State-Dependent Modulation of Neuronal Circuits in *C. elegans* Sleep

Thesis by
Julie YoungHee Cho

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Preface

> "You have brains in your head. You have feet in your shoes.
> You can steer yourself any direction you choose. You're on your
> own. And you know what you know. And YOU are the one
> who'll decide where to go…"
>
> — Doctor Seuss, *Oh the Places You'll Go*

It is always a joy to start something new. There is nothing like the initial excitement of getting a machine to work for the first time or the gratification of being surprised by the result of an experiment. It is the promise of something novel: another puzzle to solve.

In contrast, the real work is staying the course and finishing the story. Although I have provided most of the labor for the work shown here, its existence has to be attributed to others: partly to Paul who used both carrot and stick to drive me through my graduate career, partly to Michael who showed me how to start being an adult, and to my father who taught me responsibility. I hope that I'm not too far from the mark.

I am not sure how to feel about the end result, but there were definitely a lot of surprises along the way. It is shocking how four pairs of neurons and one question can elude a person for several years. Three hundred and two will take over your life... ask Paul.

ABSTRACT

C. elegans is a compact system of 302 neurons with identifiable and mapped connections that makes it ideal for systems analysis. This work is a demonstration of what I have been able to learn about the nature of state-specific modulation and reversibility during a state called lethargus, a sleep-like state in the worm. I begin with a description about the nervous system of the worm, the nature of sleep in the worm, the questions about its behavior and apparent circuit properties, the tools available and used to manipulate the nervous system, and what I have been able to learn from these studies. I end with clues that the physiology helps teach us about the dynamics of state specific modulation, what makes sleep so different from other states, and how we can use these measurements in understanding which modulators, neurotransmitters, and channels can be used to create different dynamics in a simple model system.

for my parents

and maybe Paul…

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1:

Overview of *Caenorhabditis elegans*

INTRODUCTION

In order to understand the nature of neuronal modulation, it is essential not only to know where to look in the neural network, but to know what to expect in the unaltered state. The advantage of early mapping and a small nervous system[1] is easily apparent, as characterization of functional circuits is both necessary and makes the following work possible. The nematode *Caenorhabditis elegans* with its 302 neurons and stereotyped connectivity is uniquely positioned for the study of state-dependent modulation, and the ease of handling and powerful genetics is an added bonus.

1.1 A BRIEF DESCRIPTION OF *C. ELEGANS*

C. elegans is a free-living roundworm in the Phylum *nematoda.* It lives in the soil-air interface and feeds on bacteria that grow on rotting fruit. *C. elegans* has several traits that make it a convenient and powerful tool for genetic analysis and behavioral testing. It grows from egg to adult in roughly three days and grows to 2-3mm in length (Figure 1). The adult hermaphrodite produces an average of two hundred progeny[2], and in the absence of male worms, these progeny are genetically identical plus or minus any spontaneous mutations. Considering the variability of behavior, minimal genetic variation is an advantage.

The body of *C. elegans* is an unsegmented, tapered cylinder whose structure is maintained by a tube-like arrangement of muscles attached to the hypodermis and a tough cuticle cover. It uses a series of opposing muscles to deform the cuticle and initiate locomotion. *C. elegans* crawls or swims through its environment with a characteristic sinusoidal pattern. Its trajectory is a random walk, and *C. elegans* decelerates in the presence of food or potential mates. In contrast, the frequency of reversal and directional change increases in the absence of food, ensuring that the worm will be likely to spend more time in nutrient-rich areas[2].

C. elegans is relatively easy to maintain and can be grown on media or agar plates with bacteria. It feeds by drawing the bacteria through its mouth and passing it to its pharynx. The pharynx consists of three large sets of muscles controlled by its own nervous system. It is organized into the anterior and posterior bulbs, which grinds and filters the bacteria into the intestine. The pharyngeal nervous system comprises of about 20 neurons, and autonomously controls the alimentary system. The somatic nervous system is made up of the remaining neurons.

1.2 *C. ELEGANS* NERVOUS SYSTEM

The *C. elegans* hermaphrodite nervous system comprises of 302 neurons. The male has an additional 79 neurons that are chiefly involved in the control of mating. The somatic nervous system is organized into ganglia in the head and tail (Figure 2a). The primary ganglia exists in the head of the worm (Figure 3), and the nerve ring is a synapse-rich band of processes that wraps around the phaynx anterior to the posterior bulb.

C. elegans uses chemosensation as its primary way to find food, avoid noxious conditions, find mates, and make appropriate decisions about development.[2] The main sensory ganglia, the amphid, phasmid, and labial neurons penetrate the cuticle to sense the external environment.[3] There are 32 sensory neurons in this group, and their cilia are either directly or indirectly exposed by openings made by the socket and sheath cells[3] (Figure 3b). These neurons exist in pairs, and proper development of the cilia are key to proper sensory neuron function.[4] Early studies with laser ablation allowed identification of specific sensory neurons and stimuli to which they respond.

A majority of sensory neurons reside in the head and tail ganglia. The exception is mechanosensory neurons whose cell bodies and processes are in relative proximity to areas that they innervate. The motor neurons that control locomotion are studded along the ventral midline, and can be characterized as cholinergic motor neurons that promote muscle contraction and GABAergic motor neurons that promote muscle relaxation. The two nerve cords, dorsal and ventral, carry the processes between the head and tail ganglia. It is approximated that this neural network has 6400 chemical synapses, 1500 neuromuscular junctions, and 900 gap junctions.[5]

The connections of these neurons are stereotyped and show little or no variation in the wildtype N2 strain. The computational components of the nervous system can be organized roughly into four layers: the sensory layer that is innervated by external stimuli, the first layer of interneurons that receive sensory information, the second layer of command interneurons that process sensory and interneuron input and convert them to a motor repetoire that is carried out by the fourth layer of motor neurons.[6] Although these circuits are fixed anatomically, modulation of the function of these circuits occurs through the use of neurotransmitters and neuromodulators, such as dopamine, serotonin, and acetylcholine.[7,8]

Despite its simple nervous system, *C. elegans* exhibits a wide range of behaviors.[9] In addition to more simple motor tasks, such as locomotion, pharyngeal contraction, and egg-laying, it is able to carry out complicated motor repetoires, such as mating and escape response. A worm

will exhibit preferences to certain temperature, chemical concentration, mechanosensory environment, and absence or presence of other worms. The worm can respond to a variety of negative cues and will show quick avoidance behavior in response to harsh mechanical stimulation and aversive chemicals.[2] *C. elegans* shows a type of associative learning, and when placed in a thermal, chemical, or electrical gradient, it migrates consistently and robustly towards zones associated with conditions previously associated with food or unstarved conditions.[10,11] Furthermore, basic habituation and paired conditioning are observable and are modified by the presence of food.[12,13]

1.3 SIGNAL TRANSDUCTION IN NEURONS

The *C. elegans* candidate chemoreceptors are related to the family I (rhodopsin-related) G-protein coupled receptors. Well-conserved GPCRS also encode receptors that recognize serotonin, acetylcholine, and neuropeptides, but these genes are general and are not expressed preferentially in the chemosensory neurons.[2] It is conjectured that individual sensory neurons would express a unique set of GPCRs that would bind to specific ligands and confer their functional identities. There are over a thousand predicted GPCRs, and few genes have been identified in the function of specific sensory neurons.

The ASH nocioceptive sensory neuron is one of best studied, and it is a polymodal sensory neuron that responds to mechanical stimuli as well as to metals and changes in osmolarity. Many amphid sensory neurons, in addition to the ASH, signal through transient receptor potential (TRP) channels encoded by *osm-9*[14] and *ocr-2*[15] genes (Figure 4). The TRP channel superfamily encodes vertebrate channels that sense osmosensation, pain, and pressure[2]. It is believed that these channels are downstream of the *odr-3* GPCR signaling and require synthesis of long-chain poly-unsaturated fatty acids (PUFAs).[2] Loss of *odr-3* and *gpa-3* diminish calcium transients that are mediated by the TRP channels *osm-9* and *ocr-2* in the ASH.[16]

1.4 FUNCTIONAL CIRCUITS in *C. ELEGANS*

The existence of static connectivity diagrams reconstructed from electron microscopy data is an immense advantage in any systems approach to *C. elegans* neurobiology. Analyzing these maps have helped to pinpoint specific cells and pathways for genetic and behavioral analyses and to identify motifs of connectivity.[17,18] Early studies have heavily relied upon mutant analysis and

targeted elimination of specific circuit components using laser ablation.[19] Although many of these studies indicate the necessity of both circuit and molecular components for specific behaviors, functional understanding was greatly improved by high-resolution data on the real-time processing done by these neurons.

Many functional circuits have been heavily studied over the last two decades. Of these, the best characterized are the avoidance circuit, the control of locomotion, and sensory coding.[20] The ease of studying these circuits is obvious. The avoidance circuit is the most direct and simple of all circuits in this simple system. It consists of a handful of sensory neurons directly connected to the command interneurons[16] and is easily observable as an immediate response. The control of locomotion is measurable and the role of various genes and neurons in the control of amplitude, speed and frequency of distinct features has been noted and characterized.[21] However, mechanical stimuli are often manual, and precise consistent stimulation of the same area with the same pressure is not trivial. Therefore, many chemosensory neurons and their downstream components have been heavily studied with respect to their activity in response to each other and in the presence of modulating factors.

1.5 *C. ELEGANS* IS A GOOD CANDIDATE FOR STATE-SPECIFIC MODULATION

The choice of circuit was key for my study of state-specific modulation, and there were multiple criteria in my choice: the exhibition of state-dependent change in behavior, characterization of the functional circuit that drives behavior, availability of or ability to make tools to manipulate the circuit across layers of processing, and simplicity of circuit. The avoidance circuit was the clear winner. Unlike chemosensory neurons mediating attraction, it has direct connection to the command interneurons and no obvious additional computational processing.[1] Decreased sensory arousal in response to noxious cues had been observed [22], and the primary avoidance mediating neuron, the ASH[8,16], as well as the downstream command interneurons[23,24] and motor neurons[25] had already been characterized in the adult worm.

FIGURES

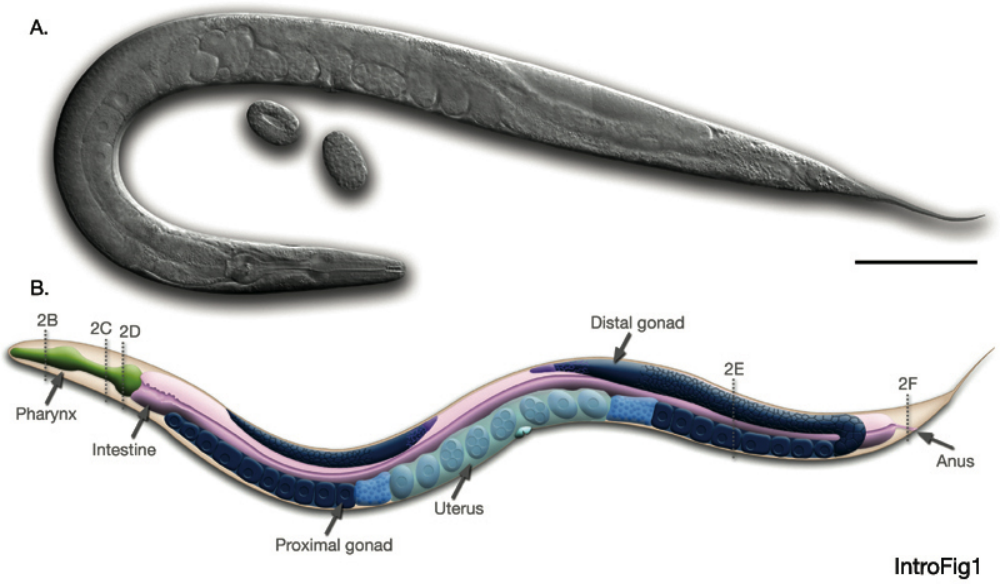

Figure 1. Anatomy of the Adult Hermaphrodite. **A.** DIC image of the adult hermaphrodite. Scale bar is 1mm **B.** Schematic drawing of the anatomical structures. (Unmodified figure from Wormatlas.[26])

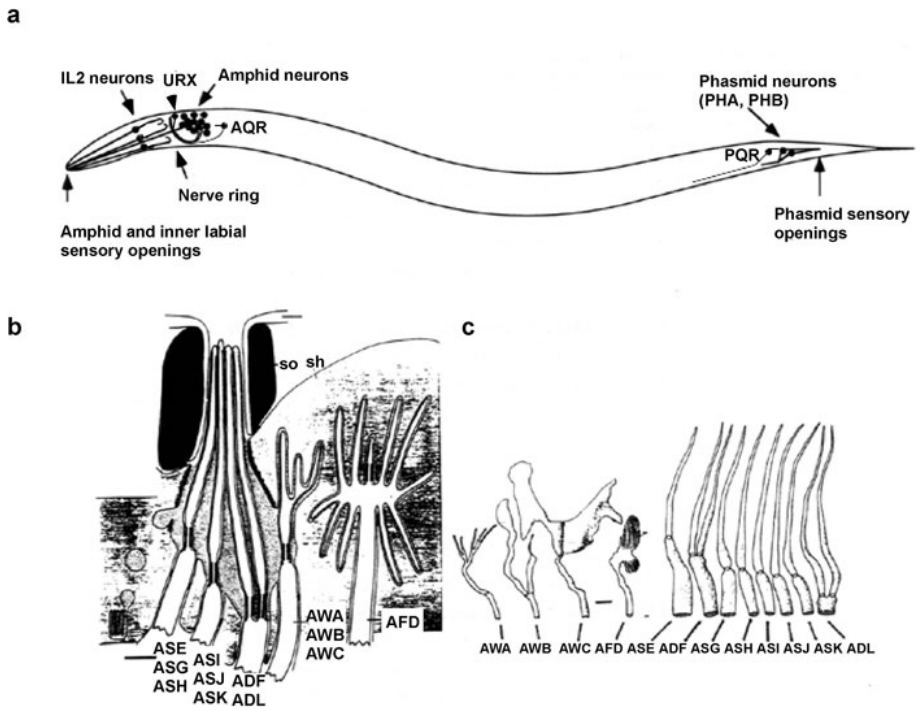

Figure 2. Structure of chemosensory organs. a. Distribution of chemosensory neurons in the animal. Amphids contain 12 associated chemosensory or thermosensory neurons. Phasmids contain two chemosensory neurons, PHA and PHB. Each inner labial organ contains one IL2 chemosensory and one IL1 mechanosensory neuron. There are two URX neurons, one AQR neuron, and one PQR neuron. **b.** Detailed structure of the amphid sensory opening showing the socket (so), sheath (sh), and ciliated nerve endings. **c.** Detailed structure of the cilia in the 12 classes of amphid neurons. (Unmodified from Wormbook.[27])

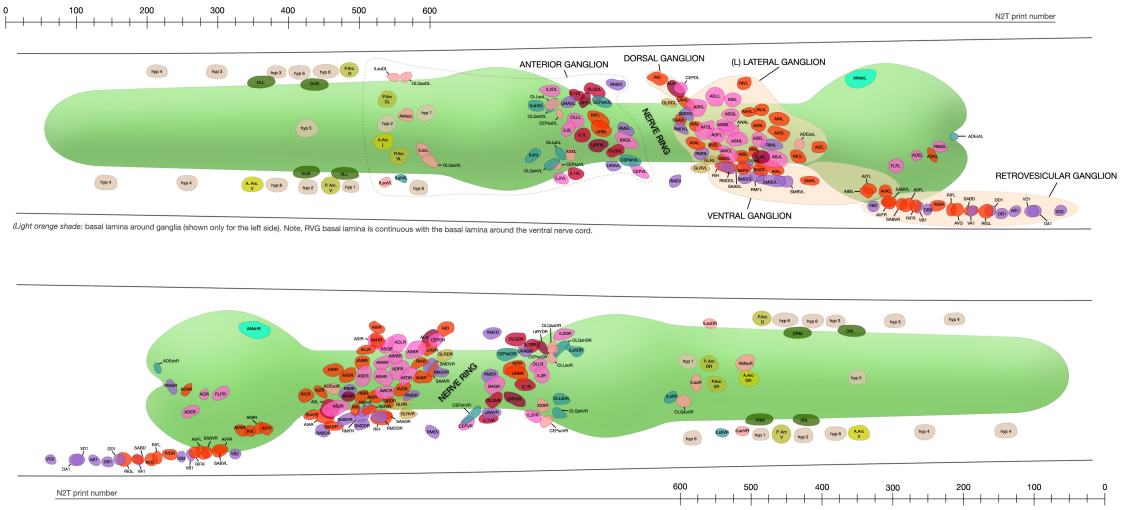

Figure 3. *C. elegans* head neurons. Schematic drawing of all head neurons in the left and right sides of the worm with respect to the pharyngeal muscle drawn in green. Note the ganglia drawn in beige in the top panel. (Unmodified figure from Wormatlas.[26])

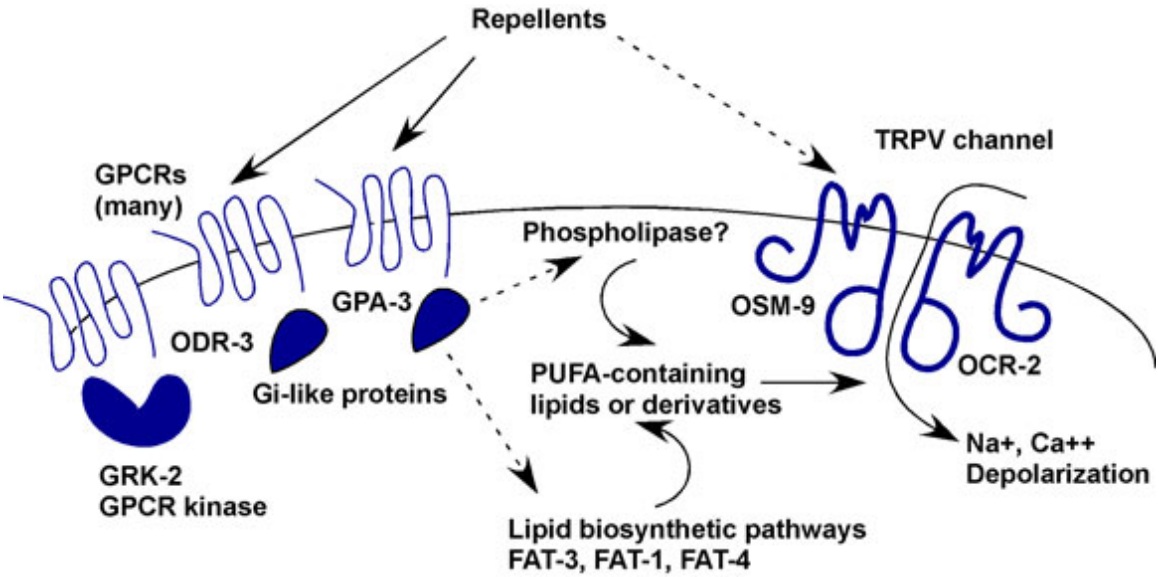

Figure 4. Potential signal transduction pathway for nociception in ASH cilia. A likely model is that repellents are detected by GPCRs and possibly by other molecules such as ion channels. GPCRs activate the Gi-like proteins ODR-3 and GPA-3, which regulate the production or consumption of phospholipids containing PUFAs (omega-3 and omega-6 polyunsaturated fatty acids). The GPCR kinase GRK-2 also promotes ASH activation. Lipid mobilization opens the TRPV channels encoded by OSM-9 and OCR-2 to depolarize the cell. TRPV channels may also be directly activated by mechanical, chemical or osmotic stimuli, perhaps with the assistance of accessory subunits such as OSM-10. (Unmodified figure from Wormbook.[27])

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CHAPTER 2:

Delving into the Sleep State

INTRODUCTION

Sleep is essential for human and animal health[1] and is behaviorally characterized by its circadian timing, behavioral quiescence, homeostasis, increased arousal threshold, and rapid reversibility.[2] Sleep is a complex process involving many facets: hardwired circadian regulation, context and activity-dependent regulation (homeostasis), global neuronal activity, specific centers for sleep, specific centers for wakefulness, and numerous regulators or sleep factors. Specific areas of the brain such as the hippocampus, cortex, and hypothalamus have been studied in detail across various species, both vertebrates and invertebrates. These studies have been illuminating in understanding the purpose of sleep and some of the processes that occur during sleep. However, integrating all this information as an overview can be a nightmare.

Additional factors such as stress, health, and the effect of genetic variation in the response of individuals to sleep regulation, both circadian and homeostatic, complicate matters further. Studying these processes in the simplest systems can help tie in the disparate pieces of information and provide a more dynamic understanding of these processes and their interactions with each other. Conveniently, sleep is conserved and present in essentially all animal species[3,4], and *C. elegans* exhibits a sleep-like state called lethargus.

Behavioral evidence suggests that our perception is dramatically dampened during sleep, and that there are physiological changes within individual neurons and their connections to each other.[1] However, there are many ways to dampen arousal[5], and despite studies spanning mammalian and non-mammalian species, the circuit modifications that promote sleep behavior are largely unknown. The conservation of neurotransmitters, several sleep regulating molecules, and sleep behaviors make anything found in *C. elegans* of potential interest in mammals, and the ease of genetics and a small nervous system allow for larger scale gene and circuit manipulations than previously possible.[5]

2.1 AN OVERVIEW OF SLEEP

Wakefulness depends on a network of cell groups in the brainstem, hypothalamus, and basal forebrain that activate the thalamus and the cerebral cortex.[1] A switch in the hypothalamus promotes γ-aminobutyric acid (GABA)-containing neurons of the ventrolateral preoptic nucleus (VLPO) to shut off this arousal system during sleep. The monoaminergic cell groups in the

arousal system fire fastest during wakefulness.[6,7] Therefore, sleep can simply be thought of as an interplay between excitatory and inhibitory centers in the central nervous system.

The VLPO projects neuronal processes to the monoaminergic cell groups that show stereotyped and coordinated changes in firing patterns associated with sleep.[8] The VLPO neurons are primarily active during sleep and contain the inhibitory neurotransmitters, galanin and GABA.[9,10,11] Cell-specific lesions showed that neuronal subpopulations in the VLPO regulate REM and NREM sleep[12] by inhibiting the monoaminergic cell groups. These cells in turn project to the VLPO and can inhibit the VLPO using both noradrenaline and serotonin.[13]

A circuit containing mutually inhibitory elements sets up a self-reinforcing loop, resulting in a "flip-flop switch", which produces discrete states with sharp transitions. Hypocretin neurons in the lateral hypothalamus stabilize this switch, and loss of these neurons cause instability of and inappropriate switching between behavioral states.[14] These neurons are mainly active during wakefulness, and especially during motor activity.[14,15]

The two-process model states that sleep results from the combination of both circadian and homeostatic processes.[16] The circadian regulation of sleep ensures that an organism rests at the appropriate and evolutionarily beneficial time, whereas homeostatic regulation is driven by the need for rest induced by the duration and intensity of activity. The earliest circadian sleep studies depended on activity patterns.[17] Now, sleep in humans and other mammals are characterized by electroencephalogram (EEG) as well as behavioral criteria to distinguish between wakefulness and sleep.[18] However, the oscillatory patterns of the EEG data are a result of underlying neural architecture that cannot be reproduced in species with different neuroanatomy. Therefore, behavioral criteria (circadian timing, behavioral quiescence, homeostasis, and increased arousal threshold) are used to identify sleep or sleep-like states in non-mammalian systems, and sleep is conserved and present in essentially all animal species tested.[3,4] Furthermore, these periods of quiescence have been shown to be associated with both circadian and homeostatic regulators of sleep.

2.2 CIRCADIAN REGULATION OF SLEEP

The circadian regulation of sleep ensures that an organism rests at the appropriate and evolutionarily beneficial time. The suprachiasmic nucleus (SCN) is the brain's "master clock", which fires in a 24-hour cycle that is driven by a transcriptional-translational loop.[19] In normal circumstances, SCN is kept in synchrony with the day-light cycle and is reset daily in response to

light detected by retina during the day, and by melatonin secretion from the pineal gland at night.[20] The SCN has some projections to the VLPO and hypocretin neurons[21], but the majority of its output is directed to the subparaventricular zone (SPZ) and the dorsomedial nucleus of the hypothalamus (DMH). Cell-specific lesions of the ventral and dorsal SPZ disrupt circadian rhythms of wakefulness and body temperature.[22] The SPZ projects to the DMH, which in turn inhibits the sleep promoting cells in the VLPO and excites wake promoting cells in the lateral hypothalamic area (LHA).[23] DMH integrates circadian input with physiological state, and DMH activity can be shifted by altered wake-sleep, activity, feeding, body temperature, and corticosteroid rhythms.[24]

The Period gene (*per*) drives the circadian clock, and PER, as well as its downstream components, has been shown to be consistent with sleep behavior. Disruption of this clock and lack of light-dark cues creates anachronistic sleep. However, a functioning circadian clock is not essential, and sleep still occurs even when circadian rhythms are disrupted or abolished.[25]

2.3 HOMEOSTATIC REGULATION OF SLEEP

Homeostatic regulation is driven by the need for rest induced by the duration and intensity of activity. Sleep homeostasis refers to the maintenance of sleep amount or depth following sleep deprivation, and is a reflection of the essential nature of sleep. The cause of this homeostasis is under debate, and there are three contending hypotheses: energy depletion hypothesis, neural plasticity hypothesis, and immune defense hypothesis.

