current understanding of the neural control of locomotion. The first part will discuss the

modern model for neural control of movement in vertebrates and gaps in our current understanding. The second part will discuss the use of *C. elegans* as a model for interrogating neural mechanisms of locomotor behavior.

PART I: VERTEBRATE LOCOMOTION

Generation of Rhythmic Motion

One of the first major insights into locomotion came from studies by Charles Sherrington in 1910. He found that dogs lacking their cerebral hemisphere maintained the ability to walk [8]. Then in 1911,Thomas Graham Brown discovered that cats who lost afferent input could still evoke rhythmic alternating limb contractions [9]. Brown's work countered an idea supported by Sherrington that a proprioceptive chain of reflexes was responsible for rhythm generation. From these pioneering studies the following conclusion could be drawn. Walking can persist independently of cerebral hemisphere input or proprioceptive input from the muscle receptor afferents. This indicated that intrinsic rhythmicity could reside within the spinal cord and from these studies the concept of a central pattern generator emerged.

Central pattern generators (CPGs) are networks of neurons capable of generating patterns of rhythmicity. The first described CPG was in locusts [10]. Later, extensive work in the lamprey has made it one of the best characterized models for CPGs (Figure 1.1) [6, 7, 11, 12]. The lamprey swims by propagating a wave of alternating muscle contractions along its body. Excitatory glutamatergic interneurons, that are projected ipsilaterally, are thought to be the

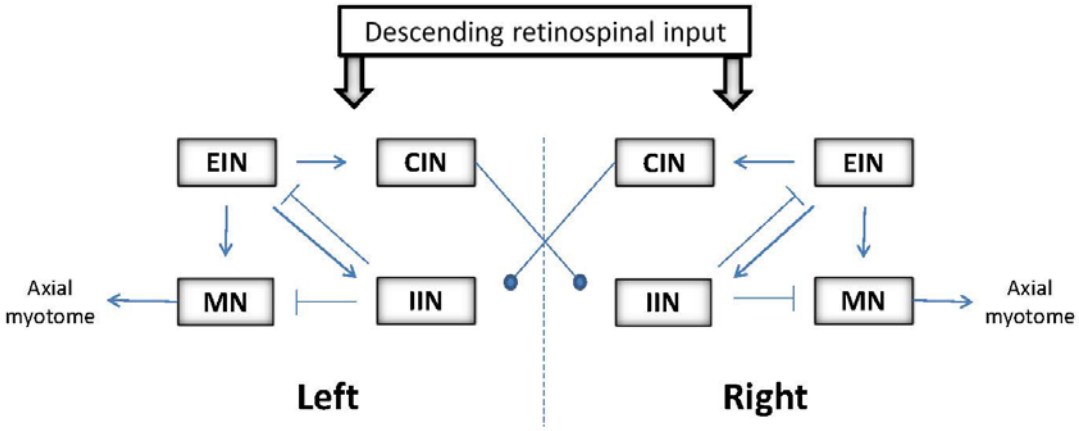

Figure 1.1 The Lamprey Central Pattern Generator

There are four classes of neurons that comprise the lamprey central pattern generator (CPG). Motor neurons (MNs) are segmentally organized and innervate the adjacent axial myotome. Glycinergic commissural interneurons (CINs) project contralaterally to inhibit opposing muscle. This ensures proper coordination of muscle during swimming. Ipsilateral inhibitory L neurons (IINs) inhibit both MN and CINs. Their role in the CPG is not understood. Excitatory glutamatergic neurons (EINs) project to all CPG cell types, display bursting, and drive the rhythmicity of the CPG during movement. Adapted from[3].

source of rhythm generation [13]. These interconnected excitatory neurons are distributed along the spinal cord and function as burst generating units to provide rhythmic glutamatergic synaptic drive to motor neurons as well as to ipsilateral and commissural inhibitor interneurons. Motor neurons are organized into segments which innervate adjacent muscle groups. Excitatory glutamatergic interneurons act on motor neurons to stimulate muscle contraction. During swimming the inhibitory commissural interneurons ensure that opposing muscles contract out of phase to facilitate movement [13, 14]. However, commissural and ipsilateral inhibitory interneurons are not required for generating oscillating patterns of activity, as rhythmicity persists in the absence inhibitory synaptic mechanisms in the lamprey [15, 16]. Similar conclusions were drawn in cat and rodent studies [17-20]. This suggests that reciprocal inhibition does not drive rhythmic behavior, but may be more important for coordination [21], and that excitatory glutamatergic neurons could be the source of rhythm generation.

Although swimming movements of the lamprey differ significantly from limbed, terrestrial vertebrates, elementary principles regarding the organization and neural makeup of rhythmic motor behavior are phylogenetically conserved [7, 13, 22, 23]. This includes the localization of the locomotor CPG to the spinal cord, and possibly the use of excitatory glutamatergic neurons for rhythm generation, and a role for inhibitory interneurons in coordinating left-right locomotor alterations [7].

Important aspects of overall network structure of the mammalian CPG have been revealed through lesion and pharmacological investigations. The

mammalian CPG, like the lamprey, is distributed along the spinal cord [24]. It exhibits a rostral-caudal, downward gradient in the potential of spinal segments to generate rhythmicity [17, 25-32]. Pharmacological perturbation suggests that the rhythmic core may be composed of ipsilaterally projecting, excitatory neurons [13]. These neurons have not been identified with certainty [7], and their role in rhythm generation has been called into question [33]. Hb9 interneurons are putative components of the mouse spinal cord central pattern generator and candidates for rhythm generation. However, when examined experimentally, Hb9 interneuron activity showed sparse activity and inconsistent firing for each cycle during fictive locomotion [34]. Furthermore, rhythm generators would be expected to precede motor neuron activity. The Hb9 interneurons, in contrast, lagged behind ventral ipsilateral motor neurons within their segment, suggesting that they are unlikely to be the rhythm generating component of the central pattern generator [34, 35]. Thus, while spinal circuits have been studied intensively for over a century, there is still no consensus as to how rhythmic activity is generated.

Walking involves alternating contractions of extensors and flexors. How this is coordinated is not well understood [7]. RCs (Renshaw Cells) and Ia-Ins (Ia Interneurons) are ipsilateral projecting inhibitory interneurons that have been identified, and are proposed to play a role in motor neuron rhythmicity [28, 36, 37]. However, genetic knockout and silencing experiments indicate that these neurons are not required for extensor-flexor coordination [38, 39], although they do seem to play a role in speed regulation. Thus for the time being the

population of interneurons controlling flexor–extensors within the mammalian CPG remains elusive.

The right-left sides of the body must be coordinated to produce proper locomotion. Out of all of the spinal circuitry, the mammalian neural networks that coordinate right versus left are the most well characterized [7]. Lesion studies indicate that commissural interneurons (CINs) facilitate this coordination [30]. CINs may be divided into two groups, intersegmental and intrasegmental. Intersegmental CINs innervate at least two segments and synchronize motor activity along the spinal cord, while intrasegmental CINs coordinate left-right activity within a given motor segment [40-50]. The role of CIN coordination of locomotion is one area that limbed vertebrates differ significantly from the lamprey. In the lamprey, inhibitory CINs are the primary mediators of left-right muscle coordination, however in limbed vertebrates this is much more complicated [7]. Mammalian vertebrates exhibit the addition of new network elements. Specifically CINs are composed of both excitatory and inhibitory interneurons and have more complicated segmentation. This increased complexity serves to bind motor synergy between limbs as well as coordinate ipsilateral inhibitor networks that modulate extensor-flexor coordination within a limb [7]. Added network complexity endows terrestrial vertebrates with increased locomotor flexibility including various gates and greater functional adaptations that permit movement across varying terrains [7].

Modulatory Inputs on Spinal Circuitry

Important insight into the role of sensory stimuli and descending input on spinal circuitry came from cat studies in the 1960s. It was found that stimulating the Mesencephalic Locomotor Region (MLR) in a decerbrate cat could initiate stepping. Moreover the strength of the stimulus not the frequency could dictate locomotor speed [51]. Work by Jankowska, *et al.* showed that electrical stimulation of skin or muscle could generate rhythmic activity in spinal cord interneurons [52]. Together these studies provided evidence that the spinal circuit rhythmic activity could be modulated by descending brain pathways and from afferent muscles.

Regulation of movement by spinal reflexes

While sensory stimuli is not required for basic walking patterns, the intrinsic spinal circuits that control walking share many of the same interneurons that are involved in flexion reflexes [7]. In general, reflexes can be thought of as coordinated, involuntary motor responses. Reflexes serve to initiate movement to escape noxious stimuli, while others automatically adopt motor patterns to achieve or maintain a behavior goal [6]. The sensory stimuli for spinal reflexes include receptors in muscle, skin and joints. Their actions are carried out by neural circuitry for motor responses entirely contained within the spinal cord. While reflexes are often thought of as stereotyped movements, they can be adjusted to serve the task at hand. This flexibility allows reflexes to be smoothly incorporated into complex movements [6].

One well known reflex is the flexion-withdrawal reflex (Figure 1.2). It serves both protective and postural functions. The flexion withdrawal reflex allows a limb to withdraw from a painful stimulus. This is brought on by simultaneous contraction of all flexor muscles in a limb. The sensory neuron senses the noxious stimulus, which acts on a motor neuron that innervates flexor muscles to induce contraction. At the same time the polysynaptic outputs act on inhibitory motor neurons to inhibit extensor muscles. This excitation of one group and inhibition of its antagonist is called reciprocal innervation and is a key principle of motor organization [53]. It ensures fast, efficient movement as contraction is not hindered by contraction of the opposing muscle. Reflexes also affect the contralateral limb. This is called the cross-extension reflex. This reflex enhances postural support during withdrawal [6].

Stimulus intensity can shape the force of muscle contraction in a reflex. Touching a hot stove will induce relatively fast withdrawal of ones wrist and elbow. However, touching a scorching hot stove will lead to rapid withdrawal of the entire limb. The duration of a reflex increases with stimulus intensity. Contractions in the flexion reflex always outlast stimulus to ensure that the limb is removed from danger [6].

Descending neurons from higher brain centers can act on the spinal cord to modulate the strength of a spinal reflex. This occurs by tonic input, which can bring a neuron closer to threshold, making them more excitable and available for action. During locomotion the level of presynaptic inhibition is rhythmically modulated. This could provide a means to modulate the strength of reflexes

during walking. Sensory afferents converge with descending brains systems at the level of the spinal cord [6]. How these signals are integrated into a smooth motor program is a fundamental principle of the neural regulation of movement that for the time being remains ill-defined.

Descending Inputs Modulate Spinal Circuits

Descending inputs from higher brain centers regulate various aspects of locomotor control including propulsion/initiation of movement, posture and steering. Normally, spinal CPGs are silent at rest [22]. An exception to this is animals that never stop moving, like the dogfish. The CPG of the dogfish is continually active, even after a complete spinal transection [54]. Within the brainstem, the mescencephalic and diencephalic locomotor regions (MLR and DLR, respectively) act as command centers for propulsion. MLR and DLR initiate locomotion in the spinal CPG through reticulospinal (RS) neurons [22]. As mentioned above, the strength of the MLR activation influences both gate and speed of the initiated movement. 5-HT (*5-hydroxytryptamine*, also known as serotonin) and norepinephrine (NE) also modulate the CPG, by stabilizing and optimizing its function [7, 13, 55, 56].

Proper control of posture is important for movement. The vestibular system allows animals to properly orient themselves in space. In the lamprey, bilateral gravistatic receptors help them to maintain their dorsal positioning during swimming (dorsal side up). If their orientation tilts, gravistatic receptors become activated and signal to the RS. The RS signals corrective signals to motor neurons and muscle to regain proper orientation [57]. Motor command centers

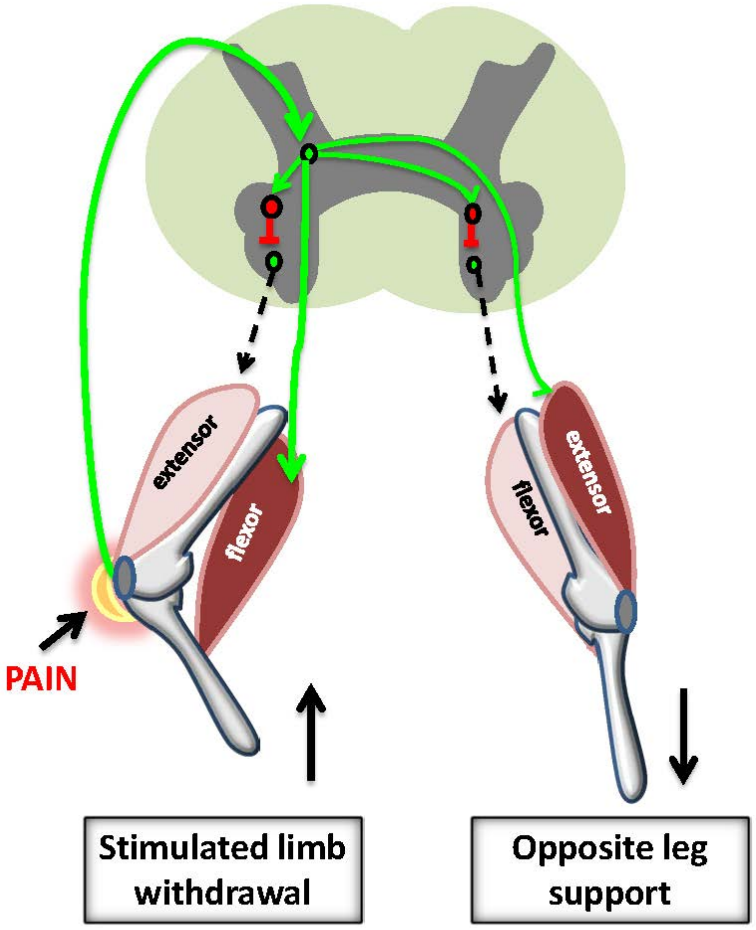

Figure 1.2 Flexion and Crossed-extension Reflex

A painful stimulus is sensed by a cutaneous afferent fiber. This excitatory pathway (green) can act on motor neurons that innervate the ipsilateral flexor muscle to remove the limb away from the noxious stimulus. In order to support limb withdrawal, the motor neurons which innervate the contralateral extensor are simultaneously activated. Inhibitory interneurons (red) ensure that motor neurons innervating antagonistic muscles are inhibited to facilitate proper reflex extension. Adapted from [7].

and vestibular systems both relay to RS neurons. Thus, the RS functions to integrate posture and propulsion commands [58]. Different populations within RS neurons permit steering during movement. In the lamprey, contralateral asymmetry, within RS populations, promotes turning [59, 60]. In limbed organisms, locomotor navigation is much more complex, but one aspect of steering is facilitated by foot orientation during the swing phase. Foot placement will direct locomotion by orienting the body in the same direction [24].

The basal ganglia play a powerful role in permitting adaptive movements in combination with the brainstem and spinal cord (Figure 1.3). Moreover, subcortical structures are sufficient to drive goal-directed movements in the absence of the cortex [61, 62]. The motor centers DLR and MLR are under tonic inhibition from the basal ganglia [22]. Within the basal ganglia reside an input layer (striatum) and an output layer (pallidium). Only strong stimulatory input from the cortex or thalamus can activate the striatum. The striatum is silent at rest and difficult to activate due to the presence of inward rectifying K+ currents. Once the striatum is activated, it goes on to inhibit the pallidium, releasing its tonic GABAergic inhibition of the MLR and DLR locomotor centers to permit movement. This disinhibitory circuitry keeps motor centers under inhibitory control preventing unintended movement. The high threshold for motor initiation serves as a filter for cortical and thalamic input and is a common feature in vertebrates [63, 64]. The striatal filter is critically dependent on dopamine innervation. Dopamine lowers the threshold for striatal activation. Thus, loss of dopamine leads to a rigid, Parkinson-like state. Excessive dopamine on the

other hand, promotes hyperkinesis and unintentional movements. The striatal pallidal complex has a prominent role in movement control. Modulation of these circuits may regulate changes in motor activity. Cortical input to the striatum may represent a site for motor learning [22]. The thalamus may directly act on striatum to promote automatic response patterns in order to bypass the cortex. The basal ganglia and connected circuitry represent a fundamental aspect of locomotor control whose function has been conserved throughout vertebrate evolution dating at least as early as 500 million years ago when the vertebrate line diverged from the lamprey [22].

A Human Perspective on Motor Control

While most of the information regarding motor control has been acquired from animal models, it is thought humans employ similar strategies for generation of rhythmic movement. A key difference between the cat, for example, compared to a human in walking, is quadruped versus biped locomotion. Bipedal locomotion requires more balance and thus more input from descending pathways onto spinal circuits. This may explain why few patients can regain normal movement following spinal lesions. In fact, it is thought that rehabilitation does not necessarily reactivate normal patterns of movement, but instead involves new compensatory strategies to replace lost function [65]. Similarly, training in decerebrate cats improves coordinated walking [6].

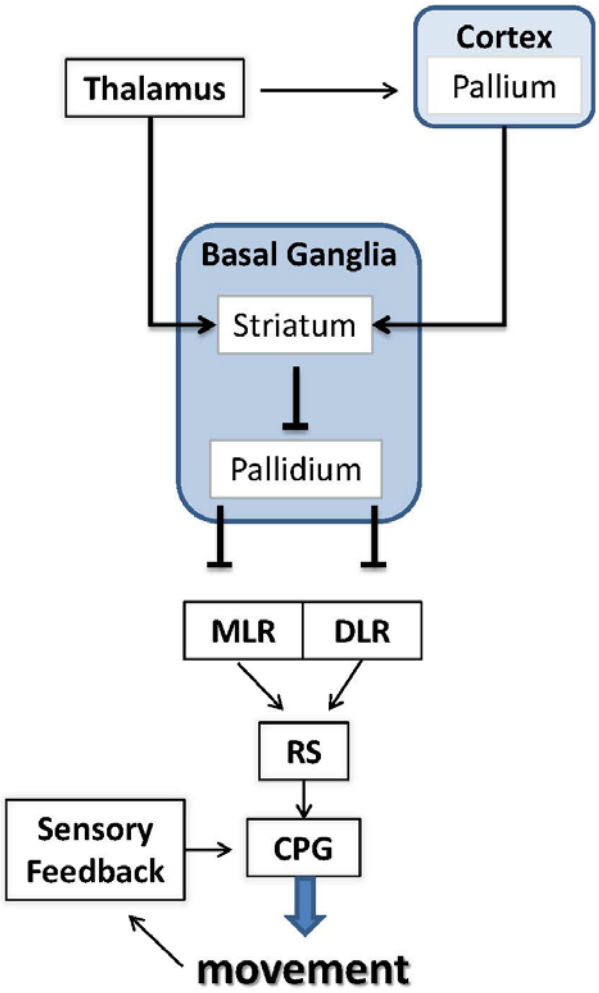

Figure 1.3 Descending Inputs Modulate Spinal Circuits.

Various supraspinal inputs modulate CPG activity. The basal ganglia (BG), selects the motor program appropriate for the task at hand. If the BG receives strong inputs from the cortex or thalamus, the striatum will become activated inhibiting the pallidium. Inhibition of the pallidium relieves its tonic inhibition of the tectum and locomotor command centers, the mescencephalic (MLR) and diencephalic locomotor regions (DLR). Together these inputs act on the CPG through the reticulo spinal (RS) neurons to initiate movement. The CPG can be further regulated by sensory feedback. Adapted from [23].

