THE UNIVERSITY OF CHICAGO

CAENORHABDITIS ELEGANS LETHARGUS IS A VULNERABLE SLEEP STATE THAT IS HOMEOSTATICALLY REGULATED BY TWO BEHAVIORALLY AND GENETICALLY DISTINCT MECHANISMS

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You have evolved from worm to man, but much within you is still worm.

- F. Nietzche, *Thus Spoke Zarathustra*

But leave the Wise to wrangle, and with me
The Quarrel of the Universe let be:
And, in some corner of the Hubbub couch'd,
Make Game of that which makes as much of Thee.

- *The Rubaiyat of Omar Khayym*

The unphilosophical and philosophical attitudes can be very sharply distinguished (with scarcely any intermediate forms) by the fact that the first accepts everything that happens as regards its general form, and finds occasion for surprise only in that special content by which something that happens here today differs from what happened there yesterday; whereas for the second, it is precisely the common features of all experience, such as characterise everything we encounter, which are the primary and most profound occasion for astonishment; indeed, one might almost say that it is the fact that *anything is experienced and encountered at all.* It seems to me that this second type of astonishment — and there is no doubt that it does occur — is itself something very astonishing. Surely astonishment and wonder are what we feel on encountering something that differs from what is normal, or at least from what is for some reason or other expected. But this whole world is something we encounter only once. We have nothing with which to compare it, and it is impossible to see how we can approach it with any particular expectation. And yet we are astonished; we are puzzled by what we find, yet are unable to say what we should have to have found in order not to be surprised, or how the world would have to have been constructed in order not to constitute a riddle!

- E. Schrdinger, *My View of the World*

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ABSTRACT

Sleep is an evolutionarily conserved biological process in essentially all animals with a nervous system, but the core function of sleep remains unknown. One of the most proven strategies for understanding complex phenomena is to perturb simple systems that model the complex phenomenon of interest. With this in mind, this dissertation uses lethargus, a 2-3 hour period of developmentally-timed sleep, in the nematode *Caenorhabditis elegans* to explore how quiescence is homeostatically regulated and how disturbances during this period affect the organism.

Here, I report that two behaviorally and genetically distinct mechanisms regulate behavioral quiescence during lethargus. I show that weak stimuli evoke homeostasis in response to extended motion by extending the subsequent bout of quiescence. I find that neuropeptides are required for this homeostatic response. Specifically, I find that the neuropeptide-Y receptor homolog NPR-1 in the RMG neuron class and inhibitory FLP-18 peptides are necessary for this "weak" homeostatic response. In contrast, strong stimuli repeated throughout lethargus result in a distinct homeostatic response: an elevation of the overall time spent in quiescence during lethargus. This "strong" homeostatic response does not require NPR-1, but instead requires the function of DAF-16/FOXO insulin-signaling transcription factor in neurons. Conversely, the "weak" homeostatic response does not require DAF-16/FOXO.

I further show that lethargus is a specifically vulnerable period during *C. elegans* development. I report that nonlethal deprivation of quiescence during lethargus beyond the regime that evokes homeostasis results in proteotoxic stress, that if left unmitigated, can cause long-lasting deficits in the animal. These deficits are anatomically, functionally, and genetically distinct. Specifically, I describe an automated protocol for depriving *C. elegans* of developmentally-timed sleep in a severe yet nonlethal manner. I then characterize three lasting effects of nonlethal deprivation of quiescence: a deficiency in pharyngeal pumping, a reduction in brood size as a result of germ cell apoptosis, and an excess of twitching in the vulval muscles of young adults. Importantly, our protocol for depriving quiescence when ap-

plied outside of lethargus does not result in deficits. I show that while both the pumping and fecundity defects are mitigated by DAF-16/FOXO, deprivation evokes distinct proteotoxic stress responses in the germline and pharynx. The unfolded protein response specific to mitochondira (UPR^{MT}) mitigates the pumping defect while the endoplasmic reticulum unfolded protein response (UPR^{ER}) mitigates the fecundity and vulval muscle defects.

Taken together, this work identifies a number of new insights about a primitive form of sleep. This dissertation shows that neuropeptides modulate quiescence during lethargus, but that strong perturbations evoke a distinct behavioral and genetic response to increase quiescence. This dissertation also shows that stronger perturbations beyond the regime of homeostasis result in the activation of distinct stress responses in distinct tissues and circuits of the animal. Importantly, these same perturbations inside and outside of lethargus evoke stress responses only during lethargus. Therefore, I show that lethargus is a vulnerable period of *C. elegans* development.

CHAPTER 1

INTRODUCTION

What distinguishes living from non-living things? One of the fundamental properties of life is the ability to respond and adapt to a dynamic environment. Indeed, to survive, animals modulate their behavior to a wide range of environmental stimuli including but not limited to temperature, volatile and soluble chemicals, and various forms of mechanical stimuli [22, 24, 53, 81, 144, 182, 392]. These responses to a changing environment beg the question of why animals respond at all. Despite being composed of the same matter as the rest of the universe, animals are distinctly different from most of matter in that they appear to be integrated entities with their own internal drives: acquiring and expending energy over time in order to maximize fitness.

Homeostasis is defined as a property of dynamic rather than static systems characterized by the ability to maintain an internally constant environment in the face of a changing external environment [45]. Framed in this way, biological drives move a systems state back to a set or target equilibrium point. From an evolutionary perspective, it therefore makes sense that a healthy animal would produce behaviors to move it toward states that maximize fitness. However, the observation of a behavior does not always lead to a clear understanding of why the behavior exists. Behavioral responses are moreover likely to be variable or stochastic [122, 126]. Despite these complexities, genetics and perturbations have been enormously successful at illuminating how animals generate behavior [29]. The purpose of this dissertation is to use genetics and perturbation experiments to cast light on the naively mundane behavior of sleep, which despite evolutionary conservation among all animals with a nervous system, scientists have not yet attributed a core function for [153, 188].

Genetics underlies much behavioral variation in animals. In humans, twin and adoption studies have shown strong correlations between genetic relatedness and the risk for many psychiatric disorders [190, 191, 192]. Can we classify the genes that affect behavior? Intuitively, genetic mutations that impact neural processes by changing proteins important for

sensation or synaptic transmission typically alter animal behavior. Supporting the conclusion that sensory genes are often the preferred targets for behavioral adaptation, sensory perception genes are among the genes under the strongest positive selection in the human genome [72, 266]. Relatedly, in model organisms, the identification of neurons and proteins involved in sensation underlie most of the prominent successes in behavioral neuroscience [25, 26, 53, 385].

Another target for behavioral adaptation are neuromodulators such as biogenic amines and neuropeptides. While neurotransmitters can only act on nearby synapses, neuromodulators can act at a distance, allowing them to act broadly to influence behavioral states. Furthermore, neuromodulators are also flexible to evolutionary pressures since they are often unnecessary for core neurotransmission and do not need the growth of new anatomical connections [29]. Supporting the hypothesis that evolution often targets neuromodulators for behavioral adaptation, neuropeptides and neuropeptide receptor expression patterns are highly variable among closely related species. For example, despite a near-invariant set of neurons in the stomatogastric ganglion of crustacean species, there is a wide divergence of neuropeptide expression [234, 382]. Oxytocin and vasopressin receptor expression is also highly variable among rodent species [165, 166].

Because screening for mutants has been the most successful strategy to find genes that influence behavior, behavioral neuroscientists study small animals with fast generation times and large brood sizes. These model organisms include the common fruit fly, *Drosophila melanogaster*, and the free-living nematode, *Caenorhabditis elegans*. As a testament to the power of simple model organisms, apart from the identification of the *Clock* mutant in a 1994 mouse mutagenesis screen by Joe Takahashi's group at Northwestern University and its later cloning in 1997, all the core clock genes, which are conserved between flies and mammals, have been first identified from the study of fruit flies [12, 197, 297, 383]. Indeed, the discovery of the first dedicated behavioral gene was the discovery of the circadian clock gene *period* by a chemical mutagenesis screen of *D. melanogaster* flies by Konopka and Benzer at Caltech

in 1971.

Despite their distant relation to mammals, it is also possible to take advantage of the unique biology of non-mammalian model organisms to understand related phenomena in the same or other organisms. Often, the molecules that affect one process or behavior are co-opted and reutilized for different processes that nevertheless share common characteristics. For example, before moving to locomotor assays to study an ongoing circadian phenomenon in single flies, the original character of Konopka and Benzers screen for *period* was pupal-adult eclosion (i.e., pupal hatching) of fly populations, taking advantage of the adaptation of wild type flies to eclose during the humid conditions of dawn in their ancestral homeland of sub-Saharan Africa [204, 297].

Another example illustrating the power of simple model organisms to study complex phenomena is the identification of mutants that affect healthspan and aging in the nematode, *C. elegans*. Due to limited resources in nature, *C. elegans* have developed a specialized diapause state during development, called dauer (enduring" in German), that promotes somatic maintenance at the expense of reproduction [47, 348]. This dauer larval stage allows the animal to survive harsh condition while seeking out new food sources. Once in a favorable environment, the worm will exit dauer stage and continue normal larval development before becoming a fertile adult. Intriguingly, dauer larvae live up to 70 days compared to the 12-18 days of normal adult worms, but animals that recover from dauer have the same lifespan of a normal worm regardless of the amount of time spent in dauer arrest. This observation led to the hypothesis that dauer larva may offer valuable insights in the controlling mechanisms of aging by Michael Klass, a postdoctoral scholar in David Hirshs laboratory at the University of Colorado who was the first researcher to screen for long-lived mutants in worms [198, 271]. As a result of studying the genetic regulation of aging in *C. elegans*, many of the first longevity genes were discovered, including *daf-2* whose activity was shown by Don Riddle and Cynthia Kenyon to trigger dauer formation when severely reduced but could bypass the dauer state while still conferring lifespan benefits when only mildly reduced [89, 115, 213].

Later, this precipitated into a discovery using RNAi that *daf-2* acts twice: once during larval development to affect dauer formation and again as an adult to exclusively affect aging [89]. Given that age is the main risk factor for cancer, cardiovascular disease, and neurodegeneration in developed countries, there is large incentive to understand the genetic regulation of aging, a phenomenon once considered to be an inexorable, entropic process [265]. However, our current knowledge that strongly conserved mechanisms influence longevity would not have even been possible without the early work studying the development of a simple, free-living worm: *C. elegans*.

In this dissertation, I will describe two projects in detail: the discovery of two behaviorally and genetically distinct mechanisms of homeostasis in *C. elegans* sleep and the discovery that nonlethal disruption of *C. elegans* sleep leads to lasting defects from proteotoxic stress in different tissues if left unmitigated. I will also briefly describe experiments suggesting that ALA, an interneuron previously implicated in regulating behavioral quiescence and recently linked to stress-induced sleep, also acts as a sensory neuron capable of responding to intense touch. In this chapter, I will first introduce the general biology of the free-living nematode, *C. elegans*. I will emphasize how this nematode is helpful to the study of neuroscience and specifically the molecular and cellular basis of behavior. Next, I will discuss what is now known and hypothesized about the behavior of sleep in vertebrates and invertebrates including *C. elegans*. Concluding the introduction, I will provide an overview of the most used tools and methods in my study of developmentally-timed sleep in *C. elegans*.

1.1 Overview of *Caenorhabditis elegans*

1.1.1 Description

Caenorhabditis elegans is a 1-mm, free-living, unsegmented roundworm that belongs to the phylum Nematoda and survives by feeding on bacteria in rotting vegetation. It shares a common urbilaterian ancestor with humans 500-600 million years ago. The animal passes

through four larval stages separated by molts before reaching sexual maturity: L1, L2, L3, and L4. For approximately 3 hours prior to each molt, the worm goes through a period of feeding and locomotion quiescence termed lethargus (Figure 1.1). Under plentiful food and at 20 degrees Celsius, *C. elegans* has a generation time of about 3.5 days. At 25 degrees Celsius, each larval stage lasts 8 to 12 hours and egg-laying begins at 62 hours [40]. Under optimal laboratory conditions, worms live for 2-3 weeks; however, if hatched in the absence of food, growth arrests at the L1 larval stage. Moreover, during the L1 and early L2 stages, environmental factors including the presence of a population-density pheromone, elevated temperature, and/or food limitation potentiates dauer formation, in which animals can live for up to 70 days with no food [292].

Two *C. elegans* sexes exist: self-fertilizing hermaphrodites (XX) and males (X0). Males compose only 0.1% of the population and arise through a spontaneous non-disjunction in the hermaphrodite germ line that occurs more often under stressful conditions. After reaching adulthood, wild type hermaphrodites produce oocytes for four days and if self-fertilized, will lay 300 eggs. The number of sperm limits the number of progeny produced. Consequently, if mated with a male during the six days after the last larval molt, *C. elegans* can lay over 1000 eggs [155].

1.1.2 Anatomy

Despite displaying a large repertoire of behaviors, *C. elegans* is anatomically simple. Typical to all nematodes, the body plan of *C. elegans* consists of an outer tube and an inner tube separated by a pseudocoelomic space. The cuticle, hypodermis, excretory system, muscles, and neurons comprise the outer tube, or body wall. The inner tube consists of the pharynx, intestine, and gonad. Hydrostatic pressure in the pseudocoelomic space maintains the animals body shape. This pressure is under osmoregulation by the excretory system, such that ablation of the excretory cell, duct cell, or the pore cell that opens to the outside of the body leads to the animal swelling with fluid and dying [261, 262].

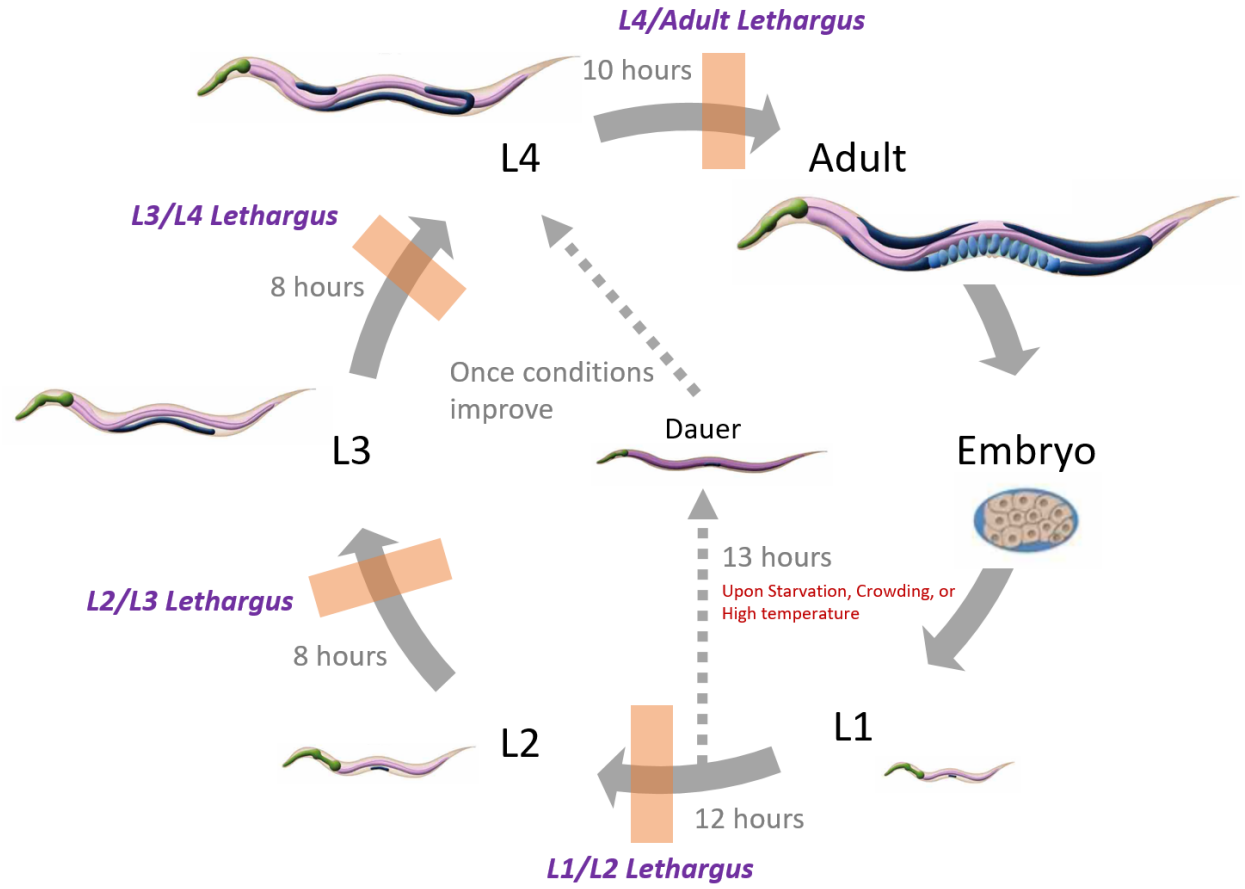

Figure 1.1: **Life cycle of *Caenorhabditis elegans* at 22 degrees C.** Preceding the molts at the end of each larval stage, *C. elegans* go through a 2 to 3 hour period of feeding and locomotion quiescence termed lethargus. Images of *C. elegans* adapted from http://www.wormatlas.org.