The concept of energy metabolism follows the idea that the neuronal activity during waking consumes energy, and the energy is restored during sleep. According to experimental evidence, the degree of arousal is decreased after prolonged wakefulness, and the propensity to sleep and the intensity of delta EEG waves during sleep are proportional to the duration of prior wakefulness.[26] One possible explanation for accumulation of sleep propensity is the accumulation of substances, sleep factors, during wakefulness. Then, logically, sleep factors should steadily increase during the waking period and decrease during sleep, their concentrations should be greater during waking than sleeping, and they should inhibit neuronal activity. Adenosine, interleukin-1, TNF-alpha, prostoglandin D2 (PDG2), and growth hormone-releasing factor (GnRH) fit these criteria.[27]

Adenosine triphosphate is the primary energy carrier in the cell. Local energy depletion through prevention of ATP synthesis and increases in the dephosphorylated products of ATP

were shown to promote sleep.[27] Extracellular adenosine in the basal forebrain (BF) increases during prolonged wakefulness and decreases during recovery sleep.[28] Neuronal activity and consumption of ATP during waking is the cause of elevated adenosine concentration[29], which in turn feeds back as an inhibitory neuromodulator to decrease activity[30]. It has been suggested that this is a self-controlling neuroprotective mechanism to preclude cell damage.[31] Adenosine is formed from AMP, catalyzed by the enzyme 5'-nucleotidase, and inactivated either back to AMP by the enzyme adenosine kinase (AK) or further to inosine by the enzyme adenosine deaminase (ADA). The enzymes that metabolize adenosine undergo circadian variation, with maximal enzymatic activity during the night and minimal activity during the day.[32]

Four types of adenosine receptors are known: A1, A2, A3, and A4. Adenosine is generally an inhibitory neuromodulator that inhibits both excitatory and inhibitory neurons. A1 and A3 receptors decrease adenylyl cyclase and cAMP whereas A2 increases cAMP.[27] The presynaptic action of adenosine is mediated by the G_{i3} subtype of G-proteins that are coupled to inhibition of N-type Ca^{2+} channels and stimulation of K^{+} channels. G_{i3} either inhibits adenylate cyclase or activates phospholipase C (PLC) as effector pathways[33]. A1 receptors are widely distributed, whereas A2 receptors are restricted to the striatum, nucleus accumbens, and olfactory bulb. The main targets for vigilance state-modulating effects of adenosine are A1 receptors[34] in the cholinergic cells in the BF, hypothalamus and cortex. Selective activation of cholinergic BF neurons using neurotensin promotes EEG gamma activity and state of wakefulness.[35]

2.4 MOLECULAR MECHANISMS OF SLEEP

Study of circadian rhythms in model organisms have helped find and understand several molecular mechanisms of sleep. Analysis of sleep in the fruit fly *Drosophila* showed that increased cAMP promotes wakefulness. Adenylate cyclase mutant rutabaga has reduced cAMP and exhibits increased sleep, whereas the phosphodiesterase mutant dunce increases cAMP and shows decreased sleep.[36] cAMP targets CREB, and CREB activity has been correlated with activity in both flies and mice.[36,37] However, there is not much mechanistic insight into how cAMP promotes wakefulness.

Epidermal growth factor (EGF) was first discovered to promote sleep through intracerebroventricular injections in rabbits.[38] These results were supported by its effects in other mammals as well as in *Drosophila*, where increased release of EGFR ligands increase sleep, while inhibition of ligand release decreases sleep bout durations.[39] The site of action in the fly is

localized to the pars intercerebralis, an area that is developmentally and functionally analogous to the hypothalamus.[39]

Several other conserved molecular components of neuronal activity and signaling have been shown to affect sleep. In Drosophila, increased PKG activity is associated with more sleep.[40] Additionally, preliminary reports suggest that PKG inhibition in the basal forebrain reduces subsequent sleep[41]. Dopamine, hypocretin, GABA, and 5'-hydroxytryptamine (5-HT) all affect the sleep wake cycle[5]. In addition, the activity of potassium channels has been shown to modulate neuronal excitability and sleep in both flies[42] and mammals[43].

2.5 LETHARGUS OR *C. ELEGANS* SLEEP

C. elegans exhibits four larval stages before it matures into the adult (Figure 1). The transition between each larval stage is marked by the molt, a process in which the worm synthesizes a new cuticle, then sheds its old one. *C. elegans* exhibits sleep-like behaviors immediately before the molt during a period called lethargus.[40,44] Lethargus is a quiescent state during which locomotion and feeding are suppressed[44] and sensory arousal is decreased[40]. Furthermore, this state shows a type of homeostasis that is similar to that seen in sleep homeostasis. Inducing spontaneous activity by increased sensory stimuli during lethargus results in anachronistic rebound quiescence.[40]

Lethargus invariably occurs during development after each of the four larval stages, and the timing of lethargus corresponds to upregulation of LIN-42, homolog of circadian regulator PER[45]. Anachronistic quiescence is induced by expression of EGF[44], a function conserved in mammals[5], and sensory arousal can be depressed by PKG[40], another well-conserved signaling protein. This conservation of molecular drivers, as well as ties to circadian and homeostatic processes, suggests that the lethargus state in *C. elegans* could prove insightful in understanding sleep regulation.

FIGURES

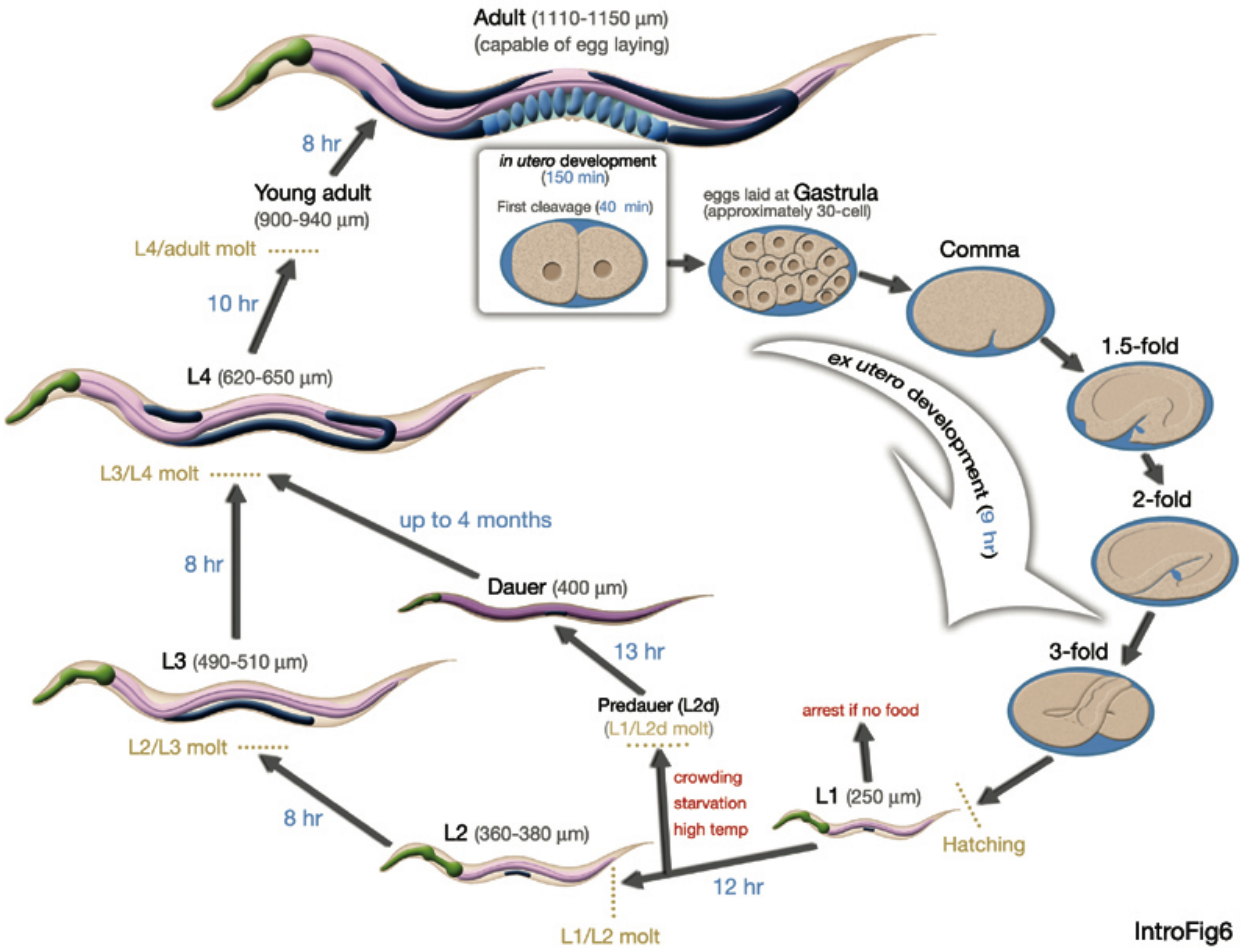

Figure 1. Life cycle of *C. elegans* at 22°C. 0 min is fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 minutes. post-fertilization. Eggs are laid outside at about 150 minutes post-fertilization and during the gastrula stage. The length of the animal at each stage is marked next to the stage name in micrometers. (Unmodified figure from Wormatlas.[46])

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CHAPTER 3:

Toolkit

INTRODUCTION

The extensive number of tools available in the *C. elegans* toolkit helps make it an attractive model system for the study of neuroscience. The most obvious is the number of genetic tools available for use. The ease of maintainence, small size, fast generation time, large numbers of identical progeny, and relatively small genome has allowed the characterization of a large mutant library, genetic mapping, and high degree of genetic control over targeting and characterizing specific neurons.[1] The transparent body of *C. elegans* allows live imaging of neuronal activity and development in live, intact animals[2], and electrophysiological methods have been developed to study neuronal activity in the form of graded potentials. In addition, optical stimulation techniques allow temporal control as wells as extent of activation in the intact freely behaving animal.[2,3]

3.1 ELECTROPHYSIOLOGY IN *C. ELEGANS*

The first nematode measured with electrophysiological techniques was *Ascaris lumbrocoides*, a worm with large neurons and graded active synaptic responses.[4] Despite their difference in size, there is striking anatomical similarity between *Ascaris* and *C. elegans*[1], and the studies of the *Ascaris* locomotor circuit provided insight into the excitatory and inhibitory function of specific *C. elegans* motor neurons.

Electrophysiology in *C. elegans* requires puncture of the external cover or cuticle, hence measurement of intact circuits is challenging. The body of *C. elegans* is an unsegmented, tapered cylinder whose structure is maintained by a tube-like arrangement of muscles attached to the hypodermis and a tough cuticle cover. The shape of the body is maintained by its high internal pressure, much like a balloon, and like a balloon, disruption of cuticle integrity results in increased likelihood of rupture. In the case of *C. elegans*, a large enough rupture also means an extrusion of organs and eventual death.

Early electrophysiological measurements in *C. elegans* focused on the neuromuscular functions of the pharynx, and these electropharyngeograms were recordings of extracellular potentials.[5] Eventually, techniques were developed and modified to record synaptic activity at the neuromuscular junction[6] and then to record individual sensory and interneurons[7]. These are high-resolution measurements that allow calculations such as transfer functions of individual synapses.[7] However, because these techniques are so intrusive and disrupt the local environment

of neuropeptides and signaling factors of the recorded neurons, it is not ideal for the study of state modulation. Measurements in the intact worm are necessary and ideal.

3.2 CALCIUM IMAGING IN *C. ELEGANS*

The discovery and use of green fluorescent protein, or GFP, has been most useful in serving as a visual marker for temporal and spatial expression of genes, identification of structure and function, as well as in opening up the field for development of a variety of new tools. Derivatives of the original GFP have been used for a variety of functions, including the fusion to proteins in order to identify compartment-specific localization and dynamics, identify co-localization of different proteins using variants that modify the excitation and emission spectra of the fluorescent protein, and split derivatives serving as detectors for the presence of various molecules or for two molecules' proximity to each other. Of course, the expression of any of these protein and products can serve as buffers and compete with the natural processes that are studied. This should be kept in mind in the context of behavior.

The calcium indicators are fluorescent detectors that increase their fluorescence in the presence of available calcium. Small molecule calcium dyes are an indirect indicator of neuronal activity, but these require injection and lack cell specificity.[8] Genetically encoded calcium indicators allow visualization in an intact animal and availability of a large number of known promoters provide cell specificity. These indicators are based on the protein *calmodulin*, which changes its conformation in response to binding of calcium. The calcium binding region of calmodulin is used to link two proteins, of which one or both are fluorescent molecules, and the changing distance between the fluorescent particles either shifts the spectra or changes the intensity of the wavelengths of light emitted.

GCamP is a calcium indicator that is a fusion of GFP, calmodulin, and M13. In the absence of calcium, the conformation of the circularized GFP allows quenching of the fluorophore, which is altered upon calcium binding.[9] Therefore, there is a dramatic and fast increase in fluorescence in response to calcium. Cameleon uses foster resonance energy transfer (FRET). FRET works through the use of two fluorophores: the donor which is excited with an external light source and an acceptor whose excitation spectra corresponds to the emission spectra of the donor. FRET requires the proximity of the donor and acceptor. Therefore, the ratio of the donor and acceptor emission that can be detected changes with alterations of the calmodulin conformation, and the acceptor to donor ratio increase upon binding of calcium[10].

Ratiometric imaging has its drawbacks and advantages. The non-FRET indicators have better dynamic range, and cameleon often shows a smaller change in response to the same stimulus in a given neuron when compared to GCamP. However, there are also many advantages to ratiometric imaging: movement and lighting artifacts can be detected and cancel out in the ratiometric calculation, altered promoter activity and expression of indicator is not an issue, and it allows for detection of fast events even in the scale of milliseconds.[11] The hardware for ratiometric imaging is a bit more complicated, but worth it. However, I find that the largest drawback to using the FRET indicator in my studies is the effect on behavior.

Light is a potent stimulant and serves a noxious cue in *C. elegans*[12], and it avoids short wavelengths of light by staying beneath the surface of the soil. Animals avoid light and it is shown that illumination with the green to ultraviolet spectra of light is detected by LITE-1 and will induce escape response as well as increase locomotion.[12] We have found that exposure to high intensity light even in the absence of *lite-1*, will induce calcium transients in the ASH nocioceptive sensory neuron of adult animals, as well as those in lethargus. Furthermore, shorter wavelengths of light elicit stronger responses.[12] Therefore, although use of low intensity light for cameleon imaging fails to elicit sensory response in lethargus, it serves as a stimulus in L4 and adult animals which is not ideal and changes sensory response to chemical stimuli.

3.3 THE OPTOGENETIC TOOLBOX

Several techniques have been developed to remotely and optically control neurons in the intact worm. Channelrhodopsin is an algae-derived cation channel that is a microbial-type rhodopsin, a seven transmembrane retinal protein that has no sequence homology to animal rhodopsins. Channelrhodopsin1 is a proton-selective light-activated channel derived from the green algae *Chalmydomonas reinhardtii.*[13] Channelrhodopsin2 is a leaky proton pump that acts as a light-gated nonselective cation channel[14] that has the ability to trigger large currents. A cofactor, *all-trans* retinal, is required for channel activity and the peak of the action spectrum is approximately 460nm. The ChR2 conductance has a large initial transient and decays to a lower steady-state level with continued illumination. The refractory period after activation is shorter at low extracellular pH and at more negative voltages. The estimated conductance of the channel is a low 50 fS. The rise time of the ChR2 current is extremely fast: less than 200 μs; decay is on the order of milliseconds and is pH sensitive.[14]

The first characterizations of ChR2 involved the control of action potential and synaptic transmission in hippocampal neurons.[15] Currently, photoactivatable channels have been used in a range of animal models including worms, flies, fish, rodents and primates.[16-20] In *C. elegans,* ChR2 can be used to drive awake behavioral responses in the presence of the cofactor all-*trans* retinal (ATR).[16] Animals can be fed ATR through the bacteria they eat, and specific promoters can be used to selectively promote ChR2 expression and activation. In addition to study of behavior, it has been used to study release at the neuromuscular junction in worms.[21]

In addition to ChR2, many other types of light-activated channels and molecules exists. Optical inhibition can be induced by using halorhodopsin, and G-protein signaling pathways can be activated by the opto-XRs. Halorhodopsin is a yellow-light activated chloride pump derived from archaebacterium *Natronomas pharaonis* that works with millisecond precision.[22] Use of optical stimulation techniques allow fast, reliable manipulation of neuronal activation and inhibition in specific subsets of neurons. Recent advances provide light-shifted varieties, as well as long-acting and subthreshold channelrhodopsins. In addition, various strategies have been used for cell-specific activation and expression of these light-activated channels.

3.4 MICROFLUIDICS

Microfluidic devices were first developed to miniaturize chemical and biochemical analyses and to make these processes more sensitive, faster, and have higher resolution.[23] Since then, microfluidic devices fabricated in polymers (polymethylsiloxane or PDMS) using soft lithography have been useful in basic and biomedical applications.[24] Microfluidics uses laminar flow and allow precise control and quick manipulation of microenvironments that are not available in conventional macro-scale methods.[25] These devices have been used for cell culture as well as neuronal stimulation[26], and *C. elegans* with its small size of 2-3mm is well-suited for this technology.

Various microfluidic devices exist for immobilization of animals, observation of locomotion, and exposure of animals with either gas or aqueous stimuli. These devices allow simultaneous control of the animals' environment while imaging, and has allowed measurement of sensory response to a variety of stimuli.[27] Manual and automated[28] controls of these devices are possible. And because they are made to conform to the shape of the animals, they allow feeding activity. Additionally, these devices allow fast and reproducible delivery of stimuli, do not rely on the use

of adhesive, and provide more consistent behavior and response than other methods of immobilization.

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CHAPTER 4:

Multilevel Modulation in *C.elegans* Sleep

Submitted for publication as Julie Cho & Paul Sternberg. “Multilevel Modulation of a Sensory Motor Circuit in *C. elegans* Sleep”

4.1 ABSTRACT

Sleep is essential for human and animal health and is behaviorally characterized by its circadian timing, behavioral quiescence, homeostasis, increased arousal threshold, and rapid reversibility. Behavioral evidence suggests that our perception is dramatically dampened during sleep, and that there are physiological changes within individual neurons and their connections to each other. However, there are many ways to dampen arousal, and despite studies spanning mammalian and non-mammalian species, the circuit modifications that promote sleep behavior are largely unknown. Here we systematically dissect components of a sensory motor circuit and identify changes in the flow of information from sensory to inter- and motor-neuron that implement sleep-like behavior in the nematode *Caenorhabditis elegans*. We demonstrate that the top two layers of sensory processing are affected, with decreases in both sensory transduction and subsequent transmission of this information to interneurons. Modulation in transmission results in a decreased likelihood of coordinated activity in the downstream interneurons, which can be reversed with sufficient prior sensory stimulation. Multilevel depression provides an elegant strategy to promote a robust decrease in arousal while allowing for rapid reversibility of the sleep state.

4.2 INTRODUCTION

Sleep behavior is conserved and present in essentially all animal species as a lack of reactivity to sensory inputs, low activity, decreased conscious awareness, and rapid reversibility to wakefulness.[1,2] Despite long-standing knowledge and characterization of these states, translation to a physiological or circuit definition has been difficult for many reasons. No single molecular or neuronal pathway has been shown to be responsible for the regulation of sleep, and incomplete knowledge of connections in sensory motor circuits of many studied species precludes interrogation of the flow of information that promotes sensory responses. Furthermore, the complexity and redundancy of the mammalian nervous system complicates the understanding of the flow of information. Here we use the nematode *C. elegans* to define sleep behavior in a simple sensory motor circuit.

C. elegans exhibits sleep-like behaviors during lethargus, a quiescent state during which locomotion and feeding are suppressed[3] and sensory arousal is decreased[4]. Lethargus lasts two to three hours, and like sleep it exhibits homeostasis: upon disruption or deprivation during the

normal resting period, there is a period of anachronistic rebound rest.[4] During lethargus, worms exhibit delayed or decreased avoidance behavior in response to both mechanical and chemical stimuli, and this response delay is reversible upon previous stimulation of the same neuron.[4] It is of note that quiescence as measured by decreased feeding and locomotory behavior is present in adult satiety behavior.[5] However, the dynamics of this state were not previously studied.

Lethargus invariably occurs during development after each of the four larval stages, and the timing of lethargus corresponds to increased expression of LIN-42, homolog of circadian regulator PER.[6] In addition, several additional conserved regulators have been identified. Anachronistic quiescence is also induced by expression of EGF[3], a function conserved in mammals[7] and Drosophila[8], and sensory arousal can be depressed by PKG[4], another well-conserved signaling protein. Conservation of sleep characteristics and molecular signaling suggests that the lethargus state in *C. elegans* could prove insightful in understanding sleep regulation.

The connections of the *C. elegans* nervous system are mapped[9,10], and functional circuits mediating avoidance defined[11]. Avoidance behaviors are mediated by mechanosensory and chemosensory neurons that activate downstream circuit components to coordinate motor neuron activity and locomotion. The ASH sensory neuron drives an avoidance circuit and promotes immediate locomotory reversal. When animals are presented with ASH-specific stimuli, ASH stimulates interneurons AVA and AVD, which in turn induces backward locomotion through stimulation of excitatory cholinergic motor neurons in the ventral cord (Figure 1a).[12,13,14] Evidence of changing arousal as measured by response delay to ASH-specific stimuli suggests modulation in the avoidance circuit during sleep behavior.

Many tools exist to probe and study neural circuits in *C. elegans*. Use of known transcriptional control regions of characterized genes drive expression of genetically encoded calcium sensors and allow measurement of activity in single or multiple cells. Cameleon is a dual channel ratiometric reporter that corrects for changes in reporter expression over states, stages and time. However, Cameleon has slower dynamics because it involves energy exchange between two fluorphores, and GCaMP, a single channel reporter, has better dynamic range. Therefore, we used both reporters: cameleon controlled for state-related changes in reporter expression and GCaMP allowed measurement of smaller calcium events. Activity patterns observed can be mimicked using light-driven cation channels such as channelrhodopsin (ChR2). Similar to the calcium sensors, ChR2 is genetically encoded and expression in select cells using specific promoters as well as combinatorial approaches that allow expression only where two promoters overlap[15] or that allow expression only in cells that are unique to one promoter in the pair[16]. These strategies allow

measurement and activation of specific components of pertinent circuits. Here we use genetically encoded calcium sensors and light-driven channels to investigate the ASH circuit during sleep and waking. We find that multiple steps in the circuit are dampened during sleep.

4.3 ASH SENSORY NEURON EXHIBITS DECREASED SENSORY RESPONSE

The amphid sensory neuron, ASH, senses multiple aversive stimuli, including mechanical stimulation at the tip of the head, and noxious chemical cues, such as copper, 1-octanol, or high osmolarity.[13] We used a chemical stimulus to characterize ASH activity during a "sleep cycle" because it is more consistent and controllable than mechanical stimuli. We fabricated a modified version of a microfluidic olfactory chip[17] to accommodate and immobilize fourth larval stage (L4), lethargus, and young adult animals (Figure 1B). These devices permitted temporally controlled delivery of chemical stimuli and simultaneous fluorescence imaging from the ASH. Individual animals were assayed for a six-hour period, during which they were subjected to a brief stimulus every 30 minutes. ASH response was measured during these intervals using the calcium indicator GCaMP3.[18] Each animal was imaged before, during and after lethargus. Chemosensory neurons use ligand-binding receptors to open ion channels[11], and ASH responds to the addition of 1mM Cu^{2+} with a robust influx of calcium in the L4 stage. During lethargus, the magnitude of ASH response decreases significantly to copper or glycerol, but full responsivity is recovered upon exit from lethargus (Figure 1C, S1A).