Summary

Animal models have provided insight into how rhythmic motor activity is generated through spinal CPGs and through reciprocal inhibition of antagonistic muscles. Due to the inherent complexities of mammalian systems, specific connectivity and functions of interneuron microcircuits in the spinal cord, sensory afferents, and descending brain inputs are not well defined. A thorough understanding of neural connectivity, function and the genetic basis for these pathways would provide insight into how movement is generated, modified in different behavioral states and how it is affected after lesion or in disease. This advancement has remained challenging in mammalian systems, due to their inherent complexity. Simpler models that are more amenable to experimentation, such as the cat and lamprey, have provided insight into conserved mechanisms for motor control. By pursuing a reductionist approach, one may be able to establish neural circuit motifs, identify key genetic components, as well as functional information about how locomotion is generated, affected by sensory stimuli and integrated into a meaningful behavior. By achieving a complete understanding of the fundamental properties within simple systems, we may be able to extend this knowledge to higher ordered systems which may use similar strategies through similar patterns of neural connectivity, albeit in greater number.

PART II: *C. ELEGANS* LOCOMOTION

Introduction

Through Sydney Brenner's life, many fundamental breakthroughs in science occurred [66]. These include deciphering how three-dimensional proteins can arise from single dimension sequences of DNA through the discovery of mRNA [67-69] to the emergence of the one gene one enzyme concept [70]. Understanding that DNA encodes amino acids, which assemble into proteins to carry out a specific function, made it clear that genes are the basis for a phenotype. After this conceptual leap, the next direction would be to examine the genetic basis of more complex phenotypes. Brenner became interested in extrapolating these ideas into higher levels of organization, asking the question, what is the genetic basis of behavior [66]? In his landmark 1974 paper, The Genetics of *Caenorhabditis elegans*, he defined behavior as, "the result of a complex ill-understood set of computations performed by the nervous system…." [71] Thus to understand the connection between genes and behavior it became necessary to achieve a thorough description of a nervous systems structure and its construction [66, 71]

Brenner eventually chose *C. elegans* as his model of choice to investigate the genetic basis of behavior for a number of reasons. *C. elegans* are small at approximately 1mm in length, transparent and exhibit a relatively compact nervous system. These properties permitted the thorough characterization of the nervous system of the worm. In 1986, Brenner, White and others, assembled a complete wiring diagram of the *C. elegans* nervous system [72]. This included

information regarding the chemical, electrical connections and processes of all 302 neurons found in the worm [72]. Meanwhile, John Sulston and Robert Horvitz, derived the complete cellular lineage of the worm [73, 74]. These heroic, descriptive endeavors established *C. elegans* as a model, in which, every neuron's position, connections, and cellular lineage are known.

From Brenner's work in the 1970's establishing *C. elegans* as a model system, research in worms has greatly expanded to more than 800 labs worldwide [75]. Aside from its well characterized nervous system, much of its popularity may be attributed to the ease of laboratory cultivation, experimentation and amenability to genetic manipulation. Worms are typically maintained on lawns of *Escheria coli* (*E. coli*) on the surface of agar in a petri dish. A rapid regeneration time of 3.5 days allows for quick generation of transgenic lines, conduction of genetic crosses, and the feasibility of conducting experiments such as lifespan in a reasonable period of time. The wide recognition of *C. elegans* as a genetic model is in part due to the ability to easily generate mutants with the chemical mutagen EMS (ethyl methane sulfonate), ionizing radiation and transposons [76]. Moreover, the diploid nature of the *C. elegans* genome allows for creation of even the most deleterious of mutations with low risk of lethality. Worms are hermaphrodites and homozygous mutants can be generated in a relatively short period of time. The advent of frozen worm stocks means that mutant lines maybe stored for many years and the ever increasing number of mutants means that there exist multiple types of mutations for many genes. The availability of complete deletions, as well as partial and conditional mutations

permits examination of genetic complexity [76]. Thus *C. elegans* has become an attractive model for dissection of the neural and genetic basis of behavior. The remainder of the introduction will focus on the current understanding of *C. elegans* locomotion, one of the most prominent of all of *C. elegans* behaviors [77].

***C. elegans* Anatomy and Current Model for Motor Control**

Worm locomotion may appear simple. In the typical lab environment, *C. elegans* can be found moving through a lawn of *Escheria coli* (*E. coli*) on the surface of agar in a petri dish, their body generating sinusoidal waves as they move forward. Most of their time is spent moving forward, but occasionally they turn or move backwards as they forage through the bacterial lawn. The neuroanatomy and musculature that enable worm movement has similar organization to that of higher ordered systems [78] in which interneurons and sensory neurons relay signals to motor neurons that act on muscle to initiate movement. While the *C. elegans* nervous system is made up of only 302 neurons, they do possess a ganglion of neurons in the head (brain) composed of sensory neurons and interneurons which modulate the actions of motor neurons and muscle. In addition, their nervous system uses a majority of the neurotransmitters found in mammals. The simple, well characterized nervous system and powerful genetics make *C. elegans* an attractive model to investigate fundamental properties in how genes and neural circuits generate behavior. This model system can, therefore, address questions that are inherently more elusive in higher ordered systems. The remainder of this chapter will focus on the

structure of the *C. elegans* nervous system, how it functions to generate movement and how it can be affected by sensory perturbations.

In 1989, John G. White and colleagues published a complete structural map of the *C. elegans* neuroanatomy [72]. This wiring description has laid the ground work for all studies describing worm behavior. While informative, it only infers function, which must be experimentally verified. Nonetheless, it is worthwhile to provide an overview of worm anatomy to understand how movement can be generated from it.

C. elegans locomotion is characterized by a sinusoidal wave which is propagated by alternating dorsal-ventral contractions of body wall muscles. There are 95 body wall muscle cells arranged in interleaved patterns of two dorsal and two ventral rows [74]. A cross section of the worm reveals a quadrant composed of two rows of muscle cells [74]. On the anterior-posterior axis, these muscles can be divided into sections based on their innervation. There are 16 head muscles which are innervated by motor neurons originating from the nerve ring (head ganglion) [74]. Head muscle innervation is unique, in that, nerve ring motor neurons map to 8 distinct regions – one for each row of cells. Ventral Nerve Cord (VNC) muscles, on the other hand, possess motor neuron projections either ventral or dorsal in nature. The more diversified connectivity of motor neurons in the head permits worms to move the head both laterally and dorsally. Whereas the VNC pattern of innervation, restricts body movement to the dorsal-ventral plane [78]. The next region is the neck, whose 16 muscle cells receive inputs from both the nerve ring and the ventral nerve cord. The body is

the largest region composed of 63 muscle cells and is innervated exclusively from the VNC motor neurons [78]. The varied connectivity between the body wall muscle regions may be rooted in developmental origin. Head muscles arise in embryonic stages whereas body muscles originate during both embryonic and larval stages [74].

113 motor neurons make up nearly one-third of the *C. elegans* nervous system. 80 of these are distributed across the VNC [74, 78]. They can be subdivided based on function and position. The five classes are: A, B, D, AS, and C. Further distinction can be made with regard to their neuromuscular junction (NMJ) locations. VA, VB, VD, VC innervate ventral body muscles, while DA, DB, DD, and AS innervate the dorsal body wall muscles (Figure 1.4). Dorsal motor neuron cell bodies reside in the VNC but extend commissures dorsally to establish the dorsal nerve cord (Figure 1.4). Each class contains multiple members that align along the anterior-posterior axis of the VNC in repeating units (*i.e.* VA-VA6) [72]. A- and B-type motor neurons are cholinergic and thought to be excitatory [78]. D-type motor neurons are GABA(γ-aminobutyric acid)ergic and inhibitory [79]. The active zones for members of each class do not overlap, although different classes may innervate the same muscle cell. This phenomenon is in contrast to mammalian systems whose motor unit consists of one motor neuron per muscle [6].

A-type motor neurons are dedicated to backward movement. This is evidenced by laser ablation, which severely impaired backward movement [80]. Their morphology is also suggestive of this behavior, as A-type motor neuron

processes are anteriorly projected [72], consistent with the idea that backward movement is accompanied by anterior moving electrical/contraction wave propagation [81, 82]. Their predominant presynaptic partners are command interneurons AVA, AVD, and AVE (Figure 1.4), which are thought to drive backward movement (more on this later). B-type motor neurons are dedicated to backward locomotion with posterior directed processes [72]. They exhibit deficits in forward movement if they are removed through laser ablation [80] and are innervated by command interneurons AVB and PVC. Further support for the roles in direction regarding A and B-type motor neurons is supported by calcium imaging [83, 84]. D-motor neurons receive input only from other motor neurons and act as cross inhibitors. For example: VD receives input from VA or VB and inhibits the contralateral muscle contraction, thus permitting the alternating dorsal ventral wave [72].

Command Interneurons

A set of interneurons thought to gate forward and backward activity was first identified by electron microscopy reconstructions [72]. These neurons (AVA, AVD, AVE, AVB, and PVC) were unique, in that, they were the only interneurons to synapse onto the motor neurons of the VNC and also span the length of the cord [72, 80]. Their role in directing forward and backward movement was first assigned through work dissecting the touch circuit [80]. Chalfie and colleagues found that elimination of these neurons, through laser ablation of their precursors, caused severe defects in movement. Specifically, ablation of AVA and AVD rendered worms incapable of backward movement while ablation of

AVB and PVC severely impaired forward movement [80]. Their critical role in gating forward and backward movement earned them the title of the command interneurons [77]. Subsequent studies have supported this notion since genetic silencing impaired forward/backward movement and gain of function mutants in the GLR-1 [AMPA(α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic)-type ionotropic glutamate receptor subunit] in the command interneurons also affected behavior [85, 86].

The aforementioned studies identified an important role for the command interneurons in behavior, but their individual activities and roles in behavior has not been examined until recently with the advent of more sophisticated technologies. These more advanced systems allow one to directly correlate calcium imaging of specific neurons to ongoing behavior [84, 87-89], as well as the advent of optogenetics which allows one to directly stimulate or inhibit neurons with light and directly examine resulting behavior [90, 91]. *C. elegans* are attractive systems for these technologies, as their cuticle is transparent and a number of promoters exist which target few and even single neurons. One of the first command interneurons to be examined was AVA. A number of calcium imaging studies demonstrated that during backward movement, calcium transients increase in AVA [84, 87-89]. Similarly, stimulation of AVA with channelrhodopsin, results in robust backward movement [92, 93]. The former study should be taken with caution, as promoters used to drive channelrhodopsin expression also drove expression in other command interneurons. Calcium imaging of AVE suggested a similar role to AVA [84, 88]. No increased activity

was seen in the command interneuron AVD [84, 88], which is not completely surprising, as AVD is a critical component of the touch circuitry and maybe active only upon mechanical stimulus [80]. The forward command interneuron circuitry has also been examined. During forward movement, calcium transients in AVB rise [84, 88]. Whereas, channelrhodopsin stimulation of PVC induced a small but appreciable increase in forward speed [93]. Thus, in the 26 years since the *Mind of the Worm* was published, the role for command in gating forward and backward movement is still the accepted model for *C. elegans* locomotion (Figure 1.4).

Propagation of the Sinusoidal Wave

While the infrastructure for worm movement is becoming better understood, there are still major unanswered questions in basic worm movement. One such question is how the pattern of sinusoidal waves is generated. It is currently not known whether a central pattern generator (CPG) for worm movement exists. The command interneurons are an attractive place for rhythmic activity to be generated, however loss of these neurons by genetic ablation does not eliminate the sinusoidal wave generation [94]. Similarly, calcium imaging data is discordant with the command interneurons acting as CPGs, as calcium transients are tonic, lacking oscillating activity [84, 87-89]. This is also true for the motor neurons that have been imaged [83, 84], although

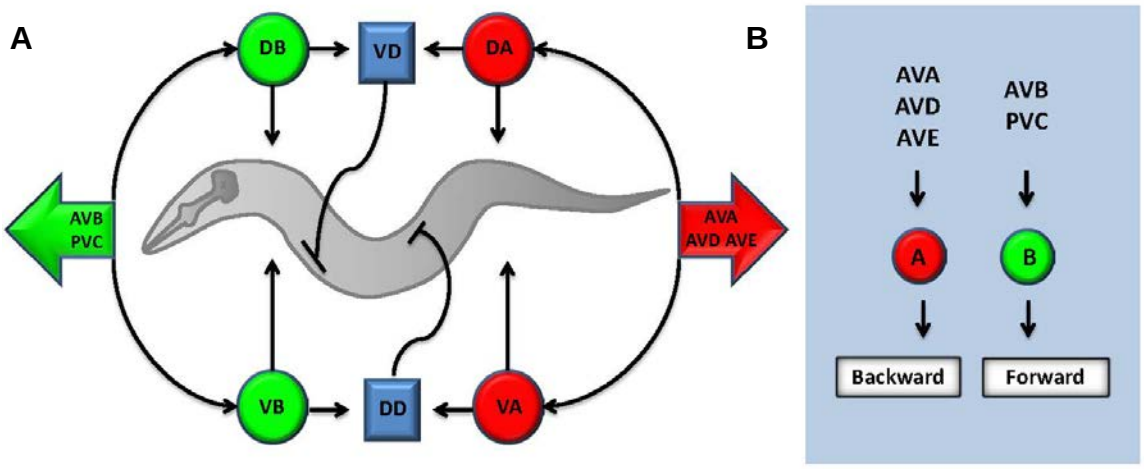

Figure 1.4 Model for *C. elegans* Locomotion

A) Command interneurons gate forward and backward locomotion. From the connectivity map and touch circuit analysis, the command interneurons have been assigned the title of gate keepers of locomotion. The command interneurons AVB and PVC gate backward movement by synapsing onto B-type motor neurons. The command interneurons AVA, AVD, and AVE drive backward movement through A-type motorneurons. There are six major classes of motor neurons distinguished by position and function. Within each class exist multiple members, for example: VA1-6. A and B-type motor neurons are excitatory and cholinergic, while D-type are inhibitory and gabaergic. DB and DA motor neurons innervate dorsal body wall muscles, while VA and VB act ventrally. VD is innervated by DB an DA motor neurons and inhibit ventral body wall muscles. DD is postsynaptic to VB and VA and inhibits dorsal body wall muscles. This reciprocal inhibition is thought to facilitate coordinated locomotion by ensuring opposing muscles are contracted out of phase. **B)** A model for forward and backward movement. Adapted from [141].

these studies did not image all motor neurons so it is possible that activity between motor neurons could be overlooked. Muscle cells display calcium oscillations that coincide with muscle contraction however, these changes in calcium are necessary for contractility. It is not clear if they have a role in initiating propagation as a pacemaker cell would be expected to have intrinsic oscillating activity and the oscillations are lost in non-moving worms [82]. Interestingly, inhibition of cholinergic motor neurons with halorhodpsin halts worm movement, but maintains their posture – suggesting that muscle cells can have contractility independent of cholinergic input [81]. The implications of this on movement are unclear. Ion channels are certain to be key components of electrical activity and a study in 2008 identified two channels important for generation of rhythmic behavior [82]. Mutations leading to loss of the transient T-type calcium channel (CCA-1) lead to abnormal bending amplitude and frequency. This is further exacerbated in double mutants that lack the NCA-1 leak channel. This channel is important for raising the membrane potential above that of the potassium equilibrium potential. Mutants lacking NCA-1 alone have a fainting phenotype that arises when they attempt to change from backward to forward movement and results in paralysis when they are placed in liquid attempting to swim. In combination with CCA-1 the dorsal/ventral wave propagation is severely disrupted in crawling worms [82]. This suggests there may be feedback between motor neuron and muscle that is required for oscillating behavior.

The cross-inhibition of GABAergic D-type motor neurons have also been proposed to play a role in wave generation [72]. This gained traction as vertebrate CPGs require negative feedback loops that coordinate patterns of contractility [78]. An organism that shares significant similarities in organization to the *C. elegans* nervous system, *Ascaris*, displays oscillations in their inhibitory ventral cord motor neurons [95]. This phenomenon seems unlikely to occur in *C. elegans* as worms devoid of GABAergic signaling are capable of movement and only display defects upon touch when they shrink as a result of simultaneous dorsal/ventral contractions [79].

One last possibility for wave propagation may be involvement of proprioceptive feedback. Mechanosensitive channels TRP-4 (transient receptor potential) have a role in establishing normal proprioception, *i.e.* posture, as well as wave amplitude and velocity during movement [96]. UNC-8(uncoordinated) also may have a proprioceptive defect as these DEG/ENAC channels display reduced bending and wave propagation [97]. TRP-4 and UNC-8, themselves are unlikely to drive rhythmicity as they still display waves, albeit with altered amplitudes [96, 97]. Therefore, it is more likely they could be modulated by a CPG, rather than being components of the CPG themselves. Thus for the time being, we have clues as to how the sinusoidal wave in *C. elegans* may be propagated, but no definitive answers.

The Transition between Forward and Backward Movement

The question of how worms transition from forward to backward movement and *vice versa*, is another critical unanswered question. Reciprocal connections between backward and forward movement exist, but is not clear how they are regulated or what their function is [72]. The role of command interneurons in gating backward and forward movement gained momentum in a study by Brockie and colleagues, in which a hyperactive GLR-1(A/T) driven in command interneurons created lurcher mutants. Lurcher mutants quickly transitioned from forward and backward movement as if they couldn't decide on a direction [94]. This suggests that feedback for direction does occur between these command interneurons, but how this is facilitated is unclear. A recent paper proposes a role for the innexins UNC-7 and UNC-9 in establishing the balance between forward and backward movement [84]. Loss of these innexins (thought to function as gap junctions), results in a kinked phenotype during forward movement, and an increased propensity towards backward movement. Normally worms spend the majority of their time moving forward and only occasionally induce backward movement. Expression analysis and behavioral studies suggest innexins act normally to suppress backward movement [84]. Their role in forward movement is unclear. Additionally gap junctions are widely expressed throughout the *C. elegans* nervous system and their potential role in electrical synchronization and how they are regulated is not known.

This section has summarized the current model for *C. elegans* locomotion. The topics discussed above included an overview of the anatomy of the neuro-

musculature system and insights into how each component contributes to movement. Major questions and gaps in knowledge regarding the motor circuit have also been addressed. This information provides the ground work for the next section which will address how sensory information can impinge on the motor circuit. Specific topics will include environmental influences on behavior state as well as the escape circuits from touch and other noxious sensory cues.

Sensory and Environmental Influences on Behavior

To better understand how the *C. elegans* nervous system generates behavior, it is important to recognize the functional microcircuits active during specific behaviors. This will provide insight into how a sensory stimulus can be transduced into a motor response. A brief overview will be presented outlining the effects of food state and the escape response that is induced by touch and other noxious stimuli. This information provides a framework for the neural circuits that permit *C. elegans* to navigate their environment and to respond to it. Furthermore it offers insight into how various stimuli can be integrated through neural networks into a programmed motor response.

Effect of Food Conditions on Behavior State

The stereotyped locomotion behavior of *C. elegans* is dependent on the availability and/or presence of food. In laboratory conditions *C. elegans* are cultured on a lawn of *E. coli* on NGM plates. Under these conditions worms spend the majority of their time foraging through the lawn. This involves slow forward movement interrupted by short reversals typically of 1-2 head swings

[98]. Worms spend most of their time on the bacterial lawn, but if they veer off they enter a state of local search behavior. The AWC olfactory neuron and ASK gustatory neuron are activated upon removal of food. Upon activation, these neurons act to stimulate the interneuron AIB, which triggers an increase in long reversals and omega turns [98]. This behavior state permits rapid change in direction allowing the worm to return to the food source. Once the food source is re-discovered, the dopaminergic sensory neurons ADE, PDE and CEP sense the presence of bacteria through the mechanoreceptor TRP-4 [96, 99, 100] and respond by slowing their basal rate of forward movement, which has been called the basal slowing response [99-102].