The lineage of somatic cells, including neurons, in *C. elegans* is essentially invariant. Taking advantage of this invariance and the transparency of the body, researchers have therefore been able to describe the complete fate map or cell lineage of the worm. 671 cells form in the embryo of which 113 undergo programmed cell death. Of the remaining 558 cells in freshly hatched L1 larva, ten percent are somatic blast cells that continue to divide through development. This process eventually gives rise to 959 somatic cells in the adult hermaphrodite and 1031 somatic cells in the adult male [340]. Of these somatic cells, adult hermaphrodites have 302 neurons which fall into 118 classes and 56 glial and support cells. Males have another 79 neurons and 36 glial and support cells, mostly due to differences in the male tail which is essential for mating. However, males are less well-studied than hermaphrodites, and new discoveries about male development and circuits are still occurring. For example, researchers recently discovered two new glia-derived interneurons in males needed for prioritizing mating behavior during adulthood [300].

1.1.3 Nervous system of C. elegans

Through painstaking reconstruction of serial electron micrographs, a complete synaptic connectivity map (i.e., a connectome) also exists for the nervous system of the adult hermaphroditic worm [397]. Although some overlap exists, the nervous systems is more-or-less organized into four layers: the sensory neurons, the first layer of interneurons that receive sensory information, and the second layer of command interneurons that integrate sensory and interneuron information before relaying it to the fourth layer, the motor neurons.

Currently, this is the only extant connectome that is complete. Given the anatomical differences between males and hermaphrodites, effort was recently expended to produce a second partial connectome: that of the posterior nervous system of the *C. elegans* male [171]. While the obvious anatomical differences between males and hermaphrodites exist in the tail, it is still unknown whether wiring differences between the nerve rings of males and hermaphrodites are responsible for many distinctly male behaviors.

Producing complete wiring diagrams remains a technical challenge to this day, but the heroic 12 person-year effort to create the first map allowed circuit-level analysis of several behaviors that for many years was not possible in any other organism [397]. The current rate-limiting step in determining a connectome is reliable extraction of all the features that define synaptic contacts from serial electron micrographs. Historically, this was done by marking synaptic densities, synaptic vesicles, and gap junctions in thousands of paper printouts by colored pens. While many questions exist about how connectivity changes in developing animals, how learning influences connectivity, how the wiring diagram changes in presumptive connectivity mutants, and how similar connectomes are between closely related species, the labor and difficulty of producing wiring diagrams has thus far prevented the determination of other complete connectomes. Computational methods to find relevant features in electron micrographs promise to simplify this process in the future [404]. Furthermore, advances in machine learning will likely expedite this process.

Aside from the challenge of constructing complete wiring diagrams, there is a question about how useful synaptic connectivity maps are. While classifying the parts of the nervous system is necessary to an understanding of how behavior is produced, it is not sufficient. From existing maps for *C. elegans*, crustaceans, fruit flies, and the vertebrate brain, it appears that the anatomical connections represent only a set of potential connections that depend on internal states and context. Neuromodulators such as neuropeptides and biogenic amines are known to modify synaptic efficiency, excitability, and neuronal dynamics [23]. Moreover, anatomically-defined synapses do not necessarily associate with neuromodulator activity. Cells release neuromodulators synaptically and extrasynaptically. Furthermore, neuromodulators act on targets locally and at long distances [23]. These caveats pose a profound challenge to identifying the neural circuits that control behavior from wiring diagrams alone. As an example of the animals internal state modifying behavioral circuits, it is known that under well-fed conditions, the ASH nociceptive neurons mediate octanol avoidance [56]. After one hour of starvation, the AWB, ADL, and ASH neurons mediate the avoidance to

octanol. Later studies showed that neuropeptides, dopamine, tyramine, octopamine, and serotonin all act to impose this shift in the circuitry [246, 402].

Despite the ambiguity of anatomical wiring diagrams given context, anatomical reconstruction of synaptic connectivity leads to new hypotheses and offers valuable information about how nervous systems produce behavior. Because only two neurons are needed for viability, one powerful method for examining the *in vivo* role of individual cells in *C. elegans* is the severing of nerve processes or complete ablation of neurons or neuron progenitors using lasers [101]. Combined with the knowledge from the wiring diagram, laser microsurgery allows the identification of roles for neurons with obvious phenotypes. In this dissertation, I use similar methods to identify a novel sensory role for an interneuron previously implicated in regulating behavioral quiescence during L4 lethargus.

As an example, much of what is known about *C. elegans* locomotion has been inferred from combining ablation experiments with knowledge of connectivity. While *C. elegans* are not segmented worms, electron microscopy reconstruction identified that six repeating units of 12 motor neurons and 12 muscles distributed along the body comprise the adult motor circuit [139, 397]. The D-type motor neurons make inhibitory GABAergic synapses with muscle cells. In contrast, the A and B-type motor neurons that are presynaptic to the D-type neuron make excitatory cholinergic neuromuscular junctions (NMJs) with muscle cells. The class A and class B neurons receive input from the AVB, PVC, AVA, and AVD interneurons in the central nervous system. Laser ablation of these neurons revealed that AVB and PVC promote forward locomotion where the AVA and AVD neurons promote backward locomotion. Originally, it was hypothesized that contralateral inhibition mediated by the GABAergic motor neurons accounts for the ability to form and propagate body bends based on laser ablation of the DD neurons during L1 [53]. However, ablation of the DD neurons later in development does not abolish bending and mutants that lack GABA have normal (albeit hypercontracted) forward locomotion [90, 243]. Taken together, these results imply that developmental remodeling takes place and that contralateral inhibition

has a modulatory rather than necessary role in adult movement.

Indeed, John White and his colleagues first noted this remodeling of the dorsal GABAergic D-type neurons in L1 larva before reconstructing the adult *C. elegans* connectome [396]. However, the remodeling of these DD motor neurons in early *C. elegans* development is still not completely understood [417]. Interestingly, without any change in the overall morphology of the neurons, the DD neurons eliminate pre-existing synapses and form new synapses starting near lethargus (a period of behavioral quiescence) at the end of the L1 stage. Genetic redundancy involving many pathways guards this process, but the timing is under control of the ubiquitous developmental timer, *lin-14* [209].

In general, whether there are critical periods of *C. elegans* development in which remodeling of neurons and synapses takes place has not been closely studied. From the few examples that exist, the molts as the end of larval stages may demarcate periods in which large development changes occur. During L4 lethargus but not as an adult, there is a reduction in transmission at GABAergic synapses at the body wall NMJ, suggestive of synaptic plasticity during the molt [83]. Moreover, like the case of DD remodeling, there is a shift in the role of GABA in the defecation cycle of males. In adult males, GABA released from the AVL and DVB motor neurons contracts the sphincter muscles and leads to expulsion in the defecation cycle. However, the same neurotransmitter released in L4 larva has the opposite effect [288]. The observation of GABAergic motoneurons adding NMJ-like structure during late L4 larval development also suggests that molts may be an essential developmental period for large synaptic changes to occur [48]. Intriguingly, the grinder of the pharynx only increases in size during molts [116] and HSN accumulates synaptic material during L4 lethargus [61]. Chapter III of this dissertation addresses this phenomenon further by examining long term defects that result from nonlethal deprivation of quiescence during L4 lethargus.

1.1.4 Genetics of C. elegans

The simplicity of maintaining *C. elegans* in addition to their relatively simple biology make *C. elegans* highly amenable to genetic analysis. *C. elegans* hermaphrodites have five pairs of autosomes (I-V) and two sex chromosomes (X/X). Males have five pairs of autosomes (I-V), but only a single sex chromosome (X/0). Because only 0.1% of the population are males and hermaphrodites self-fertilize, mutant strains can be easily isolated and propagated. For example, it is possible to isolate hermaphrodites that are homozygous for a recessive mutation without further mating. For heterozygous worms, a quarter of the F1 progeny will be homozygous for the mutation.

The genome of *C. elegans* is small and was the first multicellular animal genome to be completely sequenced. The genome itself consists of an estimated 20,000 genes spread across approximately 10^8 base pairs. Of these genes, essential genes comprise only about a tenth of the total [35]. Because *C. elegans* have a large brood size consisting of about 300 progeny per worm and about 10^5 worms can be cultivated on petri dishes, screening for mutations is highly efficient. This fact combined with the frequency of inducing mutations using chemical mutagenesis or other methods makes it possible to carry out saturation mutagenesis and find all of the genes in which loss of gene function confers a phenotype of interest. As a result, the *C. elegans* community has identified a large number of mutants that it stores and distributes at the publicly funded *Caenorhabditis* Genetics Center (CGC). Many to all of the genes involved in dauer formation, vulva development, and the mechanosensory response to light touch have been identified, and the mutants are available to researchers for a nominal fee [52, 104, 293].

In addition to classical forward genetic screening using mutagens to induce DNA lesions, a bevy of tools for reverse genetic manipulation of the *C. elegans* genome also exist. In reverse genetics, one observes the effect on the development or behavior of an organism after altering a gene's function. This method has several advantages over classical forward screening, but it requires genome sequence information and tools to target specific genes. In

the case of *C. elegans*, many such tools exist. For example, Nobel Prize winning work by the labs of Andrew Fire and Craig Mello identified a process termed RNA interference (RNAi): by injecting double stranded RNA into a worm, one can target semi-specific degradation of the corresponding mRNA [105]. Others improved upon this method by showing that it is possible to induce knockdown by soaking in dsRNA solution or feeding worms bacteria that produce dsRNA [344, 355].

The introduction of Green Fluorescence Protein (GFP) by Martin Chalfie, Osamu Shimomura, Roger Tsien and others was also transformative to the study of *C. elegans* and other model organisms [50]. *C. elegans* readily take up DNA as extrachromosomal arrays when injected into the gonad of young adult hermaphrodites. By coupling the coding sequence of GFP with the promoter of a gene, it is possible to quantify changes in gene expression over time. Moreover, by using tissue or cell-specific gene promoters, it is also possible to target expression of proteins-of-interest (such as GFP) to single cells or a set of cells. While one purpose of such a technique is to locate where a protein is needed to "rescue" a mutant phenotype, one can also use semi-specific cell reporters to target engineered proteins to a desired location. One such class of engineered proteins used throughout this dissertation and in neuroscience more generally are genetically-encoded calcium indicators (GECI) [353]. Upon depolarization, a large and rapid influx of calcium occurs in the cell body of *C. elegans* neurons. After calcium binds a calcium sensing domain, GECIs undergo a conformational change leading to an increase in the fluorescence intensity of one or two fluorescent proteins. In this dissertation, I primarily use single fluorophore sensors, the most common of which is GCaMP. Current iterations of GCaMP require less intense excitation light and therefore can be imaged for longer without phototoxicity. One caveat with the use of such molecules is that they can potentially perturb cell-signaling and lead to abnormal behavioral phenotypes. As an example, expression of iterations of GCaMP in chemosensory neurons prior to GCaMP3 result in decreased turning [354].

1.2 Overview of sleep

"If sleep does not serve an absolutely vital function, then it is the biggest mistake the evolutionary process has ever made." - Allan Rechtschaffen

1.2.1 What is sleep?

Sleep is an evolutionarily conserved biological process in essentially all animals with a nervous system. The behavioral properties that characterize sleep are reversible quiescence, increased arousal threshold, sensory gating, a specific posture, and homeostasis. In accordance with its universality, animals require sleep to survive. Sleep deprivation of rats resulted in severe pathology and death whereas the same stimuli given to non-sleep deprived rats had no effect [286]. In humans, there is an association between disturbances in sleep and a wide variety of clinical pathologies including cognitive impairment, metabolic, and psychiatric disorders [14, 55, 201, 202]. Nevertheless, there is not a clear consensus on why deprivation is harmful, and despite its importance to survival, the core function of sleep remains a mystery.

At an abstract level, sleep has been modeled as a function of two independent processes: Process C that controls the circadian rhythm of sleep and a homeostatic Process S that controls the quality and amount of sleep [34]. These two processes act independently but interact continuously. Sleep occurs when Process S reaches the upper threshold of Process C. In contrast, waking occurs when Process S reaches the lower threshold of Process C. Evidence showing that the homeostatic response to sleep persists even after disruption of the circadian rhythm supports this model [88].

More is known about the circadian clock mechanism that underlies Process C than the sleep homeostat of Process S. In invertebrates as well as vertebrates, mutations in clock genes disrupt sleep [204, 317]. In mammals, the circadian clock is maintained by transcription factors and kinases encoded by approximately ten clock genes. These clock components oscillate with a period of approximately twenty-four hours and act through a series of au-

toregulatory feedback loops in response to external cues such as light, temperature, and food. Clock genes oscillate in both the suprachiasmatic nucleus (SCN) of the mammalian brain and in peripheral tissues. Conventionally, it was thought that the SCN acts as a pacemaker and drives oscillations of clock genes in peripheral tissues, but this was later overturned by the observation that cultures of mammalian fibroblasts continue to express circadian cycles after a serum shock [21]. Several major organ systems including the liver, heart, kidney, and skeletal muscle were later shown to have their own intrinsic circadian clocks that oscillate [407]. Nevertheless, it appears that the circadian regulation of these oscillations is hierarchical given that prolonged cultures of peripheral tissues show dampened oscillations whereas SCN rhythms persist. The current model is that SCN-dependent cues synchronize and sustain circadian cycles at the level of individual organs. The pathways in which the "master clock" in the SCN control the peripheral clocks in other tissues are still ill-defined, however [140].

1.2.2 Synaptic Homeostasis Hypothesis of Sleep

In contrast to the workings of the circadian clock, the cellular and molecular mechanisms that govern the sleep homeostat (i.e, Process S that drives the need to sleep) largely remain unknown. At its core, the role of the sleep homeostat is to measure how long an organism has been awake and adjust sleep such that the total amount of sleep that an organism gets is adequate. Unlike the regulation of the circadian clock, dedicated genes that specifically function to regulate sleep have not been identified. Rather, the genes that alter sleep are also involved in normal neuronal function. This had led to several hypotheses about the core function of sleep. Because sleep also facilitates the consolidation of long-term memory, sleep may be required for nervous system plasticity [338]. The synaptic homeostasis hypothesis (SHY) proposes that sleep allows animals to renormalize synaptic strength after learning because the brain cannot sustain infinite increases in synaptic strength [359]. However, while numerous lines of evidence support the theory that sleep plays a role in synaptic downscaling,

it is unlikely to be the core function of sleep because if true, one would expect that sleep scales with nervous system complexity which does not seem to be the case. For example, elephants and giraffes sleep less than rodents [323]. Moreover, some species of birds and cetaceans during seasonal mating, migration, or birthing do not sleep for prolonged periods without any functional deficits or sleep rebound [216, 229, 230, 284, 325].