To confirm this result and control for confounding factors such as changes in GCaMP expression and baseline calcium levels, short assays were performed using ratiometric imaging with the calcium sensor Cameleon.[18] In these assays, animals of each stage were loaded into the device, imaged within an hour of loading for both baseline and stimulus measurements, and discarded. These short assays were consistent with the longitudinal analysis: there was significant decrease in calcium influx as measured by the peak fluorescence change in response to the chemical copper and glycerol (Figure 1D-E, S1A). Moreover, the availability of fluorescent indicator and baseline calcium concentration did not change sufficiently among behavioral states to account for the decrease in influx (Figure S1B-C). Therefore, the calcium imaging data indicate that there are fewer calcium channels open, and thus the same stimulus is less able to excite the ASH sensory neuron during lethargus. This result is consistent with decreased calcium dynamics observed in the mechanosensory neuron, ALM, during lethargus.[19] Furthermore, stimulus-evoked calcium transients were not ligand specific as evidenced by decreased response to both copper and

glycerol and suggest that modulation in lethargus may affect general excitability or synaptic activity of the ASH neuron.

4.4 BASAL ACTIVITY OF AVA IS SUPPRESSED IN LETHARGUS

GCaMP3 findings were confirmed with Cameleon measurements at the interneuron level. Calcium levels in AVA oscillate and increasing levels correspond with reversals.[20] We observed that oscillation of AVA activity is not regular and disappears in lethargus, but reappears in the young adult animals (Figure S3A-B). Therefore, the basal activity and the context in which AVA receives input from ASH changes during lethargus.

4.5 SYNCHRONY BETWEEN AVD AND AVA IS LOST IN LETHARGUS

To assess activity across the top two layers of the circuit, we used animals expressing GCaMP3 in ASH, AVD, and AVA. To examine stimulus-driven interneuron activity, we measured responses to ASH-specific cue (1M glycerol)[12], which has less variable dynamics (Figure S1A). Each 60-second trial consisted of a 10-second pulse of control buffer or glycerol. Trials with discernable ASH responses were chosen, and instantaneous slopes were calculated over the imaging interval to represent the magnitude of the calcium influxes. In young adult animals without stimulation, the timing of the calcium influxes showed no association with the pulse of the control buffer and did not occur during the stimulus interval (Figure S2B). However, after a glycerol stimulus, the influx of calcium in the AVD and AVA was associated with the stimulation interval (Figure 2B-C). AVD and AVA showed both decreased responsivity during lethargus as well as a loss of coordinated calcium activity. Animals in lethargus exhibited very little activity in the AVD and AVA, and fluctuating calcium levels during the glycerol stimulus were not significantly different from the period preceding the stimulus (Figure 2B). Because the magnitude of activity is considerably smaller in lethargus compared to the young adult, we had to normalize these measurements as binary values. Positive changes in fluorescence were thus counted as individual influx events. There were significantly more influxes during the glycerol stimulus in the young adult, but often little activity was seen in AVA during lethargus even when AVD activity was noted (Figure 2C-E).

To test for association of ASH-interneuron and AVD-AVA activity, we compared the timing of initiation and duration of the calcium transients between neuron pairs by performing cross

correlation analysis between each of the neurons imaged. Comparing data for timing between of calcium events between pairs allows us to assess functional correlation between neurons. This cross correlation analysis indicates that when compared to the input ASH neuron, AVD transients exhibit a lag during lethargus, whereas AVA transients do not (Figure 2D-E). This observation suggests that when awake AVD and AVA receive a signal from the ASH and exhibit simultaneous calcium transients, whereas AVD in a lethargus animal is desynchronized with AVA. We further analyzed AVD and AVA for instances of repetitive calcium transients. There was a significant decrease of coupled trains during lethargus; a majority of the traces did not show activity in both neurons, and uncoupled activity was present during lethargus (Figure 2F-G). Moreover, the number of AVA only traces did not differ significantly from the number of AVD only traces (Figure 2G), suggesting that alterations of synaptic transmission are not specific to a particular synapse.

These results indicate that coupled activity in the command interneurons occur in the awake state and may not only explain processing delay during lethargus but also suggests that synaptic transmission downstream of ASH becomes variable and differs across independent synapses. The altered efficacy of ASH-AVD and ASH-AVA synapses could be due to decreases in either presynaptic transmission or postsynaptic excitability. However, while it is possible that the ASH compartment presynaptic to AVD may vary independently from that which is presynaptic to AVA, the calcium measurements in the ASH show no indications that this is the case. In addition, synapse specific changes or lags in activity usually show a general preference for a specific synapse rather than random or equal distribution as indicated by our AVD and AVA measurements (Figure 2G). The simplest interpretation of the result is that there are postsynaptic changes in the downstream neurons.

4.6 LOSS OF SYNCHRONY IS REVERSIBLE

Delayed response could also be a way for the circuit to function in all states (both awake and lethargus) when calcium activity in the ASH in decreased. In fact, decreased avoidance response to ASH-specific stimuli has been attributed to decreasing calcium activity in dopamine-treated animals.[15] Although neuromodulator-mediated sensory dampening is seen as decreases in behavioral response in the presence of serotonin or dopamine[15], reversibility in behavior is more specific to the sleep state. Furthermore, the source of reversibility is unknown, and it is important

to identify whether reversibility is reflects as reversibility in magnitude of calcium influx or in the response of downstream components.

Therefore, we measured lethargus animals expressing GCaMP in ASH, AVD, and AVA in the microfluidic chip with and without a mechanical stimuli preceding glycerol stimulation. Individual animals showed the same trend: calcium influx in the ASH did not differ significantly between unperturbed and perturbed trials (Figure 3A-B), but the activity in the downstream interneurons was both coordinated and immediate in trials following mechanical stimulation (Figure 3A). This result indicates that although varying presynaptic input from the ASH may change circuit function and behavior, there are other postsynaptic factors that modulate transmission at these synapses. Furthermore, these changes are dependent upon previous stimulation at the level of the interneurons.

4.7 MODULATION ALSO LIES DOWNSTREAM OF ASH DEPOLARIZATION

We validated imaging data and assessed the contribution of downstream neurons to lethargus behavior using the genetically encoded light-activated cation channel Channelrhodopsin2 (ChR2). In the presence of its cofactor, all-trans retinal (ATR), ChR2 allows control of both the extent and timing of cellular depolarization through optical stimulation.[21,22,23] Changes in the sensory response to ASH-mediated noxious stimuli (such as copper or glycerol) is modulated by the presence food or exogenous application of dopamine, but these responses are modality-specific and are absent in response to mechanical stimuli.[15] Optogenetic activation of ASH was shown to be unaffected by changing conditions.[15] Direct control of ion channels and normalization of the initial current injection allows for better assessment of the downstream components of the avoidance circuit by bypassing ligand-binding and associated membrane depolarization in ASH.

Neurons classes in each level of the avoidance circuit were individually depolarized. We used animals in which ChR2 is expressed exclusively in the ASH by the use of FLP recombinase[15], and found that light-driven activation induced robust and active reversal behavior in both the L4 and young adult animals (Figure 4B). However, behavioral response delay persists upon ChR2-mediated ASH depolarization during lethargus: the average time to respond to the ChR2 stimulation increased from 2 seconds to 18 seconds in lethargus (Figure 4B). Therefore, behavioral delay is not likely due to the decrease in receptor associated depolarization at the ASH, is different from dopamine-mediated modulation, and suggests the existence of additional modulation in downstream components of the circuit.

4.8 ACTIVATION OF MULTIPLE COMMAND INTERNEURONS PROMOTES AWAKE-LIKE BEHAVIOR

We next activated the downstream command interneurons using the *nmr-1* transcriptional control region to drive ChR2 in AVA, AVD, and AVE (Figure S7A). AVA activity promotes avoidance behavior[24], and upon direct depolarization of the reverse command interneurons, animals both in and out of lethargus responded immediately (Figure 4C). We further tested animals expressing ChR2 in AVA and RIM. RIM is an interneuron associated with reversal: osmotic shock using glycerol induces a calcium influx into RIM[25] and direct depolarization of RIM with ChR2 results in robust reversal as well as calcium influx into the AVA command interneuron[14]. However, although depolarization of AVA and RIM in an awake animal induces robust reversal[26], it fails to do so consistently or quickly in lethargus (Figure 4D-E). Moreover, lethargus animals either responded similarly to awake animals or did not respond at all (Figure 4E). Thus, if and when interneurons are activated together, a rapid behavioral response followed. The lack of a rapid response upon direct ASH depolarization indicates that transmission of excitatory information from the sensory to the interneurons is decreased or delayed in lethargus.

We cannot exclude the possibility that the inability of AVA and RIM activation to generate immediate reversal during lethargus may also indicate an altered function of RIM in lethargus. RIM has been shown to exhibit either calcium influx or efflux during initiation of reverse locomotion[25], and can elicit reversals upon activation by ChR2[14] as well as inhibition by halorhodopsin[25]. It has been surmised that activity in RIM can both inhibit locomotion directly by inhibiting the muscle while activating locomotion by activating AVA. If AVA is less responsive to RIM activation during lethargus or RIM has enhanced inhibitory or decreased excitatory function, then we would expect ChR2 to be less efficient in generating reversals. Furthermore, decreasing ChR2 expression in AVA or increasing ChR2 expression in functionally modified RIM would further suppress avoidance behavior. Therefore, we measured the expression level of ChR2 in the AVA and RIM neurons and found that it did not change significantly in and out of lethargus (Figure S7B-C). Lack of altered expression suggests that either RIM function is modified or that AVA alone is not sufficient to trigger robust reversals. In either case, multiple interneuron input is a crucial component for consistent generation of immediate reversals.

Direct activation of the cholinergic motor neurons including the VA reverse motor neurons showed no significant response latency between the response of L4, lethargus, and young

adulthood (Figure 4F), suggesting that the modulation of signaling during lethargus did not occur downstream of the AVA interneuron and remained consistent with previous data indicating that the amount of contraction did not change in lethargus.[27,28] The importance of synchronous activity in multiple neurons is a reflection of the signal amplification that occurs in the circuit to promote reversal. Amplification can occur through two not necessarily distinct mechanisms: excitation of multiple interneurons by ASH and further amplification of that signal by multiple feed forward loops in the circuit (Figure 4A).

4.9 INCREASING THE EXTENT OF ASH DEPOLARIZATION CAN ELICIT IMMEDIATE RESPONSE

Previously published work describes the avoidance circuit as a coherent type 1 feed forward loop, the dynamics of which have been modeled (Figure S4A).[29] This circuit motif creates delay by preventing processing in the AVA until both ASH and AVD are active.[29] Our simulation of dynamics using the published model shows that adjusting the threshold or ratio of transfer at the ASH to interneuron synapses (even in the absence of input change) can cause a delay, which can be shortened by increasing activity at the ASH (Figures S4B; S5B). We therefore stimulated ChR2-expressing ASH neurons in a graded manner. To more strongly activate ASH, we used a 100-fold higher concentration of ATR and a 2.5-fold stronger light intensity. When subjected to this stronger stimulus, animals in lethargus displayed a reduced behavioral delay not significantly different from that of young adults (Figures 5C; S6A-B). These results are consistent with the model and suggest either the threshold or transfer at the synapse changes during lethargus, requiring significantly more presynaptic depolarization to elicit an immediate response. Increasing ASH input should increase activity at the AVD as well as the AVA, implying that the increased input to motor neurons may no longer be linear and possibly exponential. Increased current injection at RIM and AVA does not suppress response delay (Figure 5E), raising the possibility that a single command interneuron may not be able to output sufficient signal to the motor neurons to generate immediate response and that synchronous activation of the command interneurons is likely necessary for rapid reversal.

4.10 RESPONSE DELAY TO ASH DEPOLARIZATION IS REVERSIBLE

To further understand the decreased transmission between sensory and inter-neurons during lethargus, we stimulated interneurons through an ASH-independent circuit (Figure 5A). Mechanosensation at the body wall promotes reversal by activating the AVA and AVD command interneurons independent of ASH.[30,31] FLP, a mechanosensory neuron, responds to harsh touch to the anterior body.[30,32] When animals are woken by a harsh touch, they reverse immediately. After a full reversal, animals are more responsive to ChR2 stimulation, and the average response delay to ChR2-induced ASH depolarization is 2s, suppressing the response delay normally seen in lethargus (Figure 5B). Surprisingly, promoting forward locomotion using PLM/PVM-mediated tail touch, which functionally should inhibit AVD and AVA, also suppressed response delay (Figure 5B). It is thus not important whether the animals are moving forward or backward, but that they are active. Waking the animal during lethargus does not suppress response delay to AVA depolarization (Figure 5D), indicating that input into multiple command neurons is required. These results suggest that there is an awake state that determines the interneuron resistance to signal processing, and waking primes the interneurons to respond to sensory stimuli.

4.11 CIRCUIT MODULATIONS IN LETHARGUS ARE GENERAL AND DEPENDENT ON AROUSAL STATE

Behavioral quiescence can be observed outside of lethargus and in the adult stage during satiety behavior[5] and during EGF-overexpression[3]. Satiety can be induced with high nutrient food and is enhanced when a long period of starvation is followed with food[5]. We examined the behavior of animals three hours after refeeding and observed quiescence, as measured by pharyngeal pumping and locomotion (Figure 6A-B). Animals that were fasted and refed showed delayed response to ASH activation with ChR2 when compared with starved or continuously fed animals (Figure 6C). Moreover, this delay was reversible and was suppressed by previous mechanical stimulation (Figure 6D). These results show that sleep-like behavior is not restricted to lethargus and suggest the changes in the avoidance circuit is not tied to the developmental stage but physiological state of the animal.

EGF overexpression in young adult animals showed similar results. EGF signalling was implicated as a quiescence promoting component during lethargus, and anachronistic expression strongly induces quiescence.[3] Therefore, we induced quiescence by overexpressing EGF in young

adult animals (Figure 6E) and found that animals had a delayed response to ASH activation comparable to lethargus (Figure 6F). Also, delayed response due to EGF could also be reversed upon previous stimulation by a tail tap (Figure 6G). These results show that sleep-like behavior induced outside of lethargus by satiety and one component of lethargus signalling is effective in replicating the behavioral dynamics. These observations eliminate the possibility of developmental factors being the primary driver of neuronal modulation.

We also tested whether the observed sensory neuron modulation is restricted to ASH or to circuits mediating avoidance. We chose to study the gustastatory neuron, ASE, during and out of lethargus. Unlike ASH, ASE senses attractive stimuli, like preferred salt concentration, has no mechanical component, and promotes forward locomotion. We found that ASE also shows decreased calcium transients during lethargus, implying that circuit modulations are likely general across the sensory layer and similar modulation likely exist in the forward command interneurons.

4.12 DISCUSSION

We examined the well-characterized ASH avoidance circuit during sleep and waking and found that decreased arousal during sleep stems from not only the worm's inability to robustly sense the incoming stimulus but also a failure to activate components of the circuit that promote avoidance in the awake state. We found significant modulation in both sensory and command interneuron activity during lethargus and confirmed the function of observed activity patterns using channelrhopdopsin to optically control activity in single or select groups of neurons. We also find that dampened sensory activity in the ASH neuron is likely correlated with the presence of sleep drivers, is constant during lethargus, and is minimally altered in response to chemical stimuli after mechanical stimulation preceeding the measured trial duration. This neuronal activity is in stark constrast to the dynamics of behavior: sensory response to ASH activation by chemical stimuli is immediate upon previous waking with mechanical stimuli. However, changes in dynamics are well correlated with coordinated activity of the command interneurons AVD and AVA. Moreover, this coordinated activity that is lost during quiescence is recovered upon waking.

How might activation of interneurons by one sensory neuron sensitize them to other sensory neurons? One mechanism of decreasing thee threshold is to make the interneuron more receptive to presynaptic input by activity-driven changes in receptor localization.[33,34] In addition, activity-driven changes in synaptic tranmission and transfer can be readily explained by disinhibition[35] or neurotransmitter availability. The fast dynamics of the reversibility during sleep make it unlikely

that neuromodulators are directly responsible for the reversibility because unbinding from targets and breakdown or sequestration of peptides would not be possible in the time scale of behavioral dynamics (milliseconds to seconds). In addition, the mediation of reversible transmission by a secondary intermediate outside of the sensory motor circuit is precluded by the lack of candidates with the proper connectivity to all sensory neurons or even the sensory neurons published or tested in this work.[10]

Both decreased sensory transduction and reversible resistance in transmission of excitatory information downstream occurs, and the presence of these modulations pose interesting questions about the utility of having multiple levels of modulation. Decreasing the magnitude of ASH response results in decreased signal processed downstream, and theoretically, in a smaller probability of reaching threshold to relay information to downstream components of the circuit (Figures S4B, S5A). This type of sensory gating would help prolong inactivity in the sensory motor circuit and promote behavioral quiescence. However, direct alteration of the response of individual sensory neurons after waking or even all sensory neurons after waking would perpetuate a long awake state because the nervous system would not be sufficiently gated, and this might continue reponding to basal sensory stimuli as well as mechanical stimuli from locomotion. Thus, if sleep and wakefulness in the worm are regulated only by gating and lack of gating, respectively, at the sensory neuron, then the dynamics in the circuit create a positive feedback loop because activity in the circuit would sensitize the sensory neuron and promote more activity in the circuit, and so on. Furthermore, constant decrease in ASH activity makes the circuit more sensitive to small changes in signal transduction or synaptic tranmission to AVA and AVD, lengthening the range of behavioral delay and decreasing the probability of downstream synchronization (Figure S4A-B). Dynamic sensitization of the command interneurons allows for increased probability of coordinated activity upon previous stimulation, but requires sensory input to multiple command neurons (Figure 7). Therefore, the use of coordinated activity in downstream components offers flexibility and fast behavioral dynamics while simultaneously ensuring that waking leads to sensitivity to all modalities as well as sensory neurons that converge upon the command neurons.

We found that behavioral delay and reversibility is not exclusively limited to lethargus: they occur with EGF expression and with quiescence induced by satiety, indicating that these circuit modulations are due to behavioral state and not developmental stage. Thus, these results are more relevant to understanding behavioral states across species. Also, sensory dampening is likely

general in sensory motor circuits, and sensory neurons associated with food and attractive stimuli are dampened in addition to those for pain and noxious stimuli.

Past circuit understanding of sleep is limited to a general understanding of loss of synchrony between cortex and thalamus due to inability of the thalamus to relay information to the cortex.[36,37] We have demonstrated similar changes of information relay from sensory to command interneurons in a simple circuit of *C. elegans*. Decrease in sensory signaling contributes to, but is not the sole factor of, changing information relay. Excitability of the downstream command interneurons is also likely altered and serves as a point for reversible function of the circuit during the sleep-like state. Thus, small changes in dynamics at multiple levels promote and prolong quiescence while allowing reversibility of behavior upon sufficient stimulus by using components of a circuit that appear redundant, but serve to amplify or suppress input signal. The *C. elegans* nervous system is condensed when compared to a mammalian nervous system. We can then conjecture that a sensory neuron in the worm may serve to perform both sensory functions and the processing that presumably occur downstream in other less compact neural circuits. Regardless, these components of multilevel modulation serve as one strategy for generating the dynamic behaviors seen in sleep.

FIGURES

Figure 1

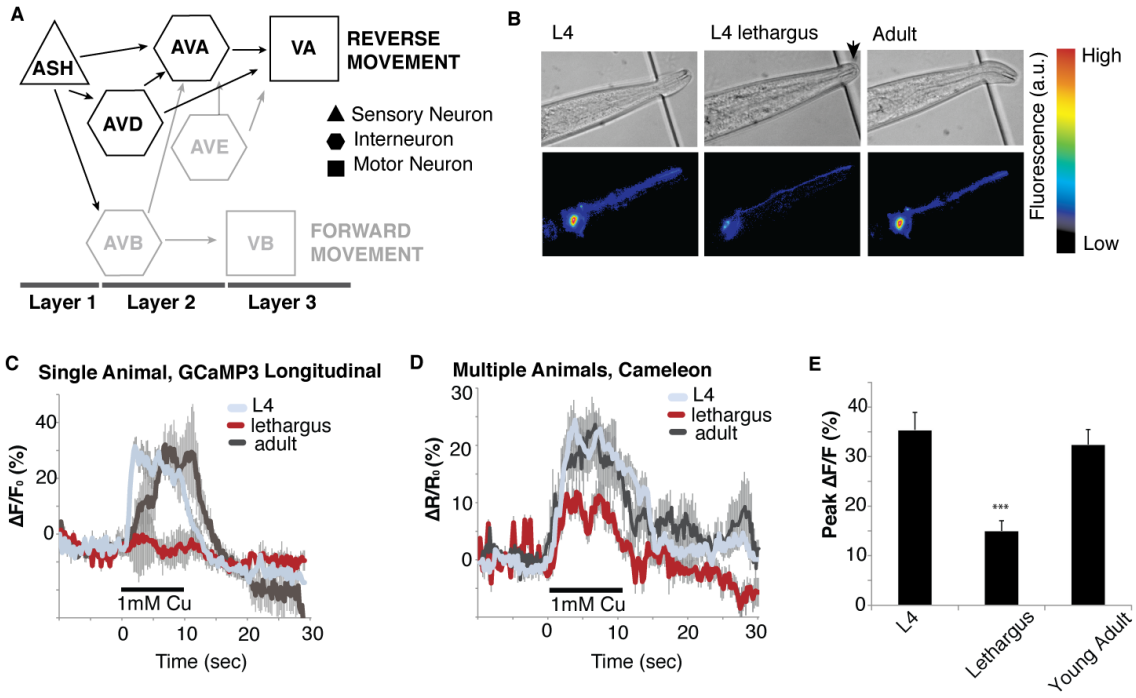


Figure 1. Sensory depression occurs in the ASH polymodal sensory neuron
(A) Functional model of the avoidance circuit. The two polymodal ASH sensory neurons work through the two AVA and two AVD interneurons to promote reversal through the VA motorneurons. Neurons tested in the study are darkened. (B) Single frames of GCaMP3 DIC and fluorescence recording in ASH over the L4, lethargus, and young adult stages. Arrow denotes the cuticle cap that marks lethargus. (C) Representative GCaMP3 traces of a single worm as it transitions from L4 to lethargus to young adult. (D) Average change in cameleon fluorescence in the ASH sensory neuron over time in a short calcium imaging assay. L4 and lethargus response to 1mM Cu^{2+} (L4 n=10; L4 lethargus n=11). (E) The calcium influx in response to 1mM Cu2+ is significantly decreased in lethargus (n=11) as compared to L4 (n=10) and young adult (n=5; ***p<0.001, Student's t-test equal variance).

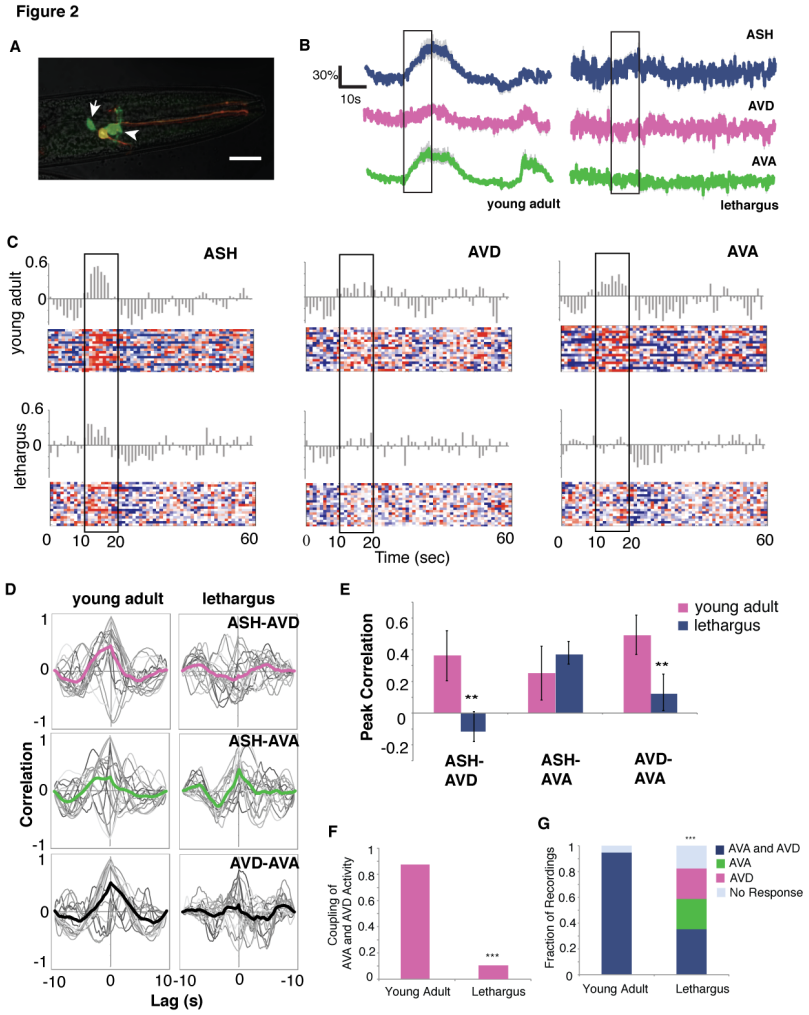


Figure 2. AVD loses synchrony with AVA in lethargus

(A) Image of the neurons in which GCaMP3 was measured. ASH is marked by the presence of both GCaMP3 and mCherry, AVD is posterior (white arrow), and AVA is anterior (white arrowhead). (B) Representative traces of a glycerol trial in a single animal in both lethargus and young adult. (C) Heat map of 60s trials denoting influx (red) and efflux (blue) in young adult (n=18) and lethargus (n=18). The proportion of influx or efflux were averaged across trials in 1s bins and are denoted by the gray bars above the heat maps. (D) Cross correlation of the stimulus interval between ASH-AVD, ASH-AVA, and AVA-AVD. Individual cross correlations are shown as gray lines, the average by the colored line. Correlation of 1 is a perfect match, and correlation of -1 denotes an inverse relationship. AVD exhibits an average lag in response and shows a decreasing correlation with AVA during lethargus. (E) Peak correlation values extracted from the cross correlation analysis. AVD loses its synchronicity with ASH and AVD only in lethargus ($p<0.01$, n=18, students t-test of unequal variance). AVA shows no decrease in correlation with ASH. (F) Quantification of trials in which GCaMP3 measurements of AVA and AVD showed corresponding trains of calcium influx during the course of the trial. The majority of trials in the young adult (0.86, n=23) showed coupled activity, but this coupling decreased significantly in lethargus ($p<0.000001$, 0.12, n=16). Many of the trials in lethargus showed activity in either AVA or AVD, while young adult animals did not show any instances of this pattern of activity ($p<0.0001$).