If worms are removed from food for more than 30 minutes they transition from local search behavior to dispersal behavior as starvation cues from the ASI neuron TGF-β (*daf-7*) and serotonin are released [98, 103, 104]. The dispersal state is characterized by long stretches of forward movement as AIB is inhibited and reversals and omega bends are suppressed by the ASI gustatory neuron and AIY interneuron [98]. After starvation, if worms return to food, they will exhibit an enhanced slowing response that is mediated by serotonin [102].

Aspects of turns and reversals are mediated through motor neurons downstream of the interneurons AIB and AIY. The motor neuron SMD innervates much of the muscles in the anterior portion of the head [72], and is involved in the high bending angle that occurs during omega turns [98]. The motor neuron, RIV, uniquely innervates only ventral neck muscles and conveys a ventral bias to omega turns [72, 98]. The motor neuron SMB may contribute to the amplitude of

the sinusoidal wave, as ablation of SMB produces worms with loopy head movements.

The focus of this section has been on how the feeding state of a worm can drastically modify aspects of basal locomotion. Thus, when analyzing behavior in an experiment, such as, the escape response to touch, it is important to take into account the behavior state of the worm since the feeding state influences the probability of turns, reversals and direction change. Furthermore, starvation can have long lasting modifications on behavior. Therefore, most well designed experiments take food conditions into consideration, and involve either performing behavior experiments on food, or only in the absence of food for less than 30 minutes.

Avoidance Behaviors Mediated by the Sensory Neuron ASH

This section will focus on how *C. elegans* avoid noxious stimuli through the sensory neuron ASH, as its role in modulating backward movement is a key aspect of Chapter 3. ASH is a polymodal sensory neuron. It is unique in the wide variety of noxious stimuli it senses and transduces into avoidance behavior. ASH has been shown to play a role in sensing the following sensory modalities: high osmolarity [105, 106], gentle touch to the nose tip [106-108], odorants [109], the heavy metals, copper and cadmium [110, 111], low pH [112], and detergents [110, 113]. Upon sensing various stimuli, worms elicit an escape response involving rapid backward movement, followed by forward movement, typically in

a new direction. The robustness of the reversal is dependent on the stimuli type and strength [89, 106].

All of the sensory cues in ASH seem to depend on the TRPV (Transient Receptor Potential channel, Vanilloid subfamily) channels OSM-9 (OSMotic avoidance abnormal) and OCR-2 (OSM-9 and Capsaicin receptor-Related) [114, 115], the G-protein α, ODR-3 (ODoRant response abnormal) [112, 116], and the biosynthetic PUFA (Poly Unsaturated Fatty Acid) enzyme [117]. How the various sensory modalities are discriminately sensed is not entirely clear. Within ASH, OSM-10 (a novel protein) appears to be specific for sensing hyperosmoloarity, but not nose touch. Similarly, GRK-2 (G-protein-coupled Receptor Kinase 2) is important for ASH response to osmolarity and octanol, but not nose touch [109, 118]. Different stimuli also elicit varied calcium responses which may reflect differences in the sensory machinery, for example chemical binding to a receptor (osmotic response) versus mechanical stimulus (nose touch response). The magnitude of ASH stimulation also appears to be reflected in the behavior response where a stronger stimulus will induce more head swings during the backward movement in the escape response [89]. The robustness of reversal behavior also is reflected in the level excitation of the command interneuron AVA which is postsynaptic to ASH [85, 89, 106, 107]. Nose touch requires non-NMDA (N-Methyl-D-aspartic acid or N-Methyl-D-aspartate) receptors as well as NMDA receptor NMR-1 (NMDA class Receptor 1). This suggests a mechanism, whereby osmotic response more strongly activates ASH leading to greater neurotransmitter release (in this case glutamate) [106, 119]. Higher levels of

glutamate, may permit activation of extra synaptic NMR-1 NMDA receptors, which may otherwise be unaffected in nose touch where less glutamate is released [106]. In addition, neuropeptides are thought to modulate the magnitude of synaptic responses. This is evidenced by the fact that mutations in the proprotein convertase, EGL-3, rescues the mechanosensory defects of GLR-1 mutants (Glutamate Receptor family (AMPA)) [120]. Proprotein convertases process proneuropeptides into active signaling molecules. Neuropeptides can either act presynaptically by altering neurotransmitter release [121], or postsynaptically on receptors [122]. With respect to osmotic and nose touch behaviors, it seems EGL-3, which is expressed in AVA elicits affects presynaptically to limit glutamate release. In the absence of EGL-3, more glutamate is released and can rescue the GLR-1 osmotic and nose touch defects, presumably by acting on NMR-1 receptors [106, 120]. Finally, feeding states also may influence ASH mediated escape responses, as food and/or serotonin have been shown to potentiate the nose touch response[123].

In summary, the polymodal sensory neuron, ASH, mediates escape responses to a wide variety of stimuli (Figure 1.5A). The varied behavior robustness is dependent on strength and genetic mechanisms within ASH which connect to the command interneurons AVA and AVD to initiate backward movement. While this model for ASH mediated escape is widely accepted and referenced, the necessity of the circuit as a whole has never been directly tested. The behavior response has been recorded in the presence of stimuli or in the absence of neurons. Moreover, all functional imaging or recordings, thus far,

have been conducted on restrained worms. No one has examined the neural activity in freely behaving worms.

Touch Circuitry

This section will briefly discuss the neural and genetic basis for touch sensation and resulting behavior. Specifically, the differences between gentle body touch and painful harsh touch circuitry and behaviors will be examined.

Gentle Body Touch

Gentle touch involves lightly stroking the body of the worm with an eyelash hair. In response to this type of mechanical stimuli, worms move away from the source of stimulus. For example, if somewhere on the anterior half of the worm is touched, the worm will move backwards. Similarly, if the posterior half of a worm is touched, the worm will move forward, or if it is already moving forward it will increase its forward velocity. The touch receptors that recognize gentle touch are the neurons ALM, AVM, PLM and PVM [80]. Based on their morphology, these neurons were first proposed to play a role in mechano-sensation. They display long processes closely associated to the cuticle and display unusually large microtubules, characteristic of touch receptors [124, 125]. Laser ablation of their neural precursors confirmed the role of these neurons in gentle body touch. Specifically ALM and AVM were important for sensing anterior touch, while PLM was found to be critical for posterior touch.

Upon sensing touch, worms are repelled from the source of the touch. Through ablation of downstream interneurons, it was found that the command interneurons AVA and AVD were required for driving worms backwards in response to anterior touch, whereas AVB and PVC were required for driving worms forward in response to posterior touch (Figure 1.5B). Anterior touch is unique from, posterior touch, in that it triggers suppression of head foraging movements [126]. During normal worm movement, worms oscillate their heads, which has been referred to as foraging [98, 126]. The motor neurons RMD, SMD, and RME are required for normal head oscillations [79, 107]. The cholinergic motor neurons RMD and SMD target different muscle sectors and their activation could stimulate muscle to induce contraction and head oscillations. RME is GABAergic and may play a role in preventing simultaneous contraction of RMD and SMD to facilitate coordinated head movements [79]. Upon tactile stimulation of ALM and AVM, the command interneuron AVD is activated which then activates AVA [80, 126]. This drives the worms backwards. In addition, AVA is thought to activate the inter/motor neuron RIM. RIM is tyraminergic and projects onto muscle, and motor neurons RMD and SMD. These downstream targets of RIM all express the tyramine gated chloride channel LGC-55 (Ligand Gated ion Channel 55). Release of tyramine, will inhibit downstream targets, hyperpolarizing muscle, RMD, and SMD [127, 128].

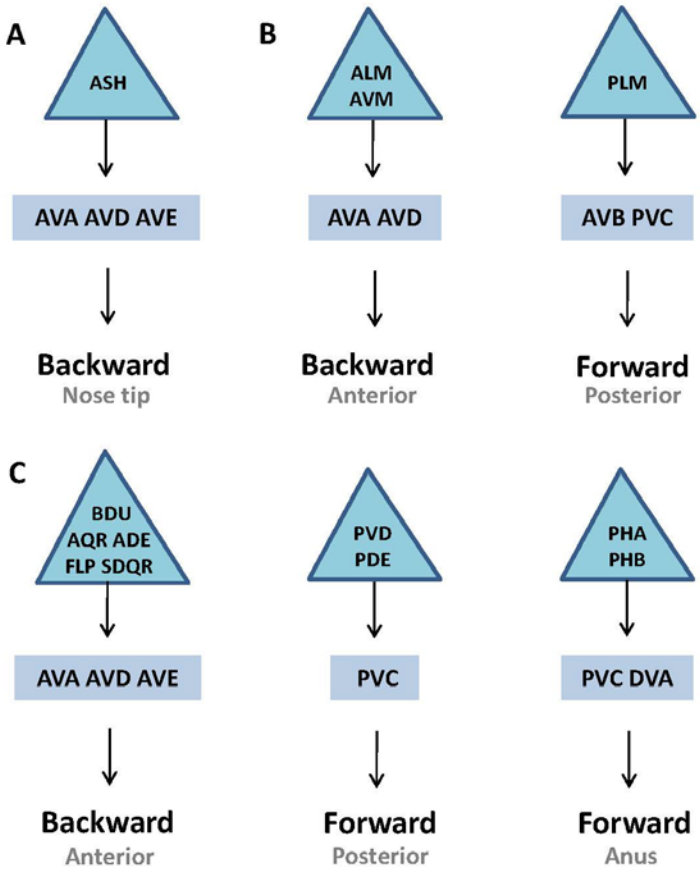

Figure 1.5 Neural Circuits Driving Sensory Induced Behavior

Sensory neurons perceive stimuli and relay this to downstream neurons to coordinate a behavior response. **A)** ASH is a polymodal sensory neuron that can sense touch to the nose tip or noxious water soluble chemicals of high osmolarity. Through postsynaptic synapses to command interneurons AVA, AVD, and AVE initiation of backward movement permits escape from noxious cues. **B)** Gentle touch involves a light stimulus such as a stroke of an eyelash upon the worm's body and will evoke movement away from the site of touch stimulus. ALM and AVM are anterior gentle touch neurons. Through gap junctions the signal to AVD which acts on AVA to initiate backward movement. PLM is the posterior gentle touch sensory neuron which acts through AVB and PVC to drive forward movement in response to touch. **C)** In the absence of gentle touch neurons, worms can still sense a strong prodding by a platinum wire and induce movement to escape this painful stimulus. This type of stimulus is called harsh touch. Sensory neurons in various regions sense harsh touch with anterior neurons acting through AVA, AVD, and AVE to move backwards and posterior and anus sensory neurons acting through PVC and DVA, PVC respectively, to move forward. A-B) Adapted from [141], C) adapted from [138].

This hyperpolarization is thought to relax the muscle and lead to suppression of head oscillatory movements in response to gentle anterior touch. Whether the muscle directly targeted from RIM or SMD/RMD is more important for this suppression is not clear. Experiments from the Alkema lab claim muscle rescue was required, whereas work from the Horvitz lab suggests neuronal targets were required [127, 128] respectively. RIM may further facilitate the escape response through inhibiting the command interneuron AVB, which also expresses LGC-55 [127]. Inhibition of forward movement, would allow for a longer reversal to occur.

Forward genetic screens for mutants defective in gentle body touch has unveiled key components of the mechanotransduction machinery. The long processes that innervate nearly one-half of the animals body length are filled with 15-protofilament microtubules that are cross-linked to one another and whose distal ends are arranged in close opposition to the cell membrane [125]. The microtubules are encoded by the α-tubulin, MEC-12 (MEChanosensory abnormal), and β-tubulin, MEC-7 [129, 130]. MEC-7 and MEC-12 are essential for mechanosensory transduction, as their absence, or loss of integrity abolishes touch sensitivity [124, 131]. MEC-2 is thought to link the intracellular cytoskeleton to the mechanotransduction channel complex [132]. MEC-4/MEC-10, members of the degenerin/ENaC (Epithelial sodium Channel) family of Na+ channels, form the pore of the mechanoreceptor. The carboxy and amino terminals of this heteromeric channel are thought to be positioned internally while the cysteine rich regions face the extracellular portion of the cell [133-136]. MEC-6 tethers the channel to the extracellular matrix and may also be part of the

heteromeric channel [132]. Together these components of the mechanoreceptor channel complex allow worms to transduce touch into a behavioral response. How the touch signal is transduced is not exactly clear. A couple of models have been put forth. It is thought that the tension created from intra and extracellular tethering of the mechanotransduction channel to the microtubules or mantle, respectively, is an important property of channel gating. Touch stimulus could perturb matrix connections, pulling the channel open and allowing Na+ to pass through the cellular membrane from the epithelium. This Na+ influx will depolarize the touch receptor neuron which acts to stimulate downstream interneuron connections primarily through gap junctions. These command interneurons will induce behavior in the direction away from the applied stimulus. This accepted model for the genetic and neural components of touch is largely inferred from genetic manipulations and neural ablation studies. Recent advances in optogenetics has allowed researchers to demonstrate direct activation of touch receptors can activate behavior responses consistent with this model [81, 93], however no one has directly examined the activity in the neurons during ongoing behavior. Furthermore, the genetic necessity has been inferred from loss of function genetic analysis, but no biochemical data has fully confirmed association of the mechanotransduction complex.

Harsh Touch

The touch receptor neurons, ALM, AVM, PVM and PLM, are essential for sensing gentle touch to the body and coordinating a proper motor output. Upon ablation of these neurons, while defective in gentle touch – worms still sense

strong prodding with a platinum wire which has been called harsh touch [80]. A comprehensive set of neural ablations has identified the neural network that mediates harsh touch sensation in various body regions (Figure 1.5C) [137].

Harsh and gentle body touch both require the interneuron PVC and seem to be distinguished by the magnitude of the PVC response. Gentle touch elicits a force between 1-10 μN, which produces a small calcium transient, while harsh touch of 100 μN or more, induces a much larger calcium transient [137]. This increased activation of PVC appears to be reflective of the behavior response as harsh touch induces a more robust behavioral response in comparison to gentle touch [137]. The channels that mediate harsh touch appear distinct from gentle touch as harsh touch is still sensed in *mec-4;mec-10* mutants defective in gentle touch [137]. TRP-4 was found to mediate the posterior touch induced mechanoreceptor current in the sensory neuron PDE, as *trp-4* null mutants lacked touch induced current [137]. Another harsh touch neuron PVD, also displayed a mechanoreceptor current [137, 138]. However there is some discrepancy in data as one group reported that MEC-10 and DEGT-1 (DEGeneration of Touch neurons 1) DEG/ENaC channels were required for this current [138], while another group recorded MEC-10 mutants and found the mechanoreceptor current to be unaffected. Thus, the mechanotransduction channel that resides in PVD still remains elusive. It does not seem to express TRP-4, but appears to be encoded by an ENaC channel as the current displayed a reversal potential close to that of Na+ at +70 mV and was sensitive to amilloride. DEGT-1 mutants were not recorded in the work by Li, W. *et al.* as the

mutant was not available [137]. Thus, DEGT-1 could potentially play a role in PVD harsh touch

CONCLUSION

Locomotion is an essential aspect of animal behavior. Fundamental elements regarding nervous system organization and rhythm generation are conserved throughout species. This suggests that various organisms may use similar strategies for motor initiation and locomotion. Through evolution, species have developed larger brains made up of increased numbers of neurons and neural circuits that facilitate greater behavior flexibility and complexity. While general principles regarding motor control have been derived, the precise neural circuitry allowing sensory stimuli to guide motor output is not well characterized. To understand how added circuitry can generate complexity it is first necessary to achieve a thorough understanding of simple circuits and their electrical and genetic properties that necessitate behavior.

The genetic model *C. elegans* possesses a simple, well characterized nervous system that has become an attractive model for delineating the genetic and molecular underpinnings of behavior. The touch circuits in particular have provided great insight into how sensory stimulus is integrated into a motor output. These studies have unveiled important aspects of touch sensation including specific genetic components required for gating mechanical transduction, sensory neurons, and importantly have identified the role of command interneurons in gating forward and backward locomotion. The accepted model

for backward movement in worms is that the command interneurons AVA, AVD, AVE are required to initiate reversals both spontaneously and in response to sensory stimuli. A limitation of this model is that the role of command interneurons has never been directly investigated. The goal of this thesis was to thoroughly examine the requirement of the command interneurons in gating backward movement both spontaneously and in response to sensory evoked stimulus. By using an integrated approach to delineate the aspects of neural circuits to initiate motor activity, we have discovered a new pathway that acts in parallel to the command interneurons. This pathway shares a striking resemblance to mammalian circuitry and suggests that throughout evolution elemental features of motor initiation may have been preserved.

CHAPTER II:

THE NEURAL CIRCUITS THAT MEDIATE SPONTANEOUS MOTOR INITIATION IN *CAENORHABDITIS ELEGANS*

C. elegans is widely used to dissect how neural circuits and genes generate behavior. During locomotion, worms initiate backward movement to change locomotion direction spontaneously or in response to sensory cues; however, the underlying neural circuits are not well defined. We applied a multidisciplinary approach to map neural circuits in freely behaving worms by integrating functional imaging, optogenetic interrogation, genetic manipulation, laser ablation, and electrophysiology. We found that a disinhibitory circuit and a stimulatory circuit together promote initiation of backward movement. This dual mode of motor initiation control is found in mammals, suggesting that distantly related organisms with anatomically distinct nervous systems may adopt similar strategies for motor control. Additionally, our studies illustrate how a multidisciplinary approach facilitates dissection of circuit and synaptic mechanisms underlying behavior in a genetic model organism.

The data from this chapter is from: Piggott, B.J., et al., *The neural circuits and synaptic mechanisms underlying motor initiation in C. elegans.* Cell, 2011. **147**(4): p. 922-33.

INTRODUCTION

One of the ultimate goals of neuroscience research is to understand how neural circuits and genes generate behavior. Despite the great diversity of their overall anatomy, the basic building blocks of the nervous systems (i.e., structural motifs/modules of neural networks) display similarity across phylogeny [1, 139]. As such, genetically tractable organisms have emerged as promising models to decode the neural and genetic basis of behavior [77].

The nematode C. elegans possesses complex behaviors ranging from motor, sensory, mating, social, sleep, and drug dependence behaviors to learning and memory [77, 140-144]. Interestingly, such a complex array of *C. elegans* behaviors, some of which were once thought to be present only in higher organisms, is mediated by a surprisingly small nervous system with merely 302 neurons and 7,000 synapses [72]. *C. elegans* also represents the only organism whose entire nervous system has been completely reconstructed by electron microscopy (EM) [72]. These features in conjunction with its amenability to genetic manipulation make *C. elegans* an attractive model for decoding the neural and genetic basis of behavior. However, even for such a simple model organism as *C. elegans*, it remains largely mysterious as to how the nervous system is functionally organized to generate behaviors.