1.2.3 Energy Conservation Hypothesis of Sleep

A major obstacle to understanding the core function of sleep is to differentiate between functions that sleep is important for versus the function that sleep originally evolved for. It is advantageous for a mechanism to evolve multiple functions over time if adaptive, but this can mar our ability to distinguish what the most essential function is. Because of this, it is beneficial to examine phlyogenetically ancient organisms that sleep. It can also be helpful to conduct comparative biology between closely related species that have different sleep behaviors. One such study examined sleep behavior of cave-dwelling versus conspecific surface-dwelling species of Blind Mexican Cavefish [92]. Interestingly, all three cave-dwelling species still slept, but they all converged to sleeping less than a quarter of the time that the surface-dwelling species slept. This suggests that sleep is an evolutionary labile phenotype, but it still does not address what function sleep serves.

Another hypothesis for sleep put forward is that inactivity itself may be adaptive depending on ecological factors [324]. However, in the case of the cavefish, it is not clear what is adaptive about wakefulness. The idea that inactivity itself may be adaptive came from associations between sleep need and the need to be vigilant from predation. Carnivores on average have the highest sleep need while herbivores have the lowest. However, the cavefish examined do not have any predators. A better explanation for the correlation might be that energy gained from food influences sleep. It is possible that the environment that cavefish occupy is not as food rich as the surface and therefore cavefish have evolved to spend more time awake in order to find food. However, it is not clear why the cavefish did not evolve to

not sleep at all. The explanation that sleep might have evolved to avoid predation also does not account for the existence of sleep rebound.

Furthermore, if it were true that sleep evolved to conserve energy when it is not efficient to forage, then one would expect that metabolism to be lower during sleep than awake. However, by monitoring changes in ATP levels, sleep has been shown to promote anabolic processes [93] and REM sleep in particular is a state of increased brain energy metabolism [285].

1.2.4 Restoration Hypothesis of Sleep

A related theory to the energy conservation hypothesis is that sleep is restorative [245]. It is not clear what is restored from sleep, however. One study found that wakefulness leads to depleted glycogen levels and sleep restores glycogen in the brains of rats [31]. Supporting this, brain glycogen levels were previously shown to increase close to the beginning of sleep and fall shortly after waking [183]. However, as sleep or wake periods continue, there was no further change in glycogen levels leaving open the question of why additional sleep is necessary. Moreover, if the restoration hypothesis were to explain why sleep exists, it would have to account for why species with the largest bodies and brains often sleep the least despite presumably requiring more cellular recovery.

1.2.5 Energy Allocation Hypothesis of Sleep

A recent theory proposes that because energy is a scarce resource, organisms evolved different behavioral states to partition competing biological processes away from each other [308]. This model proposes a unifying explanation for not only sleep, but also for different stages of sleep and torpor. Related to the well-studied life history strategies of species (e.g, r-selected versus K-selected tradeoffs in how much animals choose to invest in offspring), the energy allocation hypothesis suggests that sleep evolved as a trade-off strategy. At a given point in an animal's life history, energy is a finite resource allocated to growth (G), maintenance

(M), and reproduction (R), which together sum to 1 (G + M + R = 1) [336].

The energy allocation hypothesis states that in order to survive species must expend energy on many processes including but not limited to: predation avoidance, reproduction, foraging, sensation, movement, growth, maintenance and repair, and immune function. Because energy is a scarce resource, a conflict exists between the biological processes involved in running the machine ("waking effort": WE) and maintaining the machine ("biological investment" = BI). This conflict ultimately results in the coupling of biological operations with behavioral states. Akin to life history theory, this model suggests that waking effort and biological investment sum to 1 (WE + BI = 1) and that animals will evolve different partitioning strategies of WE and BI to maximize fitness. In other words, sleep quotas between species depend on ecology and may be explained by how much BI occurs during wake versus sleep-states.

This unifying framework addresses many of the criticisms targeted at the synaptic homeostasis, energy conservation, and restoration hypotheses while also offering interesting predictions. The energy allocation (EA) model postulates that some biological processes occur preferentially during sleep while others occur primarily during wakefulness. Consistent with this state-dependent division, genes in the brain that are upregulated during sleep or wake-states belong to distinct functional classes [67, 70]. Others have recently noted that molecules that promote organelle trafficking often are necessary for proper sleep [241]. This encompasses and extends the synaptic homeostasis hypothesis (SHY). One criticism of SHY is that sleep does not scale with nervous system complexity. The energy allocation model, however, is not exclusive to the nervous system and predicts that peripheral tissues will also show differential expression based on behavioral state. One of the few studies to examine expression differences in peripheral tissues based on behavioral state found that the liver expresses three times as many sleep-dependent transcriptional changes than the brain [236].

The energy allocation model also addresses sleep homeostasis. According to the EA model, chronic sleep deprivation will push BI processes that typically occur during sleep to

periods when the animal is awake while simultaneously having to meet the energy demands of WE. As a result, sleep deprivation induces a rise in energy requirements, and EA predicts that there will be a rising increase in hunger and food intake as the energy requirements compound. Indeed, sleep restriction in humans results in increased appetite and food intake [131, 200]. Experiments in rodents corroborate this result, and providing sleep-deprived animals with high caloric food allowed animals to better withstand the stresses of deprivation [99]. Moreover, the EA model predicts that too much sleep will also lead to lower fitness. Low amounts of sleep and high amounts of sleep are both associated with increased mortality [346]. According to EA, the lack of sleep rebound without deficits in migrating animals predicts an upregulation of BI during waking. This can potentially be tested. Similarly, EA predicts that some naturally short-sleeping animals may partition more BI during waking or are more efficient at handling the demands of BI. This can also be tested. Interestingly, elephants, which require relatively little sleep, were recently found to have 20 copies of the p53 tumor-suppressor homolog involved in repairing DNA damage [341]. Short sleeping cave populations of Blind Mexican Cavefish also show enhanced DNA repair gene expression and activity in comparison to surface populations [28].

Importantly, the EA model also addresses how injury, sickness, large developmental changes, and over-exertion are associated with behavioral quiescence. Because all biological processes carry an energy cost, trade-offs exist. It is well-established that mounting an immune response requires significant resources and that other physiological demands can lead to dampened immune responses [38, 39]. Under the EA model, mitigating cellular damage from injury, sickness, or over-exertion necessitates a shift from WE to BI. Likewise, both starvation and over-eating are expected to require more BI. As a result of these imbalances, EA predicts an increase in sleep quotas. However, the EA model also predicts that this may complicate the elucidation of universal pathways that regulate sleep. The EA model postulates there are many cellular processes that must be performed, but because there is an energetic advantage to coordinating compatible processes, partitions will naturally occur.

However, if perturbed, there will be an obligation to meet some cellular processes sooner and "sleep-like" behavior may be observed at atypical times. It is possible that the pathways that trigger these different sleep-like behaviors may be distinct. It is also likely that differences exist among species. Nevertheless, if true, the energy allocation model broadens the definition of sleep.

I favor the energy allocation model of sleep; however, the model only serves as a framework and leaves several questions unanswered. For example, what are the switches that turn on sleep? Furthermore, it will be important to identify what the distinct processes are that occur during sleep versus waking to identify biomarkers of sleep. For instance, is it possible to tell when sleep will occur or how sleepy an animal is from molecular markers? Relatedly, from such potential biomarkers, is it possible to discern how much sleep an animal needs?

1.2.6 C. elegans *sleep*

A major challenge to finding the core function of sleep is that it likely evolved adaptive functions beyond its original function that mar our ability to distinguish what sleep's essential function is across phylogeny. Because of this, it is beneficial to examine simple and phylogenetically ancient organisms that display sleep-like behavior.

In this dissertation, I use the nematode *Caenorhabditis elegans* to study the molecules and cells that regulate sleep because it is the simplest animal described to sleep. *C. elegans* display quiescence states during larval transitions [283], after stresses such as heat shock [151, 176], during satiety [411], and after infection [228]. However, the quiescence states during larval transitions (developmentally-timed sleep (DTS)) and to a lesser degrees following stress (stress-induced sleep (SIS)) are the most studied and bear many similarities to sleep in vertebrates and other invertebrates [367]. I will focus entirely on developmentally-timed sleep in this dissertation. In this introduction, I will first describe what it is known about developmentally-timed sleep, and then I will briefly describe stress-induced sleep.

C. elegans pass through four larval stages before becoming a sexually-mature adult.

Punctuating the transitions between larval stages are molts in which the worm synthesizes a new cuticle and sheds its previous one (Figure 1.1). Accompanying each molt is a 2-3 hour period of behavioral and feeding quiescence referred to as lethargus [328]. While this quiescence was first described in nematodes 100 years ago [380], it was not described as a sleep-like state until 2008 [283].

What criteria define sleep? Until the 1930s, three behavioral criteria defined sleep in all animals: reversible quiescence, decreased responsiveness, and an increased arousal threshold [199]. The invention of the electroencephalogram (EEG) led to the discovery that brain activity patterns during sleep are variable, but that slow wave activity (SWS) can act as a biomarker for sleep under certain conditions [227, 342]. EEGs are not feasible in non-mammalian animals, so sleep in *C. elegans* must be defined based on behavioral criteria and molecular conservation. Lethargus, or developmentally-timed sleep, is characterized by a typical posture, reversible behavioral quiescence, decreased responsiveness to stimuli, and as shown here, homeostatic responses to quiescence deprivation that vary depending on the strength of the stimulus [62, 91, 167, 254, 283, 367]. Moreover, deprivation of quiescence during lethargus can be lethal [91] and as shown in this dissertation, can lead to long-term deficits in distinct behaviors and tissues if left unmitigated by specific stress-response pathways.

While *C. elegans* lethargus shares many commonalities with sleep in other invertebrates and vertebrates, developmentally-timed sleep occurs with an ultradian rather than a circadian periodicity. In *C. elegans*, lethargus occurs only four times during larval development with a period of seven to nine hours [47]. Nevertheless, conserved molecular pathways that regulate sleep in other species also regulate developmentally-timed sleep. For example, LIN-42, the *C. elegans* homolog of PERIOD, cycles in phase with molting and lethargus despite PERIOD oscillating with a circadian rhythm in *Drosophila melanogaster* and mammals. Mutants of *lin-42* alter the timing and duration of molting and lethargus [249]. Moreover, molecular pathways that alter sleep in *D. melanogaster* and mammals also alter quiescence

during lethargus. For example, similar to other species, EGF signaling in worms has been shown to promote sleep [376], and PDF [63], cAMP [167, 283, 326], and dopaminergic signaling [327] have all been shown to promote waking. In this dissertation, I further show conservation of neuropeptide-Y signaling in sleep homeostasis and the conservation of the endoplasmic reticulum UPR in mitigating the effects of sleep deprivation.

Because of the simplicity of the *C. elegans* nervous system, some of the cells required to turn on developmentally-timed sleep have also been identified. A common theme among these neurons is that they are all peptidergic. The GABAergic interneuron, RIS, and the paired glutamatergic RIA interneurons have been implicated in regulating behavioral quiescence during lethargus with RIS having a stronger effect [264, 373]. Previously, a peptidergic interneuron called ALA was implicated in regulating developmentally-timed sleep, but recent studies including work done in this dissertation suggest that it is not linked to developmentally-timed sleep but rather stress-induced sleep.

C. elegans stress-induced sleep is a state of behavioral and feeding quiescence that results from cellular stress during any developmental period [151, 176]. While stress-induced sleep is associated with reversible behavioral quiescence and an increased arousal threshold, it has not been documented whether there is a homeostatic response to deprivation or if there is a typical posture. Nevertheless, deprivation of sleep-induced sleep does increase mortality [151]. Stresses that result in stress-induced sleep in *Drosophila melanogaster* and *C. elegans* include heat shock, cold shock, osmotic shock, UV light, and bacterial pathogens [151, 215]. One unanswered question that has not yet been addressed is whether prolonged periods of waking are themselves stressful and might trigger stress-induced sleep. Longitudinal imaging of adult *C. elegans* behavior may address this question in the future.

How does stress-induced sleep in *C. elegans* differ from developmentally-timed sleep? In addition to the neurons that trigger sleep in the two types, the signaling molecules identified so far that promote quiescence differ between the two types of sleep. In stress-induced sleep, ALA neuropeptides encoded by *flp-13*, *flp-24*, and *nlp-8* are collectively required to induce

sleep [260]. Alone, each mutant has small or no effect on quiescence. While it is possible that some or all of these neuropeptides are important for quiescence in developmentally-time sleep as well, only FMRFamide peptides encoded by *flp-11* and released from the GABAergic RIS neuron have been shown definitively to reduce locomotion quiescence during lethargus. A common theme among these "behavioral switches" that trigger the two types of sleep is that they are all neuropeptides. Possibly, this similarity is a consequence of the unique ability of neuropeptides to diffuse broadly throughout the animal and affect the coordination of multiple circuits. In Chapter II and Chapter III of this dissertation, I present data suggesting that the action of neuropeptides may be important for such coordination.

1.3 Toolkit

Caenorhabditis elegans has been a successful model organism not only due to its biological simplicity, transparent body, invariable cell lineage, small genome, fast generation time, large brood size, ease of maintenance, but also the presence of a collaborative community of worm researchers that freely develop and share tools and resources. Here, I will discuss some of the tools extensively used throughout the work presented in this dissertation. Specific methods are discussed at the end of chapters. When not noted as gifts from other labs, mutant strains used in this dissertation were obtained from the publicly funded *Caenorhabditis* Genetics Center operated at the University of Minnesota.

1.3.1 Microfluidics as an imaging tool

To image *C. elegans* for several hours, we make use of polydimethylsiloxane (PDMS) microfluidic chambers with evenly spaced posts (Figure 1.2). These chambers, also known as "artificial dirt", provide a transparent, structured substrate for *C. elegans* to crawl as they would on agar while allowing the experimenter to image with a fixed field of view and focal plane [226]. As an advantage, artificial dirt chambers can be mounted to slides thereby per-

mitting the use of high magnification, high numerical aperture microscope objectives. This dramatically increases image quality versus imaging on agar, especially when imaging *C. elegans* neurons engineered to express fluorescent probes. To improve the signal-to-noise ratio of such experiments, nearly all the expression reporter and calcium imaging experiments in this dissertation (with the exception of the ALA experiments in Appendix I) were performed using artificial dirt chambers sealed by a coverslip with VALAP (a biologically inert 1:1:1 mixture of Vasoline, lanolin, and paraffin wax). All behavior imaging is similarly performed in artificial dirt chambers. Animals inside the chambers swim in OP50 bacteria suspended in Nematode Growth Media (NGM). To facilitate the analysis described in the next section, each observation chamber contains a single animal.

Another benefit of soft lithography is that one can easily cast arbitrarily shaped features to constrain the worm. In the optical and electrophysiological recordings of *C. elegans* pharyngeal pumping, we make use of such devices to immobilize the worm while providing a constant flow of OP50 or exogenous serotonin.

1.3.2 Image analysis

Two complementary methods are used throughout the experiments discussed in this dissertation to determine motion and quiescence: (1) a frame-subtraction method and (2) PyCelegans, a posture-based method. While frame-subtraction is fast, it conveys information only about motion rather than the finer details of behavior. To address this, our lab developed PyCelegans which leverages parallel computing resources to identify the body of the animal in each frame, segment the midline, and determine the position and propagation of bends based on changes in the angles of the midline over time. This posture-based method allows us to classify motion as forward, backward, dwelling, or quiescence at the cost of lower throughput. Because these methods are extensively discussed, I will briefly describe them here.