Figure 3

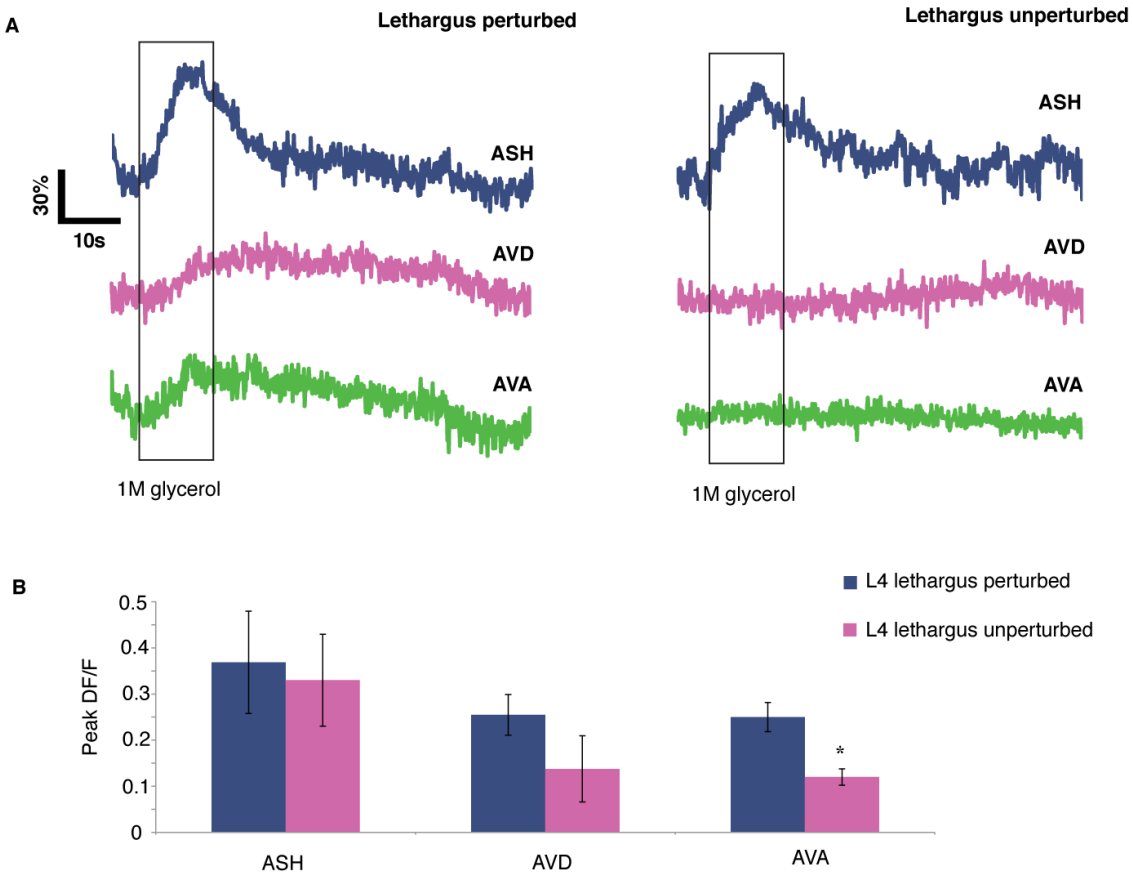


Figure 3. Loss of synchrony is reversible and can be separated from magnitude of calcium influx in the ASH

Representative GCaMP3 measurements of ASH, AVD, and AVA of a single worm in response to 1M glycerol with and without previous mechanical stimulation during lethargus. (B) Perturbation does not significantly affect peak increase in ASH as measured by GCaMP3, but it does cause significant increase in responsivity at the AVA (*$p<0.01$, $n=4$, students t-test of unequal variance).

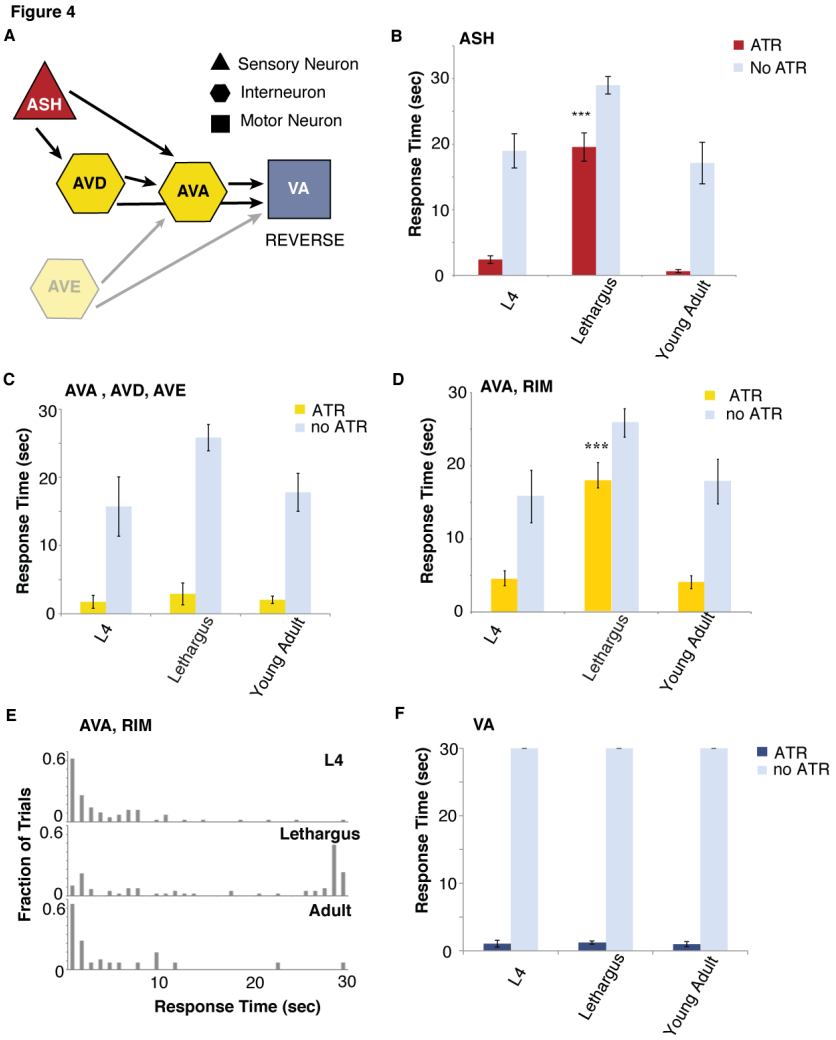


Figure 4. **Sensory modulation occurs downstream of ASH depolarization**
(A) Schematic diagram of the neurons manipulated to generate reversal. ASH promotes activity at the AVD and AVA interneurons. (B) Behavioral response times to ASH depolarization using channelrhodopsin. The mean ± s.e.m. values are shown: L4 (n=13), lethargus (n=9), adult (n=7); ***p=0.0001, ANOVA. All ATR treated animals were compared to their paired non-ATR treated controls L4 (n=13), lethargus (n=9), adult (n=7); p<0.0001, Student's t-test of unequal variance. (C) AVA, AVE, and AVD were depolarized using channelrhodopsin. Avoidance behavior did not differ significantly between worms in and out of lethargus. L4 (n=7), lethargus (n=6), adult (n=6); ANOVA. All ATR treated animals were compared to their paired non-ATR treated controls L4 (n=7), lethargus (n=6), adult (n=6); p=<0.0001, Student's t-test equal variance. (D) Depolarization of AVA without the other command interneurons showed a significant delay in lethargus (n=15) when compared with L4 (n=16) and adult (n=8); ***p<0.0001, ANOVA. All ATR treated animals were compared to their paired non-ATR treated controls L4 (n=15), lethargus (n=16), adult (n=8); p<0.0001, Student's t-test of unequal variance. (E) Distribution of individual trials show two different populations during lethargus, making reversal behavior in lethargus significantly different; ***p<0.0001, Mann-Whitney test. (F) Depolarization of cholinergic motorneurons with channelrhodopsin. L4 (n=5), lethargus (n=5), adult (n=5), ANOVA. All ATR treated animals were compared to their paired non-ATR treated controls L4 (n=13), lethargus (n=9), adult (n=7); p<0.0001, Student's t-test equal variance

Figure 5

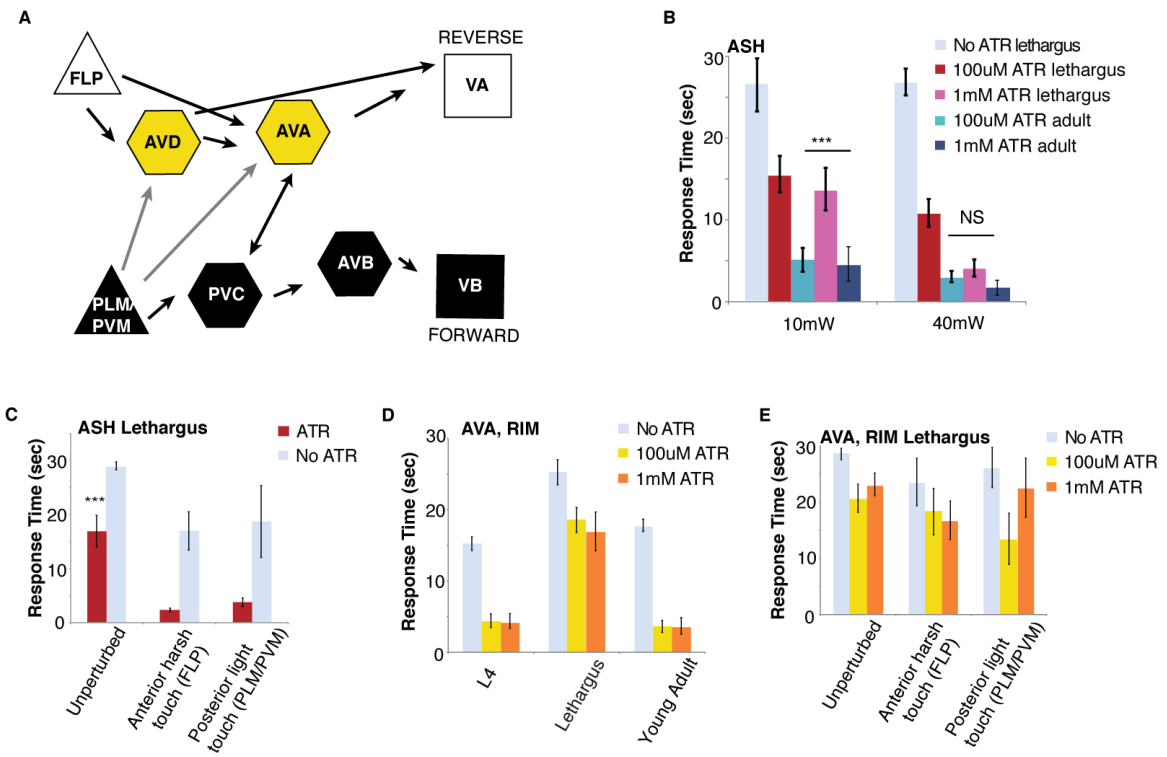


Figure 5. Reversibility arises from interneuron activation but requires simultaneous input to both AVD and AVA

(A) Schematic diagram of the neurons manipulated to generate reverse locomotion. Multiple sensory cues and neurons can induce reversal, including ASH, and FLP.[12,30] FLP neurons are activated in response to harsh mechanical stimulation at the body wall and act independently of ASH to promote activity at the AVD and AVA interneurons. The PLM and PVM mechanosensory neurons work through the PVC interneuron but are also synaptically connected with the AVA and AVD to generate forward locomotion in response to tail touch. (B) ASH of lethargus animals were depolarized with ChR2 unperturbed (n=21), after harsh touch at the body wall (n=9), and after tail touch (n=12). Both perturbations suppressed the delay in reversal; ***p<0.0001, ANOVA. All ATR treated animals were compared to their paired non-ATR treated controls unperturbed (n=21), after harsh touch at the body wall (n=9), and after tail touch (n=12); p=<0.0001, Student's t-test of unequal variance. (C) Behavioral response to 10mW and 40mW of optical stimulation of ChR2 in lethargus and young adult animals at 100µM and 1mM ATR concentrations. (D) AVA of lethargus animals grown in 0, 100µM, or 1mM ATR were depolarized with ChR2 unpertubed (n=23), after harsh touch (n=10), and after tail touch (n=13). No significant difference was seen between animals grown on 100µM and 1mM ATR. All ATR treated animals were compared to their paired non-ATR treated controls unperturbed (n=23 after harsh touch at the body wall (n=10), and after tail touch (n=13)); p=<0.0001, Student's t-test of unequal variance. (E) Even with increased ATR concentraion at 1mM, depolarization of AVA in animals continued to show a significant delay in lethargus (n=11) when compared with L4 (n=13) and adult (n=9); ***p<0.0001, ANOVA. No significant difference was seen between animals grown on 100µM and 1mM ATR.

Figure 6

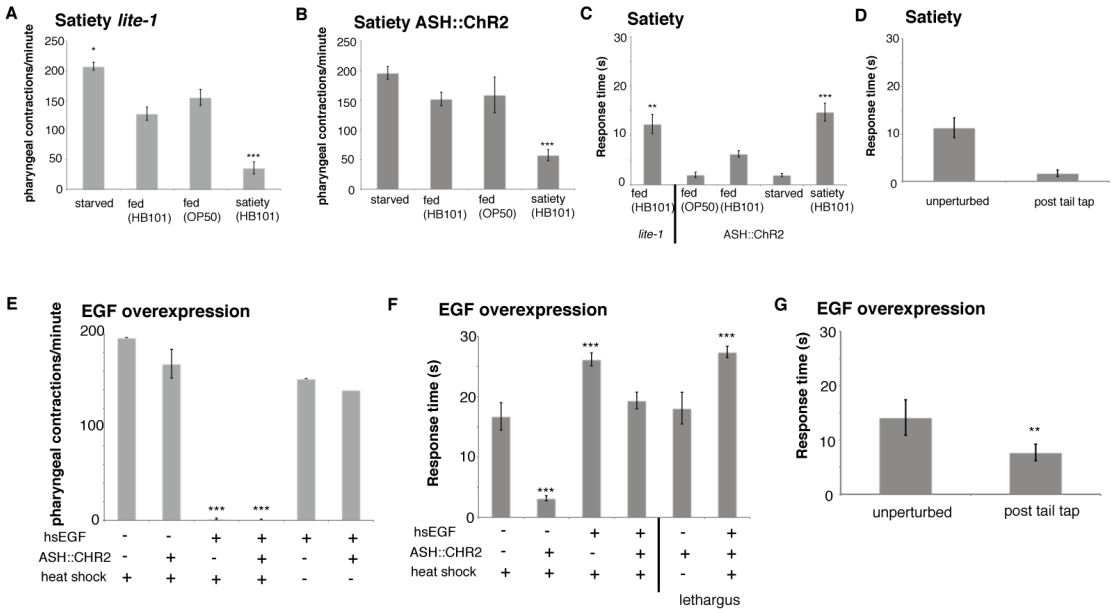


Figure 6. Circuit modulations in lethargus are dependent on arousal state

(A) Feeding activity of *lite-1* animals (which has decreased response to blue light) as indicated by pharyngeal contractions in response to starvation (n=8), feeding on OP-50 (n=9), feeding on HB101 (n=5), and feeding after 12 hours of starvation, i.e. satiety assay (n=14). The mean ± s.e.m. values are shown, *p=0.01, ***p=0.0001, ANOVA. (B) Feeding activity of ASH::ChR2 animals as indicated by pharyngeal contractions in response to starvation (n=8), feeding on OP-50 (n=5), feeding on HB101 (n=13), and feeding after 12 hours of starvation, i.e. satiety assay (n=17). The mean ± s.e.m. values are shown, ***p=0.0001, ANOVA. (C) Reversal following light activation of *lite-1* (n=8), ASH::ChR2 animals that were subjected to starvation (n=20), feeding on OP-50 (n=13), feeding on HB101 (n=8), and feeding after 12 hours of starvation, i.e. satiety assay (n=17). The mean ± s.e.m. values are shown, **p=0.001, ***p=0.0001, ANOVA. (D) Reversal following light activation of ASH::ChR2 animals following the satiety assay before perturbation (n=11) and after tail tap (n=11), ***p=0.0001, Student's t-test of unequal variance. (E) Feeding activity as indicated by pharyngeal contractions were measured to assess activity levels of the animals following EGF overexpression by heat shock. Only animals with the heat shock EGF transgene (hsEGF) that were treated with heat shock showed decrease in activity. The mean ± s.e.m. values are shown: heat shock *lite-1* (n=5), heat shock ASH::ChR2 (n=5), heat shock hsEGF (n=11), heat shock hsEGF ASH::ChR2 (n=7), hsEGF (n=5), ASH::ChR2 (n=5), ***p=0.0001, ANOVA. (F) Reversal following light activation of heat shocked animals. The mean ± s.e.m. values are shown: heat shock *lite-1* (n=11), heat shock ASH::ChR2 (n=17), heat shock hsEGF (n=18), heat shock hsEGF ASH::ChR2 (n=16), heat shock ASH::ChR2 in lethargus (n=10), heat shock hsEGF ASH::ChR2 in lethargus (n=9), ***p=0.0001, ANOVA. (G) Reversal following light activation of hsEGF ASH::ChR2 animals following before perturbation (n=11) and after tail tap (n=11), ***p=0.0001, Student's t-test of unequal variance.

Figure 7

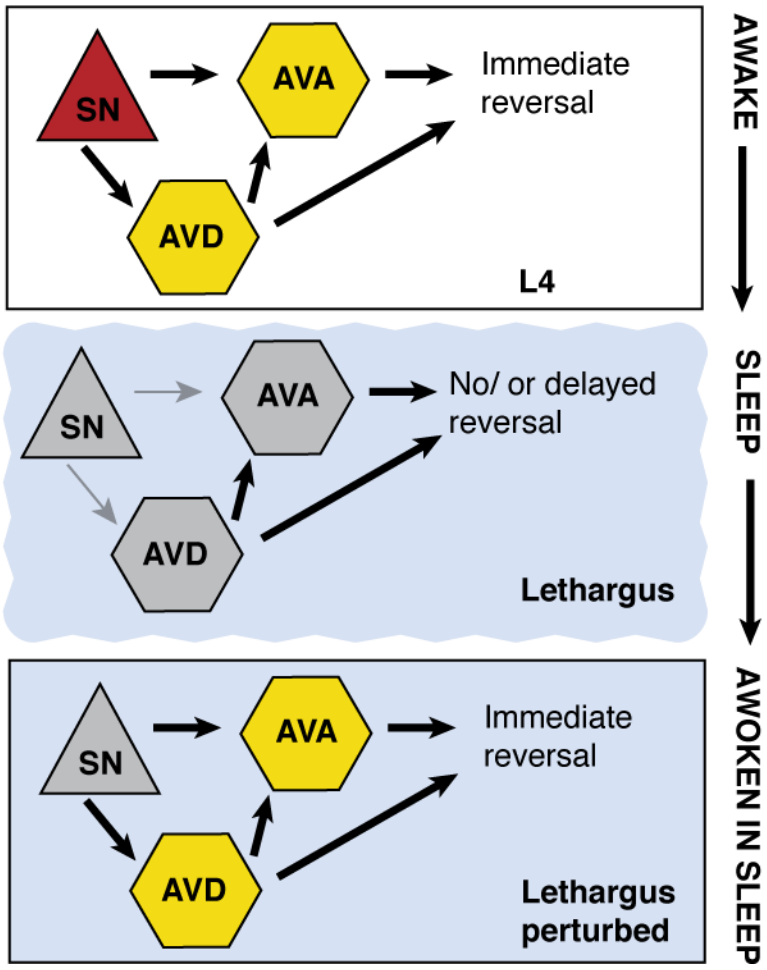
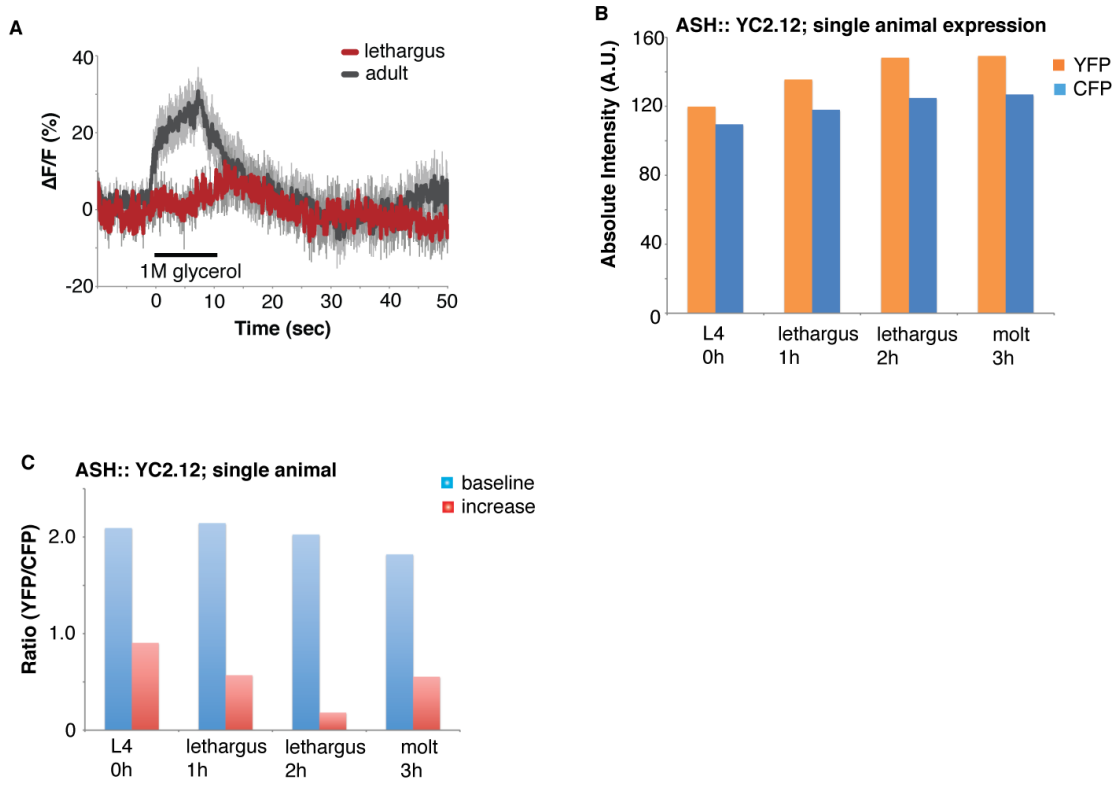


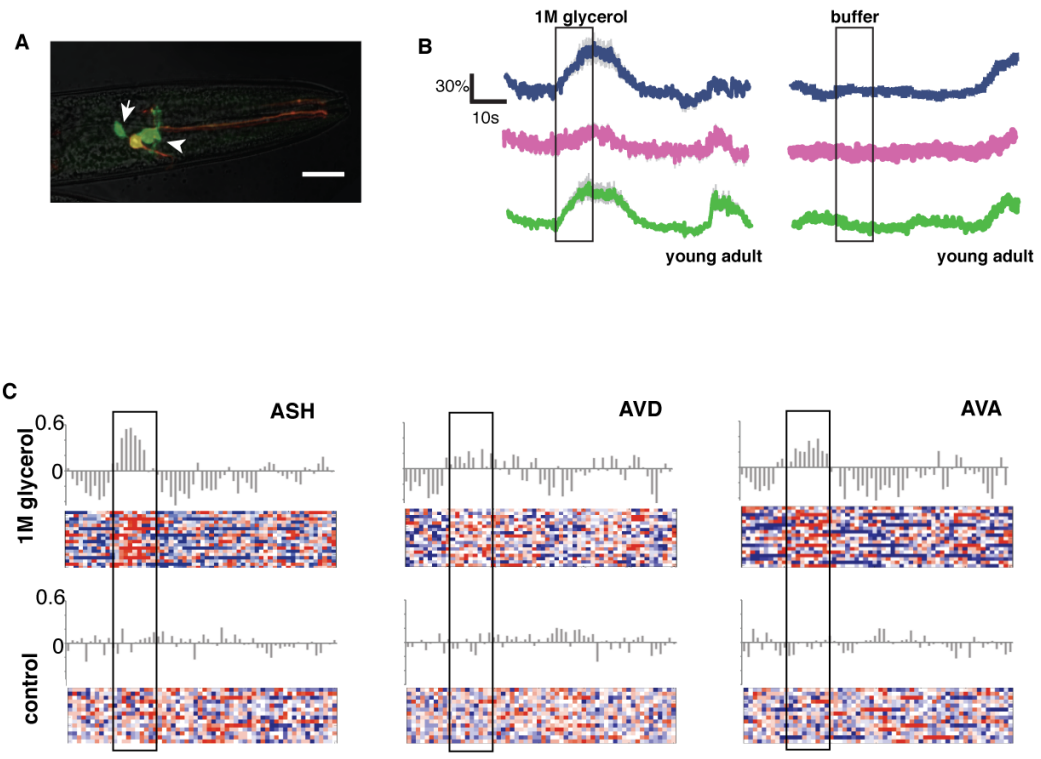
Figure 7. Waking sensitizes the interneurons for coordinated activity
Schematic model of activity during lethargus and waking during lethargus. Greyed neurons indicate decreased excitability: sensory neuron (SN) and the decreased excitability of or synaptic transmission of interneurons (AVD and AVA). Arrows mark synaptic connections between the neurons and the gray thin lines denote decrease in transmission across the sensory interneuron synapses in lethargus.