One of the most prominent behaviors in C. elegans is its locomotion behavior [77]. Locomotion forms the foundation of most, if not all, C. elegans behaviors (e.g., sensory, social, mating, sleep, and drug-dependent behaviors, and learning and memory) because these behaviors all involve locomotion and

are, to varying degrees, manifested at the locomotion level. During locomotion, worms often initiate backward movement (i.e., reversals) to change the direction of locomotion either spontaneously or in response to sensory cues [77]. Previous work from a number of labs has identified several key components in the neural circuitry that controls the initiation of reversals [85, 86, 98, 108, 126]. In particular, a group of command interneurons (AVA, AVD, and AVE) was found to be essential for the initiation of reversals, as laser ablation of the precursors to both AVA and AVD rendered worms incapable of moving backward (Chalfie et al., 1985). Based on the structural map, these command interneurons receive inputs directly from sensory neurons and also from upstream interneurons (first and second-layer interneurons), and send outputs to ventral cord motor neurons (A/AS type) that drive reversals [72, 80]. Activation of sensory neurons by sensory cues would directly or indirectly excite these command interneurons, leading to the initiation of reversals [77]. This constitutes a feed-forward stimulatory circuit (Figure 2.1A). However, it is not clear whether this circuit, though widely accepted, truly accounts for all of the reversal events seen in this organism.

In this study we applied a multidisciplinary approach to map neural circuits in freely behaving animals. Using this approach, we interrogated the locomotion circuitry and found that our current view of the circuitry needs to be significantly revised. We identified a disinhibitory circuit acting in concert with the command interneuron-dependent stimulatory circuit to control the initiation of reversals. Interestingly, the activity patterns of these two circuits are differentially regulated

by sensory cues. Notably, such a dual mode of motor initiation control has also been identified in mammals, suggesting that morphologically distinct nervous systems from distantly related organisms may adopt similar strategies to control motor output. Our study also highlights the value of applying a multidisciplinary approach to dissect the neural and genetic basis of behavior.

EXPERIMENTAL PROCEDURES

The CARIBN System and Calcium Imaging

As diagramed in Figure 2.1B, the automated CARIBN system consists of an upright microscope (Zeiss M2Bio), EMCCD camera (Andor), dual-view beamsplitter (Optical Insights), Xenon light source (Sutter), motorized stage, and computer (Dell). A C-mount (0.633) is used to couple the camera to the beamsplitter. A dual-band excitation filter (Chroma) simultaneously excites G-CaMP and DsRed at 488 and 560 nm, respectively. This system can be readily adapted to monitor fluorescent signals from Cameleon that has also been extensively used for imaging calcium transients in C. elegans neurons and muscles [88, 145, 146]. In this case a different set of filters is needed. We used a 20x objective in conjunction with a 1.63 zoom lens to acquire images. A home-developed software package controls the system and follows fluorescent objects (neurons of the worm) in dark field by their size and brightness. Specifically, a feedback loop system is introduced to track the object (neurons of the worm) by instructing the stage to move the object to the center of the camera field (re-centering) every half second (2 Hz). Under this setting we very rarely

(<1%) lose track of the worm over a 10 min window. Images were acquired with 10–30 ms exposure time (depending on fluorescence intensity of the transgene) at up to 22 Hz without binning. To facilitate identification of neurons for ratio computation, a mask image was generated for each frame by applying the following digital filters: a spatial filter to sharpen the image by correcting the motion blur; and an intensity filter and size filter to single out the neuron of interest from other neurons and the nerve ring. None of these digital filters would alter the ratio of G-CaMP/DsRed fluorescence because the ratio computation was solely based on the raw images. Nevertheless, there are always a few frames, particularly those captured during stage movement, that are of poor image quality; thus, these frames are not processed and are marked with dotted lines in the traces. A series of digital spatial filters and morphological filters were used to selectively enhance the autofluorescence emitted from the worm body, such that the outline of the worm body (head and a portion of the anterior body) can be identified to derive behavioral parameters such as backward/forward movement, speed, and trajectory. To compute the ratio change during a reversal event, we first determined the precise starting and ending frame numbers of the reversal. The image data 2 s before the starting frame were used as the basal line, and the mean ratio value of this basal line was used to compute the ratio change. The first peak or trough within the reversal period was identified to calculate the ratio change.

Calcium imaging was performed on day 1 adult worms under the standard laboratory condition where worms were allowed to freely move on the surface of

an NGM plate covered with a thin layer of bacteria (OP50) without any physical restraint. To image the activity of RIM in response to ChR2 stimulation by ChR2, worms were first tracked under the DsRed channel excited with yellow light and then switched to the G-CaMP/DsRed channels excited with both blue and yellow light. To control intrinsic phototaxis responses [147], imaging was performed on lite-1(xu7) worms insensitive to blue light [148].

Optogenetics

Worms grown on NGM plates supplied with 5 mM all-trans retinal were tested on retinal-free NGM plates spread with a thin layer of OP50. ChR_2 experiments were carried out in lite-1(xu7) worms lacking intrinsic phototaxis responses [148]. Unless otherwise indicated, a 5 s pulse of blue (470 ± 20 nm; 0.1–0.2 mW/mm^2) or yellow light (575 ± 25 nm; 25 mW/mm^2) was delivered from an Arc lamp (EXFO) by a 10x objective (Zeiss M2Bio) to the head of a forward-moving worm to turn on ChR^2 or NpHR, respectively. A positive response was scored if the worm stopped forward movement and also initiated a reversal of at least one-half of a head swing or more. We only scored the reversals initiated during the 5 s of light illumination. Each worm was tested five times with a 5 min interval between each test, and a percent score was tabulated for each worm. Because worms exhibit spontaneous reversals, a basal level of reversals was observed in controls. This number shows some variation, which may be contributed by temperature, humidity, and quality of NGM plates. Because worms reared on retinal-containing plates show a slightly higher frequency of spontaneous reversals under our conditions, transgene-free siblings

(rather than worms grown on retinal-free plates) were used as controls in behavioral tests.

Laser Ablation

Laser ablations were performed on L1 or L2 worms [149]. The transgene Pnmr-1::gfp was included in worms to help identify AVA, AVD, AVE and RIM [150]. To quantify reversals, we assayed day 1 adult worms (10 min) on NGM plates spread with a thin layer of OP50 bacteria using an automated worm tracking system described previously [141]. A positive score was assigned if both the head and the tail of a worm moved backwards for at least half of a head swing. Most ablations shown in the same panel were done in parallel. To make data collected from different days comparable, the same mock controls were repeated every time. Only those ablations with similar control data were considered comparable.

Molecular Genetics

Plasmids encoding G-CaMP and DsRed2 (Clontech) driven by the nmr-1 promoter were co-injected to generate transgenic lines co-expressing G-CaMP and DsRed, which were used for imaging of AVA [150]. The transgenes AIB::ChR2::YFP, AIB::G-CaMP/DsRed, AIB::glr-1, AIB::eat-4(RNAi) were driven by the npr-9 promoter (Bendena et al., 2008), and the transgene RIM::NpHR::YFP and RIM::avr-14 were controlled by the gcy-13 promoter [151]. To capture all the avr-14 iso-forms, the avr-14 rescuing construct was made as a mini-gene by fusing a cDNA fragment encoding the first six common exons with a genomic fragment encompassing the rest of the gene. The transgene expressing

eat-4 RNAi was generated by injecting two separate (sense and anti-sense) constructs encompassing a fragment of eat-4 gene in N2 as described previously [152]. The cex-1 promoter was used to drive expression in RIM of the transgene RIM::G-CaMP/DsRed [153]. By cell position, we confirmed that the cex-1 promoter is expressed in RIM. The transgene ASH::eat-4 was expressed under the sra-6 promoter [109]. The two transgenic arrays AIB::ChR2::YFP and RIM::G-CaMP/DsRed were crossed together for calcium imaging of RIM in response to AIB stimulation using the CARIBN system. For patch-clamp recordings, ASH, AVA, AIB and RIM was each marked with a transgene expressing mCherry (or DsRed) under the sra-6, nmr-1, npr-9 and gcy-13 promoter, respectively. These transgenes were crossed together with others (e.g. rescuing, ChR2 and RNAi transgenes) to mark neurons for recording. G-CaMP3.0 was used in all cases for calcium imaging except in RIM (G-CaMP1.3).

RESULTS

Role of Command Interneurons in the Initiation of Reversals during Spontaneous Locomotion

The current model is that the command interneurons AVA, AVD, and AVE, particularly AVA, mediate the initiation of reversals (Figure 2.1A). As a first step, we imaged the calcium activity of AVA during spontaneous locomotion by expressing in AVA a transgene encoding G-CaMP3.0, a genetically encoded calcium sensor [154]. DsRed was coexpressed with G-CaMP3.0 to enable ratiometric imaging. To reliably correlate behavior and neuronal activity, we

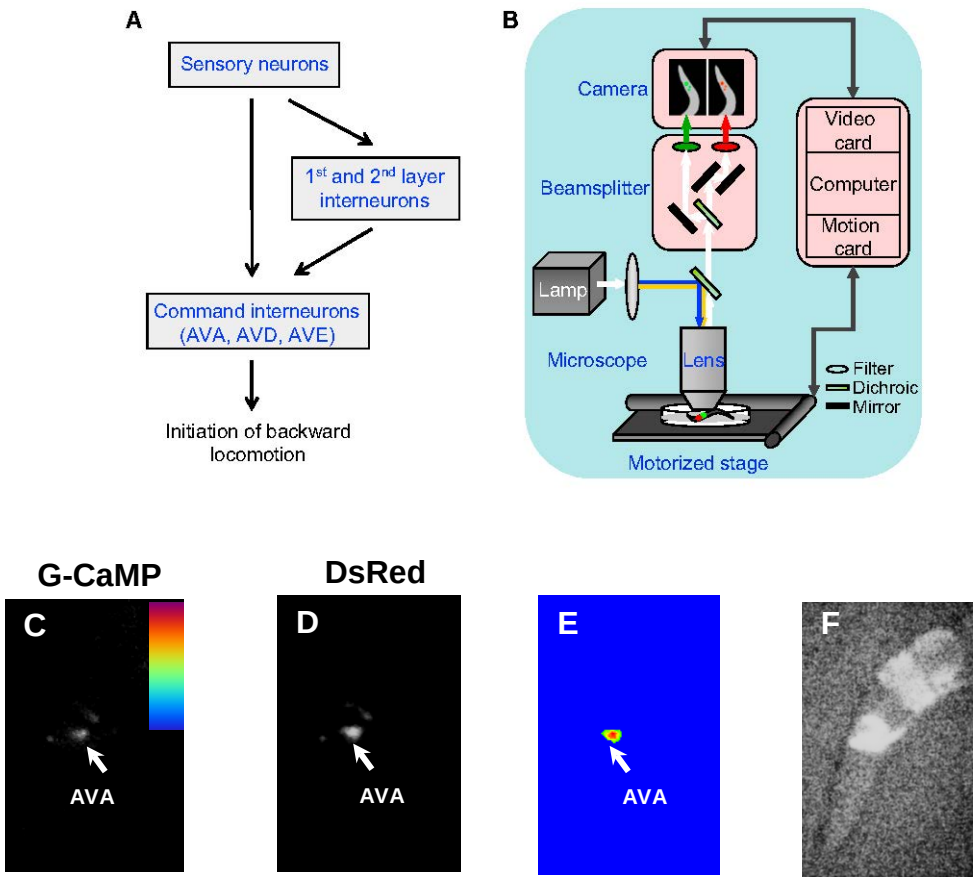

Figure 2.1 The Current Model of the Locomotion Circuitry that Controls the Initiation of Backward Movement A) In this model the command interneurons AVA/AVD/AVE receive input from sensory neurons and interneurons (first layer, AIB/AIA/AIY/AIZ; second layer, RIM/RIA/RIB) and directly synapse onto downstream motor neurons (A/AS, not drawn) to drive backward locomotion. **B)** A schematic drawing of the CARIBN system that enables simultaneous imaging of neuronal activity and behavioral states in freely behaving worms.**C)** A snapshot raw image of a freely-moving worm under the G-CaMP channel. The arrow points to the AVA neuron. The worm harbors a transgene co-expressing G-CaMP and DsRed2 under the nmr-1 promoter. Images were acquired with 5 ms exposure time at 22 Hz without binning. **(D)** Raw image of the same animal from the same frame under the DsRed channel. **E)** G-CaMP/DsRed ratio image processed from (A) and (B). Digital filters were applied to mask other neurons and the nerve ring in the image to facilitate ratio computation. Pseudocolor was generated to indicate the relative ratio. **F)** An enhanced image of (B) allowing for visualization of the worm body. Digital filters were applied to selectively enhance the autofluorescence of the worm body.

developed an automated calcium imaging system that allows simultaneous imaging of behavior and neuronal calcium transients in freely behaving animals (Figure 2.1B and Figure 2.1C). We named it the CARIBN (Calcium Ratiometric Imaging of Behaving Nematodes) system.

We used the CARIBN system to perform imaging experiments on worms moving on the surface of an NGM (nematode growth media) plate in an open environment without any physical restraint, which is the standard laboratory condition under which nearly all behavioral analyses in C. elegans are conducted. Consistent with previous results obtained with a similar system [87], we found that AVA exhibited an increase in calcium level during reversals (Figures 2.2A and 2.2B), indicating that AVA is involved in controlling backward movement during spontaneous locomotion.

Command Interneurons Are Not Essential for the Initiation of Reversals

To further evaluate the role of the command interneurons AVA/ AVD/AVE in reversal initiation, we ablated these neurons individually and in combination. Although worms lacking AVA exhibited a reduced reversal frequency, ablation of AVD or AVE did not result in a notable defect in reversal frequency (Figure 2.2C), consistent with the view that AVA plays a more important role in triggering reversals than do AVD and AVE [86, 98]. Surprisingly, worms lacking AVA, AVD, and AVE altogether can still efficiently initiate reversals, albeit at a reduced frequency (Figure 2.2C). These results demonstrate that whereas the command interneurons AVA/AVD/AVE are important for initiating reversals, they are not essential for this motor program.

Thus, there must be some unknown circuits that act in parallel to the command interneuron-mediated circuit to regulate the initiation of reversals during locomotion.

RIM Inhibits the Initiation of Reversals, and Its Activity Is Suppressed during Reversals

To identify such circuits, we first examined the wiring pattern of the worm nervous system. RIM, RIA, and RIB are classified as the ‘‘second-layer’’ interneurons that are suggested to act upstream of the command interneurons in the locomotion circuitry (Figure 2.1A) [98]. In particular the inter/motor neuron RIM sits at a unique position. It receives input from a number of interneurons and also sends output to downstream head motor neurons and neck muscles [72]. Importantly, consistent with previous reports [86, 98, 126], laser ablation of RIM greatly increased reversal frequency (Figure 2.2C). This suggests that RIM inhibits the initiation of reversals during locomotion. By contrast, laser ablation of RIA and RIB does not show a significant effect on reversal frequency during spontaneous locomotion [98] and [data not shown], though these neurons regulate certain sensory behaviors [143]. Therefore, we imaged the activity of RIM during spontaneous locomotion using the CARIBN system. If RIM suppresses the initiation of reversals as suggested above, one would predict that each reversal event should be accompanied by a down regulation of RIM activity. Indeed, RIM activity was downregulated during reversals (Figures 2.2D and 2.2E). This result is consistent with the model that RIM inhibits reversal initiation,

implying that relieving such inhibition by suppressing RIM activity should trigger reversals.

Suppression of RIM Activity Can Initiate Reversals Independently of AVA/AVD/AVE

To test this, we took an optogenetic approach by expressing halorhodopsin (NpHR) as a transgene specifically in RIM. NpHR is a light-gated chloride pump, and its activation by light suppresses neuronal activity [91]. Inhibition of RIM by NpHR effectively triggered reversals in freely moving worms (Figure 2.2F), suggesting that RIM tonically suppresses reversals during locomotion, and relieving such suppression triggers reversals.

To ascertain whether the role of RIM in reversal initiation depends on the command interneurons AVA/AVD/AVE, we checked worms lacking these neurons. Inhibition of RIM by NpHR can still initiate reversals in AVA/AVD/AVE-ablated worms (Figure 2.2G). Thus, suppression of RIM activity can trigger reversals independently of the AVA/AVD/AVE-mediated stimulatory circuit. This finding reveals the presence of an RIM-mediated parallel circuit in promoting reversals.

As a control, we performed the converse experiment. If inhibition of RIM can turn on the parallel circuit, stimulation of RIM should not. To test this, we expressed channelrhodopsin-2 (ChR2), a light-gated cation channel [90, 155], as a transgene specifically in RIM. To specifically interrogate the role of the parallel circuit, we killed AVA/AVD/AVE to eliminate the stimulatory circuit because it could be artificially turned on by its connections with RIM [92]. In these worms,

stimulation of RIM by ChR2 cannot trigger reversals (Figure 2.2G). This is in sharp contrast to the observation that inhibition of RIM by NpHR can trigger reversals in the same type of worms (Figure 2.2G). Thus, RIM inhibition, rather than stimulation, can turn on the parallel circuit to initiate reversals. Collectively, the aforementioned data suggest that RIM acts in a circuit in parallel to the command interneurons AVA/AVD/AVE to tonically suppress reversals during forward movement, and inhibition of RIM relieves such suppression, leading to reversal initiation.

AIB Acts Upstream of RIM to Trigger Reversals

We next asked which neurons act upstream of RIM to initiate reversals. The wiring map of C. elegans nervous system reveals that though over a dozen neurons synapse onto RIM, most of them merely form sparse connections with RIM. Among them, AIB is quite unique in that it is a first-layer interneuron and forms unusually dense synaptic connections with RIM by sending over 30 synapses to RIM [72, 156]. In addition, AIB regulates reversals in olfactory behavior [157]. Laser ablation of AIB suppressed the reversal frequency to a level similar to that of AVA/AVD/AVE ablated worms (Figure 2.3I). These observations raise the possibility that AIB may regulate reversal initiation by modulating RIM activity. Thus, we imaged AIB activity during reversals using the CARIBN system. AIB activity increased during reversals (Figures 2.3A and 2.3B), suggesting a role for AIB in promoting the initiation of reversals during spontaneous locomotion. If AIB promotes reversal initiation, then stimulating AIB

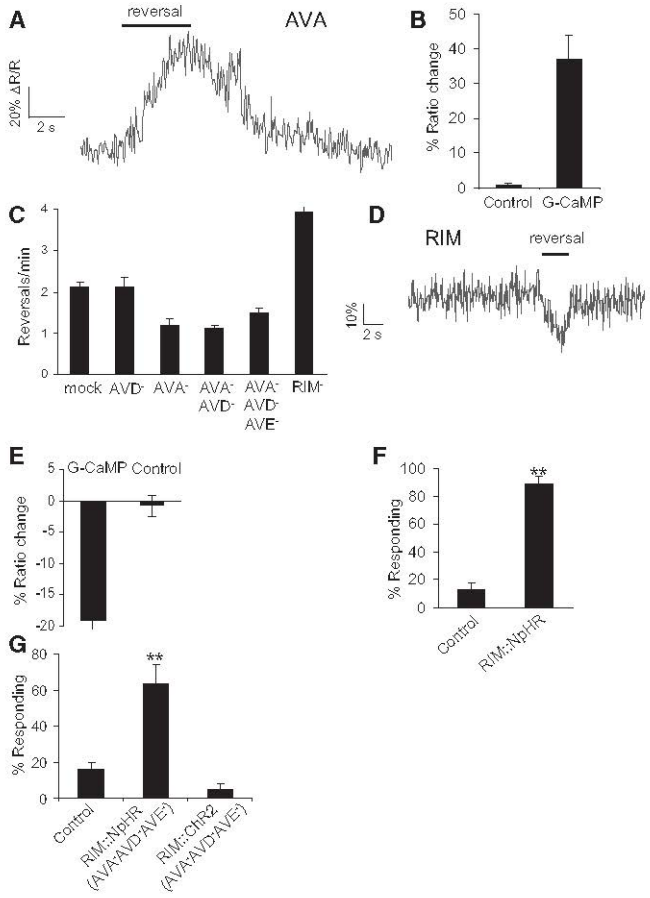

Figure 2.2 The RIM Neuron Acts to Inhibit the Initiation of Backward Locomotion, and Relieving Such Inhibition Triggers Backward Locomotion

A) AVA exhibits an increase in calcium level during spontaneous reversals. The bar on top of the trace denotes the time window during which the worm underwent backward movement. **B)** Peak percent change in the ratio of G-CaMP/DsRed fluorescence in AVA during reversals (n = 40). Control, transgenic worms express-ing YFP and DsRed under the same promoter. **C)** Laser ablation of AVA, AVA/D and AVA/D/E-ablated worms were uncoordinated during reversals (n R 5). **D)** RIM is inhibited during reversals. **E)** Peak percent change in the ratio of G-CaMP/DsRed fluorescence in RIM during reversals (n = 37). **F)** Inhibition of RIM by NpHR triggers reversals. NpHR was expressed as a transgene specifically in RIM. Control worms (transgene-free siblings) showed a basal level of spontaneous reversals. **p < 0.0001 (t test). n = 10. **G)** Inhibition of RIM by NpHR triggers reversals by turning on a parallel pathway. ChR2 was expressed as a transgene specifically in RIM and was turned on with a flash of blue light (2.5–5 mW/mm^2) (n R 5). **p < 0.0001 (ANOVA with the Bonferroni test). All error bars, SEM.

should trigger reversals. To test this, we expressed ChR2 as a transgene specifically in AIB. Stimulation of AIB by ChR2 effectively triggered reversals, providing further evidence for a role of AIB in promoting reversal initiation (Figure 2.3C).