The frame-subtraction method counts the number of pixels that change for each pair of

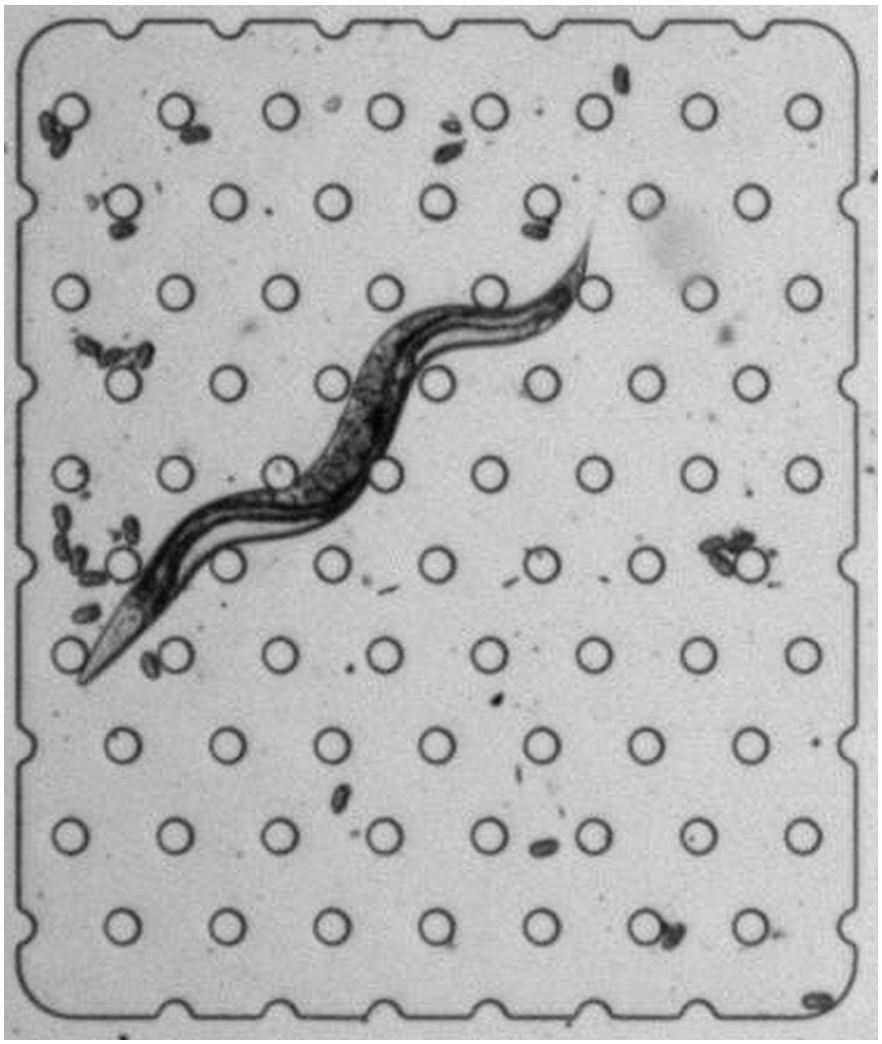

Figure 1.2: **An example of "artificial dirt".** An example image of a polydimethylsioxane (PDMS) microfluidic chamber with an adult *C. elegans* worm approximately 1mm in length. Image was taken from an experiment assaying the integrity of the egg-laying circuit after sleep deprivation by optogenetically stimulating the HSN motor neurons that control egg-laying.

consecutive image frames and smooths the resulting time series using a Gaussian filter with a width of 1 frame. A pixel is considered to have changed when its intensity changes by a value >30, an experimentally determined threshold. Quiescence is then defined when <2 pixels change in the smoothed time series. To calculate the quiescence fraction of lethargus, we find the fraction of frames that the worm is non-motile over a 10-minute running window as previously described [283]. For this method, we image at 2 frames per second at a 1.25X magnification for 12 hours. By adjusting lighting conditions, background pixel intensity is set to approximately 220 using a greyscale range of 0-255.

In contrast, PyCelegans, a posture-based method, requires imaging at 10 frames per second at a 4.2X magnification. To emphasize the contrast of the worm's body, one should set the background level higher than that for frame-subtraction (230-250 on a greyscale range of 0-255). The midline of the animal is divided into 20 equal segments, and the dynamics of angles formed by the inner 18 segments is calculated. Forward locomotion is defined by the propagation of bends in the anterior to posterior direction for 3 or more consecutive midline segments. Backward locomotion is similarly defined in the posterior to anterior direction. Dwelling is defined as non-directional propagation of body waves. Quiescence was defined when the dynamics of 17 angles did not exceed 0.01 radians/sec.

1.3.3 Physiology

While it is standard to monitor neural activity by recording electrical activity from neurons, these techniques are less feasible in *C. elegans* because (1) the neurons of *C. elegans* are only 2-3 microns in diameter and (2) the tough proteinacious cuticle enclosing the pressurized pseudocoelom makes penetration by electrodes difficult. However, because the cuticle is transparent and it is relatively straightforward to generate transgenic animals expressing reporters in neurons of interest, genetically-encoded calcium probes such as GCaMP are often used as a less invasive substitute to record neural activity in intact, freely-behaving worms. Nematodes lack voltage-gated sodium channels, therefore calcium entry through voltage-

gated channels is thought to be essential for excitability of *C. elegans* neurons. While only acting as a proxy for neural activity and limited by their inability to detect subthreshold membrane potential changes, genetically-encoded calcium indicators have distinct advantages other than ease of use. For example, recent developments in tracking have permitted "brain-wide" imaging of *C. elegans* neurons simultaneously using GCaMP [185, 310, 381]. Impressively, this pan-neuronal imaging is possible not only in immobilized but also freely moving animals, thereby potentially leading to more complete descriptions of *C. elegans* behavior at the the neuronal level. In the ALA work presented in this dissertation, I make extensive use of GCaMP to characterize ALA's physiological responses to picking-like touch. Other than neurons, GCaMP can also be used to image muscles. In Chapter III of this dissertation, I make use of GCaMP to image the vulval muscles of the egg-laying circuit.

1.3.4 Mechanical perturbations

The most used method for depriving *C. elegans* of developmentally-timed sleep in this dissertation is mechanical deprivation with piezo buzzer elements (Figure 1.3). In brief, a standard worm plate (60mm x 15mm) containing 10mL of Nematode Growth Medium with agar is placed in a clamp mounted with four piezo buzzer elements. Stimuli are delivered in the form of 1kHz vibrations whose timing and duration are controlled using a custom Matlab script. For "weak" stimuli described in homeostasis experiments, we use 0.4 seconds of 1 kHz vibrations repeated every 15 minutes throughout lethargus. For "strong" stimuli in similar experiments, we use 15 seconds of 1 kHz vibrations repeated every 15 minutes throughout lethargus. We determined weak and strong based on locomotion responses outside of lethargus. In contrast to the homeostasis experiments, to investigate long-term deficits from sleep deprivation beyond the regime that evokes homeostasis we used a 1 kHz square wave stimulus with a period of 6 minutes and a duty cycle of 50%.

In addition to mechanical stimuli, we used blue light (λ = 475±15nm) as a "weak" stimulus in Chapter II. The light was supplied by a Luxeon Star 7-LED assembly with a

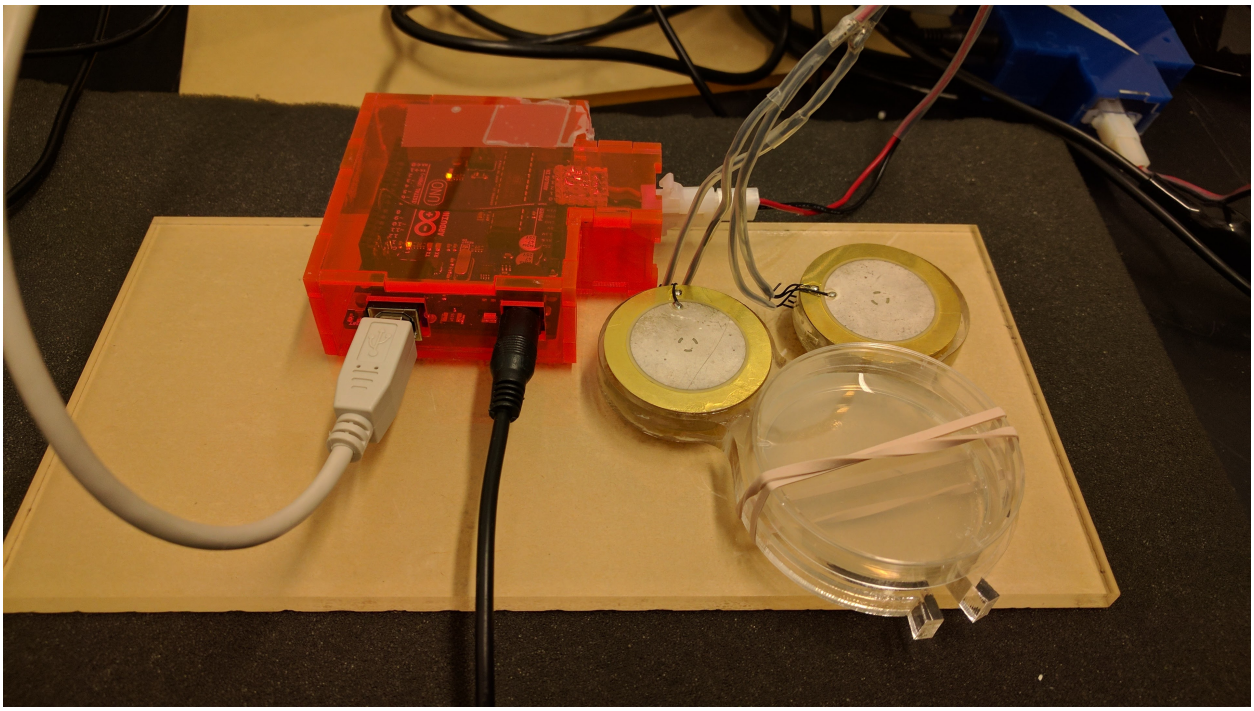

Figure 1.3: **Clamp to deliver mechanical stimuli to *C. elegans*.** Example image of the clamps used in this dissertation to deliver mechanical stimuli to *C. elegans*. An acrylic clamp with "Mickey Mouse ears" was laser cut and mounted with four piezo buzzer elements (Digikey part no. 668-1190-ND). A standard worm plate (60mm x 15mm polystyrene petri dish) is shown inside the clamp. The timing and duration of the stimuli are controlled using a custom Matlab script.

diffused optic array driven by a 700mA FlexBlock driver. This assembly was mounted to the microscopes approximately 7cm from the animals. Light intensity was measured at the location of the animals before experiments, and the timing of the stimuli was controlled using LabView (National Instruments Inc., Austin, TX).

1.4 Scope of the dissertation

Sleep is an evolutionarily conserved biological process in essentially all animals with a nervous system, and total sleep deprivation can result in death. Disruptions to sleep, especially during development, can lead to lasting deficits in chordates and arthropods, but there is not a clear consensus on the mechanisms that govern sleep or why sleep deprivation is harmful. Furthermore, the core function of sleep remains a mystery despite its importance to

survival. This dissertation uses the simplest animal known to sleep, the free-living nematode *Caenorhabditis elegans*, to dissect how a phylogentically ancient form of sleep is regulated in response to perturbations.

Chapter II identifies two behaviorally and genetically distinct homeostatic mechanisms that regulate behavioral quiescence during *C. elegans* lethargus. In addition to showing conservation of molecular mechanisms that govern sleep with other animals, this work is the first to suggest that the routine homeostatic stabilization of sleep differs from the homeostatic compensation to strong sleep disturbances. Moreover, this work illustrates the importance of neuropeptides in modulating sleep and suggests that common stress-response pathways may play an essential role to *C. elegans* sleep.

Chapter III extends the work in Chapter II by exploring long-term deficits in various tissues and circuits that result from deprivation of *C. elegans* lethargus beyond the level of homeostatic compensation. We find that nonlethal deprivation of *C. elegans* sleep can result in proteotoxic stress leading to pumping deficits and a reduced brood size as a result of germline apoptosis. As suggested in Chapter II, common stress-response pathways mitigate this proteotoxic stress, but intriguingly, we find that the stress responses can vary between circuits and tissues. Importantly, our deprivation protocol does not trigger these stress responses when applied outside of lethargus.

Chapter IV summarizes the conclusions of the work in this dissertation, and I speculate about this work and the work of other studying *C. elegans* lethargus implies about the phenomenon of sleep.

Lastly, experiments performed in Appendix I suggest that a *C. elegans* interneuron originally implicated in developmentally-time sleep but recently found to be essential for stress-induced sleep also acts as a nociceptor.

CHAPTER 2

HOMEOSTASIS IN *C. ELEGANS* SLEEP IS CHARACTERIZED BY TWO BEHAVIORALLY AND GENETICALLY DISTINCT MECHANISMS

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This chapter is adapted from Nagy et al., eLife 2014. JS contributed to the conception and design of the study, performed, analyzed, and interpreted behavioral assays, screened and identified the molecular and cellular mechanism for the behavioral response to weak stimuli, and performed the fluorescent reporter expression experiments (Figures 2.10, 2.11, 2.12, 2.13, 2.14, 2.19, 2.20, 2.21, 2.22 C). SN contributed to the conception and design of the study, performed, analyzed, and interpreted posture-based behavioral assays, and identified the molecular mechanism for the behavioral response to strong stimuli (Figures 2.5, 2.6, 2.7, 2.9, 2.15, 2.16, 2.22 ABD). NT contributed to the conception and design of the study, performed, analyzed, and interpreted behavioral assays, and characterized the behavioral response to weak stimuli (Figures 2.1, 2.2, 2.3, 2.4, 2.17, 2.18). SI contributed to the conception of the study. IAS contributed to the conception and design of the study and the acquisition and analysis of data for the behavioral response to mechanical stimuli. EL contributed to the conception and design of the study. DB contributed to the conception and design of the study and helped analyze and interpret the data. All authors contributed to writing and editing of drafts, and approved the final manuscript. An asterisk (*) denotes equal contribution.

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2.1 Abstract

Biological homeostasis invokes modulatory responses aimed at stabilizing internal conditions. Using tunable photo- and mechano-stimulation, we identified two distinct categories of homeostatic responses during the sleep-like state of *C. elegans* (lethargus). In the presence of weak or no stimuli, extended motion caused a subsequent extension of quiescence. The neuropeptide Y receptor homolog, NPR-1, and an inhibitory neuropeptide known to activate it, FLP-18, were required for this process. In the presence of strong stimuli, the correlations between motion and quiescence were disrupted for several minutes but homeostasis manifested as an overall elevation of the time spent in quiescence. This response to strong stimuli required the function of the DAF-16/FOXO transcription factor in neurons, but not that of NPR-1. Conversely, response to weak stimuli did not require the function of DAF-16/FOXO. These findings suggest that routine homeostatic stabilization of sleep may be distinct from homeostatic compensation following a strong disturbance.

2.2 Introduction

Sleep architecture the duration, timing, and order of individual stages of sleep is derived from a combination of internal timekeeping pathways, a drive towards an appropriate baseline (sleep pressure), and external constraints. Collectively, the use of both mammalian and non-mammalian models has suggested that sleep is phylogenetically ancient and evolutionarily conserved [44, 263, 316]. The key behavioral hallmarks of sleep are episodic reduced motion, reversibility, typical postures, sensory gating, and homeostasis [44]. Generally, the homeostatic drive underlies correlations between the strength and duration of a disruption and the subsequent duration and quality of sleep. Behavioral signatures of homeostasis include faster time-courses of wake-to-sleep transitions, prolonged periods of sleep, and increased arousal thresholds following a period of deprivation that increases sleep pressure [5, 147, 251, 283, 357].

The nematode *Caenorhabditis elegans* is the simplest model organism that has been shown to exhibit a sleep-like state to date [62, 263, 283]. The 2-3 hour period of lethargus, a developmental stage that precedes the termination of each larval stage, is characterized by behavioral quiescence, a cessation of feeding, reduced or delayed responses to external stimuli, a distinct posture, and compensation following deprivation [62, 167, 283, 314, 376]. The *C. elegans* homolog of the circadian clock protein PERIOD is required for synchronization of lethargus, and its mRNA levels track the developmental/molting cycle [6, 173, 249, 350]. Additional conserved signaling pathways that exhibit functional similarities in mammalian, insect, and nematode sleep include the epidermal growth factor (EGF) [107, 205, 331, 376, 421], the cyclic GMP-dependent protein kinase PKG [212, 283, 376], cAMP-dependent signaling [124, 148, 283], G_s signaling, and genes acting downstream of dopamine signaling [327].