Supplementary Figure S1



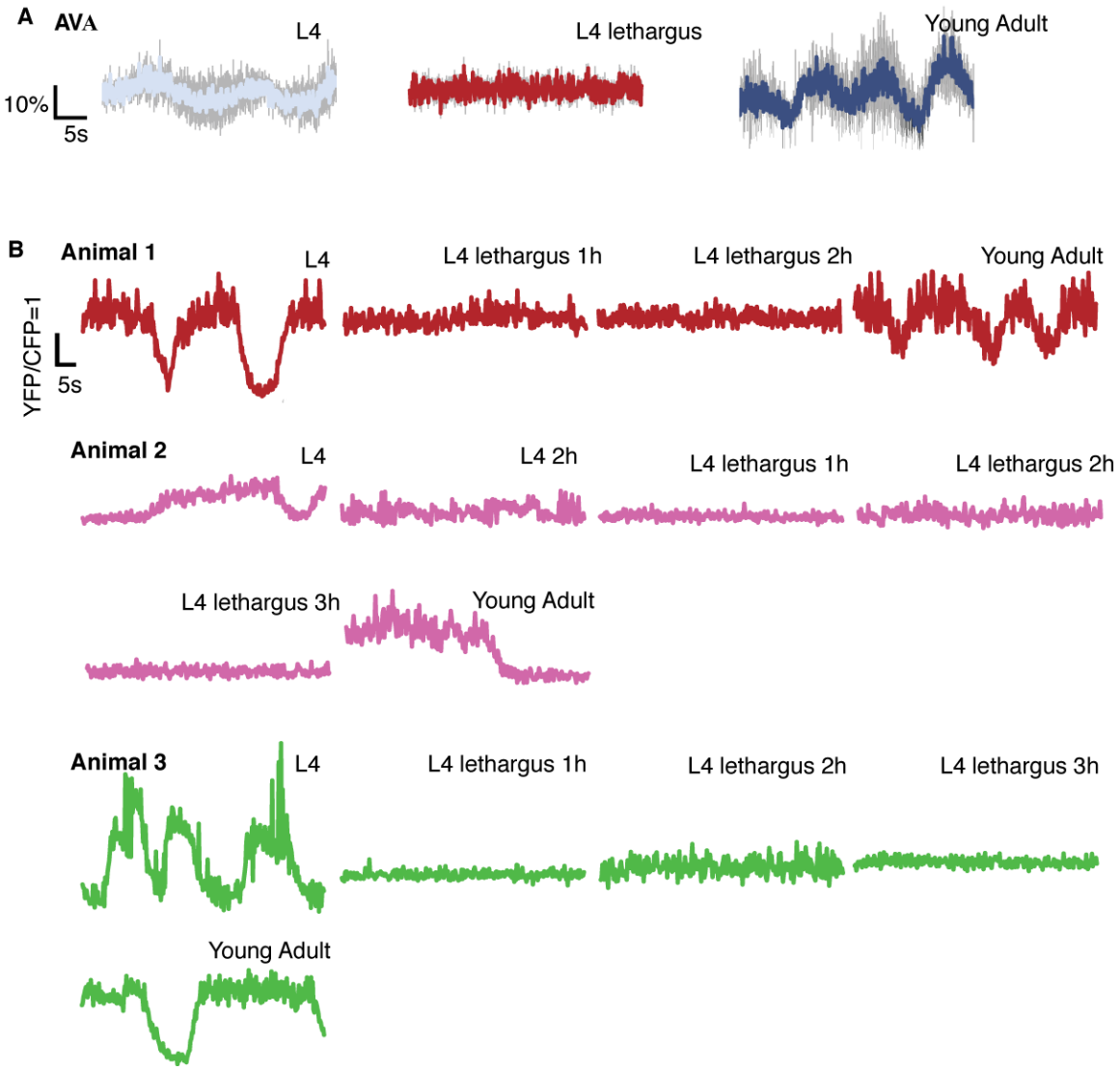
Supplementary Figure S1.
(A) Average Response 1M glycerol as measured by GCaMP3 in the ASH. Lethargus (n=25) and young adult (n=18). (B) Quantification of YFP and CFP expression in the ASH neuron of ASH::YC2.12 of one animal over time. **(C)** Quantification of baseline YFP/CFP ratio and amount of peak increase in ratio in response to 1mM Cu^{2+} in the ASH neuron of the same ASH::YC2.12 animal measure in B.

Supplementary Figure S2



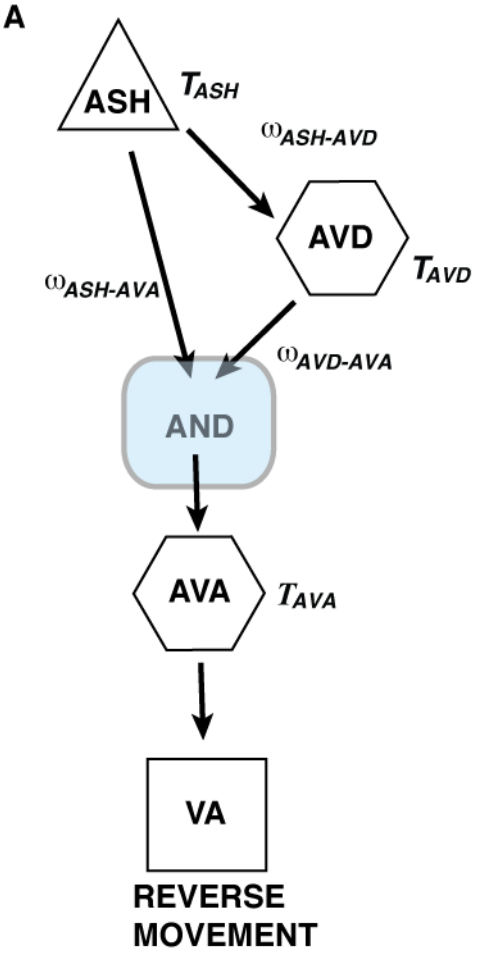
Supplementary Figure S2.
(A) Image of the neurons in which GCaMP3 was measured. ASH is marked by the presence of both GCaMP3 and mCherry, AVD is posterior (white arrow), and AVA is anterior (white arrowhead). (B) Representative traces of a glycerol trial in a single animal and representative traces of the control buffer trial. (C) Heat map of 60s trials denoting influx (red) and efflux (blue) in glycerol (n=18) and buffer (n=14). The proportion of influx or efflux were averaged across trials in 1s bins and are denoted by the gray bars above the heat maps.

Supplementary Figure S3



Supplementary Figure S3.
(A) AVA activity was measured by ratiometric FRET imaging. Representative individual traces are shown. The signal was calculated as the ratio of YFP to CFP emission at each time point. Intrinsic activity was measured during the 30s interval, and there are discernable calcium transients in the L4 worms. (B) AVA activity measured in individual worms over time. Intrinsic activity was measured during the 30s interval longitudinally as the worms went into and came out of lethargus.

Supplementary Figure S4

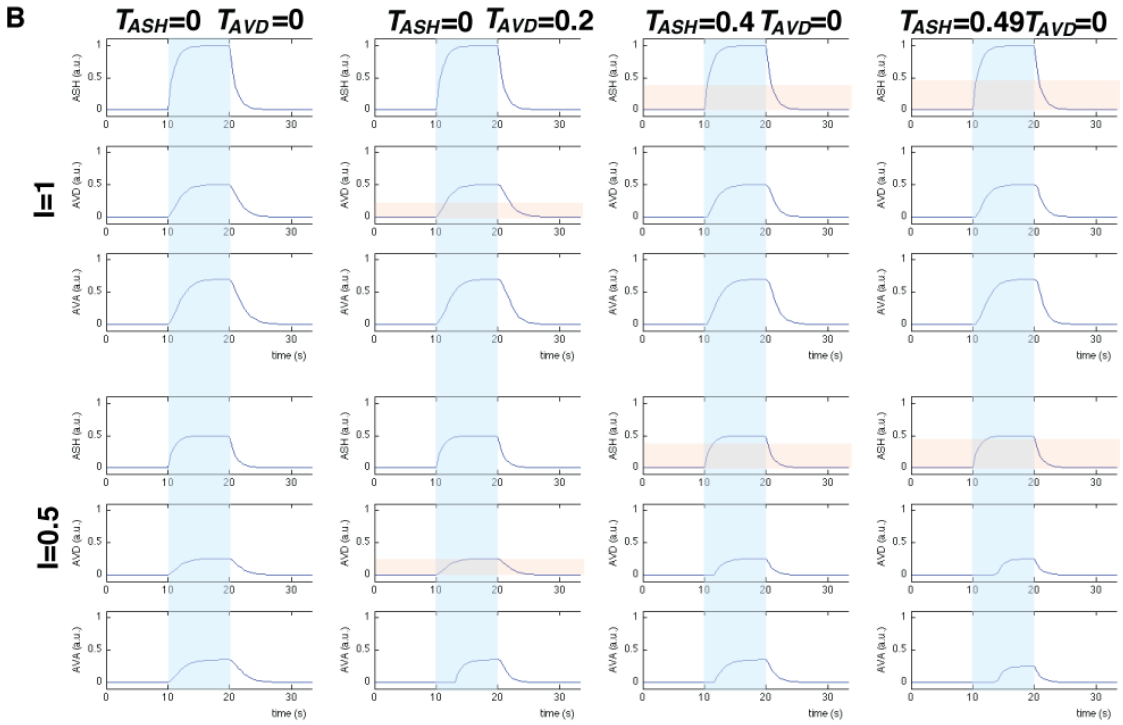


$dASH/dt = \beta(I > T_{ASH}) - \alpha\, ASH$

$dAVD/dt = \beta\,(ASH\,\omega_{ASH\text{-}AVD} > T_{AVD}) - \alpha\, AVD$

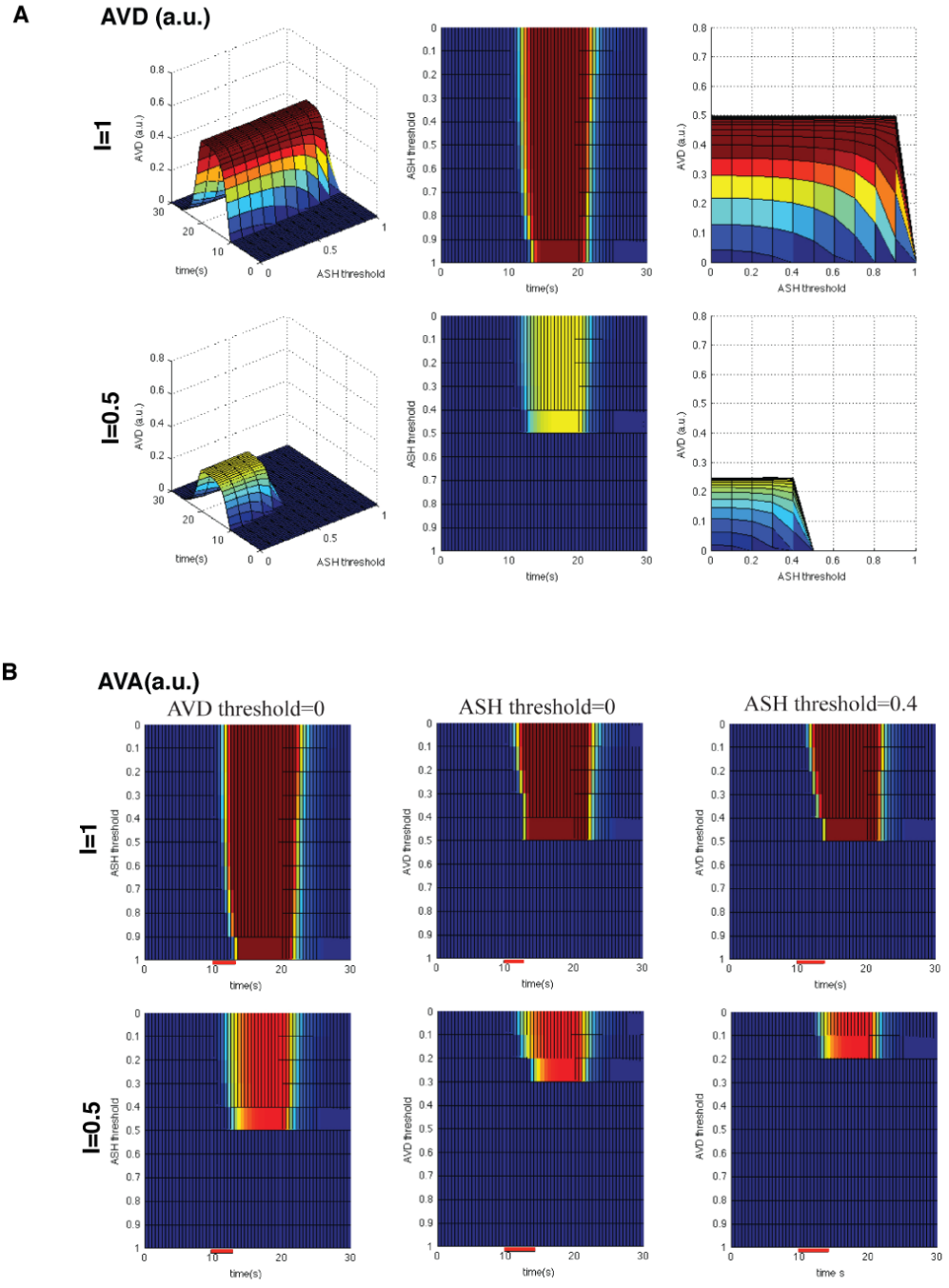
$dAVA/dt = \beta(ASH\;\omega_{ASH\text{-}AVA} + AVD\,\omega_{AVD\text{-}AVA} > T_{AVA}) - \alpha AVA$

$\alpha=1, \beta=1, \omega_i=1$



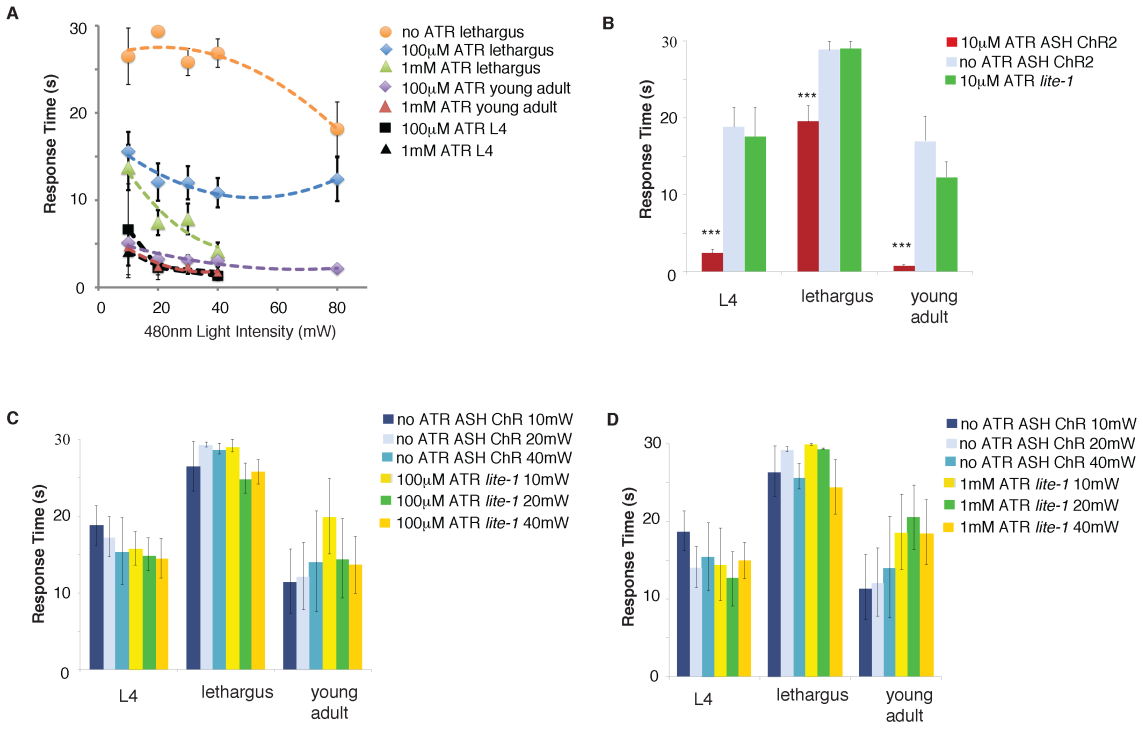
Supplementary Figure S4.
(A) Schematic of the feed forward loop. Equations from the model were adapted from those published.[29] (B) Simulation of dynamics built from the equations shown in (a). Threshold at ASH and AVD are adjusted to illustrated changes in dynamics at the downstream neurons. Input values were entered at I=1 and I=0.5. Previous published values for α=1 and β=1.[29]

Supplementary Figure S5



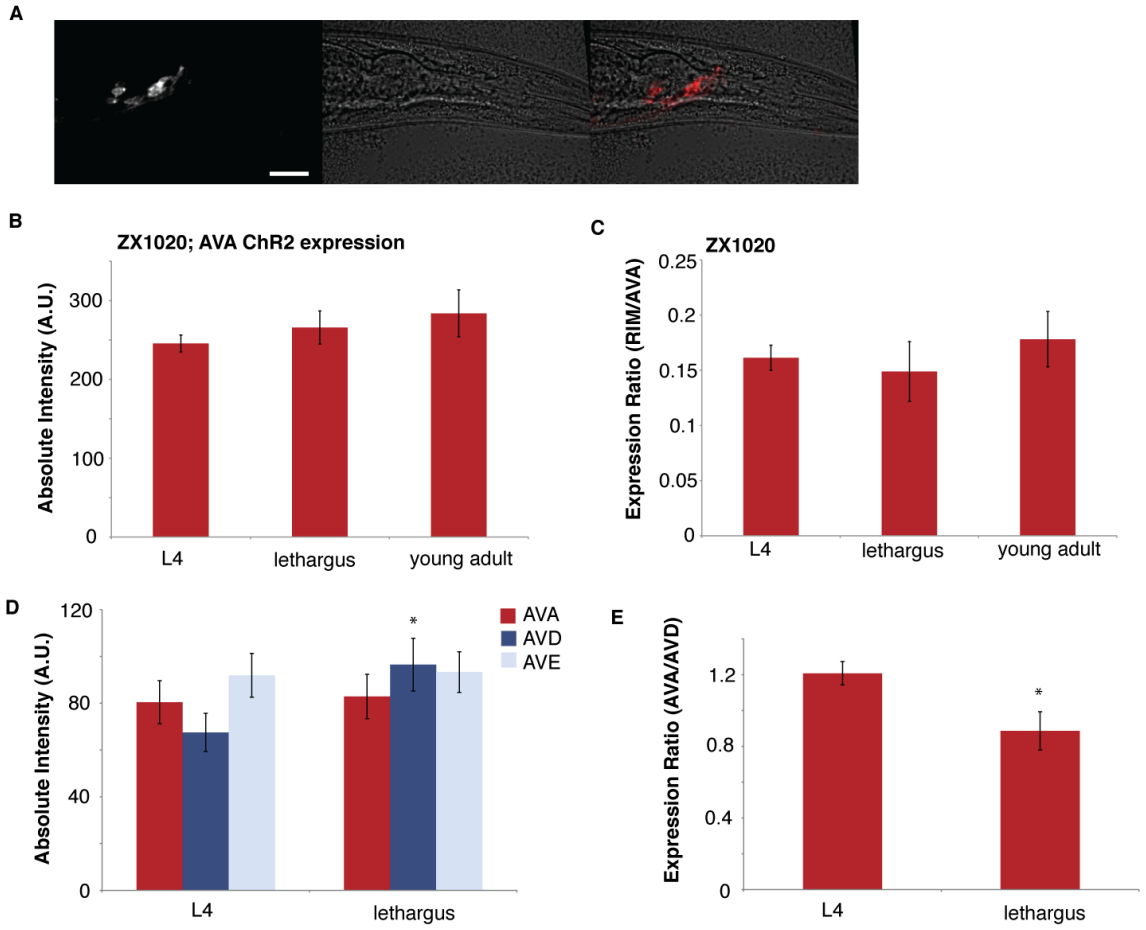
Supplementary Figure S5.
(A) Changes in timing and magnitude of AVD activity in response to input *I*=1 and *I*=0.5 at varying ASH thresholds. (B) Changes in timing and magnitude of AVA activity in response to input *I*=1 and *I*=0.5 at varying AVD and ASH thresholds.

Supplementary Figure S6



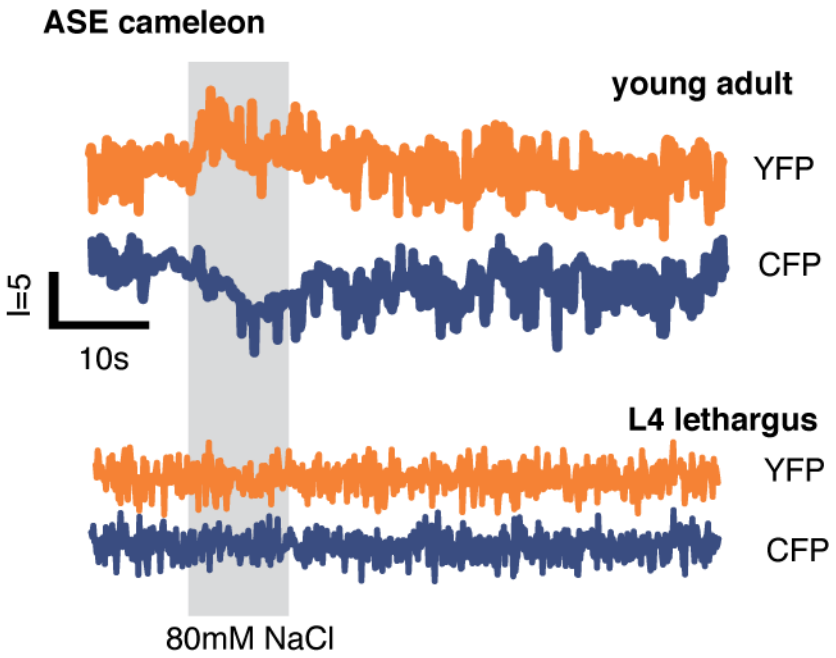
Supplementary Figure S6.
(A) Behavioral response to graded intensity of optical stimulation of ChR2 in L4, lethargus and young adult animals at 100µM and 1mM concentrations. (B) Reversal following light activation of the ASH neuron in animals grown on 10µM ATR as compared to no ATR control and *lite-1* grown on 10µM ATR. The mean ± s.e.m. values are shown: L4 (n=9), lethargus (n=12), adult (n=8), ***p=0.0001, ANOVA. (C) Control behavioral response to graded intensity of optical stimulation of ChR2 in L4, lethargus, and young adult animals at 100µM ATR concentration. The mean ± s.e.m. values are shown: L4 (n=19), lethargus(n=12), adult (n=8), NS, ANOVA. (D) Control behavioral response to graded intensity of optical stimulation of ChR2 in L4, lethargus, and young adult animals at 1mM ATR concentration. The mean ± s.e.m. values are shown: L4 (n=9), lethargus(n=7), adult (n=8), NS, ANOVA.