The fact that AIB extensively synapses onto RIM suggests that AIB may act through RIM to promote the initiation of reversals. However, AIB also makes synaptic connections with other neurons, including AVA [72]. Thus, the possibility that AIB acts through AVA rather than RIM to promote reversals cannot be ruled out. Thus, we repeated the ChR2 experiments on RIM-ablated worms and found that stimulation of AIB by ChR2 can no longer further stimulate reversals in these worms (Figure 2.3D). By contrast, worms with AVA/AVD/AVE ablated still initiated reversals in response to AIB stimulation by ChR2 (Figure 2.3E). These results suggest that under this condition, AIB acts through the RIM-dependent parallel circuit, rather than the AVA/AVD/AVE-dependent stimulatory circuit, to promote the initiation of reversals.

AIB Triggers Reversals by Inhibiting RIM

We considered that AIB may inhibit RIM to trigger reversals. This model predicts that stimulation of AIB should result in inhibition of RIM. To test this, we recorded the activity of RIM in response to AIB stimulation by ChR2. Although optogenetics has been applied to stimulate neurons in freely behaving worms [81, 93], it has not been possible to simultaneously record neuronal activity in the same animal. The CARIBN system allows us to stimulate one neuron by optogenetics while recording the activity of another neuron on freely

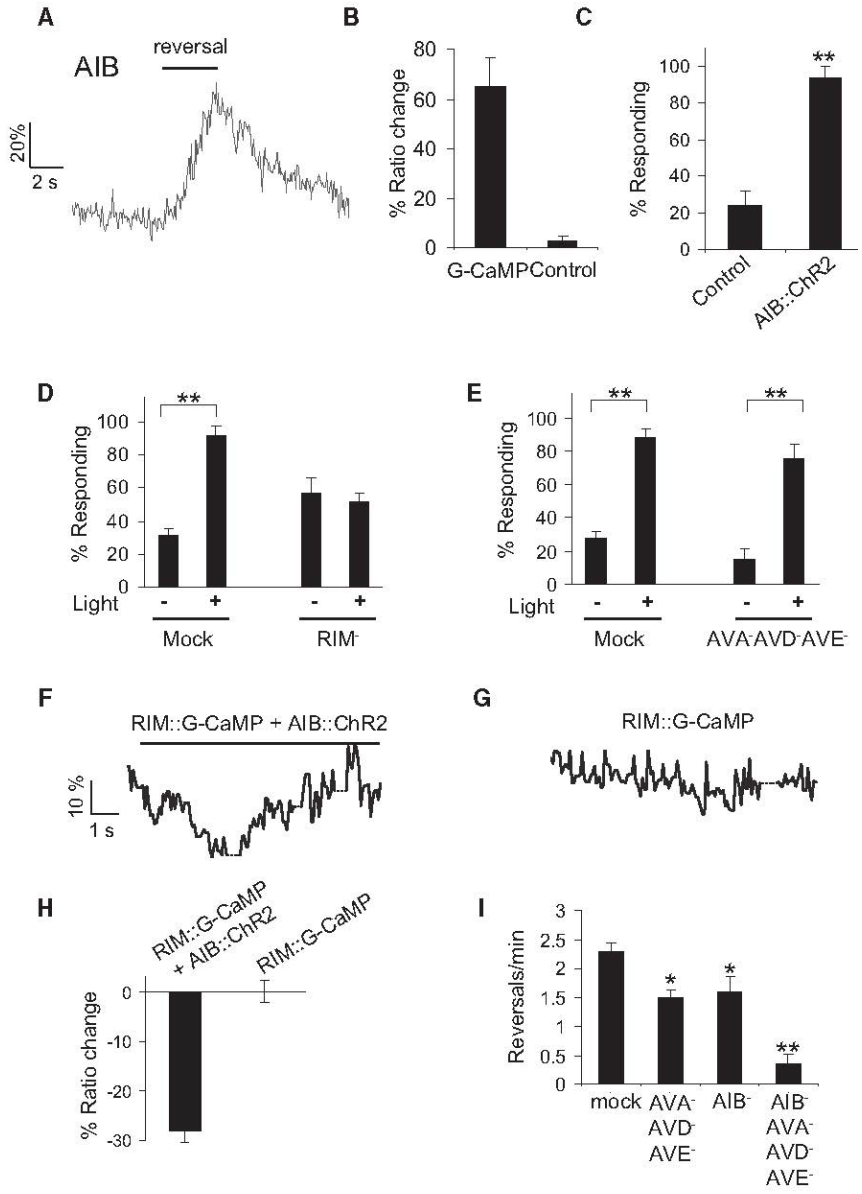

Figure 2.3 The AIB Neuron Promotes the Initiation of Backward Locomotion by Inhibiting the Activity of RIM

A) AIB fires during reversals. G-CaMP and DsRed were co-expressed as a transgene specifically in AIB. **B)** Peak percent ratio change in G-CaMP/DsRed fluorescence in AIB during reversals (n = 21). Control worms express YFP and DsRed under the same promoter. **C)** Stimulation of AIB by ChR2 triggers upstream of RIM to promote reversals. A flash of blue light was used to trigger revers-als in worms express-ing ChR2 specifically in AIB. Control worms (transgene-free siblings) showed a basal level of spontaneous reversals. **p < 0.0001 (t test). n = 10.**D)** AIB acts upstream of RIM to promote the initiation of reversals. As RIM suppresses reversals, worms lacking RIM showed a higher basal level of spontaneous reversals. **p < 0.0001(t test). n R 7. **E)** AIB triggers reversals in an AVA/AVD/AVE independent manner. **p < 0.0001 (t test). n = 9. (**F** and **G**) Calcium imaging of RIM shows that RIM is inhibited by stimulation of AIB. The dotted lines in the traces represent those few missing frames with low image quality, which are refractory to image processing. The bar on top of the trace in **F)** denotes the reversal. **H)** Peak percent change in RIM calcium level in response to AIB stimulation by ChR2 (n R 6). **I)** Simultaneous ablation of AVA/AVD/AVE and AIB abolished nearly all reversal events during spontaneous locomotion. AVA/AVD/AVE data are a duplicate from Figure 2.2C. *p < 0.03, **p < 0.0001 (ANOVA with the Bonferroni test). n R 5. All error bars, SEM.

behaving animals. Specifically, the blue light used to image G-CaMP calcium signals in RIM can also turn on ChR2 expressed in AIB, making it possible to image the activity of RIM in response to stimulation of AIB on freely behaving worms. Upon light stimulation, RIM exhibited a sharp decrease in calcium level (Figures 2.3F–2.3H). As predicted, worms initiated reversals (Figure 2.3F). The decrease in RIM activity depended on AIB stimulation because no such response was observed in worms lacking the ChR2 transgene in AIB (Figures 2.3G and 2.3H). These data, together with the results from electrophysiological recordings (see below), strongly suggest that AIB triggers reversals by inhibiting RIM activity.

Taken together, our results suggest a model in which AIB acts upstream to inhibit RIM, an inter/motor neuron that tonically inhibits reversals during locomotion; activation of AIB suppresses RIM activity, which in turn relieves the inhibitory effect of RIM on backward movement, thereby triggering reversals. In other words, backward locomotion inhibited by RIM can be ‘‘disinhibited’’ by AIB. This would constitute a disinhibitory circuit that promotes the initiation of reversals (Figure 3.6I).

The Disinhibitory and Stimulatory Circuits Together Form the Primary Pathways Promoting Reversal Initiation during Spontaneous Locomotion

Is this disinhibitory circuit important for the initiation of reversals during spontaneous locomotion? If so, then simultaneous elimination of both the disinhibitory and stimulatory circuits should result in a severe defect in reversal initiation. Indeed, whereas ablation of AVA/AVD/AVE or AIB only reduced

reversal frequency, ablation of AVA/AVD/AVE and AIB together abolished nearly all reversal events during spontaneous locomotion (Figure 2.3I). These results suggest that the AIB-RIM-dependent disinhibitory circuit and the command interneurons AVA/AVD/AVE-dependent stimulatory circuit together form the primary pathways to control reversal initiation during spontaneous locomotion.

DISCUSSION

C. elegans has emerged as a genetic model to study motor control and sensorimotor integration [77]. In this study we interrogated the circuit and synaptic mechanisms underlying the initiation of reversals in spontaneous locomotion and some sensory behaviors by applying a multidisciplinary approach integrating calcium imaging, optogenetics, genetic manipulation, laser ablation, and electrophysiology. Performing calcium imaging and optogenetic assays on freely behaving worms allowed us to reliably associate circuit activity with behavior. Genetic manipulation and laser ablation facilitated the interrogation of the role of individual genes and neurons in the circuitry. A combination of these approaches permits a rigorous dissection of the neural and genetic basis of behavior. To our knowledge, such a comprehensive approach has not been applied to map neural circuits underlying behavior in other organisms.

We found that our current model of C. elegans locomotion circuitry needs to be significantly revised. In particular we showed that the command interneurons AVA/D/E, which were long believed to be essential for the initiation of reversals, are in fact not required for this motor program. Genetic ablation of

these neurons and others also suggested a similar conclusion [86]. Importantly, we identified an RIM inter/motor neuron-dependent disinhibitory circuit acting in concert with the command interneuron-mediated stimulatory circuit to promote the initiation of reversals (Figure 2.3I). RIM may control reversal initiation by regulating the activity of its downstream motor neurons and/or muscles, and possibly the command interneurons that control forward movement (e.g., AVB and PVC).

Interestingly, the disinhibitory circuit identified in this study is functionally analogous to those found in the mammalian basal ganglia that facilitate the initiation of motor programs. These circuits allow the brain to suppress competing or non-synergistic motor programs that would otherwise interfere with sensory and goal-directed behaviors [158]. In the case of C. elegans, because its pharynx cannot efficiently take up surrounding bacteria (i.e., worm food) during backward locomotion, such a circuit would provide a potential mechanism for the animal to suppress reversals; in doing so the animal would be able to spend most of its time moving forward or dwelling to facilitate feeding and only initiate reversals stochastically (spontaneous reversals) or in response to sensory cues.

Stimulatory circuits have also been widely employed by mammals to control motor initiation [158]. For example, in response to painful sensory stimuli, nociceptive dorsal root ganglion (DRG) neurons can bypass the basal ganglia and the upper motor nervous system to trigger a limb withdrawal response by directly activating the local circuitry in the spinal cord [158]. This would ensure that animals can rapidly escape from painful stimuli [158]. Our results suggest

that despite the great diversity of their anatomy, the nervous systems from distantly related organisms may adopt similar strategies to control motor output.

CHAPTER III:

THE NEURAL CIRCUITS AND SYNAPTIC MECHANISMS MEDIATING AVOIDANCE BEHAVIOR IN *C. ELEGANS*

For the last 26 years the standard model for initiation of backward movement in *C. elegans* was that it required the command interneurons AVA/AVD/AVE. By directly examining the role of these neurons through calcium imaging in freely moving worms and laser ablation analysis, we found that while important for coordinating backward movement they were not absolutely required. Moreover we identified a disinhibitory circuit that acts in parallel to the stimulatory circuit to drive backward movement. Further characterization of the disinhibitory circuit revealed that, like the stimulatory circuit, it is regulated by sensory cues. In addition, the dynamics of these circuits are differentially modified by the type of stimulus. Through electrophysiological interrogation we characterized the synaptic mechanisms governing these circuits and discovered that both circuits require glutamatergic transmission, but depend on distinct glutamate receptors.

The data from this chapter is from: Piggott, B.J., et al., *The neural circuits and synaptic mechanisms underlying motor initiation in C. elegans.* Cell, 2011. **147**(4): p. 922-33.

INTRODUCTION

Within the brain, neural circuits exist that are specific for both sensory perception and motor commands. In mammalian systems these interconnections are not well characterized. How neurons integrate sensory stimuli into motor output is a fundamental question in neuroscience. It is from the patterns of interconnection that distinct aspects of behavior arise [6]. From its development as a model, *C. elegans* has been used to identify the neural and genetic correlates of behavior. The touch circuit, in particular, has provided a wealth of information regarding key genetic machinery for mechanotransduction as well as the underlying neural circuitry that produces behavior in response to touch [80, 132, 134]. In fact, this work identified the role of the command interneurons AVA/AVD/AVE as drivers of backward movement. Subsequent work has further characterized the synaptic interactions between the polymodal sensory neuron ASH and the command interneuron AVA [107]. ASH expresses EAT-4, a vesicular glutamate transporter indicating that ASH is glutamatergic [159]. In response to nose touch and solutions of high osmolarity like glucose or fructose, ASH is activated and goes on to activate the neuron AVA [106]. Nose touch requires AMPA-type glutamate receptors GLR-1 and GLR-2, while osmotic response requires the NMDA receptor NMR-1 in addition to GLR-1 and GLR-2 [106].

We identified a disinhibitory circuit composed of AIB and the motor/interneuron RIM, which act in parallel with the command interneurons to drive backward movement [89]. As AVA has been shown to be activated in

response to ASH-dependent sensory stimuli We wondered if the disinhibitory circuit could be affected by ASH sensed stimuli, as ASH has synaptic inputs onto both AIB and RIM [72]. Using an integrative approach, we examined the role of these circuits in mediating behavior in response to nosetouch and osmotic shock and characterized the key synaptic components that govern this circuitry.

EXPERIMENTAL PROCEDURES

Behavior Assays

Nose touch stimulus was delivered as described [108]. A small drop of 2 M glycerol was placed in the path of a forward-moving worm to induce osmotic avoidance response as described [106]. OP50 was not included in the osmotic assay. A positive response was scored if the worm stopped forward movement and also initiated a reversal lasting at least half of a head swing. We only scored the reversals initiated within the first 3 s after the animal encountered the drop. Each worm was tested five times with a 5 min interval between each test, and a percent score was tabulated for each worm.

Electrophysiology

Patch-clamp recordings were performed under an Olympus microscope (BX51WI) using an EPC-10 amplifier and the Pulse software (HEKA) as previously described [99]. Briefly, we glued worms to a Sylgard coated coverglass covered with bath solution and then carefully cut a small of cuticle in the head to expose head neurons while keeping the nose tip intact. The animal was kept alive during recording. To preserve synaptic functions, it is important to

avoid displacing neurons from their original position during dissection; otherwise, chemical synapses may get disrupted/depressed, and their activity may also quickly run down (though electric synapses tend to be preserved). Blue light pulses (0.2 mW/mm^2; 470 ±20 nm; 0.5–1 s) were delivered from an Arc lamp (EXFO Xcite) coupled to a mechanical shutter (Sutter) triggered by the amplifier. A glass probe driven by a piezo actuator (PI) mounted on a micromanipulator was used to deliver nose touch stimuli (10 mm) toward the nose tip. The normal bath solution contains: 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM dextrose, and 5 mM HEPES (330 mOsm; pH adjusted to 7.3). The pipette solution contains 115 mM K-gluconate, 15 mM KCl, 5 mM $MgCl_2$,10 mM HEPES, 0.25 mM $CaCl_2$, 20 mM sucrose, 5 mM EGTA, 5 mM Na_2ATP, and 0.5 mM NaGTP. When recording nose touch- and ChR2-evoked responses, supernatant from freshly grown OP50 culture was diluted (1:10) into the bath solution to mimic the conditions of behavioral assays and also to help prevent the run down of synaptic functions. In the high Cl- pipette solution, 115 mM K-gluconate was replaced with KCl. Cells were mostly recorded by current clamp, and currents were clamped at 0 pA unless otherwise indicated.

When recording nose touch- and ChR2-evoked responses, supernatant from freshly grown OP50 culture was diluted (1:10) into the bath solution to mimic the conditions of behavioral assays and also to help prevent the run down of synaptic functions. In the high Cl- pipette solution, 115 mM K-gluconate was replaced with KCl. Cells were mostly recorded by current clamp, and currents were clamped at 0 pA unless otherwise indicated.

RESULTS

Both the Disinhibitory and Stimulatory Circuits Are Recruited to Promote the Initiation of Reversals in Response to Nose Touch

Upon discovering that both the command interneuron stimulatory circuit and the newly identified disinhibitory circuit were the main drivers of backward movement, we then wondered how sensory cues impinge on these two circuits. In addition to spontaneous reversals, worms initiate reversals in response to various sensory stimuli, particularly aversive cues. As a consequence, these animals are able to avoid unfavorable or hazardous environments, a behavioral response essential for their survival. We focused on nose touch behavior, one of the best-characterized avoidance behaviors [108]. In this behavior, touch delivered to the worm nose tip triggers reversals (Figure 3.1A). The polymodal sensory neuron ASH is the primary sensory neuron detecting nose touch stimuli because its ablation leads to a severe defect in nose touch behavior [108]. In addition, nose touch can stimulate this neuron in calcium imaging assays [114]. Notably, ASH sends synapses to both AIB and AVA [72], and nose touch can excite AVA in electrophysiological assays [106]. This suggests a model in which ASH may engage both the disinhibitory and stimulatory circuits in this avoidance behavior.