Homeostatic regulation within *Caenorhabditis elegans* lethargus was previously examined by manually depriving the animals of quiescence. After a deprivation period of 30 minutes during lethargus the onset of long response latencies to chemical stimuli was accelerated. In addition, mechanical stimulation for 60 minutes at the time that the onset of lethargus was expected resulted in increased subsequent peak quiescence [283]. Recently, quiescent behavior and homeostatic rebound were also seen when a sleep-like state was induced anachronistically in adult animals, suggesting that developmental factors are not essential for neuromodulation during *C. elegans* sleep [62].

C. elegans can locomote forward or backward by propagating dorsoventral body-bends from anterior to posterior or vice versa, respectively. Alternatively, they move in a variety of non-directional manners collectively referred to as dwelling [111, 117, 125, 384]. During lethargus, *C. elegans* prominently exhibit quiescence the complete absence of dynamic muscle contraction. Alternating bouts of locomotion and quiescence comprise the simple architecture of *C. elegans* sleep [167, 283]. In a previous study we have shown that the durations of these bouts are correlated [167] but the mechanisms underlying this process of routine stabilization were not examined. Here we analyze the behavioral responses of sleep-

ing nematodes under undisturbed, weakly disturbed, and strongly disturbed conditions. To do so we continuously assayed the locomotion of *C. elegans* from the mid fourth intermolt stage (L4int), through the fourth lethargus stage (L4leth), and into the mid young adult stage (YA).

We found that weak photo- or mechano-stimulation transiently skewed the dynamics of bouts while preserving the characteristic pairwise correlations. Thus, under unperturbed or weakly perturbed conditions, homeostatic compensation manifested as a transient extension of quiescence bouts (and shortening of motion bouts under some conditions) in response to prolonged motion. This form of compensation under low noise conditions, termed micro-homeostasis [167, 263], required the function of the neuropeptide Y (NPY) receptor homolog, NPR-1.

In contrast, strong stimuli induced a qualitatively different homeostatic response: the animals moved continuously for several minutes, after which quiescence monotonically returned to its baseline level. Compensation for the motion induced by a strong stimulus manifested as an upshift in the baseline fraction of time spent in quiescence, rather than a transient extension of quiescence bouts. The homeostatic responses to strong stimuli required the function of the DAF-16/FOXO in neurons (see also [91]) but not the function of NPR-1. Conversely, micro-homeostasis was not abolished in *daf-16* mutants.

In addition, we show that neuropeptidergic signaling is not strictly required for maintaining high levels of mean quiescence during lethargus. Loss of function of UNC-31/CAPS, a calcium-dependent activator protein required for dense core vesicle exocytosis [18, 57], resulted in a minor reduction of overall quiescence. In contrast, quiescence was strongly suppressed by the loss of the subsets of mature neuropeptides that were processed by the EGL-3 proprotein convertase or the EGL-21 carboxypeptidase E (CPE) [162, 164, 168, 184]. As previously suggested [335], this apparent discrepancy can be resolved: collectively, our data indicates that a balance between inhibitory and excitatory contributions from different peptides modulates the duration of bouts of quiescence.

Our findings support a model in which locomotion during lethargus is coupled to a measure of increased sleep pressure. Quiescence serves to ameliorate this pressure and homeostatic regulation dynamically maintains an appropriate quiescence baseline. Interestingly, the homeostatic routine stabilization of motion and quiescence in low-noise environments is mechanistically distinct from homeostatic responses following strong, stressful disruptions. To our knowledge, the analysis presented here is the first to identify this distinction.

2.3 Results

2.3.1 Motion plays a causal role in prolonging quiescence during lethargus

Homeostatic regulation of lethargus was previously examined using manually delivered strong mechanical stimuli, after which baseline levels of responsiveness were regained in four minutes [283]. However, even undisturbed animals compensate for spontaneous prolonged motion with prolonged quiescence during lethargus [167]. Therefore, a mechanism that dynamically stabilizes lethargus behavior may be invoked by motion in quiet or weakly noisy environments. If so, weak stimuli should transiently skew the bout architecture by elongating motion bouts and causing a subsequent (compensatory) extension of quiescence bouts. To test this, we first exposed wild type animals at the fourth intermolt larval stage, L4int, to pulses of blue light of intensities ranging from 0.3-100 mW/cm^2 and measured their responses using the frame subtraction method. In brief, this method consists of digitally recording the behavior of the animals and assessing the levels of motion and quiescence based on the number of pixels that change their brightness between consecutive frames (see [253]). The observed responses depended on the light intensity and the duration of the stimulus, and we determined that a 15 sec pulse of light at an intensity of 20-40 mW/cm^2 evoked weak, reproducible responses (Figures 2.1, 2.2, 2.3). Interestingly, we noted that 5 sec pulses failed to produce a sharp response specifically during lethargus. This suggested that the animals were less responsive during lethargus and that reduced responsiveness could be assayed separately from delayed

responsiveness [283].

The response of L4int larvae and post lethargus young adult (YA) animals to weak blue light stimuli consisted of elevated levels of locomotion, which persisted for 15-25 sec after the end of the pulse, followed by a two-minute decline back to baseline locomotion levels. In contrast, during L4leth the average level of locomotion crossed its baseline one minute after it peaked, proceeded to fall below it for 2-3 additional minutes ($p<0.01$), and only then stabilized at baseline levels (Figure 2.1 A). The transient trough in locomotion resulted from an increase in the fraction of time the animals were quiescent rather than from slower motion (Figure 2.1 B).

The presence of a weak light stimulus terminated bouts of quiescence prematurely and extended bouts of motion (Figure 2.1 C, $p<0.01$ in both cases). Identical responses were observed whether the onset of the stimulus interrupted a bout of quiescence or motion (Figures 2.1, 2.4). The increase in the fraction of time spent in quiescence after the stimulus was removed could have been caused by an extension of quiescence bouts, shortening of motion bouts, or both. To distinguish between these possibilities we turned to an accurate and computationally intensive behavioral analysis. This previously described approach was based on continuous measurements of the dynamics of body posture at high temporal and spatial resolutions [167, 253]. Using this analysis and a 15 sec weak light stimulus, we measured the durations of bouts of motion and quiescence after the stimulus was turned off. We found that, during the first half of lethargus, the compensatory response was comprised of an increase and a decrease in the durations of quiescence and motion bouts, respectively (Figure 2.1 D, $p<0.01$). During the second half of lethargus, a compensatory increase in the durations of quiescence bouts was still observed. Taken together, these findings revealed that motion during non- or weakly-interrupted lethargus, but not during the L4int or YA stages, caused a compensatory transient increase in quiescence.

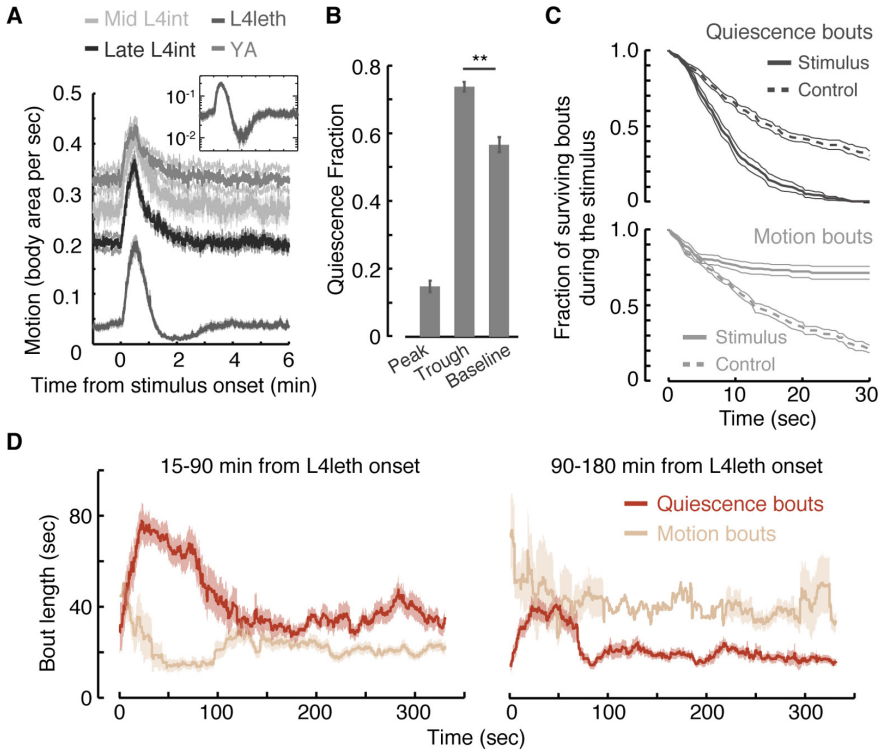

Figure 2.1: **Motion plays a causal role in determining the duration of subsequent quiescence during lethargus.** (A) wild type animals at the mid L4int, late L4int, L4leth, and YA stages were exposed to 30 s light stimuli at an intensity of 20 mW/cm^2. All stimuli were initiated at t = 0. Outside lethargus, locomotion monotonically decayed to baseline levels in 2 min. During lethargus, the peak in locomotion was followed by a trough prior to returning to baseline. Insets: the responses during lethargus shown on a semi-log scale. (B) The fractions of quiescence were calculated for 1 min intervals centered at the times of the peak and trough of the L4leth responses, as well as for their respective pre-stimulus baselines. Plots and bars depict mean ± s.e.m obtained from datasets of N = 4050 animals per condition. Asterisks indicate p<0.001. (C) Survival curves of quiescence and motion bouts of wild type animals exposed to a 30 s, 20 mW/cm^2, blue light stimulus during the first hour of L4leth. Bouts were identified using the frame subtraction method and control data were obtained from the same animals, but 8 min after the stimulus (non-stimulated control animals were also assayed, analyzed the same way, and found to be indistinguishable from this control group). Mean ± s.e.m, N >200 bouts for each condition. (D) The dynamics of bouts obtained from a posture-based analysis following a 15 s, 20 mW/cm^2, blue light stimulus. Left and right panels correspond to the first and second halves of L4leth, respectively. Plots depict mean ± s.e.m, smoothed using a 30 s running window average. N = 40 animals.

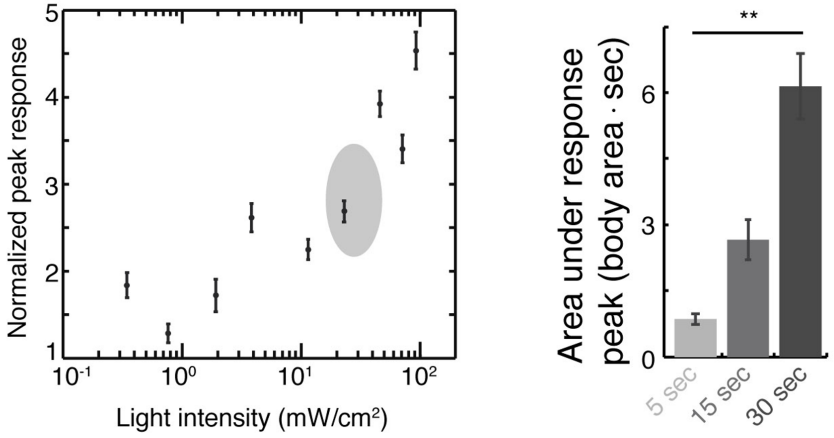

Figure 2.2: **Calibration of weak blue light stimuli.** Responses to blue light stimuli were defined as the peak value of overall motion, as measured using the frame subtraction method, normalized by the baseline average motion during a 1-min period prior to the stimulus. Left: L4int larvae were exposed to 15 s pulses of blue light at different intensities. Locomotion responses increased as a function of the light intensity in the 2100 mW/cm^2 range. The plot depicts mean ± s.e.m responses, N = 2030 animals. Shaded area emphasizes the range of stimuli used throughout the rest of the manuscript. Right: the total amount of motion induced by the stimulus was defined as the area under the response peak. Three durations of a 20 mW/cm^2 light stimulus were assayed (N>200 trials per condition, error bars depict s.e.m, p<0.001).

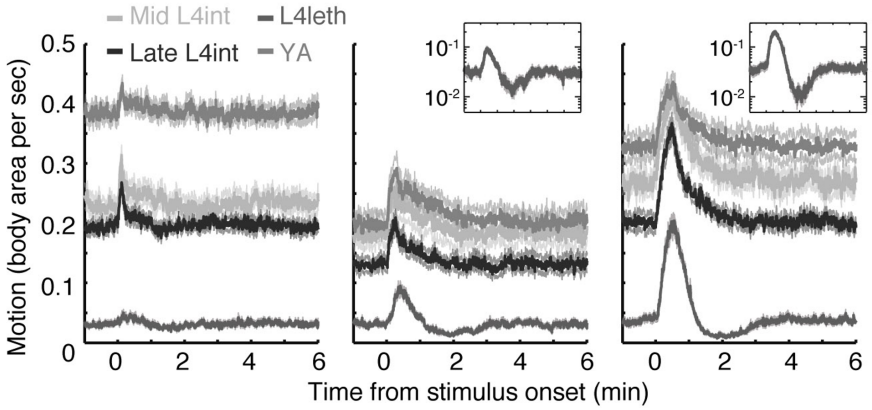

Figure 2.3: **Responses to weak light stimuli.** wild type animals at the mid L4int, late L4int, L4leth, and YA stages were exposed to 5 (left), 15 (middle), and 30 s (right) light stimuli at an intensity of 20 mW/cm^2. All stimuli were initiated at t = 0. A sharp peak in locomotion in response to a 5 s stimulus was observed outside lethargus, but not during lethargus. Both 15 s and 30 s stimuli evoked a transient increase in locomotion. Outside lethargus, locomotion monotonically decayed to baseline levels in 2 min. In contrast, during lethargus the peak in locomotion was followed by a trough prior to returning to baseline. Insets: the responses during lethargus shown on a semi-log scale.

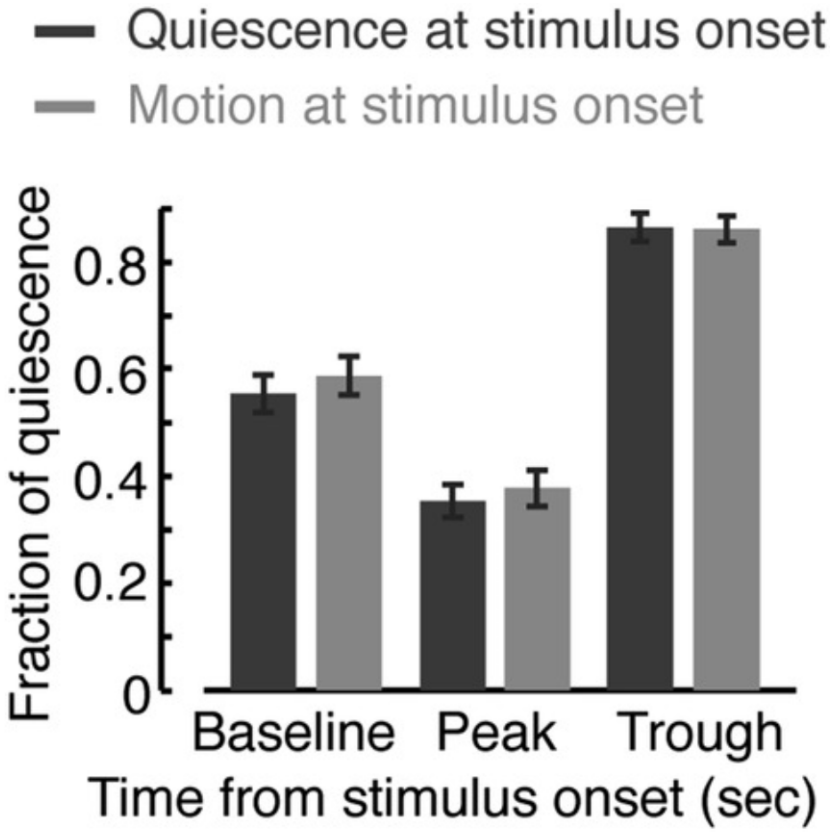

Figure 2.4: **Responses during quiescence and motion.** The fraction of quiescence during 1 min intervals centered at a time point prior to the onset of the stimulus (baseline), at the peak of the locomotion response, and at the trough of the response. Dark or light bars correspond to data obtained from instances when the animals were quiescent or motile, respectively, at the time of the stimulus onset. Responses assayed using the frame subtraction method appeared indistinguishable based on the type of bout at the time of stimulus onset. Error bars depict s.e.m. Plots and bars in all panels depict mean ± s.e.m obtained from datasets of N = 4050 animals per condition. Asterisks indicate $p<0.001$.