Supplementary Figure S7



Supplementary Figure S7.
(A) Image of the *pnmr-1::ChR2::mcherry* expression (left=*mcherry*, middle= DIC, right= overlay). Expression is seen in 3 sets of neurons (AVA, AVD, and AVE). Scale bar is 20µm. **(B)** Quantification of ChR2::GFP expression in AVA of the ZX1020 animals during L4, lethargus, and young adult. The mean ± s.e.m. values are shown: L4 (n=10), lethargus (n=11), young adult (n=13), NS, ANOVA. **(C)** Quantification of the ratio of ChR2::GFP expression in the RIM and AVA of ZX1020 animals during L4, lethargus, and young adult. The mean ± s.e.m. values are shown: L4 (n=10), lethargus (n=6), young adult (n=12), NS, ANOVA. **(D)** Quantification of ChR2::mcherry expression in AVA, AVD, AVE of the *nmr-1* animals during L4, lethargus, and young adult. The mean ± s.e.m. values are shown: L4 (n=5), lethargus (n=5), *p<0.05, ANOVA. **(E)** Quantification of the ratio of ChR2::mcherry expression in the AVD and AVA of *nmr-1* animals during L4, lethargus, and young adult. The mean ± s.e.m. values are shown: L4 (n=5), lethargus (n=5), *p<0.05, Student's t-test of unequal variance.

Supplementary Figure 8



Supplementary Figure S8.
Representative traces of Cameleon measurements in the young adult and lethargus ASEL sensory neuron in response to 80mM NaCl.

METHODS

Strains. *Caenorhabditis elegans* strains were maintained under standard conditions at 20ºC (Brenner 1974). The following strains were used in this study:
ASH ChR2: AQ2235 *lite-1(ce314)*; *ljIs114[Pgpa-13::FLPase; Psra-6::FTF::ChR2::YFP]* [15],
AVA, RIM ChR2: **ZX1020** *lin-15(n765ts); lite-1(ce314); zxEx704[pflp18::loxP::LacZ::STOP::loxP:: ChR2::mCherry::SL2::GFP* (80 ng/µl); *pgpa-14::Cre* (80 ng/µl); *lin-15+]*[26],
Cholinergic motor neuron ChR2: **EG5096** [*Punc-17*:*ChR2*::*mCherry*] [27],
ASH ChR2 with ASH, AVD, and AVA GCaMP3: SRS85 *sraIs49 [nmr-1p::G-CaMP; unc-119(+)]; lite-1(ce314); sraEx80 [sra-6p::chop-2(H134R)::mCherry; osm-10p::G-CaMP; unc-122p::mCherry]* [14],
AVA cameleon: PS5955 (*Prig-3:: cameleon*),
ASH cameleon: *ljEx95[Psra-6::YC2.12]*[13] .

Molecular biology. The genetically encoded light-activated channelrhodopsin gene *ChR2* (a gift from the Deisseroth lab) was cloned into the pPD96.52 Fire vector (Addgene) to generate a *ChR2::YFP* fusion with the *unc-54 3'utr*. This construct was fused to a 5kb sequence from upstream of the *nmr-1* gene previously demonstrated to drive expression in AVA and AVD. Expression was confirmed in AVA, AVD, and AVD interneurons.[14]

Microfluidic device fabrication. We designed chips in AutoCAD (Autodesk) and sent the design to a mask-making service (Photosciences), which provided the chrome masks. We created the master molds by spin casting at 3,000 r.p.m and patterning a 19-µm-thick layer of SU-8 photoresist (MicroChem) on bare silicon wafers. We used previously published procedures to prepare the master molds for use with polydimethylsiloxane (PDMS) and perform the soft lithography to make the PDMS replicas. [38] The PDMS replicas were treated with air plasma (40W for 12s) to activate the PDMS surface and manually bond it to a coverslip. The PDMS devices were ethanol treated and flushed with FU-18 before use.

Calcium imaging. Calcium imaging was performed in a microfluidic device essentially as described.[17] The device was altered in only the part of the chip that constrains the worm to fit different developmental stages. Several versions were made in which the dimensions were scaled

to fit an animal 600µm in length and 30-60µm in diameter. The flow of stimuli and buffers in the device was controlled using an external valve system to regulate pressure at the various inlets, which modified the pattern of flow. External components were built according to published protocols to automate the valve system (Rafael Gómez-Sjöberg, Microfluidics Lab, Lawrence Berkeley National Laboratory), and valves were controlled using LabView software (National Instruments). Fluorescence time-lapse imaging (100-ms exposures, 5Hz) was performed on a Zeiss Axioscope inverted microscope with a 40x air objective and an Andor EMCCD camera. Animals were presented with alternating streams of S-basal complete buffer and stimulant (Cu2+ or glycerol) in S-complete buffer. All image analysis was done using a custom script written in Matlab: after background subtraction, total fluorescence intensity was measured from the individual regions of interest (ROIs) corresponding to the cell body. No adjustments were done for photobleaching as there was minimal evidence of such with the low intensity light exposure.

Analysis of Calcium Events. Exponential smoothing of the calcium imaging data was performed in Matlab (alpha value of 0.05) and instantaneous time derivatives were calculated for each time point. These values were plotted in a raster plot (Figures 2c, S4c) and were characterized as an influx (>0) or efflux (<0). These events were further binned into one-second bins to quantify the probability of these events during the duration of the imaging and response to buffer or glycerol. Cross correlation was performed on the data of corresponding ASH-AVD, ASH-AVA, and AVD-AVA traces. The value at lag time=0 or peak correlation was used to assess differences between different neuron sets and conditions.

Behavior. Unless otherwise stated, AQ2235 animals were grown in the dark with 10µM ATR and were illuminated with blue light (425–475 nm) from an LED device (Phillips LumiLEDS) at 12mW intensity at the level of the plate. EG5096 and ZX1020 animals were grown in the dark with 100µM ATR, picked onto a new ATR plate, allowed to rest for 10 minutes, and were illuminated with blue light (425–475 nm) from an LED device (Phillips LumiLEDS) at 12mW and 30mW intensity at the level of the plate. Animals were stimulated with an LED device when stationary or exhibiting forward locomotion, and imaged during the assay for behavioral analysis using a Leica stereomicroscope and a Unibrain camera using Unibrain software. Reversal was scored as posteriorwards movement that was greater than the length of the worm's head. Three trials were performed for each worm, and the interval between trials was 30 seconds.

Worms were perturbed for the waking assays by harsh touch with a worm pick immediately anterior to the middle of the worm (marked by the vulva), or by tail touch with an eyelash drawn across the tail of the worm. ChR2 assays were performed when the animals exhibited stationary behavior or forward locomotion (all stimulations were performed within 30 seconds of the perturbation).

Modeling. All simulations were performed using custom scripts in Matlab with parameters and equations previously published.[29] Individual variables (e.g. ASH activity), were generated by allowing the script to generate values at the various input values where other variables (ASH threshold, AVD threshold, AVA threshold) were fixed. The script ran variations of different combinations to generate the data plotted in the activity vs. time plots and the surface plots that are shown in Figures S2 and S3.

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CHAPTER 5:

What Physiology Tells Us About the Regulation of Sleep

INTRODUCTION

In the previous chapter, we have made efforts to understand circuit mechanisms that promote sleep-like behavior in *C. elegans*. We found that calcium influx at the sensory neuron is dampened. However, there are many ways to dampen arousal[1], and although neuromodulators such as dopamine[2] and serotonin play key roles in the regulation in the starved and satiated states, their differing effect on select neurons are not always consistent with the sleep-like state. Therefore, we further test for physiological changes within other amphid sensory neurons to understand if sleep is global as well as to find better candidates for neuromodulation of the sensory neurons. The abundance of data concerning the expression of genes in individual neurons and large mutant and RNAi library makes it possible to test potential candidates.

In addition to sensory dampening, we found that the downstream effectors of the ASH sensory neuron were desynchronized during lethargus and that the primary interneuron, that drives the reverse motor neurons, AVA, was less responsive to channelrhopsin activation. In our preliminary results, we find that modulation of responsivity in the command neurons mediates the reversibility of sleep behavior. Careful stimulation with multiple inputs at the same or different sensory neurons allows visualization of activity in the circuit during waking and offer insights into what happens in the awake state.

5.1 SEARCHING FOR SLEEP REGULATORS

The first step for suppression of lethargus phenotypes was to understand the lethargus phenotypes to measure. The first studies of *C. elegans* sleep relied on measurement of locomotion, feeding behavior, and arousal.[3,4] It was through these studies that the conservation of regulators such as EGF[3], PKG[4], and cAMP[4] were confirmed. Because many of the neurotransmitters and signaling pathways are conserved, we began with an initial candidate screen to look for variability in the features that can be detected. Although more time-consuming to measure, the observation of locomotor activity is able to offer more subtle and numerous features in characterizing the mutant lethargus phenotype.

We began by perturbing protein kinase G, PKG, a conserved molecule shown to affect sleep. In Drosophila, increased PKG activity is associated with more sleep.[4] Additionally, preliminary reports suggest that PKG inhibition in the basal forebrain reduces subsequent sleep.[5] In *C. elegans*, PKG is shown to decrease sensory arousal and increase activity in lethargus.[4] We

tested the locomotor phenotype of these mutants in our system to compare our methods and assess interaction with the ALA, a sleep promoting interneuron (Figure 1-2). We confirm that PKG plays a role in promoting lethargus independent or downstream of ALA activity and that duration of lethargus remains unchanged. We also assay a set of neurotransmitter and signaling candidates (Figure 3), and found mutant phenotypes where lethargus duration was shortened.

5.2 MECHANISMS FOR SENSORY DEPRESSION

Calcium influx at the ASH and ALM[6] sensory neurons are dampened during lethargus. These are two disparate neurons: one is a polymodal nocioceptor that senses volatiles, aqueous chemicals, and mechanical stimuli, and the other a simple mechanosensor. It is possible that sensory regulation may occur only in these two neurons or in neurons with mechanosensory functions in general, but confirming modulation of subsets of neurons will narrow the candidates to those components present in these select cells.

Single cell studies of individual neurons have identified gene transcripts present in a handful of sensory neurons (Schwarz & Sternberg et al., unpublished data), and testing them for functional changes in lethargus was logical. We found that sensory dampening as measured by calcium imaging occurred in both the AWC and ASE sensory neurons (Figure 4). These are chemosensory neurons and do not share the same receptors with the ALM, indicating that modulation of these cells cannot be occurring at individual ligand receptors but rather at conserved signaling elements or components that induce changes in membrane potential.

We used channelrhodopsin to induce current in the ASH sensory neuron and assessed calcium changes in the absence of ligand-activated G-protein signaling. To do this experiment, we imaged the cell body using low light that did not induce changes in cellular calcium[7], and additionally illuminated a small area of the sensory cilia to activate channelrhodopsin (Figure 5). This decreased the amount of light contamination that occurs with whole field and allowed better control of the area of activation. Using these methods, we found that there was no exponential increase in calcium in response to channelrhodopsin during lethargus (Figure 6).

An increase in calcium in response to calcium influx suggests recruitment through voltage-gated or calcium-gated calcium channels. We have selected an available set to measure for arousal. The control wild-type animals showed immediate response to ASH:ChR2, whereas the negative controls, *unc-13* and *unc-31*, showed no significant response to ChR2 activation (data

not shown). *cca-1* and *ccb-1,* voltage-gated calcium channels, show delayed reversal in response to channelrhodopsin (Figure 7).

These long-term changes in the sensory neurons do not change much in preliminary experiments of perturbed animals (Figure 8). Sensory response remains relatively the same, but the response to the sensory input varies dramatically. This observation suggests that long-term regulation occurs at the sensory level, but there are additional dynamic changes that occur in the sensory-interneuron synapses.

FIGURES

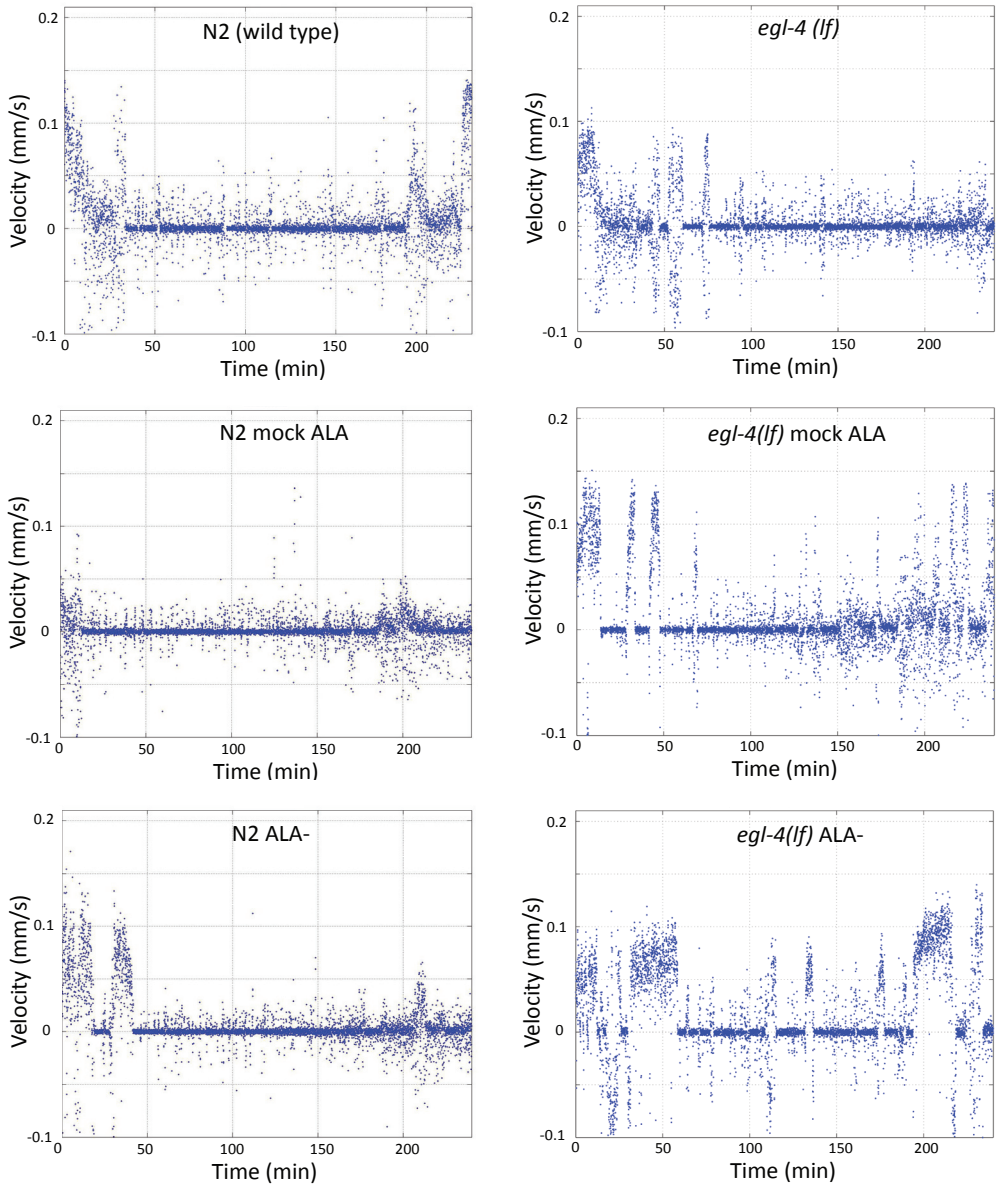

Figure 1. Representative velocity tracks of the N2 and *egl-4(lf)* lethargus are shown. *egl-4(lf)* mutants exhibited increases in locomotion in response to mock and ALA-ablation.

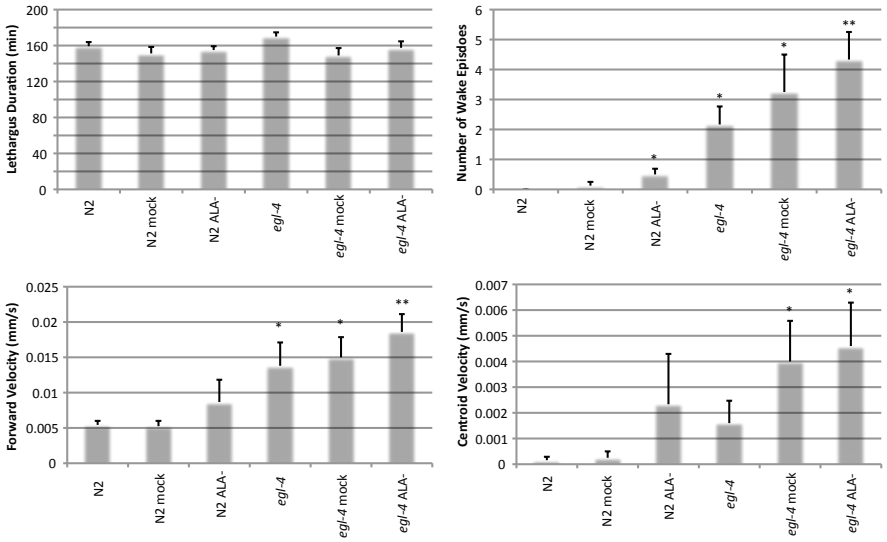

Figure 2. Quantitation of velocity data of the N2 and *egl-4(lf)* lethargus are shown. *egl-4(lf)* mutants exhibited increases in locomotion in response to mock and ALA-ablation.

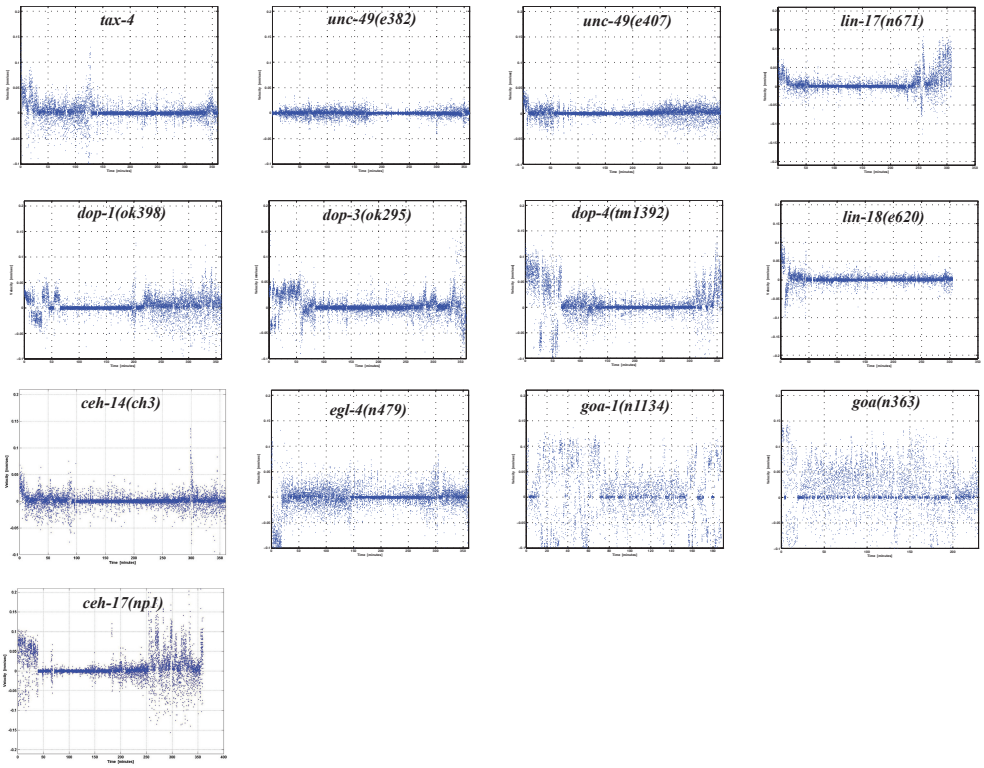

Figure 3. Velocity tracks of mutant animals with genes affecting conserved neurotransmitter and signaling pathways.

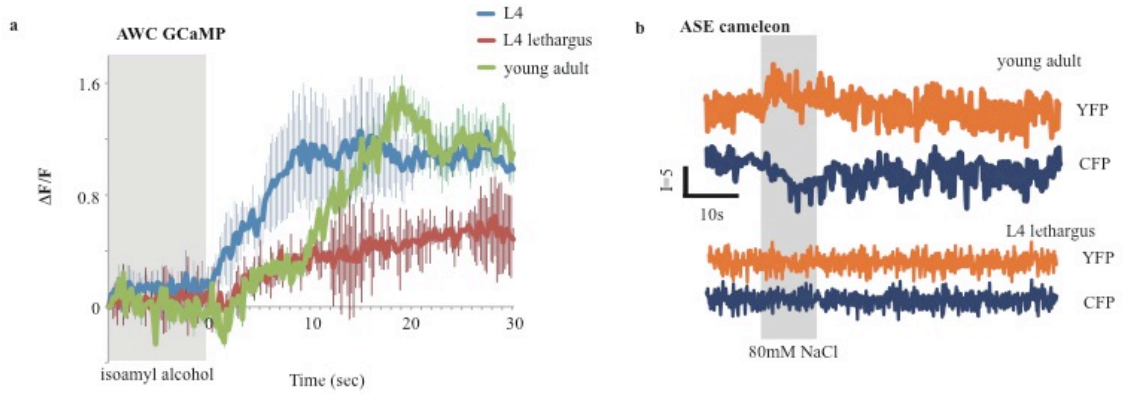

Figure 4. (a) Calcium influx as measured by GCamP in the AWC sensory neurons in response to isoamyl alcohol. (b) Calcium influx as measured by cameleon in the ASE sensory neuron in response to increased NaCl concentration in a single animals during lethargus and adult stages.

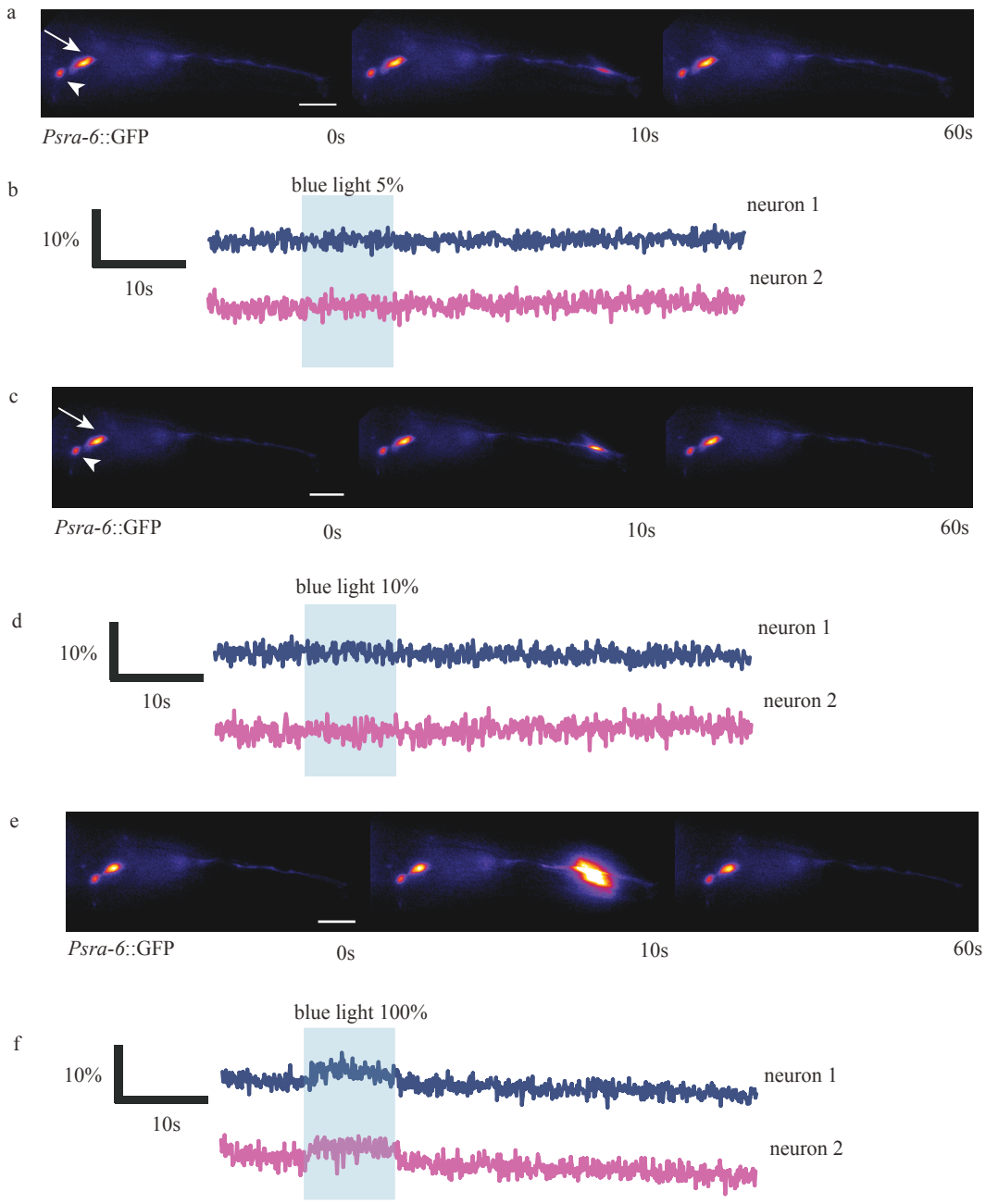

Figure 5. Technical tests for light contamination at the neuronal cell body in response to regional illumination. Chosen images of the GFP expressing animals before, during, and after regional illumination (a)(c)(e). There is no apparent increase in GFP intensity at lower levels of regional stimulation and a small increase when the highest intensity is used. Note arrowhead indicating neuron 1 and arrow indicating neuron 2.