To test the aforementioned model, we first employed our CARIBN system to image the activity of the nose touch circuits. Because this imaging system

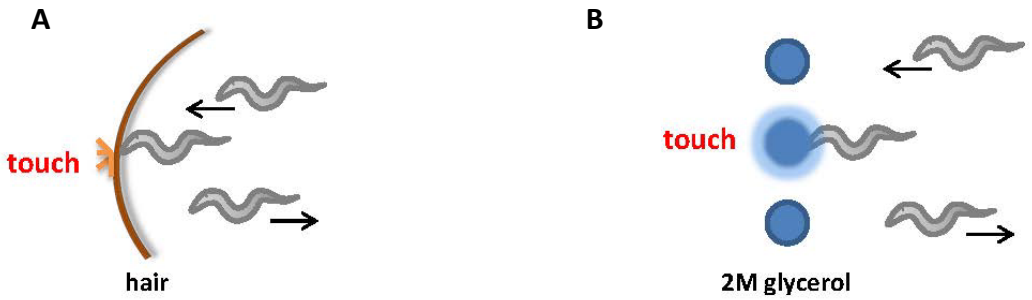

Figure 3.1 Nose touch and Osmotic Avoidance Behavior

Both nose touch and osmotic avoidance behavior are mediated by the polymodal sensory neuron ASH. **A)** The nose touch behavior assay is conducted by placing an eyebrow hair in front of a worm moving forward, perpendicular to the hair. When the worm encounters the hair with its nose tip, it will stop and then initiate backward movement. The backward activity in response to nosetouch is typically a short reversal of 0.5-1 head swings in length. During this short reversal, head foraging movements are still intact. This experiment is performed on a thin lawn of bacteria. **B)** Osmotic avoidance behavior is examined by challenging worms with a drop of 2M glycerol. This drop of high osmolarity is noxious to the worm and they rapidly reverse upon encountering it with their nose tip. In response to high osmolarity, worms typically induce a long reversal of 2 or more head swings ending in an omega bend. During this long reversal the majority of worms will suppress head oscillations. This assay is conducted on a NGM (normal growth media) agar plate devoid of bacteria. Worms are tested in 30 minutes or less to ensure they do not develop starvation behavior.

performs recording in an open environment, we were able to deliver touch stimuli directly to the nose tip of freely moving worms while simultaneously monitoring their neuronal activities and behavioral states. Our model predicts that nose touch should stimulate AVA, but inhibit RIM via stimulating AIB. Indeed, upon nose touch, AVA showed an increase in calcium activity during reversals (Figures 3.2A and 3.2C). Similarly, nose touch also stimulated AIB during reversals (Figures 3.2B and 3.2C). By contrast, RIM was inhibited during reversals (Figures 3.2D and 3.2F). Importantly, in AIB-ablated worms, RIM was no longer inhibited during reversals, indicating that the inhibition of RIM requires AIB (Figures 3.2E and 3.2F). This is consistent with the model that sensory information flows to RIM via AIB. These observations suggest that nose touch may trigger reversals by recruiting both the disinhibitory and stimulatory circuits.

To provide additional evidence, we killed AIB, RIM, and the command interneurons. Laser ablation of AIB, RIM, or AVA/AVD/AVE all led to a significant reduction in reversal frequency (Figure 3.2G), indicating that both the disinhibitory and stimulatory circuits contribute to nose touch behavior. More importantly, simultaneous elimination of both circuits by killing AVA/AVD/AVE together with AIB or RIM virtually abolished all reversals triggered by nose touch (Figure 3.2G). Thus, the disinhibitory and stimulatory circuits together form the primary pathways through which worms initiate reversals to avoid nose touch cues.

The Disinhibitory Circuit Cooperates with the Stimulatory Circuit to Promote the Initiation of Reversals in Response to Osmotic Shock

Similar to nose touch, osmotic shock delivered to the worm nose also triggers reversals by stimulating the same sensory neuron ASH (Figure 3.1B) [114]. Notably, osmotic shock is known to be much more noxious than nose touch [106], and unlike nose touch, a failure to avoid high osmolarity environment (e.g., 4 M fructose) leads to death. As a result, osmotic shock suppressed head oscillations during reversals, whereas nose touch did not; nor was this phenomenon observed during spontaneous locomotion [126] (Figure 3.3G). Suppression of head oscillations is believed to facilitate efficient escape from noxious cues such as osmotic shock, and this behavioral strategy requires stimulation of RIM [126]. As was the case with spontaneous locomotion and nose touch behavior, both AVA and AIB were stimulated by osmotic shock (Figures 3.3A–3.3C); however, RIM was stimulated rather than inhibited by osmotic shock (Figures 3.3D and 3.3F), an observation distinct from that observed in the other two behaviors. This indicates that whereas the stimulatory circuit was clearly functional in osmotic avoidance behavior, the disinhibitory circuit was instead recruited to promote suppression of head oscillations in this behavior.

To further characterize the osmotic avoidance circuits, we performed laser ablation experiments. Worms lacking the disinhibitory circuit (AIB or RIM ablated) only exhibited a slight, but insignificant, reduction in reversal frequency in osmotic avoidance behavior (Figure 3.3H). As expected, worms with RIM ablated no longer suppressed head oscillations during reversals, consistent with the role

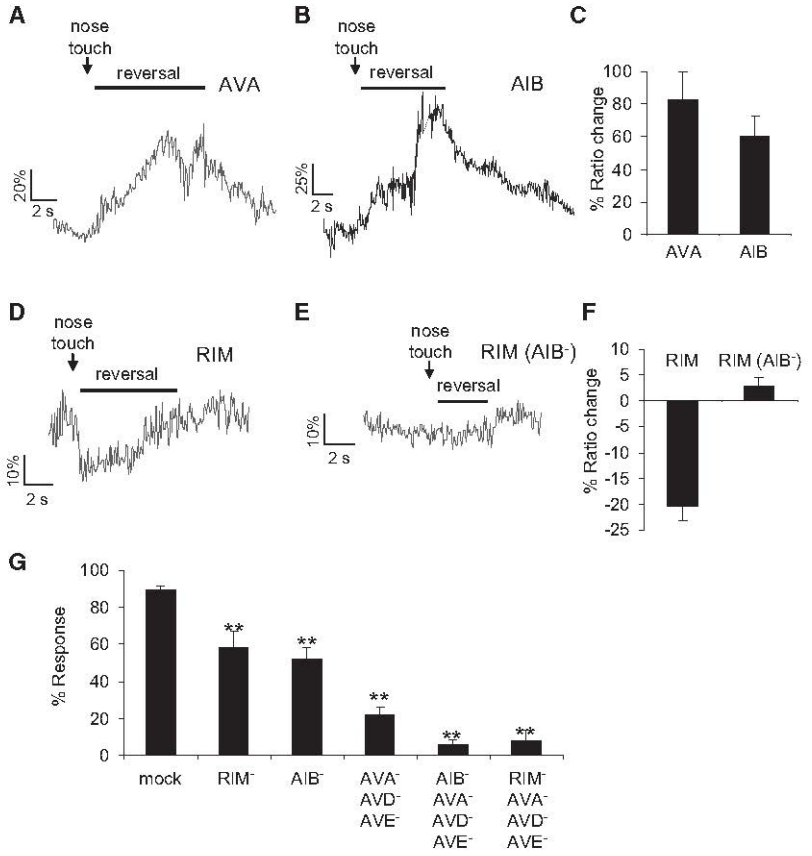

Figure 3.2 Worms Employ Both the Disinhibitory and Stimulatory Circuits to Trigger Backward Locomotion in Nose Touch Avoidance Behavior

A) AVA is stimulated during reversals in nose touch behavior. **B)** AIB is stimulated during reversals in nose touch behavior. The dotted lines in the trace represent missing frames. **C)** Bar graph summarizing the data in A) and B) (n=11). **D)** RIM is inhibited during reversals in nose touch behavior. **E)** Inhibition of RIM depends on AIB. **F)** Bar graph summarizing the data in D) and E) (n=12). **G)** Simultaneous ablation of both the disinhibitory and stimulatory circuits abolished nearly all reversal events triggered by nose touch (nR5). **$p < 0.0001$ (ANOVA with the Bonferroni test).
All error bars, SEM.

of RIM in this function (Figure 3.3G). By contrast, worms lacking the stimulatory circuit (AVA/AVD/AVE ablated) displayed a significant defect in osmotic avoidance behavior (Figure 3.3H); notably, osmotic shock can still trigger reversals in these worms, albeit at a reduced frequency, indicating that additional circuits are functional in the absence of the stimulatory circuit (Figure 3.3H).

We considered that the remaining reversal events in AVA/AVD/AVE-ablated worms could be mediated by the disinhibitory circuit. Indeed, in AVA/AVD/AVE-ablated worms, osmotic shock no longer stimulated RIM but, instead, inhibited RIM during reversals, which is similar to that observed in the other two behaviors (Figures 3.3E and 3.3F). This demonstrates that the disinhibitory circuit is functional in worms lacking the stimulatory circuit, suggesting that the disinhibitory circuit is responsible for the remaining avoidance response in these worms. This also suggests that the excitatory input to RIM was derived from AVA/AVD/AVE in osmotic avoidance behavior, consistent with the fact that these command interneurons form synaptic connections with RIM [72]. Finally and importantly, simultaneous ablation of both the disinhibitory and stimulatory circuits rendered worms virtually incapable of initiating reversals in response to osmotic shock (Figure 3.3H). Thus, in osmotic avoidance behavior worms employ the stimulatory circuit as the primary pathway and the disinhibitory circuit as the salvage pathway to trigger reversals; in addition, worms recruit neurons in the disinhibitory circuit to suppress head oscillations to facilitate efficient escape from high osmolarity environment. This illustrates an example in which the two circuits cooperate to promote avoidance responses to noxious.

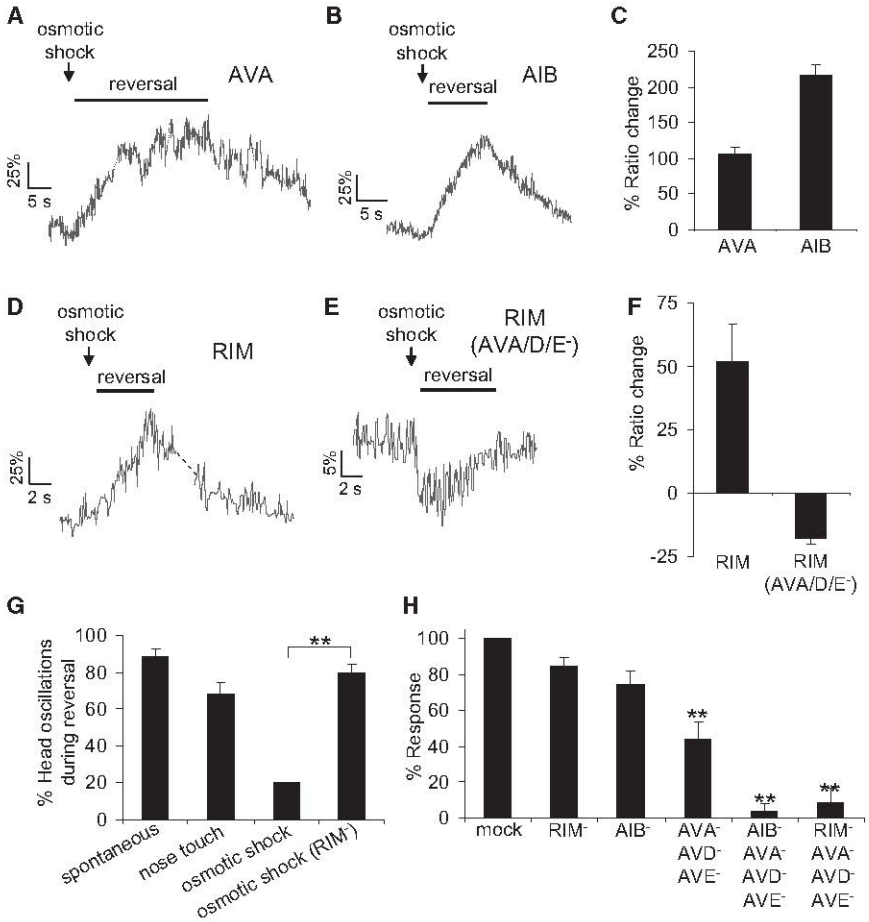

Figure 3.3 The Role of the Disinhibitory and Stimulatory Circuits in Triggering Backward Locomotion in Osmotic Avoidance Behavior

A–C) AVA and AIB are stimulated during reversals in osmotic avoidance behavior. Stimulus, 2 M glycerol. n = 11. **D)** RIM is stimulated during reversals triggered by osmotic shock. **E)** RIM is inhibited during reversals in worms lacking AVA/AVD/AVE. The dotted lines in this trace and in A) and D) represent missing frames. **F)** Bar graph summarizing the data in (D) and (E) (n = 7). **G)** Head oscillations occur during reversals in spontaneous locomotion and nose touch behavior, but are suppressed in osmotic avoidance behavior (n = 5). **p < 0.0001 (ANOVA). **H)** Simultaneous ablation of both the disinhibitory and stimulatory circuits abolished nearly all reversal events triggered by osmotic shock (n R 5). **p < 0.0001 (ANOVA with the Bonferroni test). All error bars, SEM.

stimuli. This also shows that sensory cues (nose touch versus osmotic shock) differentially regulate the activity patterns of these two circuits.

Electrophysiological Recording of the Activity of the Disinhibitory and Stimulatory Circuits

Having identified the circuits that promote reversal initiation, we then set out to investigate the synaptic mechanisms by which the circuits process information. Although our CARIBN system can record the circuit activity in freely behaving animals, this assay is indirect because it measures the calcium level but not the membrane excitability of a neuron, and also lacks the capacity to resolve synaptic events in the circuitry. Thus, we decided to employ electrophysiological approaches to record the circuit activity by patch clamping. However, the small size of worm neurons (2 μm in diameter) makes this type of recording technically challenging [160].

We focused on the nose touch circuits due to the relative ease of delivering touch stimuli with precision in whole-cell recording. This was achieved by using a glass probe driven by a piezo actuator to press the nose tip (Figure 3.4A). We recorded all of the four major neurons in the two circuits: the sensory neuron ASH and the interneurons AVA, AIB, and RIM (Figure 3.6I). We focused on recording voltage signals through current clamp, due to the high input resistance of worm neurons (typically 2–5 GU) [148, 160]. Nose touch evoked a depolarizing voltage response in ASH (Figure 3.4B). Similarly, a depolarizing voltage signal (i.e., EPSP) was detected in AVA and AIB upon nose touch (Figures 3.4C–3.4F). By contrast, in the RIM neuron, nose touch triggered a

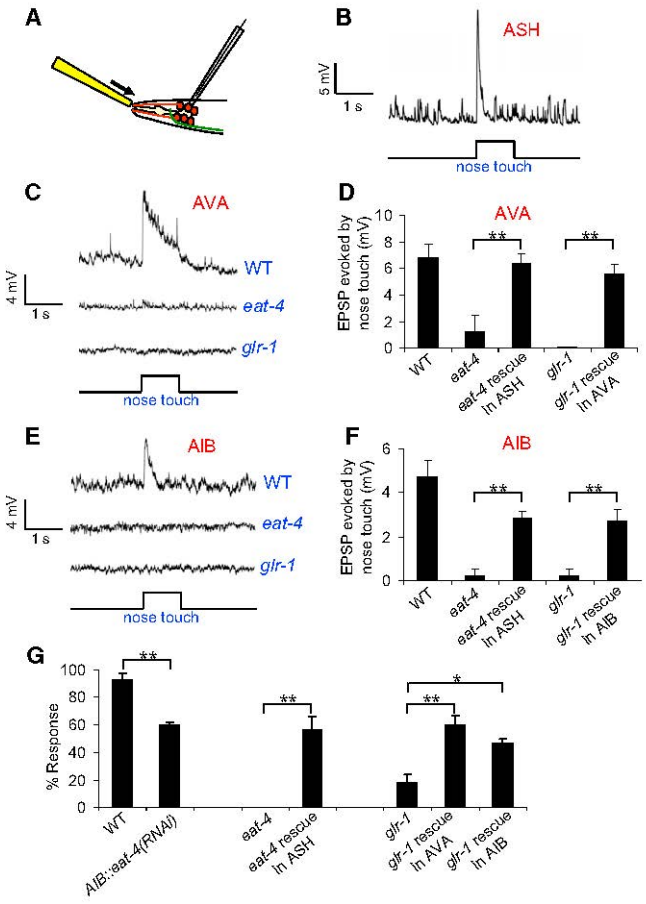

Figure 3.4 Electrophysiological Characterization of the ASH-AVA and ASH-AIB Synapses of the Stimulatory and Disinhibitory Circuits in Response to Nose Touch

A) A schematic illustrating the setting of whole-cell recording (not drawn toscale). **B)** Nose touch depolarizes the sensory neuron ASH. The miniature upward spikes represent spontaneous activity of ASH. Clamping current, 0 pA. **C** and **D)** AVA is depolarized in response to nose touch in wild-type but not in *eat-4(ky5)* or *glr-1(n2461)* mutants (n = 7). Clamping current, 0 pA. **p < 0.005 (t test). **E** and **F)** AIB is depolarized in response to nose touch, which requires EAT-4 and GLR-1 (n = 5). Clamping current, 0 pA. **p < 0.005 (t test). **G)** Nose touch behavior (n = 10). *p < 0.02; **p < 0.005 (t tests used for two-group comparisons; ANOVA with the Dunnett test used for multi-group comparisons). All error bars, SEM. See Figure 3.5 for sample traces.

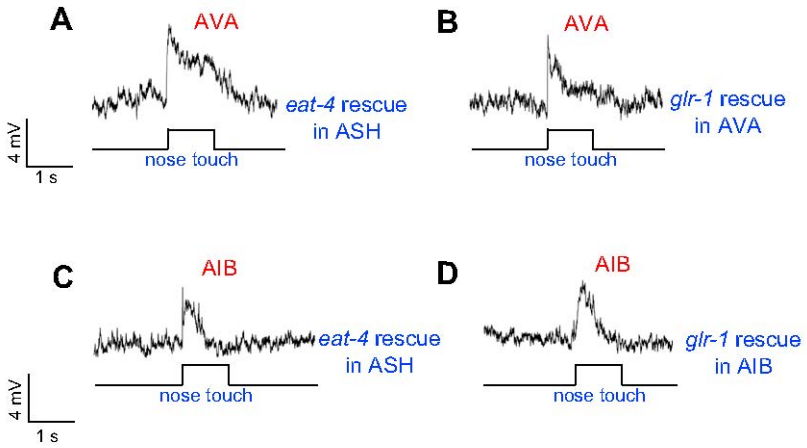

Figure 3.5 Sample Traces of AVA and AIB EPSP Responses Evoked by Nose Touch, Related to Figure 3.4

A and **B)** AVA traces. Expression of wild-type eat-4 gene in the presynaptic neuron ASH under the sra-6 promoter rescues nose touch-evoked EPSP response in AVA of *eat-4* mutant worms **A)**. Expression of wild-type glr-1 gene in AVA rescues nose touch-evoked EPSP response in AVA of glr-1 mutant worms **B)**. See data summary in Figure 3.4D. **C** and **D)** AIB traces. Expression of wild-type eat-4 gene in the presynaptic neuron ASH under the sra-6 promoter rescues nose-touch evoked EPSP response in AIB of *eat-4* mutant worms **C)**. Expression of wild-type glr-1 gene in AIB under the npr-9 promoter rescues nose touch-evoked EPSP response in AIB of *glr-1* mutant worms (D). See data summary in Figure 3.4F.

hyperpolarizing voltage response (i.e., IPSP) (Figures 3.5A and 3.5B). Finally, we directly recorded the synaptic events between AIB and RIM by stimulating AIB with ChR2 (Figures 3.6A and 3.6B), and then recording postsynaptic responses in RIM. AIB stimulation by ChR2 led to a hyperpolarizing response (IPSP) in RIM (Figures 3.6C and 3.6D). These results are well consistent with our calcium imaging data from freely behaving animals. Thus, activation of ASH by nose touch can turn on both the disinhibitory and stimulatory circuits, providing further evidence for our model. It is worth noting that the resting potential of RIM was around + 20 mV, much higher than that of AIB (- 50 mV), indicating a more depolarized state for RIM. This is consistent with our model that RIM remains in an active state to tonically inhibit the initiation of reversals during locomotion.