2.3.2 The character of behavior during a motion bout affects subsequent quiescence

Posture-based analysis allowed for improved measurements of pairwise correlations between durations of bouts of motion and subsequent bouts of quiescence in undisturbed animals (Figure 2.5 A, $R=0.47\pm0.03$, $p<0.001$) [167]. This approach revealed that these correlations gradually decayed as lethargus progressed (Figure 2.5). Moreover, it enabled us to compare groups of motion bouts that contained different qualities of motion despite having similar overall durations. We could thus address the question of whether vigorous or directed motion in and of itself might affect the subsequent bout of quiescence.

To compare between groups of motion bouts of equal durations, we binned the bouts recorded during the first 90 minutes of L4leth of non-stimulated wild type animals in 2 seconds wide bins. For each bin, we calculated the median vigor of locomotion as measured by the rate of change of body-curvature (Figure 2.6 B, left panel). The motion bouts (from each bin) were then separated into two groups: those exhibiting higher-than-median or lower-than-median vigor with respect to their bin of origin. Each of the two groups therefore contained bouts of all durations and, importantly, the average duration of a motion bout was the same in both groups (Figure 2.6 B, middle panel). Having controlled for the mere durations, we found a significant effect of the level of locomotion on the duration of the subsequent quiescence bout (Figure 2.6 B, right panel). A similar analysis, performed exclusively on bouts that contained directed motion, considered the separation between the two groups based on the fraction of the bout spent in directed motion and produced similar results (Figure 2.6 C). We thus conclude that enhanced or directed locomotion during a bout of a given duration positively affects subsequent quiescence.

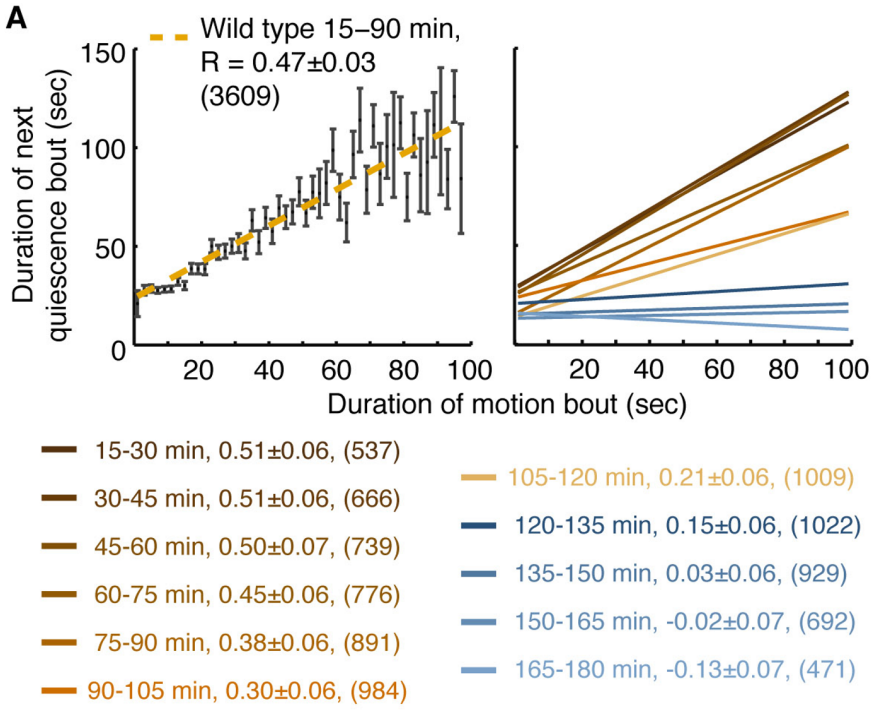

Figure 2.5: **Correlation between motion bout duration and the duration of the next quiescence bout.** (A) Posture-based analysis improved the measurement of pairwise correlations between the durations of motion bouts and those of subsequent quiescence bouts in undisturbed wild type animals ($R = 0.47 \pm 0.03$, $N = 3609$ bouts from 40 animals, $p<0.05$). As a guide to the eye, motion bouts were grouped according to their durations in 2 s wide bins. The mean $\pm$ s.e.m duration of the subsequent quiescence bouts for each bin was plotted and these mean values were fitted to a line. In addition, pairwise correlation coefficients were calculated for each 15 min interval of L4leth separately. As a guide to the eye, linear fits to the binned data are depicted. In all cases, the errors were defined as the 95% confidence intervals and the number of bouts is given in parentheses.

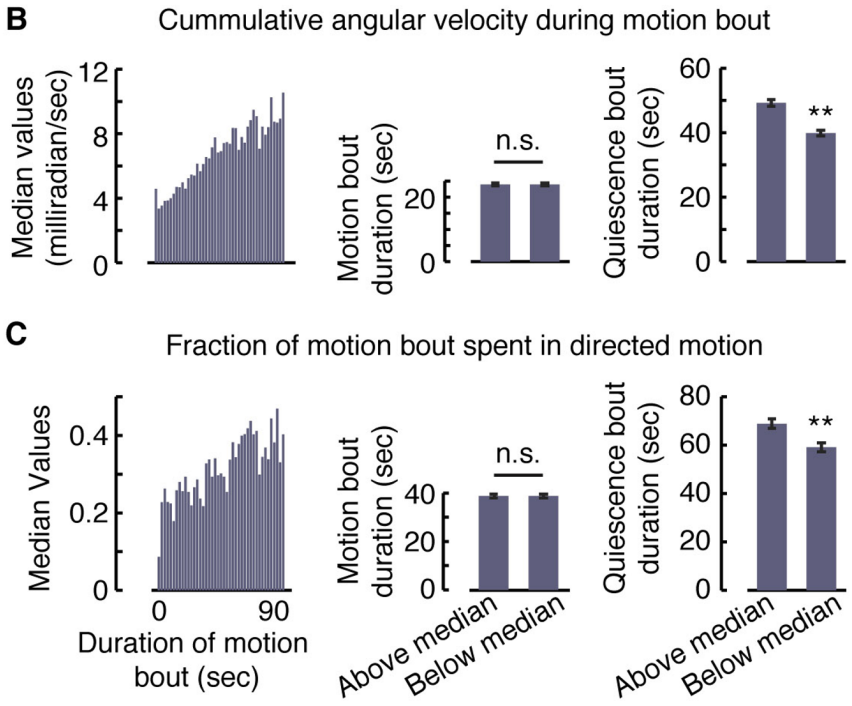

Figure 2.6: **The overall levels of motion and the fraction of directed motion during a motion bout have a significant effect on subsequent quiescence.** Overall motion was defined as the mean time derivative of the absolute values of 18 angles along the body and directed motion was defined as either forward or backward locomotion, as opposed to dwelling ([253]). Left: the median values of the overall vigor of motion (B) and the fraction of directed motion (C) as a function of the duration of the motion bouts (binned in 2 s bins). Middle (right): the durations of motion (quiescence) bouts calculated separately for the group of bouts that was above or below the median of its respective bin. The durations of quiescence bouts differed significantly between the two groups. N = 40 animals, error bars depict s.e.m, $p<0.01$.

2.3.3 Behavioral responses to external stimuli are distinct during lethargus

Dwelling behavior during a motion bout appears similar to dwelling behavior outside of lethargus. However, although locomotory responses were shown to be delayed during lethargus [62, 283], they were not previously examined in detail. We asked whether responses to a weak stimulus during a bout of motion were distinct from responses during a bout of quiescence, from responses outside of lethargus, or from both.

To examine behavioral responses to external perturbations throughout this study, we used a recurrent stimulus assay: animals were repeatedly exposed to a stimulation regime of brief, widely spaced, photo- or mechano-stimuli. The duration of each individual stimulus was 0.4 or 15 sec, depending on the type of assay, and the spacing between consecutive stimuli was 15 minutes. Animals were continuously assayed for 10 hours from the L4int stage to the mid YA stage. A diagram outlining the design of these assays is depicted in Figure 2.7 A. Since multiple 15-minute cycles were aligned and averaged, the resulting data had periodic boundaries. For instance, the same one minute period could be referred to as the 15^{th} minute after the stimulus or the one minute just prior to the stimulus (see Figure 2.7, Figure 2.8).

In our hands, during lethargus, the transient changes in behavioral dynamics that constituted the short-term response to a stimulus were limited to a period of 3 minutes immediately following stimulation. After this short-term response was complete, behavioral dynamics returned to a steady state characteristic of the conditions of the experiment. Consequently, baseline behavior for each set of experimental conditions was defined as the steady state measured during a 5-minute period starting 10 minutes after a stimulus and 5 minutes prior to the subsequent stimulus (labeled explicitly in Figure 2.7 A and Figure 2.8, and depicted as t=-50 min in subsequent panels).

Outside of lethargus, the onset of a weak light stimulus evoked a sharp rise in the propensity for forward locomotion while backward locomotion was suppressed (L4int) or unchanged (YA). From the time of the offset of the stimulus, forward locomotion monotonically declined

and reversals returned to baseline levels (L4int) or were briefly elevated (YA). The probability of forward locomotion decayed to its baseline value in 3 min as the baseline balance between directed motion and dwelling was re-established (Figure 2.7 B left and right panels).

During L4leth the onset of the stimulus evoked a sharp rise in the propensities for both forward and backward locomotion. The offset of the stimulus did not reverse the increasing propensity for moving forward. Rather, forward locomotion persisted for 20 sec after the light was turned off, and subsequently fell below its steady state value while quiescence levels exceeded their baseline (Figure 2.7 B middle panel). Similar features were observed for responses throughout L4leth (Figure 2.7 C), and regardless of whether the onset of the stimulus occurred during a motion or a quiescence bout (data not shown). Thus, responses to weak stimuli revealed similar locomotory responses during bouts of motion and quiescence and differentiated both types of bouts from the L4int and YA stages (Figure 2.8.

2.3.4 Homeostatic responses to weak and strong stimuli are distinct

The compensatory extension of quiescence bouts after a weak light stimulus was distinct from previously reported responses to manually delivered strong mechanical stimuli [91, 283]. To test whether the modulation of bout duration was specifically evoked by light, we assayed animals that were exposed to a mechanical stimulus: vibrations at a frequency of 1kHz [253]. The strength of the stimulus was tuned by varying its duration. Outside lethargus, a 0.4 sec stimulus elicited a transient increase in reversals followed by a brief enhancement of the propensity for forward locomotion, while a 15 sec stimulus elicited a similar initial recoil followed by an enhancement of forward locomotion that lasted for 10 minutes (Figure 2.9 A, B, left). We thus refer to the short stimulus as weak and the longer stimulus as strong.

During L4leth, weak mechanical stimuli induced transient backward locomotion, followed by enhanced quiescence. Specifically, the first bout of quiescence after the recoil was elongated, and the architecture of locomotion and quiescence returned to baseline one minute after the stimulus was delivered (Figure 2.9 A, right). In contrast, the strong stimulus dis-

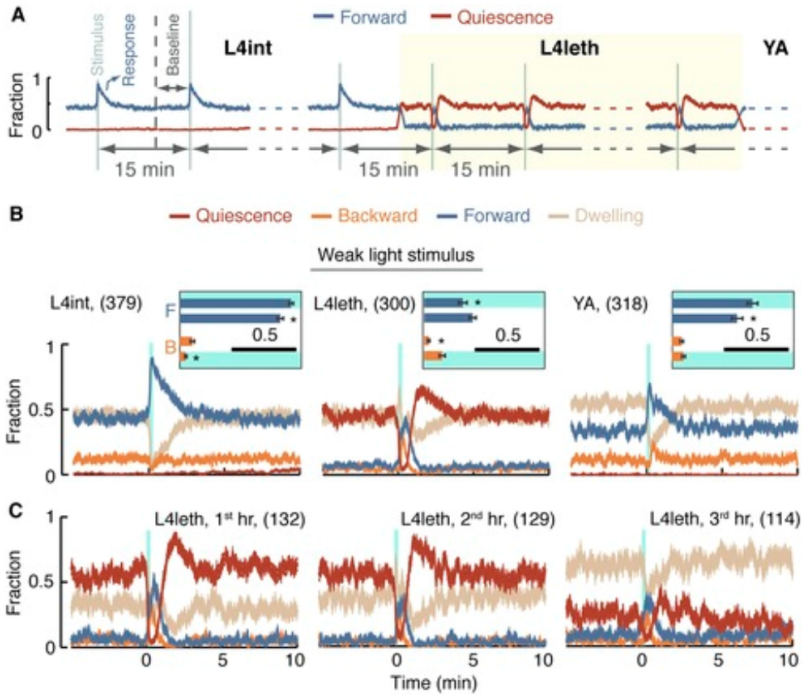

Figure 2.7: **A posture-based analysis of locomotion responses to weak light stimuli.** (A) A diagram describing the repeated stimulus assay, in which a generic brief stimulus (vertical lines) was repeatedly delivered at 15 min intervals (long horizontal arrows). Each assay started at the mid L4int stage, continuously progressed through L4leth (shaded area), and ended at the mid YA stage. For the purpose of illustration, the blue and red lines symbolize tentative probabilities of forward locomotion and quiescence, respectively. Baseline behavior was measured during the 5-min period starting 10 min after a stimulus, or equivalently, 5 min prior to the subsequent stimulus. The beginning of the first baseline period is depicted by a dashed vertical line. (B) The fraction of forward locomotion, backward locomotion, dwelling, and quiescence before, during, and after a weak (15 s, 20 mW/cm^2 blue light) stimulus provided at the L4int (left), L4leth (middle), and YA (right) stages. A compensatory post-stimulus enhancement of quiescence, as well as enhanced reversals during the stimulus, and a rising propensity for forward locomotion after the stimulus was turned off were uniquely observed during lethargus. Insets: the fraction of forward locomotion before and after the offset of the stimulus (top) and the fraction of backward locomotion before and after the onset of the stimulus. Shading denotes the presence of the light stimulus. All fractions were calculated from the 7.5-s period (half of the duration of the stimulus), the scale bars represent a fraction of 0.5, and asterisks denote $p<0.05$. (C) The data from the middle panel of (B) plotted separately for the first, second, and third hours of L4leth. Enhanced quiescence was observed in all three cases, although it was less prominent during the third hour. Plots in panels (B, C) depict mean ± s.e.m and the number of stimuli assayed is noted in parentheses for each condition.