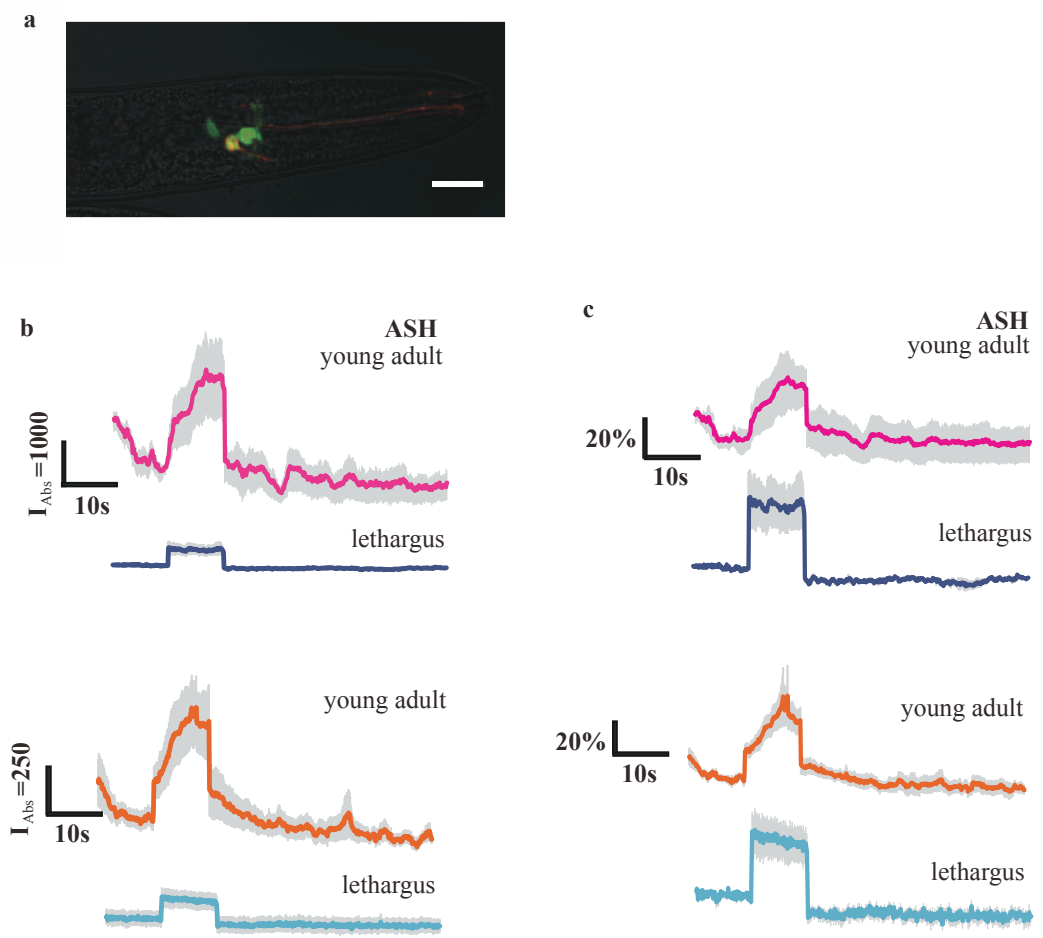

Figure 6. (a) Image of strain in which ASH GCaMP3 was measured. ASH is marked by the presence of both GCaMP3 and mCherry. (b) (c) (d) (e) Additional influx of calcium is only present in young adult animals. Lethargus animals show a flat pulse which does not look physiological.

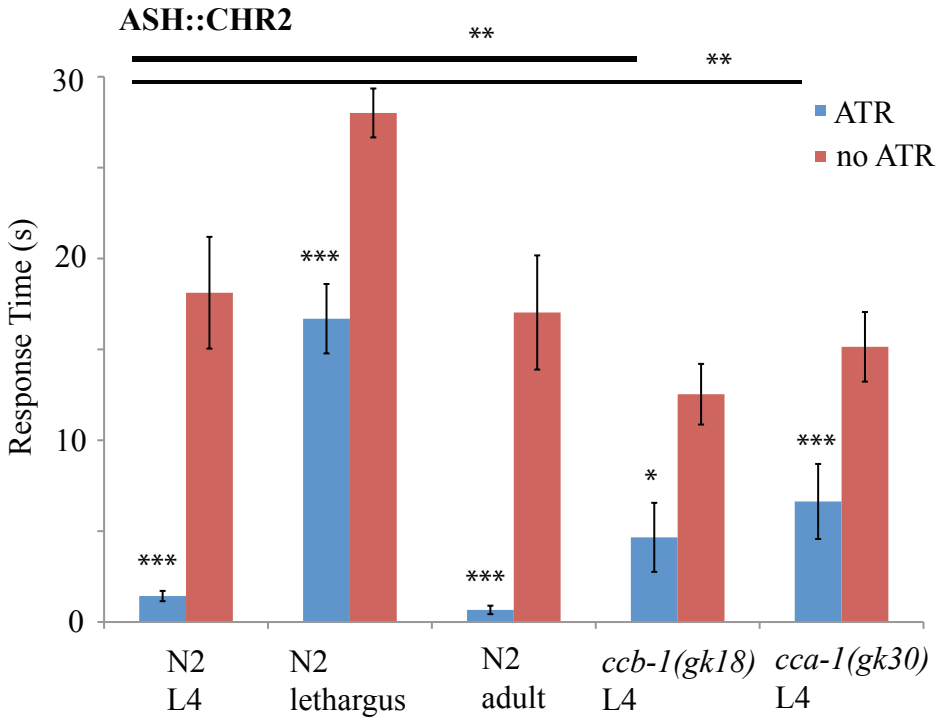

Figure 7. Time of sensory response of L4 N2, *cca-1*, and *ccb-1* animals. N2 lethargus and adult animal responses are also shown. All animals showed a significant response in the presence of the cofactor ATR as compared to conditions without (*p<0.01, ***p<0.0001). In the presence of ATR, *cca-1* and *ccb-1* animals showed a significant (p<0.001) difference from N2 animals in response to light-activated ChR2.

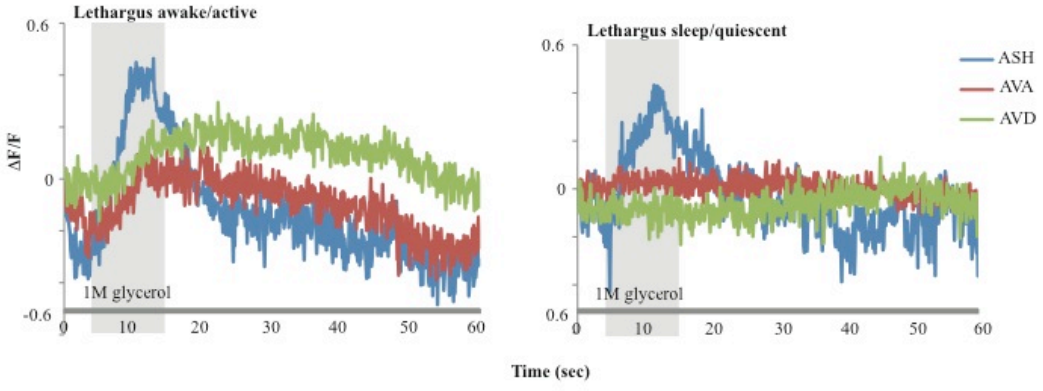

Figure 8. Activity of ASH, AVD, and AVD in response to chemical stimulus after perturbation and the following rest.

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CHAPTER 6:

Conserved Molecular Mechanisms of Sleep Homeostasis

6.1 ABSTRACT

Rest and repair of any system from a single cell to a large organism is essential for proper maintenance and function. The idea of sleep as a homeostatic process is based on this general concept and is bolstered by its necessity in human and animal health.[1] Deprivation studies in both mammalian and non-mammalian species have shown that a prolonged period of wakefulness results in increased sleep pressure that can only be dissipated during sleep. There are changes in gene expression corresponding to deprivation and the following recovery sleep.[2] However, many questions still remain about the cause of this homeostasis, the pertinent molecular correlates of sleep propensity, and the origin of these signals. Here we explore issues of rest and homeostasis during lethargus, the sleep-like state in the nematode *Caenorhabditis elegans*. We find that aspects of adenosine signaling, an important regulatory pathway of sleep homeostasis, is conserved. By measuring and altering the downstream signaling components of this pathway, we explore the ideas of the specificity and the multi-faceted effects of targeted depression in the neuronal network.

6.2 INTRODUCTION

The two-process model of sleep regulation asserts that sleep pressure results from the combination of both circadian and homeostatic processes. The circadian regulation of sleep ensures that an organism rests at the appropriate and evolutionarily beneficial time, whereas homeostatic regulation is driven by the need for rest induced by the duration and intensity of activity.[3] Sleep homeostasis refers to the maintenance of sleep amount or depth following sleep deprivation, and is a reflection of the essential nature of this sleep. The cause of this homeostasis is under debate, and there are three contending hypotheses: the energy depletion hypothesis, the neural plasticity hypothesis, and the immune defense hypothesis.

The concept of energy metabolism is that neuronal activity during waking consumes energy while sleep allows energy restoration.[4] Adenosine triphosphate (ATP) is the primary energy carrier in the cell, and local energy depletion through prevention of ATP synthesis and increase in the degradation products of ATP were shown to promote sleep. Adenosine is one of these products, and extracellular adenosine in the basal forebrain (BF) increases during prolonged wakefulness and decreases during recovery sleep.[5] Neuronal activity and consumption of ATP during waking is the cause of elevated adenosine concentration, which

in turn feeds back to adenosine receptors primarily as an inhibitory neuromodulator to induce sleep.[6,7] Adenosine binds to the A1, A2, A3, and A4 adenosine receptors. The primary mediator of sleep is the adenosine A1 receptor, which works through the G_{i3} G-protein signaling to activate potassium channels, inactivate calcium channels, and inhibit adenylate cyclase (Figure 1a).

The main targets for vigilance state-modulating effects of adenosine are the cholinergic cells in the basal forebrain. Selective activation of cholinergic BF neurons using neurotensin promotes EEG gamma activity and state of wakefulness.[8] Corresponding cell-specific lesions of cholinergic cells in the BF abolished extracellular adenosine.[9]

Sleep is conserved and present in essentially all animal species tested.[10,11] One sleep-like state in the nematode *C. elegans* is lethargus.[12,13] Lethargus is a quiescent state during which locomotion and feeding are suppressed[12] and sensory arousal is decreased.[13] Lethargus reliably occurs during *C. elegans* development before each of its four larval molts, and the timing of lethargus corresponds to upregulation of LIN-42, homolog of circadian regulator PER.[14] Lethargus exhibits characteristics similar to sleep homeostasis, and following deprivation of rest by increased sensory stimulus and motor activity during lethargus animals show anachronistic rebound quiescence.[13] Daf-16/FOXO has been shown to play a role in the regulation of homeostatic sleep in the muscle.[15] Anachronistic quiescence can also be induced by expression of epidermal growth factor (EGF)[12], a function conserved in mammals[16], and sensory arousal can be depressed by PKG[13], another well-conserved signaling protein. This conservation suggests that the lethargus state in *C. elegans* could prove insightful in understanding sleep regulation.

6.3 ADENOSINE RECEPTOR ANTAGONIST PARTIALLY SUPPRESSES LETHARGUS

Adenosine is an inhibitory neuromodulator that binds to the adenosine receptors to promote sleep. The primary mediator of sleep is the adenosine A1 receptor. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) is a selective adenosine A1 receptor antagonist[17] that has been shown to increase activity and arousal during sleep when injected into the mouse prefrontal cortex[7]. We performed six to eight hour incubations with DPCPX or control (1% DMSO) during the L4 stage preceding lethargus, and found that DPCPX treatment significantly increased activity (Figure 1b) and sensory arousal as measured by 1-octanol response (Figure

1c) at higher concentrations. Acute assays immediately before or during lethargus showed no significant change (data not shown), but it was unclear whether it was because feeding activity was required for entry of the drug or because entry and equilibration of drug concentration is a slow process.

6.4 DOWNSTREAM EFFECTORS OF A1 RECEPTOR SIGNALING PROMOTE HYPERACTIVITY DURING LETHARGUS

The A1 receptor works through G_{i3} G-protein signaling to activate potassium channels, decrease activity of calcium channels, and inhibit adenylate cyclase. This induces hyperpolarization of the neurons, decreases excitatory response to neurotransmitters that act through $G_{\alpha s}$ signaling, and decreases transcription of genes related to cAMP, which help potentiate excitation. In *C. elegans*, *goa-1* encodes both the main G_o and G_i class of G-protein signaling, and the cAMP catalyzing adenylate cyclase is *acy-1* (Figure 2a).[18]

Loss of downstream effectors of A1 receptor signaling (G_i) should repress neuronal depression mediated by the A1 receptor; we find that loss of *goa-1* indeed results in increased activity and exhibits similar phenotypes to drug treatment. Although *goa-1(lf)* results in hyperactivity in the adult animals[19], there was only a moderate increase in velocity of the animals during lethargus, and this increase was not significant when compared to the wild-type N2 animals (Figure 2b-c). However, the *goa-1(lf)* animals showed a significantly short lethargus whose duration was roughly half that of wild-type N2 animals (Figure 2d). In addition multiple alleles of the *goa-1(lf)* showed increased sensory response during lethargus (Figure 2e), indicating that although animals were not active, they were responsive and had increased sensory arousal.

G_i signaling inhibits adenylate cyclase, which catalyzes conversion of ATP to cAMP. In contrast to the *goa-1(lf)* animals, the mutants of adenylate cyclase, *acy-1(gf),* and mutants of cyclic nucleotide phosphodiesterase, *pde-4(lf)*, increase cAMP (Figure 3a) and showed more general hyperactive phenotypes. The *acy-1(gf)* animals were significantly more hyperactive during lethargus (Figure 2c, 3b-c), as well as during the adult stage[20], and both *acy-1(gf)* and *pde-4(lf)* animals showed increased feeding behavior during lethargus. These animals also exhibited a shorter quiescent period (Figure 2d, 3e), but these periods were not as dramatically short as those seen in the *goa-1(lf)*. Furthermore, we confirmed *acy-1(gf)* and *pde-4(lf)* animals showed no decrease in sensory arousal during lethargus (Figure 2e).[13] The

discrepancy between the two mutants can be explained as increased specificity of lethargus regulation by G_i or *goa-1* and the integration of all general excitatory components by adenylate cyclase or ACY-1 activity. The *goa-1* gene should exhibit more dramatic phenotypes with regard to lethargus-specific phenotypes, whereas *acy-1* should be less specific and exhibit hyperactive phenotypes in general. In addition, the partial suppression of lethargus by *pde-4(lf)* indicate that there is cAMP being produced in the nervous system in the period prior to and possibly during lethargus. It is possible that although adenosine inhibits ACY-1 activity, other pathways that activate ACY-1 may continue to be active.

We wanted to further test this reasoning by measuring the contribution of G_q subtype of G protein signaling. G_q signaling also excites cell signaling and is known to be inhibited by the G_o/G_i class signaling in *C. elegans.*[19] Therefore, overexpression of G_q, *egl-30*, also results in hyperactive phenotype in adult animals.[21] If suppression of activity during lethargus is general and acts through all pathways that decrease neuronal excitability, then overexpression of G_q should also change lethargus phenotypes. Furthermore, since these mutants show a much more dramatic phenotype than the *goa-1(lf)* animals in the adult stage, they should show a stronger effect. Surprisingly, both locomotion of *egl-30(OE)* animals during lethargus and lethargus duration were not significantly different from wildtype (Figure 2b-c). These results suggest that dampening of neuronal activity during lethargus is primarily through the G_i pathways.

6.5 SUPPRESSION OF SENSORY AROUSAL IS MEDIATED THROUGH THE NERVOUS SYSTEM

GOA-1, EGL-30, ACY-1, and PDE-4 are expressed almost ubiquitously in the neurons and muscles of *C. elegans.*[18] Selective expression of ACY-1(gf) in either muscle or neurons result in adult hyperactivity.[22] However, we found that, although expression of ACY-1(gf) resulted in increased feeding behavior, there was a smaller increase of locomotion that was not significant when compared to the wildtype N2 animals (Figure 4a,c). Furthermore, no significant change in lethargus duration could be detected (Figure 4b). However, there was a significant increase sensory arousal in animals with neuronal expression of ACY-1(gf), and a small trend toward sensory depression in muscle expression of ACY-1(gf) (Figure 4d). These data suggest that sensory arousal is mediated through activity in the nervous system and fatigue induced by increased muscle activity.

cAMP acts through CREB phophorylation to mediate transcription through the CRE enhancer. Measuring CRE-mediated transcription with the CRE::GFP can help suggest neuronal changes of cAMP during lethargus. We used a previously developed marker using multiple CRE domains fused to a destabilized GFP, and confirmed that CRE-mediated transcription is primarily in head neurons and pharyngeal muscle in the adult (Figure 5a-b).[23] We also found that expression persists during lethargus, but changes in intensity of expression varied in neuronal subsets suggest their modulation during lethargus (Figure 5c).

6.6 INTERACTION WITH OTHER SLEEP REGULATORS

The presence of EGF is correlated with activity in the SCN to drive circadian rhythms in mammals.[24] EGF is known to induce anachronistic sleep in *C. elegans* through the ALA interneuron.[12] EGF overexpression suppresses activity, feeding behavior, and sensory arousal. Furthermore, this sensory arousal is reversible, much like it is during sleep. We assessed activity of mutations increasing cAMP in the context of the ALA and EGF overexpression. We found that removal of ALA does not result in additional hyperactivity in the mutant *acy-1* animals. In fact, loss of ALA causes no significant change in velocity or lethargus duration, although there is a small trend toward extending lethargus duration (Figure 6a-b). Locomotor (Figure 6b) but not feeding (Figure 6d-e) phenotype of LIN-3 or EGF overexpression is suppressed by *acy-1(gf)*. In contrast, *pde-4(lf)* no animals showed no suppression of the LIN-3 overexpression phenotypes. The lack of feeding activity as measured by pharyngeal contractions in the *acy-1(gf)* and *pde-4(lf)* mutants with LIN-3 overexpression indicates that LIN-3 regulates this behavior independently by affecting components that are downstream of *acy-1* and *pde-4* signaling. This suggests that *acy-1,* but not *pde-4,* is downstream of *lin-3*. Perhaps in neurons where the LIN-3 receptor (*let-23*), *acy-1* and *pde-4* are expressed together, LIN-3 decreases neuronal excitability by inhibiting genes that promote ACY-1 activity, indirectly decreasing cAMP. With little cAMP to break down, the role of PDE-4 decreases significantly in these cells. It is also possible that *lin-3* and *acy-1* work in parallel pathways that inhibit or potentiate common downstream components of neuronal transmission. In this case, loss of *pde-4* should also suppress LIN-3 overexpression. The fact that it does not suggests that *lin-3* signaling directly or indirectly affects cAMP levels and the activity of *acy-1*.

6.7 DISCUSSION

Here we show conservation of adenosine signaling in the nematode *C. elegans* and its conserved role in promoting quiescence. We find that the downstream effectors of the adenosine signaling pathway show phenotypes consistent with decreased sleep drive: increased activity, lack of sensory depression, and decreased lethargus duration. Downstream effectors specific to the adenosine signaling pathway show more lethargus-specific phenotypes, whereas commonly shared components show more general hyperactive phenotypes. Furthermore, G_i or GOA-1 has been shown to interact with potassium channel, *unc-103*[25,26], which has been shown to be one of the components necessary for quiescence during lethargus (Van Buskirk et Al., unpublished).

The use of G-protein pathways in the regulation of sleep has two advantages: long-term regulation and integration of activity in context with environmental and internal stimuli. G_i interaction with the potassium channel ensures long-term hyperpolarization of the cell, but inhibition of adenylate cyclase ensures general dampening while continuing to allow integration of excitatory signals in the nervous system. If there are sufficient excitatory drivers at a given neuron, then there will be suppression of lethargus with respect to that behavior. However, the identity and the site of activation are important and only suppression of adenosine signaling will allow partial suppression of all lethargus-specific behaviors. Another interesting point is that increasing motor activity by bypassing the nervous system does not increase sensory response, showing that muscle hyperactivity and potential receptivity does not equate to fast response. In fact, animals with muscle-specific ACY-1(gf) show a trend toward further decrease in sensory arousal as compared to wild-type N2. This bolsters the idea that fatigue in the muscles may feed back on the nervous system.

Furthermore, we show a strategy for the integration of EGF signaling with that of the adenosine pathway through downstream effector, *acy-1*. ACY-1 is a good starting point for understanding the molecular components of sleep drive integration by the two-process model.

FIGURES

Figure 1

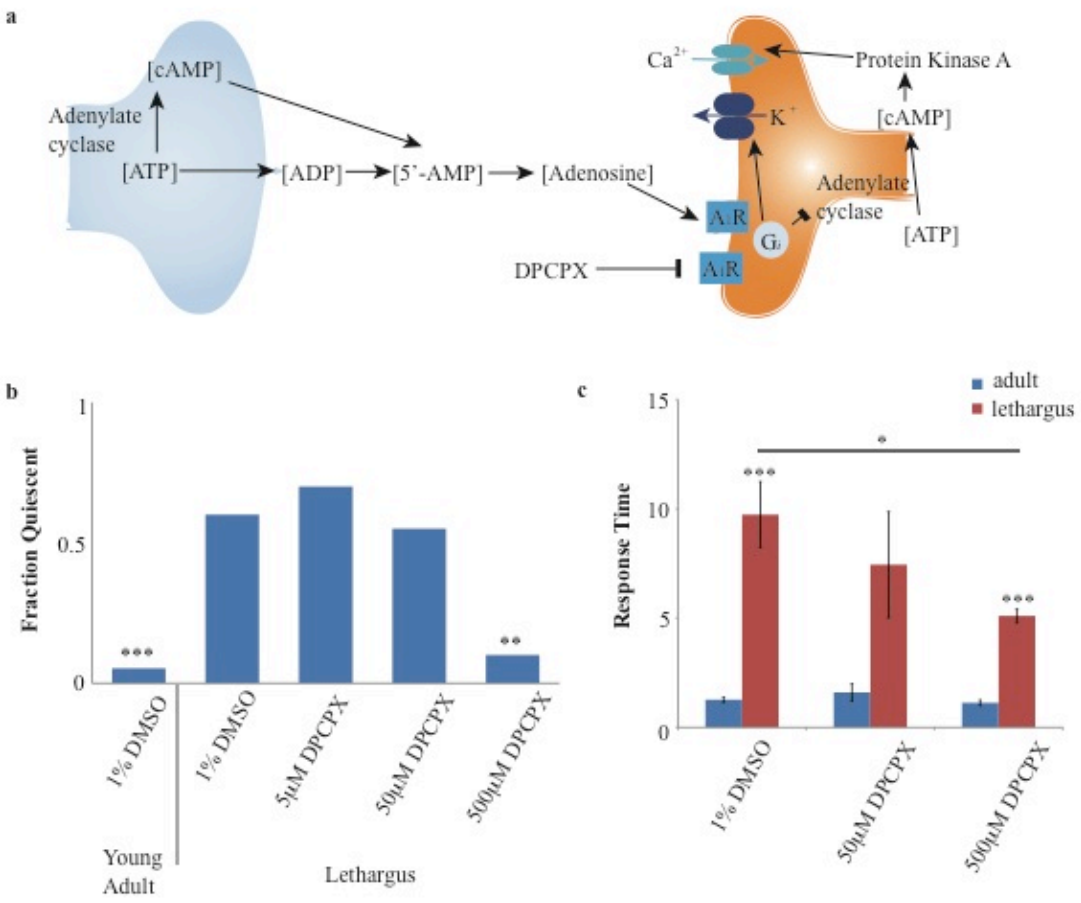


Figure 1. Adenosine anatagonist suppresses the sleep-like state.
(a) Schematic of adenosine signaling**.** (b) Quiescence measurements of animals incubated in labeled concentrations of the adenosine A1 antagonist DPCPX. Fraction of the population exhibiting suppression of locomotion (n=20). Fisher's exact test, **p<0.001, ***p<0.0001. (c) 1-octanol response assays in adult and lethargus animals incubated with 1% DMSO (n=25), 5μM DPCPX (n=15), 50μM DPCPX (n=18), 500μM DPCPX (n=20). Student's t-test *p<0.01, ***p<0.0001.