The ASH-AVA and ASH-AIB Synapses Are Glutamatergic and Require an AMPA/Kainate-Type Glutamate Receptor

We first characterized the presynaptic mechanisms of the nose touch circuits. Initially, we focused on the ASH-AVA and ASH-AIB synapses. ASH is known to be glutamatergic, and worms deficient in glutamatergic transmission are severely defective in nose touch behavior [106]. Thus, we performed recordings on eat-4 mutant worms where glutamatergic transmission is deficient. *eat-4* encodes a vesicular glutamate transporter [159]. Nose touch evoked EPSPs in AVA and AIB were severely defective in eat-4 mutant worms (Figures 3.4C–3.4F). Furthermore, expression of wild-type eat-4 gene in ASH restored nose touch-evoked EPSPs in AVA and AIB (Figures 3.4D, 3.4F, 3.4A, and 3.4C), as well as nose touch behavioral response in eat-4 mutant worms (Figure 3.4G).

These results support the view that the ASH-AVA and ASH-AIB synapses are glutamatergic.

We then turned our attention to the postsynaptic receptors, asking which glutamate receptors are required for the EPSP responses in AVA and AIB. GLR-1 is the closest C. elegans homolog of AMPA/kainate-type glutamate receptors and has been reported as the primary excitatory glutamate receptor in AVA and AIB [85, 106, 107, 157]. Consequently, worms lacking GLR-1 are severely defective in nose touch avoidance behavior [85, 107]. We recorded the activity of AVA and AVB in response to nose touch in glr-1 mutant worms. No EPSP signals could be evoked by nose touch in AVA or AIB of mutant worms (Figures 3.4C–3.4F), indicating that GLR-1 is required for EPSPs in these two interneurons. Furthermore, expression of wild-type glr-1 gene in AVA or AIB restored nose touch-evoked EPSP responses in AVA or AIB of glr-1 mutant worms, respectively (Figures 3.4D, 3.4F,3.5B, and 3.5D), as well as nose touch behavioral responses (Figure 3.4G). Thus, GLR-1 is an essential subunit of the postsynaptic receptors mediating EPSPs in AVA and AIB.

The AIB-RIM Synapses Are Also Glutamatergic and Require a Glutamate-Gated Cl- Channel

Finally, we characterized the AIB-RIM synapses. Notably, AIB also appears to be glutamatergic because it expresses eat-4 [161]. As expected, nose touch can no longer trigger IPSPs in RIM of eat-4 mutant worms (Figures 3.4A and 3.4B). However, this can also be explained by a defect in the sensory neuron ASH because eat-4 is expressed in ASH as well. Therefore, we knocked down

eat-4 specifically in AIB by IPSP in RIM (Figures 3.6B and 3.7C). This RNAi treatment also resulted in a significant defect in nose touch behavior to an extent similar to that caused by AIB ablation (Figures 3.4G and 3.2G). These data suggest that the AIB-RIM synapses are glutamatergic. To provide further evidence, we directly interrogated the AIB-RIM synapses by recording the activity of RIM in response to AIB stimulation by ChR2 in eat-4 mutant worms. No IPSP was detected in RIM following stimulation of AIB by ChR2 in mutant worms (Figures 3.6C and 3.6D), further suggesting that the AIB-RIM synapses are glutamatergic.

The question arises as to how glutamate, a well-known excitatory neurotransmitter, triggers an inhibitory response (IPSP) in RIM. In addition to glutamate-gated cation channels such as GLR-1, the C. elegans genome encodes at least half a dozen glutamate-gated Cl- channels [162]. Notably, the IPSP response in RIM reversed its sign around -50 mV, close to the equilibrium potential of Cl-, suggesting that it is mediated by a Cl- channel (Figure 3.7D). Moreover, using a high Cl- pipette solution, we detected an EPSP rather than IPSP response in RIM (Figure 3.7E), further suggesting that it is carried by a Cl- channel.

To provide additional evidence, we directly perfused glutamate toward RIM. Glutamate evoked a hyperpolarizing current in RIM with a reversal potential around -50 mV (Figures 3.6E–3.6G). Increasing the Cl- concentration in the pipette solution shifted the reversal potential close to 0 mV (Figure 3.6G).

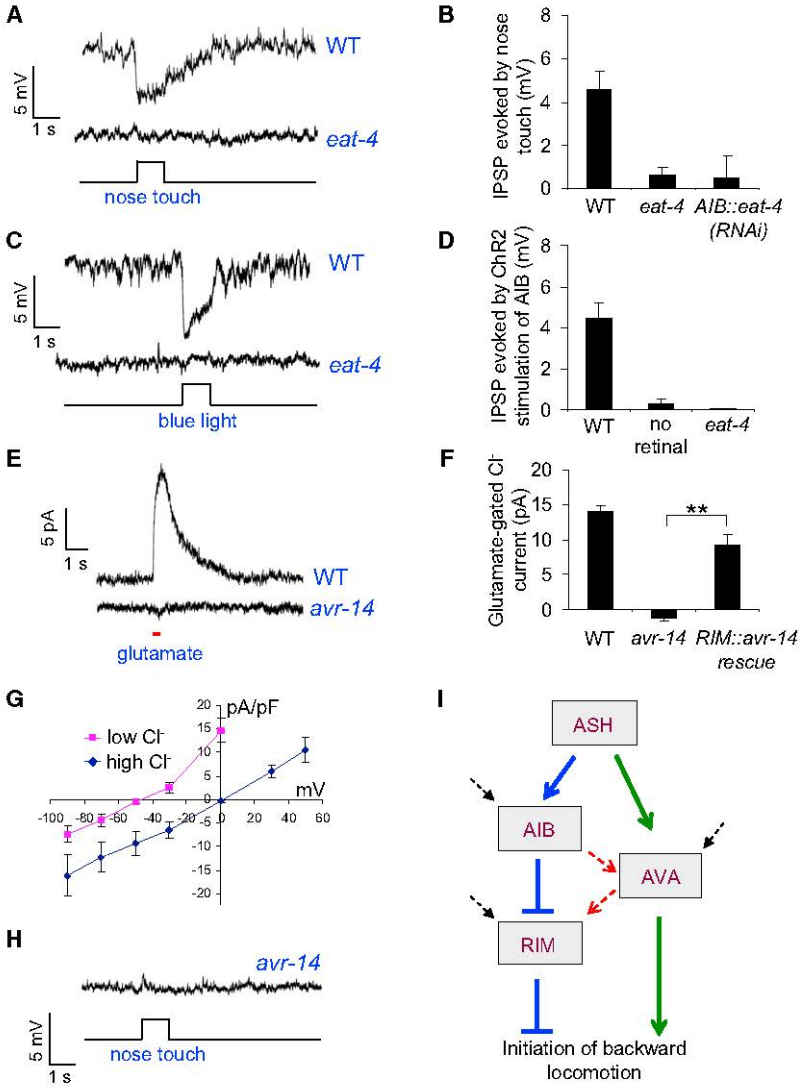

Figure 3.6 Electrophysiological Characterization of the AIB-RIM Synapse of the Disinhibitory Circuit in Response to Nose Touch A and **B)** RIM is hyperpolarized in response to nose touch, which depends on eat-4. n = 9. Clamping current, 0 pA. **C** and **D)** AIB stimulation by ChR2 leads to inhibition of RIM. See AIB traces in Figure 3.7A. n = 6. Clamping current, 0 pA. **E** and **F)** Glutamate (1 mM) perfusion evokes a hyperpolarizing outward current in RIM, which was absent in *avr-14(ad1302)* mutant worms. Voltage clamp, 0 mV. The small inward current in *avr-14(ad1302)* mutant was carried by an unknown glutamate-gated cation channel whose activity was masked by the predominant anion channel AVR-14 in wild-type worms (n = 6). **p < 0.001 (t test). **G)** Glutamate-gated currents are carried by a Cl- channel (n = 5). **H)** No IPSP signal was detected in RIM of *avr-14(ad1302)* mutant worms in response to nose touch. Clamping current, 0 pA. **I)** A schematic model illustrating the disinhibitory and stimulatory circuits. The dotted arrows in red indicate crosstalk between the two circuits. AIB, if overstimulated by ChR2 (with >103 brighter blue light), also sends output to AVA (B.J.P., J.L., and X.Z.S.X., unpublished data). The dotted arrows in black indicate that other unknown sensory neurons and interneurons may regulate the two circuits by sending output to AVA, AIB, and RIM. All error bars, SEM. See also Figure 3.7.

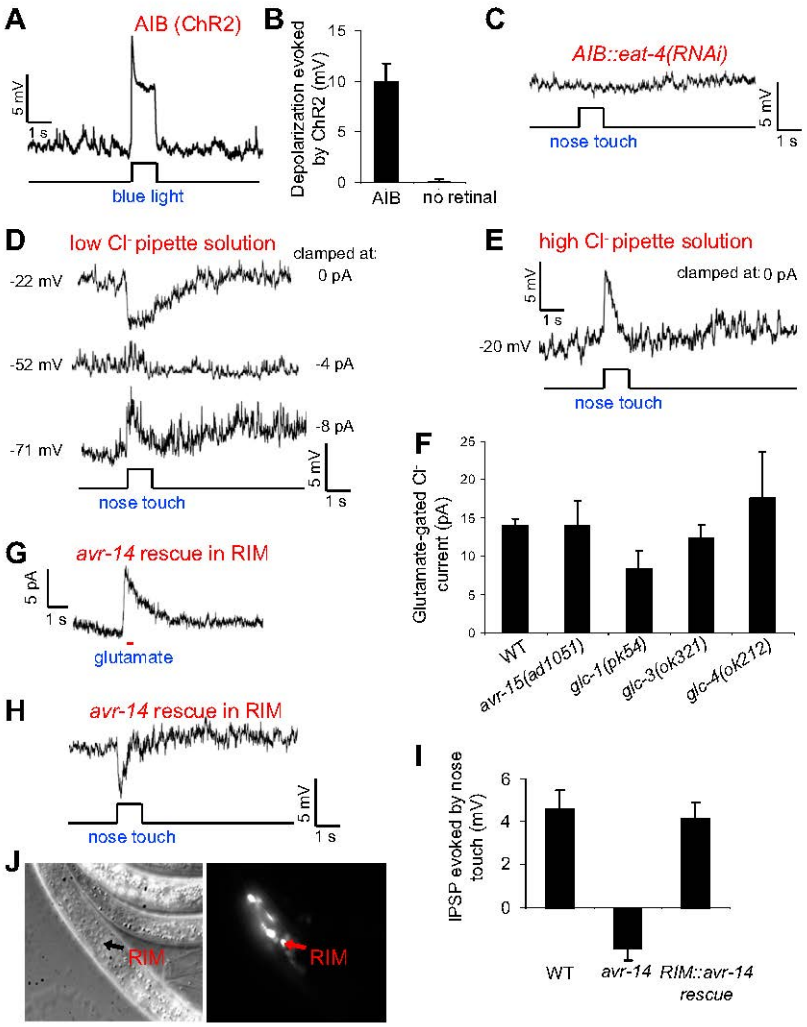

Figure 3.7 Additional Characterizations of the AIB-RIM Synapses of the Disinhibitory Circuit in Response to Nose Touch, Related to Figure 3.6

A and **B)** ChR2 stimulation by blue light depolarizes AIB. ChR2 was expressed as a transgene specifically in AIB under the npr-9 promoter. Error bars: SEM. n=7. No depolarization response was observed in control worms (no retinal). n=5. Error bars: SEM. Clamping current: 0 pA. **C)** RNAi of eat-4 in AIB abolishes the RIM IPSP signal evoked by nose touch. Sample trace. Clamping current: 0 pA. RNAi was expressed as a transgene in AIB under the npr-9 promoter. See data summary in Figure 3.6. **D** and **E)** The IPSP response in RIM is mediated by a Cl- channel. **D)** The IPSP response in RIM reversed its sign around -50 mV under low Cl- pipette solution. Clamping currents were indicated to the right of the traces, and the resulting voltages were marked to the left. **E)** Nose touch evoked an EPSP rather than IPSP under high Cl- pipette solution. **F)** Glutamate-gated Cl- currents in RIM of mutant worms lacking genes encoding the alpha-subunits of glutamate-gated Cl- channels. Recordings were performed as described in Figure 3.6. Clamping voltage: 0 mV. Error bars: SEM. **G** and **H)** Sample traces of rescued RIM responses in avr-14 mutant. **G)** Glutamate-gated Cl- current in RIM of avr-14 mutant worms was rescued by expression of wild-type avr-14 gene in RIM under the gcy-13 promoter. Clamping voltage: 0 mV. See data summary in Figure 3.6F. **H)** The same transgene rescues the RIM IPSP response evoked by nose touch. Clamping current: 0 pA. **I)** Bar graph summarizing the data in **H)** and Figure 3.6H. Similar to that described in Figure 3.6F, avr-14 mutant worms showed a small depolarizing response which was carried by an unknown glutamate-gated cation channel whose activity was masked by the predominant anion channel AVR-14 in wild-type. nR8. The WT data from Figure 3.6B is also included. Error bars: SEM. **J)** avr-14 is expressed in RIM. A 7 kb PCR product encompassing 2.9 kb 5UTR and 4.2 kb coding region of the avr-14 gene was amplified from genomic DNA and fused to YFP driven by a SL2 trans-splicing site. Left: DIC image; right: fluorescence image. Arrows point to RIM.

These data together suggest that the IPSP response in RIM is mediated by a glutamate-gated Cl^- channel.

Finally, we sought to identify the glutamate-gated Cl^- channel genes required for IPSPs in RIM. We focused on the a subunits of glutamate-gated Cl^- channels because they can form functional channels on their own [162]. Five such genes are present in the C. elegans genome, including avr-14, avr-15, glc-1, glc-3, and glc-4 [162]. Although avr-15, glc-1, glc-3, and glc-4 mutant worms all expressed glutamate gated Cl- currents in RIM (Figure 3.7F), mutations in avr-14 abolished such currents (Figures 3.6E and 3.6F). As a result, nose touch can no longer evoke IPSPs in RIM of avr-14 mutant worms (Figure 3.6H). AVR-14 was expressed in RIM (Figure 3.7J), and expression of wild-type avr-14 gene in RIM rescued glutamate-gated Cl- currents (Figures 3.6F and 3.7G), as well as nose touch-evoked IPSP response in RIM (Figures 3.7H and 3.7I). Furthermore, AVR-14 can form a functional glutamate-gated Cl- channel in heterologous systems [163]. These observations indicate that AVR-14 is an essential subunit of the postsynaptic receptor(s) mediating the glutamate-gated Cl- current underlying IPSPs in RIM.

DISCUSSION

We found that both the stimulatory circuit and the disinhibitory circuit respond to nose touch and osmotic shock. Interestingly, the activity of these circuits, are differentially regulated by distinct sensory stimuli. This is reminiscent of flexion-reflex circuits that produce more robust behavior responses to strong stimulus and under necessary conditions [164] can completely bypass

supraspinal circuits. Osmotic shock is known to represent a stronger stimulus then nose touch [106]. In response to nose touch, both the disinhibitory and stimulatory pathways generate backward movement in a manner similar to that of spontaneous backward movement. Osmotic shock elicits higher activation of AVA, which leads to depolarization of RIM overriding AIB's normally inhibitory action. Activation of RIM suppresses head foraging activity and suppresses AVB to facilitate rapid escape from the noxious stimulus [126, 127].

The existence of both a stimulatory and disinhibitory circuit may help ensure that backward movement, a critical motor program, is efficiently executed, and also provide flexibility for its modulation by sensory inputs and perhaps by experience. These two circuits apparently do not act in isolation and are regulated by sensory cues. In addition to ASH, other sensory neurons may impinge on these circuits. Other interneurons may also modulate these circuits via AVA/D/E, RIM, and AIB (Figure 3.6I). For example AIZ and AIY form connections with RIM and may regulate RIM activity. Finally, the two circuits may regulate each other through crosstalk as shown in osmotic avoidance behavior. It should also be noted that our data do not exclude the possibility that additional circuits may function in parallel to regulate reversals. One interesting observation is that though connected by gap junctions, the activity patterns of RIM and AVA are not synchronized in spontaneous locomotion or nose touch behavior, suggesting that these electrical synapses are dynamically regulated under different physiological contexts. Similar observations have been observed in vertebrate retinal circuits [165]. This presents an example in which distinct

sensory inputs (nose touch versus osmotic shock) differentially regulate the dynamics of motor circuits. Future studies will elucidate whether and how other sensory cues, sensory neurons, and interneurons regulate these two circuits, how they regulate each other through crosstalk, and whether and how they are modulated by experience.

CHAPTER IV:

DISCUSSION

OVERVIEW

One of the most challenging frontiers remaining in the biological sciences is understanding how our nervous system enables perception and response to environmental stimuli [6]. Most organ systems are understood in terms of how their structure facilitates their function. An exception to this is the brain whose structure-function relationship is rudimentary at best [166]. Since Cajal's discovery that neurons are functionally interconnected and relay signals with specific directionality [167], it has been appreciated that neural connectivity holds the key to function, however we still know very little about the connectivity itself. Traversing this divide is extremely difficult within the nervous system for a couple of reasons. The mammalian brain is composed of billions of neurons, with thousands of cell types, which are assembled into circuits through trillions of synapses [6, 168]. Moreover, describing large brain volumes (some pyramidal neurons branches put end to end can extend to more than a meter in length) at resolutions sufficient to resolve synapses is a major technical challenge [166].

Surprisingly, formal analysis of EM reconstructions, suggest that neural circuits share basic organizational principles [2].

C. elegans is the only organism whose entire wiring diagram is known [72]. While the wiring diagram of the worm's nervous system was published over a quarter of a century ago functional aspects of how a worm senses and responds to its environment is largely unknown. A major goal of this thesis work was to thoroughly address this question by examining the neural circuitry responsible for backward movement. The CARIBN system facilitated calcium imaging in freely moving worms. This enabled the direct correlation of neural activity to behavior. Optogenetics permitted direct, temporal modulation of specific neurons to elucidate the effect of a neurons state on behavior. Laser and genetic ablation provided insight into the necessity of particular genes and neurons in behavior. Lastly, electrophysiology provided the means to resolve synaptic mechanisms and neural properties in detail. Alone, each of these techniques has limitations, but when used in combination they can weave together an illustration of the neural circuit function and dynamics underlying behavior generation.

We used this integrative approach to dissect the neural basis for backward movement. We found that although the command interneurons were important for coordination of backward movement, they were not absolutely required for initiation. The classic model for backward movement established in 1985, was largely indirect and based on structural connectivity (Figure 4.1A) [80], emphasizing the point that function cannot be directly inferred from connectivity.

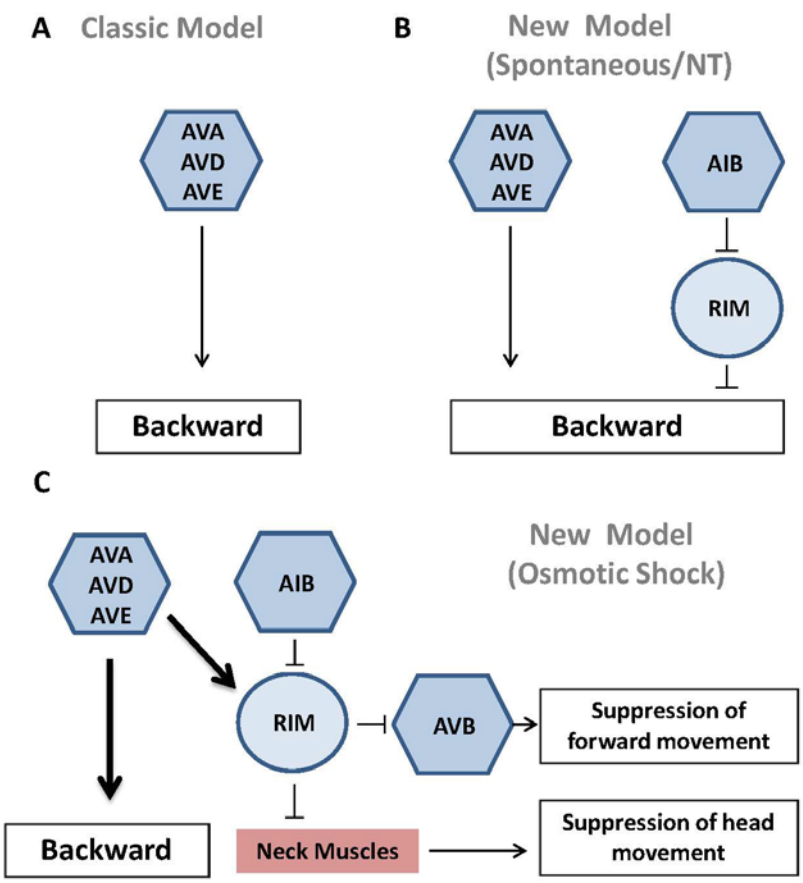

Figure 4.1 Revised Model for Backward Motor Initiation.