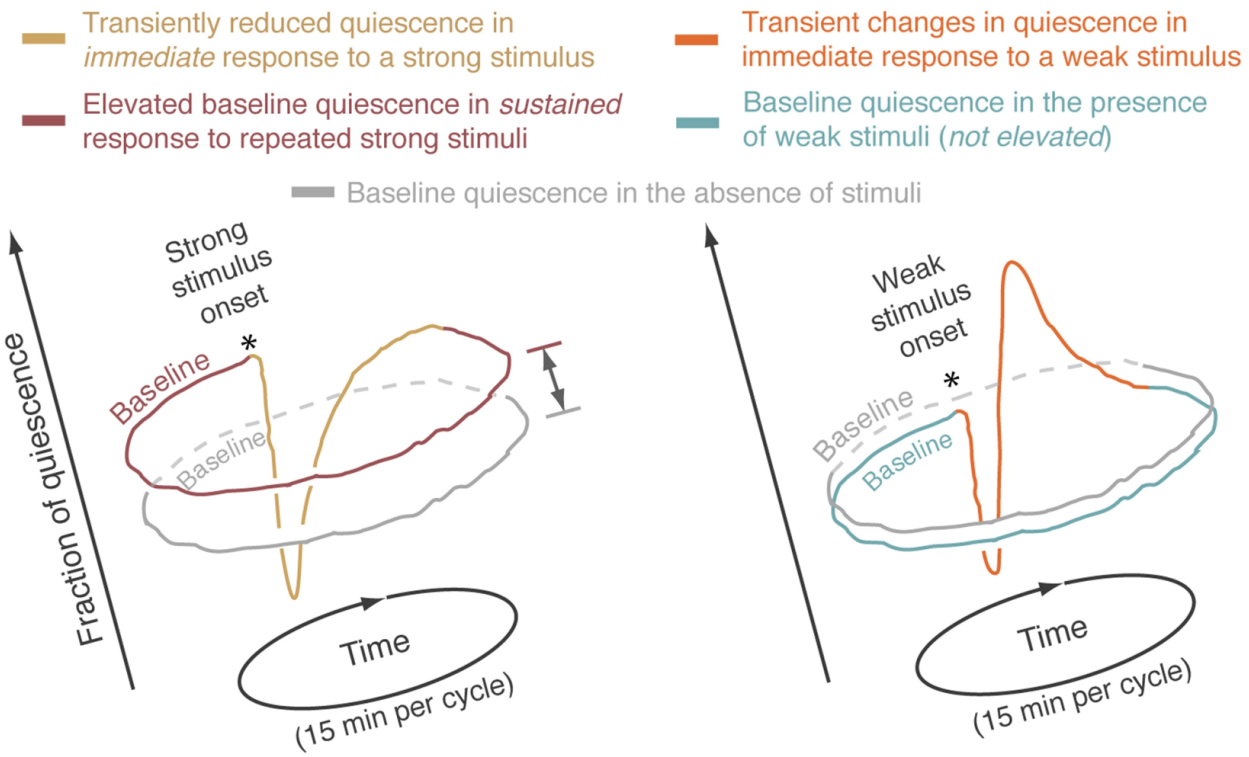

Figure 2.8: **The averaged behavior data have periodic boundaries.** Each sketched closed curve represents the average fraction of quiescence as a function of time for a particular set of experimental conditions. Since repeated 15-min cycles were aligned and averaged, the mean behavioral dynamics have periodic boundaries. Graphically, this can be represented by plotting the data on a circular time axis, where the onset of the stimulus is denoted by an asterisk. During lethargus, the immediate response to a stimulus lasted no more than 3 min. After this short-term response was complete, the fraction of quiescence returned to a steady state that was characteristic of the conditions of the experiment. The fraction of time spent in quiescence during this steady state was defined as the baseline level of quiescence for the relevant experimental conditions. In this framework, baselines were compared between different conditions. Left: responses to strong stimuli. The immediate response to a strong stimulus is depicted by the yellow curve and the baselines for periodically stimulated and undisturbed animals are depicted by brown and grey curves, respectively. Elevation of the steady state quiescenceas compared to the mean quiescence of undisturbed animalsconstituted the sustained response to the presence of repeated strong stimuli. This elevation is emphasized by a double-headed arrow. Right: responses to weak stimuli. The immediate response to a weak stimulus is depicted by the orange curve and the baselines for periodically stimulated and undisturbed animals are depicted by blue and grey curves, respectively.

rupted the architecture of behavior during lethargus: it was followed by several minutes of enhanced motion and a monotonous relaxation to baseline quiescence levels. Upon return to baseline, quiescence bouts were not transiently extended such that a peak in quiescence was not observed. However, compensation took on a different form: the overall level of baseline quiescence was elevated (Figure 2.9 B, C). This overall elevation of quiescence was consistent with previously reported compensation after strong stimulation [91, 283]. These results suggested that there were two regimes of disruption and compensation. Weak perturbations resulted in a transient modulation of bout durations that did not disrupt (and could even enhance) the characteristic correlations of the bouts architecture. In contrast, a strong perturbation abrogated the routine dynamics of bouts for several minutes and increased the baseline fraction of quiescence thereafter.

2.3.5 The neuropeptide Y receptor homolog, NPR-1, plays a role in modulating quiescence in both unperturbed and weakly stimulated animals

Neuropeptide Y (NPY) and its receptors have been implicated in the regulation of sleep (albeit in different manners) in humans, rats, fruit flies, and nematodes [13, 63, 94, 253, 361, 378]. To test their role in mediating micro-homeostasis we assayed animals carrying two mutant alleles of the *C. elegans* NPY receptor homolog gene, *npr-1*. In our hands, overall quiescence in animals carrying the *npr-1(ky13)* allele, a glutamine to ochre nonsense mutation at codon 61, was only mildly different from wild type [253]. However, bout correlations in these mutants were significantly reduced. The *npr-1(ad609)* null allele induced similarly reduced bout correlations and a more pronounced defect in the durations of quiescence bouts throughout lethargus (Figure 2.10 A).

We next assayed the responses of *npr-1* mutants to weak blue light stimuli. Using the frame subtraction method, we could not detect significant compensation following the excess

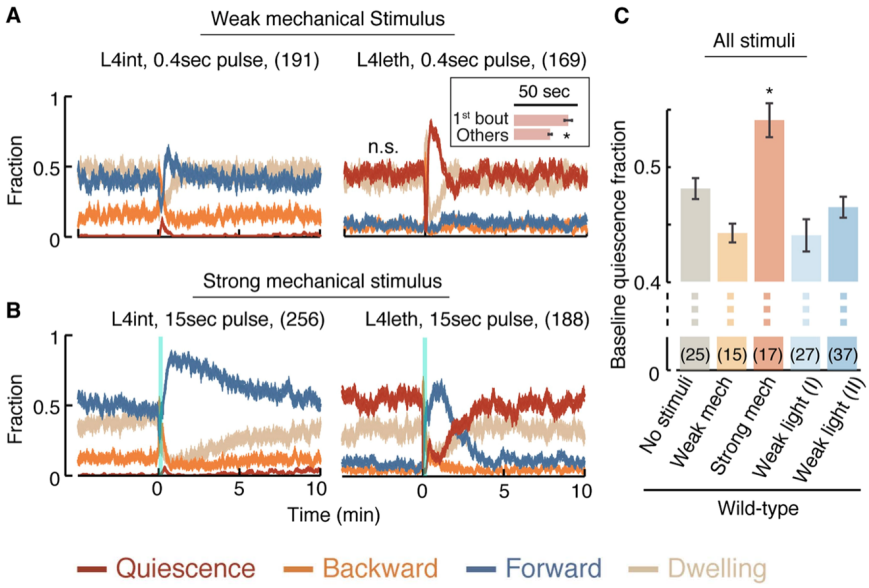

Figure 2.9: **A posture-based analysis of locomotion responses to weak and strong mechanical stimuli.** (A) A weak mechanical stimulus (0.4 s of 1 kHz vibrations) produced a reversal followed by a small elevation of forward locomotion in L4int larvae (left) and a brief reversal followed by enhanced quiescence during L4leth (right). Inset: the first quiescence bout after the stimulus was longer than subsequent bouts ($p<0.05$). (B) A strong mechanical stimulus (15 s of 1 kHz vibrations) produced reversals followed by a prolonged (10 min) elevation of forward locomotion in L4int larvae (left) and a brief reversal followed by elevated levels of directed motion for 45 min during L4leth (right). Notably, quiescence returned to its baseline value without transiently exceeding it. (C) Mean baseline fraction of quiescence was measured during the baseline period (see Figure 2.7 A). The baseline fraction of quiescence was significantly higher in strongly stimulated animals as compared to unstimulated and weakly stimulated animals. Weak light I and II labels refer to stimulus strengths of 20 and 40 mW/cm^2 blue light, respectively. Plots in panels (A, B) depict mean $\pm$ s.e.m and the error bars in panel (C) depict $\pm$s.e.m and asterisks denote $p < 0.05$. The number of stimuli assayed is noted in parentheses for each condition.

motion induced by the stimulus in either of the two mutant strains (Figure 2.10 B). The posture-based analysis confirmed their severe defect in modulation of bout durations (Figure 2.11 C, Figure 2.11 D). The overall activity and, in particular, the initial response of *npr-1* mutants to the weak stimulus were similar to wild type. This indicated that the mutants were not defective in sensing the stimulus or in their locomotory capabilities, but specifically in their ability to compensate for a weak disturbance. In contrast, *npr-1* mutants exhibited wild type-like compensation following strong mechanical stimuli: when animals carrying either of the two mutant alleles were exposed to a strong mechanical stimulus their baseline fraction of quiescence was elevated as compared to non stimulated or weakly stimulated animals (Figure 2.11 E). Thus, in addition to the phenotypic differences described above, homeostatic compensation during undisturbed or weakly disturbed lethargus was affected by NPR-1, while homeostatic compensation for strong stimuli was not. Therefore, the routine stabilization of lethargus behavior in low-noise environments and the homeostatic compensation for stressful disturbances are mechanistically separable.

2.3.6 The neuropeptide Y receptor homolog, NPR-1, may act through G_o signaling to dynamically modulate quiescence

NPY can act through G_i/G_o signaling pathways to inhibit cAMP formation and to modulate calcium and potassium channels [157]. In *C. elegans*, NPR-1 was suggested to act through G_o signaling or, when expressed in the pharynx, through G_q signaling [206, 294]. While EGL-30 promotes enhanced locomotion, GOA-1 reduces locomotion levels [210, 244, 269, 275, 290, 315]. We therefore examined *goa-1* and *unc-43* mutants, lacking the function of the only *C. elegans* ortholog of mammalian G_i/G_o alpha subunits and a calmodulin protein kinase II (CAMKII) acting upstream of *goa-1*, respectively [275, 287, 294]. Both mutants exhibited reduced (but not abolished) quiescence, and defects in their ability to dynamically augment quiescence in response to prolonged motion (Figure 2.13). Although understanding the precise role of G_o signaling in this process is outside the scope this work, these findings

are consistent with a model in which NPR-1 acts through G_o signaling to dynamically modulate quiescence during lethargus in response to spontaneous or induced mild variations in locomotion.

2.3.7 Rescue of the neuropeptide Y receptor homolog, NPR-1, in the RMG neuron class restores micro-homeostasis

To identify where NPR-1 acts to mediate micro-homeostasis, I employed a rescue strategy by expressing *npr-1* cDNA in the strongest NPR-1 expressing cells. NPR-1 is predominately expressed in 20 neuron types, and particularly in the AQR, PQR, and URX neurons that are exposed to the body fluid [73]. However, expression of NPR-1 is also strong in the RMG neurons, which form extensive gap junctions with sensory neurons to regulate locomotion and social behavior [232]. Using the *gcy-32* promoter to rescue NPR-1 in AQR, PQR, and URX failed to restore micro-homeostasis (data not shown) [232]. However, *npr-1* transgene expression specifically in the RMG neurons using intersectional expression of the *flp-21* and *ncs-1* promoters could significantly rescue the defect in micro-homeostasis of *npr-1(ad609)* mutants following perturbation with light (Figure 2.12). Therefore, *npr-1* likely acts in the RMG neurons to regulate micro-homeostasis.

2.3.8 Peptidergic signaling is required for micro-homeostasis

NPR-1 is a predicted neuropeptide receptor and the FMRFamide-like neuropeptides encoded by *flp-18* and *flp-21* were shown to be two of its ligands [85, 194, 206, 296]. We therefore asked whether peptidergic release from dense core vesicles (DCVs) was required for micro-homeostasis. To answer this question, we assayed the loss of function of UNC-31, the sole *C. elegans* ortholog of mammalian calcium-dependent activator protein for secretion (CAPS) required for DCV exocytosis [18, 57]. To confirm that the observed phenotype was explained by the mutation of interest, we tested a strong loss of function allele, *unc-31(e169)*, and a

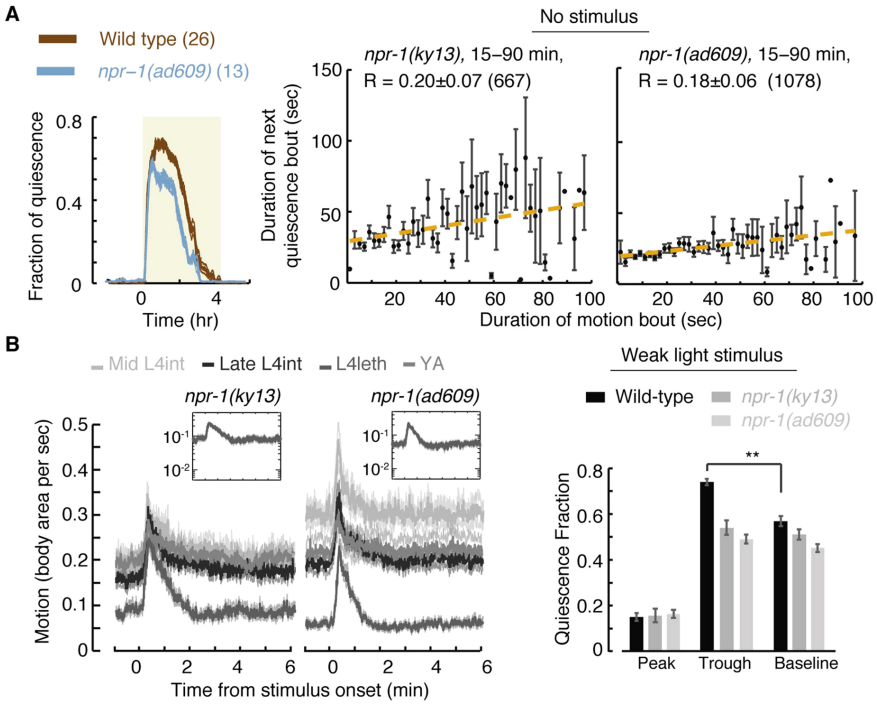

Figure 2.10: **NPR-1 is required for micro-homeostasis but not for homeostatic responses to strong stimuli (Frame Subtraction).** (A) Undisturbed behavior of *npr-1* mutants. Left: the fraction of quiescence of wild type animals and *npr-1(ad609)* mutants during L4leth (shaded area). The fraction of quiescence of *npr-1* mutants was recently published ([253]) and plotted here for comparison. Plots depict mean ± s.e.m, the numbers of animals assayed are denoted in parentheses. Middle and right: pairwise bout correlations and plots of binned bouts (see Figure 2.5 for details). Pairwise correlations were significantly reduced in *npr-1* mutants ($p < 0.05$). All correlations are given with 95% confidence intervals and error bars depict ± s.e.m. The number of bouts in each case is denoted in parentheses. (B) L4int, late L4int, L4leth, and YA *npr-1* mutants were exposed to weak (15 s, 20 mW/cm^2 light) stimuli. All stimuli were initiated at t = 0. In *npr-1* mutants assayed using frame subtraction, a trough did not follow the transient increase in locomotion before returning to baseline. Insets: the responses during lethargus shown on a semi-log scale. For each strain, the quiescence fraction was calculated during 1 min intervals centered at the times of the peak and trough of the L4leth responses, as well as for their respective pre-stimulus baselines. Quiescence was not enhanced following the peak in locomotion in *npr-1* mutants. Plots and bars depict mean ± s.e.m obtained from datasets of N = 5060 animals per condition. Asterisks and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively.