Figure 2

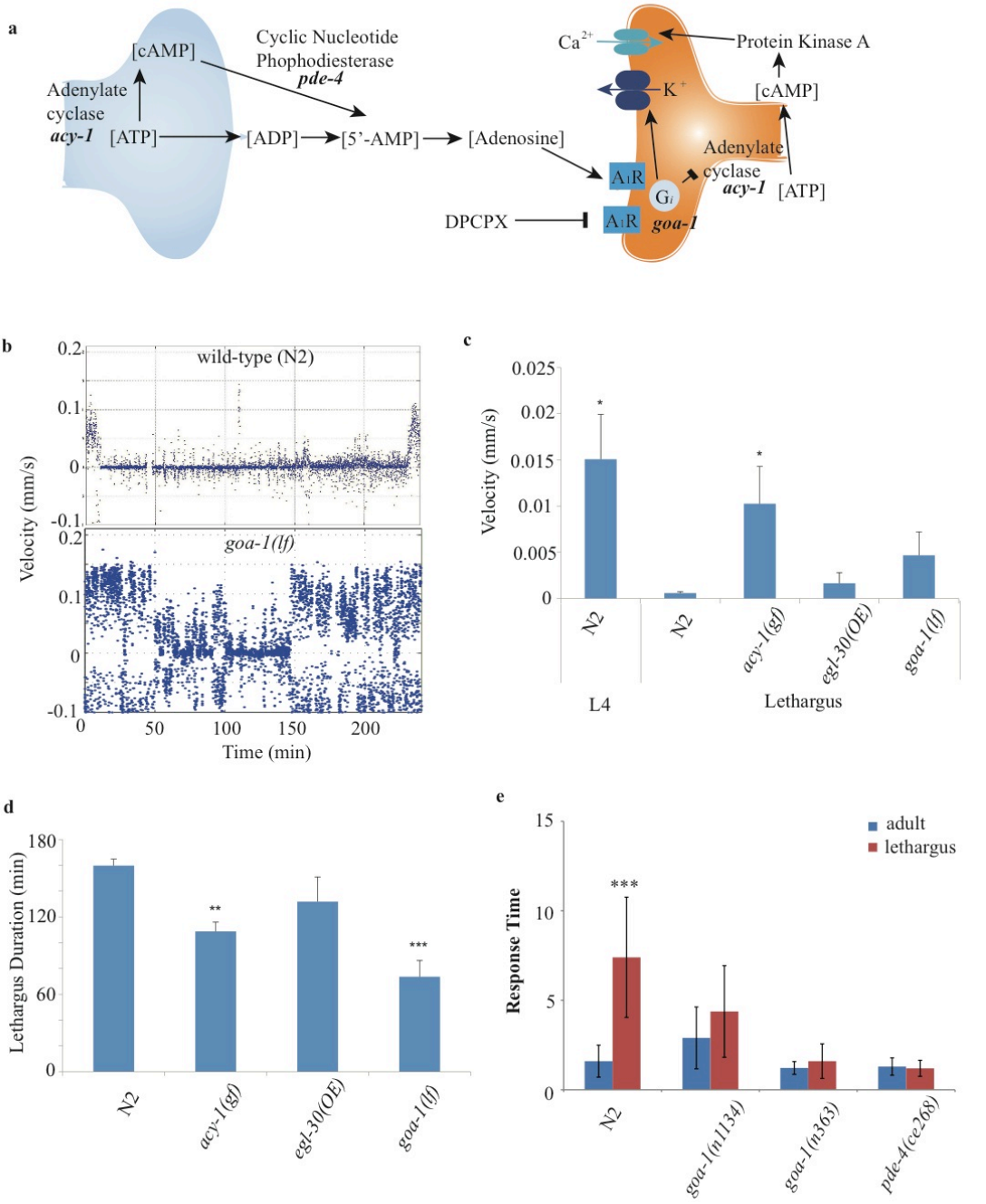


Figure 2. Lethargus phenotypes are preferentially promoted by the $G_{i/o}$ pathway.
(a) Schematic of candidate *C. elegans* genes in the adenosine signaling pathway. (b) Representative activity traces during lethargus in both wildtype N2 and *goa-1* animals. (c) Velocity measurements of wildtype N2 animals in the L4 stage (n=10), lethargus (n=10) as well mutants in lethargus: *acy-1* (n=9), *egl-30* (n=16), and *goa-1* (n=9). ANOVA *p<0.01. (d) Lethargus duration in wildtype N2 (n=20), *acy-1* (n=9), *egl-30* (n=16), and *goa-1* (n=9). ANOVA **p<0.001, ***p<0.0001. (e) 1-octanol response assays in adult and lethargus animals: wildtype N2 (n=20), *goa-1(n1134)* (n=14), *goa-1(n363)* (n=9), and *pde-4(ce268)* (n=9). Student's t-test of unequal variance ***p<0.0001.

Figure 3

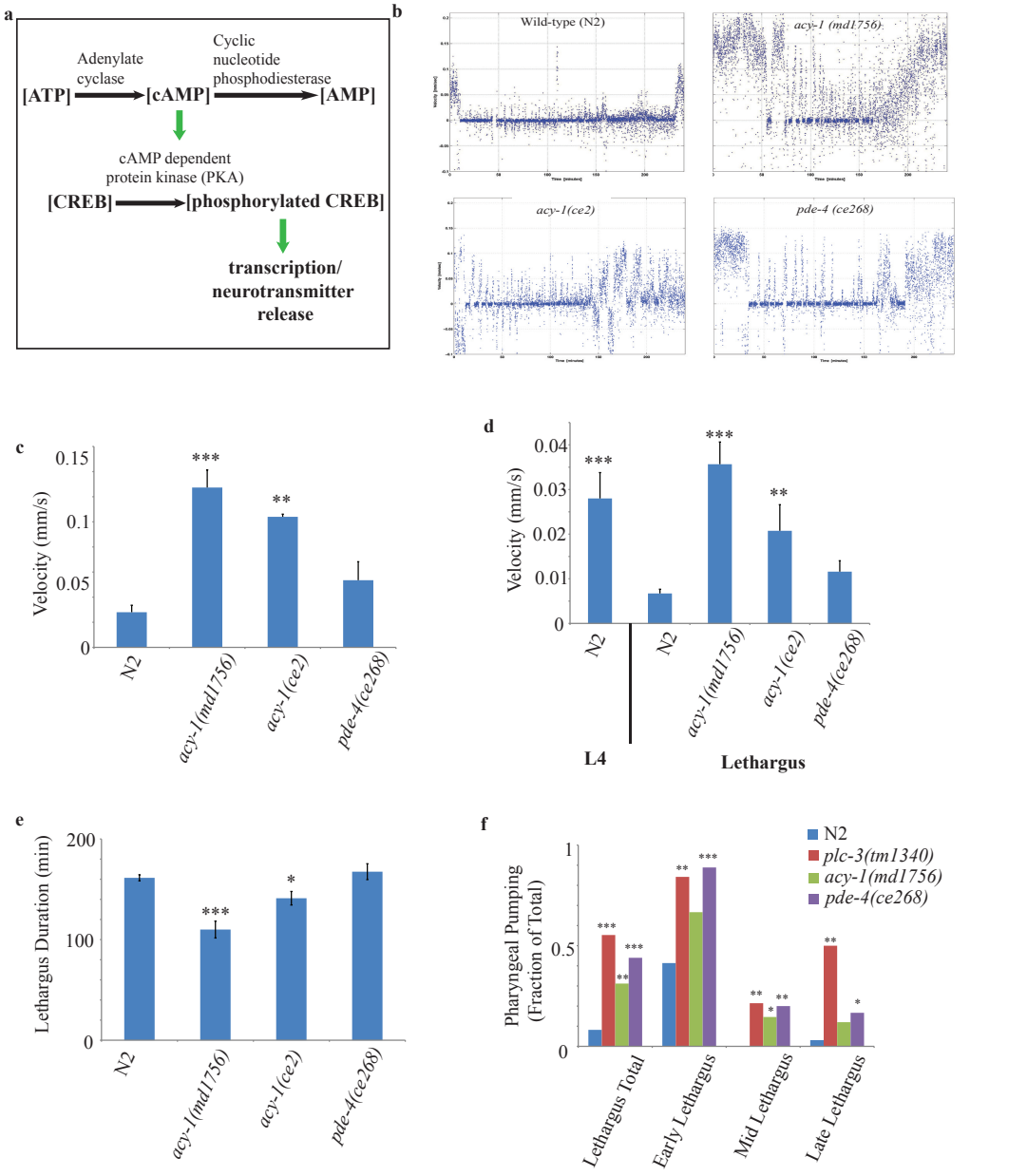


Figure 3. cAMP increase general locomotor and feeding activity during lethargus.
(a) Schematic of candidate *C. elegans* genes in the adenosine signaling pathway. (b) Representative activity traces during lethargus in both wildtype N2, *acy-1(gf)* and *pde-4(lf)* animals. **(c)** Velocity measurements of wildtype N2 (n=25), *acy-1(md1756)* (n=9), *acy-1(ce2)* (n=10), and *pde-4(ce268)* (n=9) animals. ANOVA **p<0.001, ***p<0.0001. (d) Velocity measurements during lethargus of wildtype N2 (n=25), *acy-1(md1756)* (n=9), *acy-1(ce2)* (n=10), and *pde-4(ce268)* (n=9) animals. ANOVA **p<0.001, ***p<0.0001. (e) Lethargus duration in wildtype N2 (n=25), 1-octanol response assays in adult and lethargus animals: wildtype N2 (n=25), *acy-1(md1756)* (n=9), *acy-1(ce2)* (n=10), and *pde-4(ce268)* (n=9) animals. ANOVA *p<0.01, ***p<0.0001. (f) Pharyngeal contractions during lethargus in wildtype N2 (n=171), *plc-3(tm1340)* (n=47), *acy-1(md1756)* (n=109), and *pde-4(ce268)* (n=25) animals. Fisher's exact test *p<0.01, **p<0.001,***p<0.0001.

Figure 4

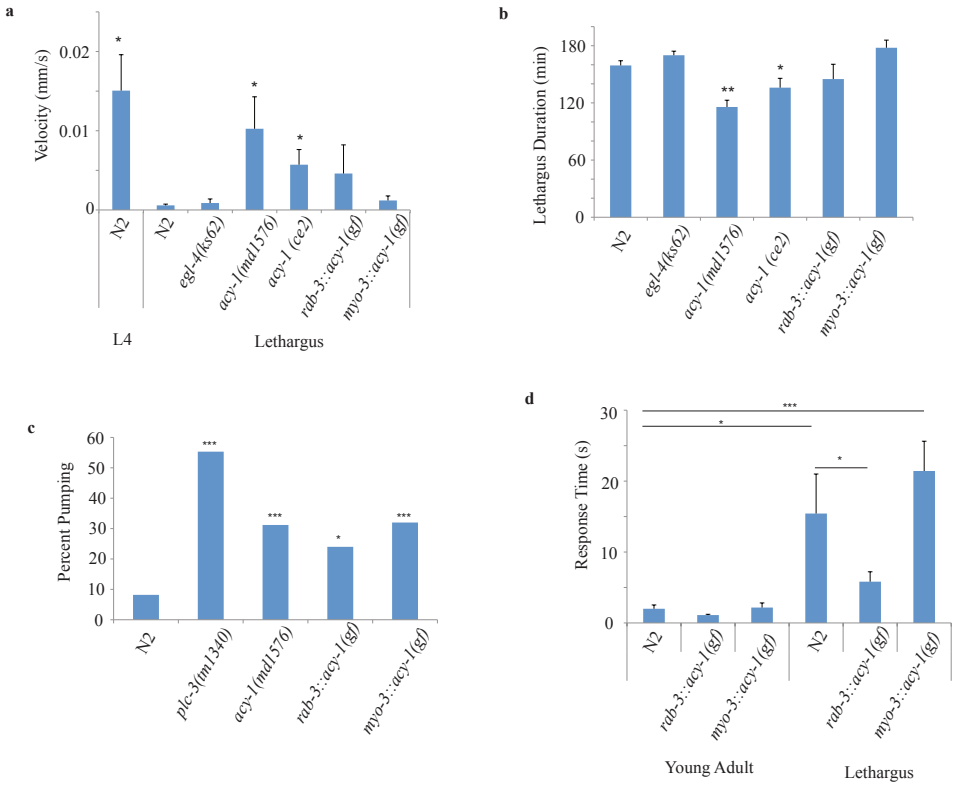


Figure 4. cAMP driven muscle excitability increase hyperactivity but not arousal.
(a) Velocity measurements during lethargus of wildtype N2 (n=10), *egl-4(ks62)* (n=7), *acy-1(md1756)* (n=9), *acy-1(ce2)* (n=10), neuronal expressing *rab-3::acy-1(gf)* (n=6) and muscle expressing *myo-3::acy-1(gf)* (n=8) animals. ANOVA *p<0.01 (b) Lethargus duration in wildtype N2 (n=10), *egl-4(ks62)* (n=7), *acy-1(md1756)* (n=9), *acy-1(ce2)* (n=10), neuronal expressing *rab-3::acy-1(gf)* (n=6) and muscle expressing *myo-3::acy-1(gf)* (n=8) animals. Chi-square test *p<0.01 (c) Pharyngeal contractions during lethargus in wildtype N2 (n=171), *plc-3(tm1340)* (n=47), *acy-1(md1756)* (n=109), neuronal expressing *rab-3::acy-1(gf)* (n=25) and muscle expressing *myo-3::acy-1(gf)* (n=25) animals. Chi-square test *p<0.01, **p<0.001,***p<0.0001. (d) 1-octanol response assays in adult and lethargus animals in wildtype N2 (n=25neuronal expressing *rab-3::acy-1(gf)* (n=25) and muscle expressing *myo-3::acy-1(gf)* (n=25) animals. ANOVA *p<0.01, ***p<0.0001.

Figure 5

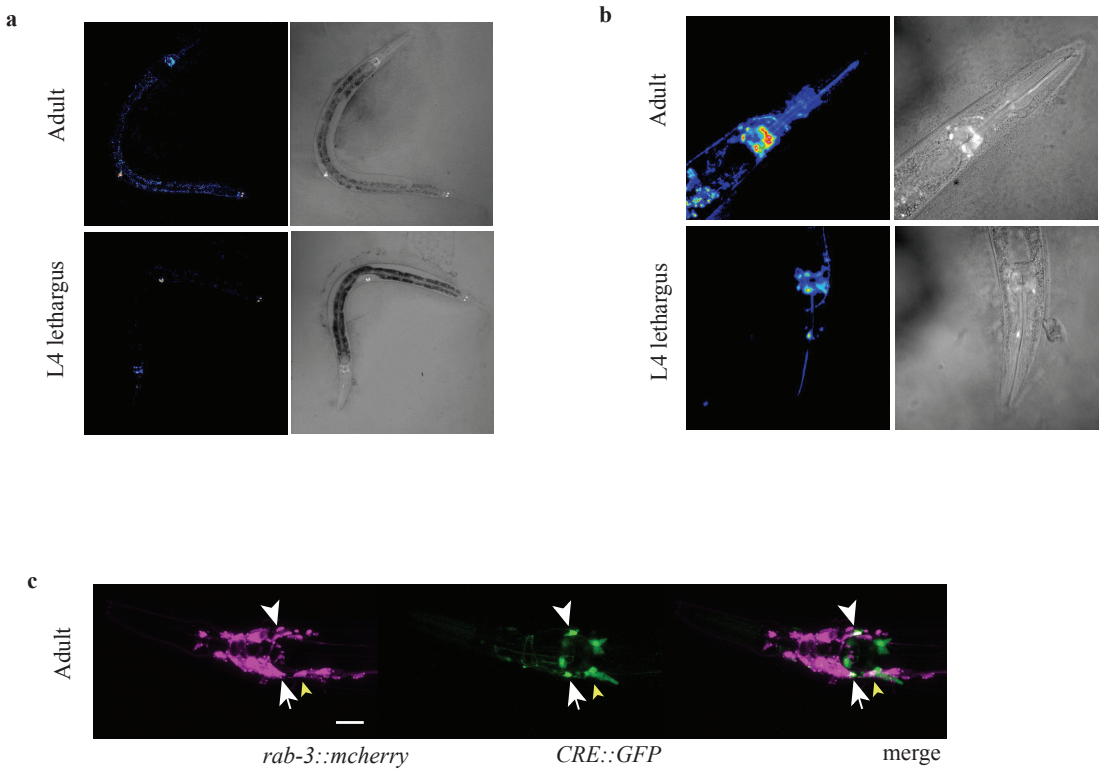
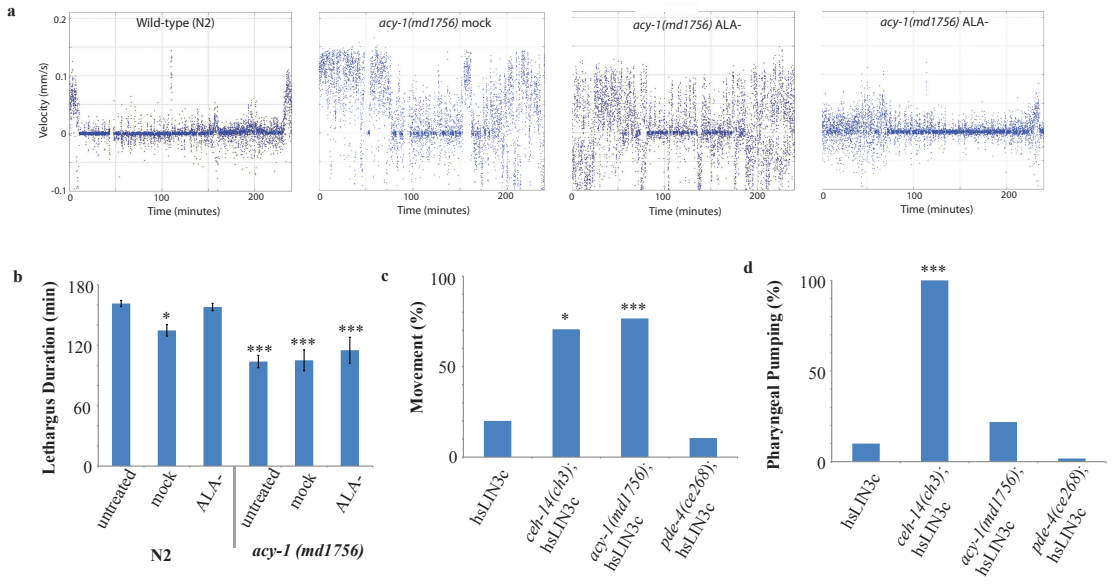


Figure 5. Distribution of neuronal cAMP is altered in lethargus.
(a) Confocal images of worms in adult and lethargus and of the distribution of CRE::GFP expression in the body. Note comparison expression of non-CRE GFP in the vulval cells. (b) Representative images indicating intensity of expression in head neurons of worms in adult and lethargus. (c) cAMP acts through CREB and CRE primarily through a small set of head neurons and pharyngeal muscle cells. Note arrow indicating colocalization of GFP with neuronal mcherry. Scale bar is 20μm.

Figure 6



e

Genotype	Pumping (%)	Moving (%)	n	Pumping (p-value)	Moving (p-value)
hsLIN3c	10	20	20		
ceh14(ch3); hsLIN3c	100	70.588	17	<0.0001	0.003
acy-1(md1756)	100	100	16	<0.0001	<0.0001
acy-1(md1756); hsLIN3c	21.875	76.563	64	0.3359	<0.0001
pde-4(ce268)	100	81.25	16	<0.0001	<0.0001
pde-4(ce268) ; hsLIN3c	1.754	10.526	57	0.1636	0.275

Figure 6. Selective suppression of EGF overexpression phenotypes.
(a) Representative activity traces during lethargus in wildtype N2, *acy-1(md1756)* mock ablated, hyperactive *acy-1(md1756)* ALA-ablated and lethargus rescued *acy-1(md1756)* ALA-ablated. (b) Lethargus duration in wildtype N2 (n=20), wildtype N2 mock ablated (n=15), wildtype N2 ALA-ablated (n=15), *acy-1(md1756)* (n=9), *acy-1(md1756)* mock ablated (n=5), and *acy-1(md1756)* ALA-ablated (n=8) animals. Chi-square test *p<0.01 (c) Locomotion phenotype of wildtype N2 and mutant animals with induced EGF overexpression. Percentage of animals exhibiting locomotion after heat shock EGF/LIN-3. Chi-square test *p<0.01, ***p<0.0001. (d) Feeding phenotype of wildtype N2 and mutant animals with induced EGF overexpression. Percentage of animals pharyngeal contractions after heat shock EGF/LIN-3. ***p<0.0001. (e) Phenotype of mutants after heat shock induction.

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CHAPTER 7:

Findings and Discussion

DESCRIPTION OF FINDINGS

C. elegans and its avoidance circuit proved useful for the study of state-specific modulation. We found that almost all genes affecting neuronal activity and excitability had some degree of impact on the sleep phenotypes that were assayed. Therefore, in understanding the regulation of behavior, it was necessary to understand what components of the nervous system were in fact being regulated.

Behavioral evidence suggests that our perception is dramatically dampened during sleep, and that there are physiological changes within individual neurons and their connections to each other.[1] We studied a sensory-motor circuit of *C. elegans* and found that there is long-term dampening at the sensory neuron during lethargus, with allowed dynamic change in the downstream interneurons following previous activation. Furthermore, our data suggest that reversal of sensory dampening is not likely the cause of behavioral reversibility, and it is rather the efficiency of signal transmission from sensory to interneuron that is altered. Changes in dynamics are well correlated with coordinated activity of the command interneurons AVD and AVA. The mechanism for changing signal transmission across the synpase is unclear, and can be due to either transmitter release or activity of the downstream receptors in the interneurons. We also showed that although there is remodeling at the neuromuscular junction during development, there is no suppression of activity downstream of the command interneurons.

Although these studies were not able to pinpoint the modulators that regulate the sleep-like state, we propose that channels and modulators of these channels are likely to be important in the sensory depression exhibited during lethargus. Functional data suggest that these channels are not active in lethargus, and loss of individual channels result in delay of sensory response to channelrhodopsin. It is important to note that these responses are significantly different from their controls, indicating that signal transduction in response to activation occurs.

In addition, we have confirmed adenosine signaling is likely conserved in *C. elegans.* We suggest that the signaling pathways that mediate adenosine signaling are conserved in their function[2]. It would be of interest to see how these mutants affect the deprivation of quiescence during lethargus.

The work in this thesis clarifies how circuit studies can be directly applied to understand behavior and pinpoint components of the *C. elegans* sensory-motor circuit that are important in modulating arousal. Much of the findings in other model organisms have been confirmed, and we have demonstrated how some of these technological methods can be modified for additional

physiological insights and also used for faster more specific screens. The next step is to use them in conjunction with new sequencing technologies and classic genetics to find some amazing new things.

FUTURE DIRECTIONS

Our study of the sensory motor circuits in lethargus is the first to follow processing of sensory information from input stimulus through the sensory neurons and interneurons to its conversion to behavior through the motor neurons. This is a powerful approach that allows characterization of key components of modulation while identifying circuit components do not contribute to changing behavioral dynamics. Our results indicate some intriguing questions regarding the molecular and circuit mechanisms that contribute to sleep behavior. For one, it would be interesting to separate the contribution of molecular drivers that decrease the efficiency of signal transmission at the sensory-interneuron synapses from the effect of long-term sensory neuron inactivity on the excitability of command interneurons. Furthermore, these functional characterizations involving the identity of modified sensory neurons and careful characterization of physiological and behavioral dynamics allow for new hypotheses involving the key molecular regulators involved in sleep-like behavior. We would expect that dampened neurons would be those that express receptors, modulating components, and functional channels in common that allow these neurons to respond to the signals that promote lethargus. Similarly, neurons that are not dampened should not express these genes. Ease of genetics in *C. elegans* allow fast and insightful analysis of these candidates and would better allow us to understand what it means to sleep at the level of the circuit.

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