A) In the classic model for backward movement, the command interneurons AVA, AVD, and AVE represented the only pathway for backward movement and were absolutely required for initiating all reversal events. **B)** From this thesis work, we have established a new model for backward movement involving the stimulatory command interneuron pathway as well as a new disinhibitory pathway composed of the interneuron AIB as well as the inter/motor neuron RIM. RIM tonically inhibits backward movement, but during spontaneous or nose touch (NT) evoked reversal events, AIB is stimulated and inhibits RIM, relieving inhibition of RIMs downstream targets to permit backward movement. Headshaking is present during these short reversals. **C)** Stronger stimulus, like osmotic shock, differentially affects these parallel pathways. AVA is strongly activated by ASH and also may receive additional input from AIB. This higher level of activation stimulates RIM through its electrical synapse with AVA. Stimulation of RIM inhibits neck muscles to suppress head foraging movements. Inhibition of AVB from RIM suppresses forward movement, permitting a long reversal event to facilitate rapid escape from the noxious stimulus.

We discovered a disinhibitory circuit that acts in parallel to the command interneuron stimulatory circuit to drive backward movement and both pathways can be regulated by sensory stimulus (Figure 4.1 B-C). The stimulatory circuit represents a direct pathway to drive backward movement and AVA activity is necessary to sustain long periods of backward movement that may be necessary for a worm to get far away from a noxious sensory cue that could potentially be life threatening. The disinhibitory circuit provides a pathway to suppress unwanted motor programs to facilitate goal-oriented behaviors. Worms spend the majority of their time moving forward during feeding and their backward motor program is suppressed through tonic inhibition of RIM. During backward movement AIB is stimulated, it inhibits RIM allowing for initiation of backward movement. Dual modulation of motor activity is a key aspect of basal ganglia motor control. Within the dorsal striatum lie the direct and indirect pathways. Activation of the direct pathway leads to motor initiation, while activation of the indirect pathway suppresses unwanted movements [169]. Thus the basal ganglia functions to ensure selection of the proper motor program for the task at hand.

DISORDERS AFFECTING THE BASAL GANGLIA

The importance of the basal ganglia in motor control is illustrated in diseased states like Huntington's disease (HD), Parkinson's Disease (PD), and obsessive compulsion (OC) spectrum disorders [169]. In PD, progression of midbrain dopamine loss leads to reduced dopamine levels in the striatum. This results in severe hypokinetic motor deficits which are hallmarks of PD. Animal

models and human patients have provided insight into basal ganglia dysfunction in PD. Striatal depletion of dopamine leads to increased activity in the indirect pathway and decreased activity in the direct pathway [169]. This generates increased inhibition of unwanted movement and possibly even suppression of desired movement. Furthermore, suppression of the direct pathway makes motor initiation more challenging in patients with PD [169].

Huntingtons Disease is caused by polyglutamine expansion of Huntington proteins, which can lead to degeneration of specific subsets of neurons. The indirect pathway of the basal ganglia is selectively vulnerable [164]. Preferential loss of the indirect pathway results in loss of suppressed movements producing hyperkinesia and chorea which are prevalent characteristics of HD.

Obsessive Compulsion Spectrum disorders are characterized by repetitive thoughts, and uncontrolled urges to perform rituals. These include Obsessive Compulsive Disorder (OCD) and Tourette's Syndrome (TS) and basal ganglia circuitry has been implicated in these disorders [169]. OCD may be attributed to an enhanced propensity to form motor habits. TS may involve excessive activation of the indirect pathway as DR2 (Dopamine Receptor 2) antagonists represent an effective treatment [169]. Thus like many disorders or diseases involving the nervous system, the basal ganglia plays a critical role in proper motor control, but the specificity of the circuitry underlying this function and how it is dysregulated in diseased states is not well understood. As similar strategies for motor control are conserved in lower systems, perhaps information regarding neural motifs and functional aspects of behavior could provide insights into

fundamental properties for movement and insight into how therapies could aide dysfunction in motor circuits.

POTENTIAL ROLE OF DISINHIBITORY CIRCUIT IN LEARNING AND MEMORY

Behaviors can be adapted over time upon experience. Learning requires the alteration of synaptic activity within neural circuits. A major challenge for neuroscience is understanding how these changes at the molecular and cellular level translate into altered neural circuit function and behavior [6]. *C. elegans* has a remarkable capacity to learn and remember environmental features that predict conditions conducive to survival. They have a tendency to prefer conditions similar to those they were cultured on with respect to temperature, gustatory cues, and familiar bacteria [170, 171]. They can also display associative learning, preferring ions paired with food or developing an aversion to pathogenic bacteria [170, 171]. Interestingly, components of the disinhibitory circuit, AIB and RIM, have been linked to neural circuitry implicated in learning and memory.

Temperature can affect *C. elegans* behavior. Worms are preferentially attracted to their cultivation temperature if it is between 15-25◦C [170]. When placed on a temperature gradient the sensory neurons AFD, AWC, and possibly another, unknown neuron perceive temperature [172]. Downstream interneurons (AIB, AIY, AIZ, RIA, RIB, RIM) integrate these signals into a motor response [170]. These neurons are also implicated in navigation behaviors such as

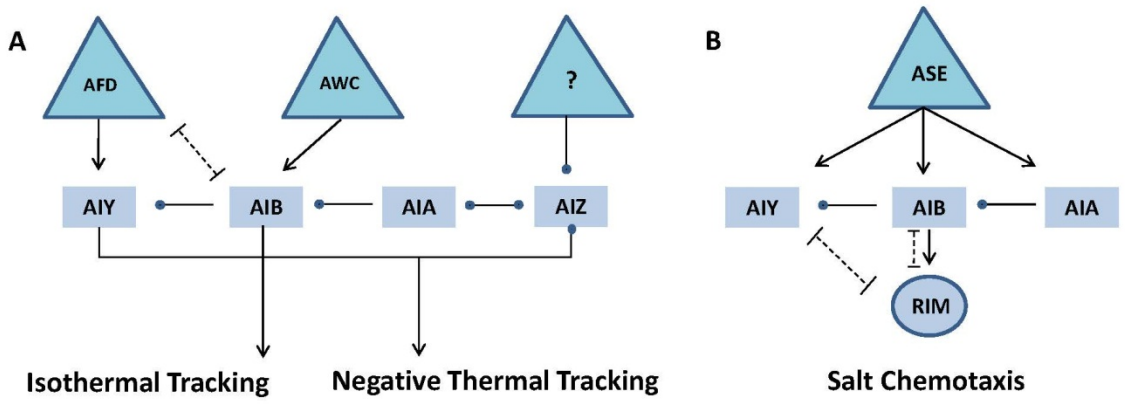

Figure 4.2 Neural Networks Underlying C. elegans Thermosensation and Gustatory Sensation.

A) Isothermal tracking occurs as worms stay around a region similar to their cultivation temperature (T_c). Negative thermotaxis occurs as worms are at a temperature higher than T_c and migrate down the temperature gradient to reach their T_c. Sensory neurons are represented by triangles, while interneurons are rectangles. Arrows indicate excitatory synapses, while dashed bars indicate gap junctions and closed circles represent chemical synapses of unknown sign. Adapted from [173]. **B)** The ASE pair of gustatory neurons is responsible for salt sensation. ASER is preferential to Cl^- while ASEL in preferential to Na^+. They act on downstream interneurons to migrate up the chemical gradient of particular ions and this circuitry can be modified to avoid ions after associative learning Adapted from [177].

turning, omega bends and reversals [98]. While the details of how they are regulated in response to temperature are not known, they potentially could modulate the locomotor aspects of thermotaxis.

A change in cultivation temperature or starvation can modulate *C. elegans* thermotaxis. This behavioral plasticity can be used to model learning and memory by dissecting the cellular and molecular aspects that mediate these changes [170]. Information regarding feeding state is not encoded by AFD sensory neurons [173-175]. However, interneurons AIZ, AIY and RIA have been implicated in starvation mediated plasticity [176]. As AIZ has numerous connections to both AIB and RIM, and AIY has gap junctions with RIM, the disinhibitory circuit represents an enticing target for synaptic plasticity.

Worms chemotax to various water soluble attractants like salt. This behavior is primarily mediated by ASE gustatory neurons [177]. Na^+ and Cl^- are chemical attractants. Na^+ is sensed by ASEL and Cl^- is sensed by ASER. Normally when given a choice between these ions, worms are equally distributed. When paired with food, worms will show preference toward the ion associated with food. If Na^+ or Cl^- is paired with an aversive stimuli like garlic extract they avoid that particular ion [178]. These experiments demonstrate that worms can exhibit associative learning. Both sensory neurons ASE and its downstream targets AIY, AIB and AIA have been implicated in mediating associative learning [176]. Furthermore it has been proposed retention of learned Na^+ aversion requires the NMDA receptors NMR-1 and NMR-2 and expression of NMR-1 in RIM alone was sufficient to rescue wildtype behavior in

NaCl conditioning assay [179]. Salt chemotaxis learning also occurs in response to starvation. Worms will avoid salt after exposed to it during a period of starvation. This type of learning requires insulin signaling [180-182]. INS-1 (INSulin like peptide 1) is released from the interneuron AIA, while downstream signals DAF-2 and AGE-1 affect ASER. How exactly the neural plasticity responsible for generating associative starvation induced salt memory is not known. Recent work in the Iino lab suggests a role for ASER, AIA and AIB neurons in the integration of salt and starvation during salt chemotaxis learning [183]. In response to prolonged NaCl exposure and found that it induced increased Ca^{2+} transients in ASER, while it decreased Ca^{2+} transients in ASEL, and both ASER/L neuron activity was dependent on insulin signaling[183]. Imaging downstream targets AIA and AIB also exhibited decreased neural activity after sustained exposure to salt. AIB, in particular, exhibited a large decrease that was insulin independent [183]. As AIB has a role in modulating reversal and turning behavior, salt conditioning could alter its activity to alter the behavior state of the worm. How insulin regulates this learning is not clear. Regardless, AIB and RIM are proposed to have important roles in gustatory learning and future examination of these circuits will provide insight into behavioral plasticity.

DOWNSTREAM TARGETS OF RIM

A final future direction of this thesis work is to delineate the downstream targets by which RIM exerts tonic inhibition to suppress backward movement and how this may relate to the balance between forward and backward movement.

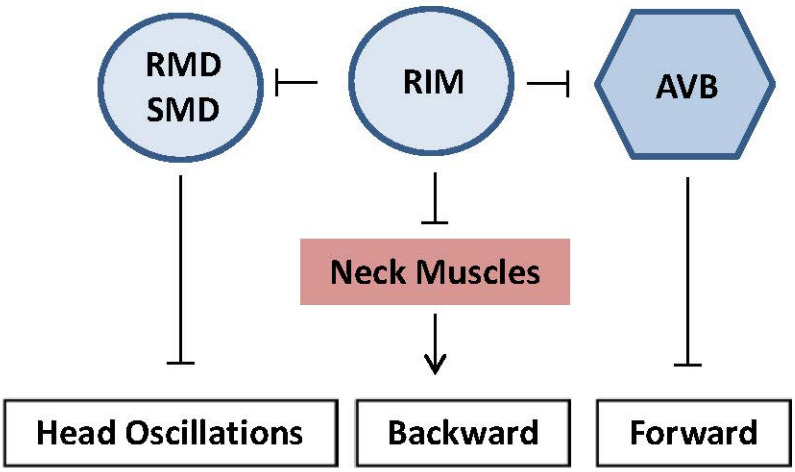

Figure 4.3 Downstream Pathways of the Inter/Motor Neuron RIM

A model for the downstream circuitry of the inter/motor neuron RIM and their proposed functions. Stimulation of RIM induces suppression of head foraging movement and AVB evoked forward movement to facilitate long reversals. Inhibition of RIM releases suppression of RMD and SMD motor neurons to trigger backward movement, with head swings. This is typically a short reversal as AVB is not inhibited and can switch to forward movement.

The major downstream targets of RIM include the motor neurons RMD and SMD, neck muscle and AVB. Each of these targets expresses the lgc-55 tyramine gated chloride channel. The role of RIM to suppress head oscillations likely occurs either through inhibiting either RMD/SMD [128] or through inhibiting neck muscles [127]. Activation of RIM is thought to inhibit AVB, a command interneuron involved in driving forward movement, to facilitate extended backward movement for escape. When considering these neural activities it is important to consider the context of the behavior.

Our work demonstrates that during spontaneous reversals and in response to gentle sensory stimuli (nose touch) AVA was activated as was AIB, while RIM was inhibited. These reversals tended to be short, no more than 1-2 head swings. However, during strong sensory stimulus like osmotic shock, AVA was more highly activated and this activity spilled over onto RIM through their electrical synapse, overriding suppression from AIB. Activation of RIM could facilitate suppression of head movement a phenomenon that did not occur in shorter reversals under our conditions. Therefore, our model is that under normal freely moving conditions when the worm is moving forward, RIM is tonically suppressing the activities of RMD/SMD and possibly neck muscles that could make initiation of backward movement occur if they are active. How exactly this could occur is not clear and is a future direction of this project. Motor neurons throughout the worm are electrically coupled so activation of RMD and SMD could trigger a reflex to initiate propagation of the electrical wave from the tail to the head to facilitate backward movement. To test this hypothesis will

require specific promoters for these neurons (which are currently unavailable) to drive expression of ChR2. This would permit examining the effect of temporally stimulating the activity of these neurons and noting the resulting behavior. Calcium imaging these neurons and muscle could also provide insight into the function of downstream targets of RIM.

CONTROVERSY OVER RIM ACTIVITY

There is some controversy regarding the role of RIM in motor activity. Recently a group has reported that RIM activity was directly coupled to AVA, or in other words they found RIM to be stimulated during most reversal events [84]. They proposed that RIM provides an essential link between the important AVA command interneurons for driving backward movement and the neuron AVB that drives forward movement [84]. Thus, they proposed AVA activations leads to inhibition of the forward pathway through RIM. This conclusion is in contrast to our data, in which we only see RIM activated under strong stimulus conditions. During all other reversal events we see RIM inhibition. The reason for this discrepancy between our results and those from the Zhen lab may be due to experimental conditions. The Zhen lab uses a coverslip, essentially sandwiching the worm between the cover glass and agar surface [84]. This sandwich serves to slow worms during movement and to restrict their z-axis. While this may provide a means to more easily image worms, the disadvantage to this approach is that worms are semi-restrained, not freely moving and thus exhibit altered behavior. Upon imaging our lines under sandwiched cover glass conditions, we too found RIM activation during reversals. We do not believe this is attributed to

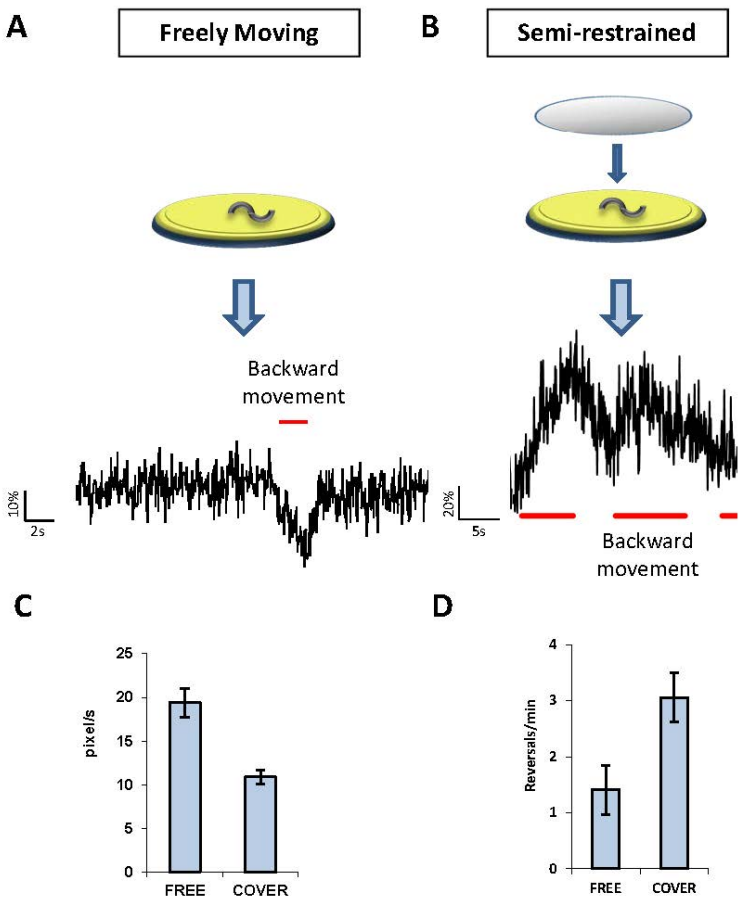

Figure 4.4 Imaging *C. elegans* Under Freely Moving Versus Semi-restricted Conditions

Restraining worms with a cover glass affects their behavior and alters RIM activity. **A)** In freely moving worms, RIM is inhibited during the majority of reversal events. **B)** When worms are semi-restrained, in a sandwich between a cover glass and an agar surface, RIM is activated during reversal events. This is likely attributed to an increase in neural activity required to drive muscle contraction under semi-restricted conditions. **C)** The difficulty in moving is manifested as a decrease in velocity. **D)** An increased reversal rate is also indicative of rapid transitions between forward and backward movement as worms struggle to maintain long durations of directional movement.

Increased resolution, as the signal to noise ratio remained constant (data not shown). We propose instead, that RIM activation is due to over excitation of neural circuits under restrained conditions (Figure 4.4B). Worms under these conditions move slower, travel smaller distances and rapidly transition between forward and backward movement (Figure 4.5C-D). Thus, we believe that RIM activation reflects over stimulus of AVA, which does not occur in freely moving conditions (Figure 4.4A), the conditions used for the majority of behavior assays conducted on *C. elegans*.

CONCLUSIONS

As the only organism with a structural map of the entire nervous system available, *C. elegans* has emerged as a model to dissect how genes and neural circuits generate behavior [77]. Nevertheless, much of the information regarding motor circuits was inferred from the structural map and, thus, has not been extensively tested at the experimental level. It has become increasingly clear that a structural map of the nervous system, though highly informative, cannot be directly transcribed into a functional map [77]. Apparently, an understanding of the functional map requires rigorous interrogation of the functional roles of individual neurons in the circuitry in the context of behavior and of how genes, environment and experience regulate circuit dynamics and hence behavioral output. Our study illustrates an example of how a multidisciplinary approach can be employed to study these questions in a genetic model organism.

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