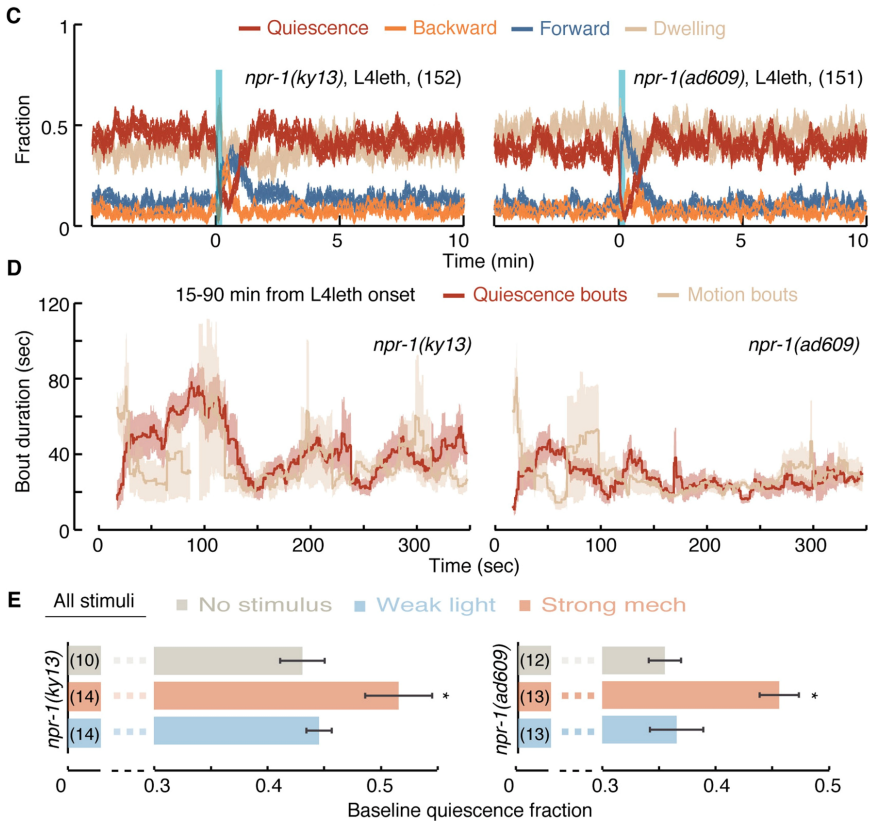

Figure 2.11: **NPR-1 is required for micro-homeostasis but not for homeostatic responses to strong stimuli (Posture-based).** (C) A posture-based analysis of behavior of L4leth *npr-1* mutants: the fraction of forward locomotion, backward locomotion, dwelling, and quiescence before, during, and after a weak (15 s, 20 mW/cm^2, blue light) stimulus. The data were aligned by the time of the onset of the stimulus and then averaged. Plots depict mean ± s.e.m. In agreement with the frame subtraction measurements, the compensatory enhancement of quiescence fraction shortly after the stimulus was nearly abolished in *npr-1* mutants. N = 14 and 13 animals (*ky13* and *ad609*). (D) A posture-based analysis of bout dynamics of *npr-1* mutants following a weak stimulus. N = 14 and 13 animals, plots depict mean ± s.e.m, smoothed using a 30 s running window. (E) The mean baseline fractions of quiescence during the 5 min intervals prior to each stimulus tested. Similar to wild type, baseline quiescence fraction was significantly higher in strongly stimulated animals as compared to non-stimulated and weakly stimulated *npr-1* mutants. Error bars depict ± s.e.m and asterisks denote p <0.05. The number of stimuli assayed is noted in parentheses for each condition.

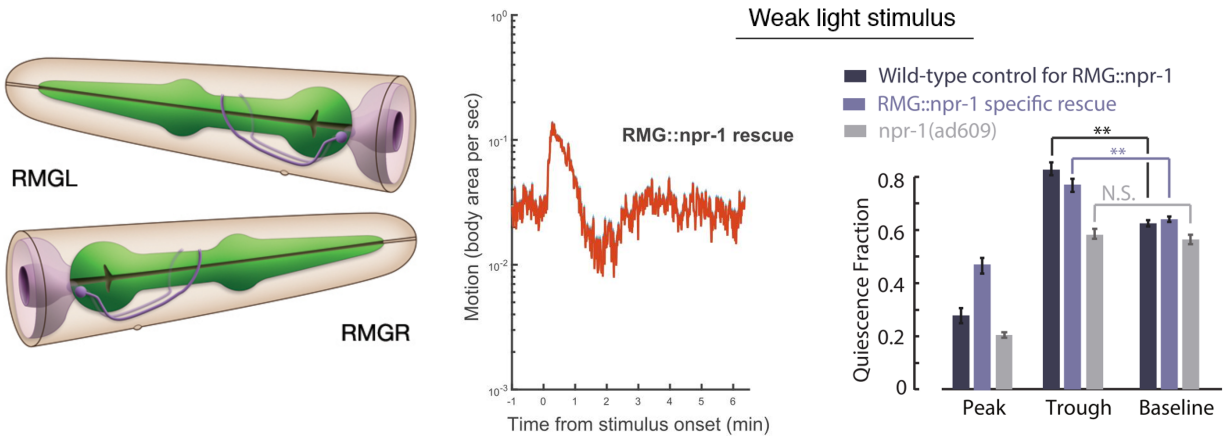

Figure 2.12: **Rescue of NPR-1 in RMG neurons is sufficient to restore microhomeostasis.** Using Cre-Lox recombination to rescue *npr-1* specifically in the RMG hub-and-spoke neurons (shown in purple wrapping around the pharynx bulb; image adapted from http://www.wormatlas.org) was sufficient to restore microhomeostasis in *npr-1(ad609)* mutants (N=24 animals)

putative null allele, *unc-31(e928)*, [57, 334].

Under undisturbed conditions, the quiescence bouts of *unc-31* mutants were shorter than wild type, but the overall amount of quiescence was only weakly reduced in these mutants (Figure 2.15 A, B). Moreover, *unc-31* mutants did not exhibit paralysis or anachronistic quiescence outside of lethargus and their overall locomotory behavior during lethargus was similar to wild type ([255] and data not shown). Nevertheless, pairwise correlations between subsequent bouts in these mutants were abolished (Figure 2.15 B). This could indicate that the absence of a group of functional neuropeptides impaired the dynamic extension of quiescence bouts in response to variations in durations and compositions of motion bouts. Alternatively, the quiescence bouts of *unc-31* mutants may be too short to sustain detectable correlations. We favor the first explanation for two reasons. First, the Hawaiian strain (a wild isolate of *C. elegans*) exhibited quiescence bouts that were comparable in duration to those of *unc-31* mutants but nevertheless maintained wild type correlations during minutes 45-120 from the onset of L4leth (Figure 2.17, Figure 2.18). Second, when bout pairs containing longer quiescence bouts were excluded from the wild type dataset, such that the mean duration of the remaining quiescence bouts equaled that of *unc-31* mutants, the pairwise

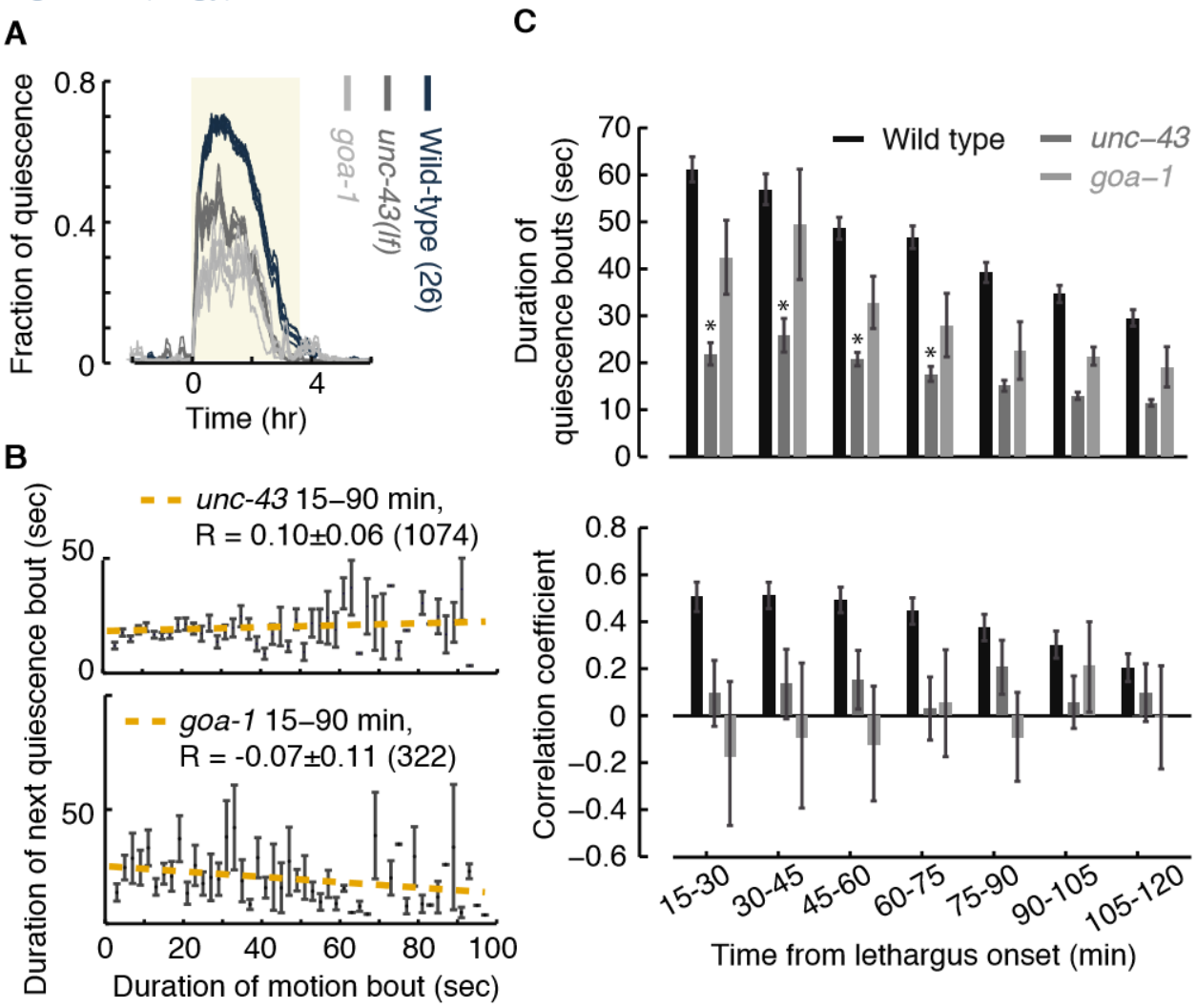

Figure 2.13: **Pairwise correlations are abolished in G_0 signaling mutants.** (A) The fraction of quiescence of wild type animals, *unc-43* mutants, and *goa-1* mutants during L4leth (B) Pairwise bout correlations and bplots of binned bouts. Pairwise correlations are mostly abolished in *unc-43* mutants and abolished in *goa-1* mutants. All correlations are given with 95% confidence intervals ($p<0.05$) and error bars depict ± s.e.m.The number of bouts in each case is denoted in parentheses. (C) Top: mean durations of quiescence bouts for 15 min intervals during the first two horus of L4leth. Error bars depict ± s.e.m. Bottom: pairwise correlations of bout durations for 15 min intervals during the first two horus of L4leth. Error bars depict 95% confidence intervals.

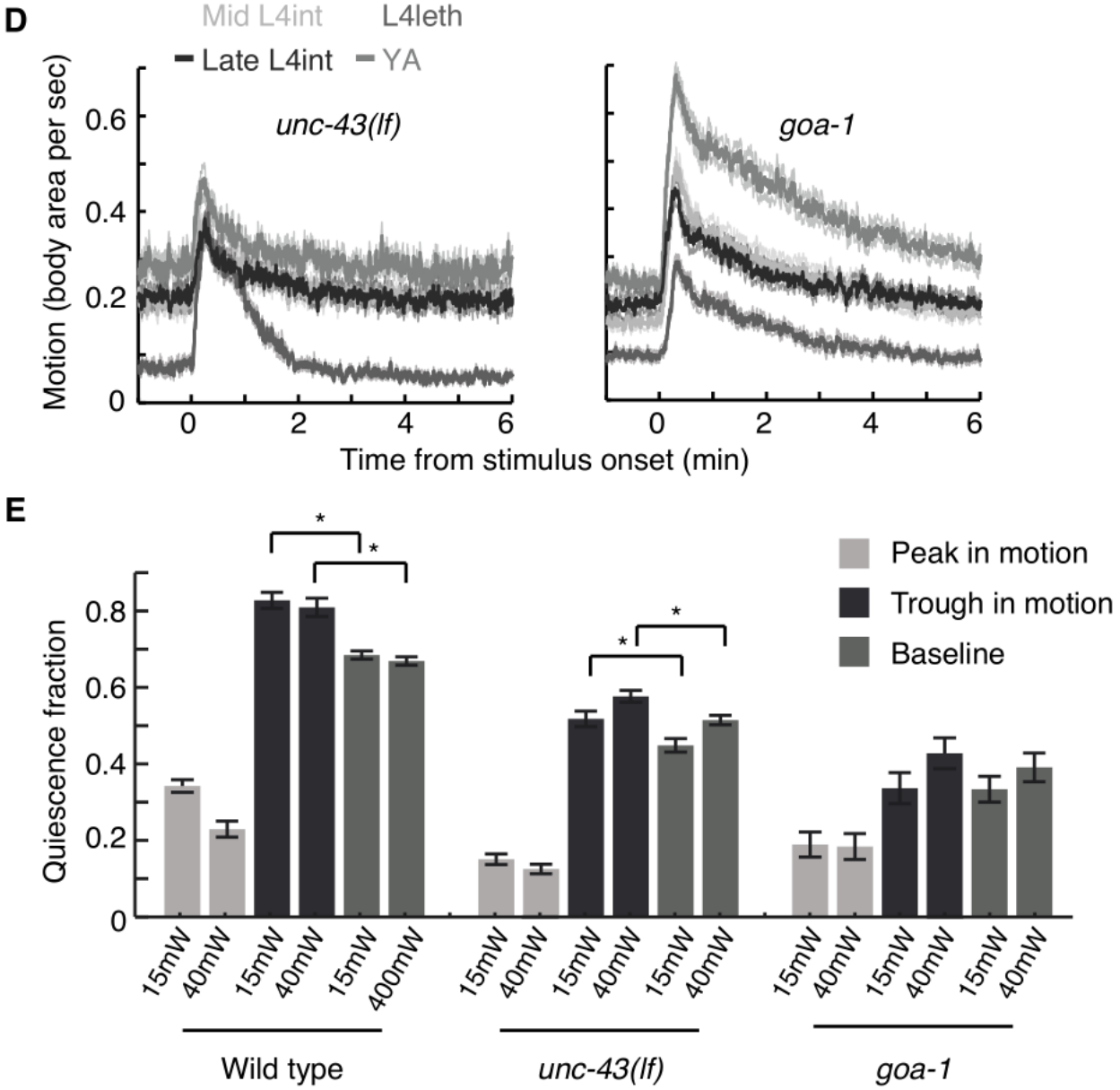

Figure 2.14: **G_0 signaling may be required for micro-homeostasis.** Mid L4int, lateL4int, L4leth, and YA animals carrying mutant alleles for *unc-43* and *goa-1* were exposed to 15 sec light stimuli at an intensity of 15 and 40 mW/cm^2. All stimuli were initiated at t=0. The transient increase in locomotion was not followed by trought that was visually detectable by frame subtraction analysis before returning to baseline. (E) The fractions of quiescence were calculated for 1 minute intervals centered at the times of the peak and trough of the L4leth responses, as well as for their respective pre-stimulus baselines. Quiescence was enhanced following the peak in locomotion in *unc-43* mutants, bt to ta lesser degree than in wild type animals. Quiescence was not enhanced following the peak in *goa-1* mutants. Plots and bars depict mean ± s.e.m. obtained datasets of N=60-70 animals per condition.

correlation between the remaining bouts was reduced to R=0.2±0.03, but not abolished.

We next assayed the responses of *unc-31* mutants to weak (light) stimuli. Animals carrying the *unc-31(e928)* null mutation, as well as animals carrying the *unc-31(e169)* loss of function mutation, exhibited a diminished ability to prolong quiescence bouts in response to prolonged motion. The stronger defect was observed in *unc-31(e928)* mutants (Figure 2.16 C, D). Collectively, these findings suggest that peptidergic signaling plays a key role in regulating micro-homeostasis.

In addition to *unc-31*, we assayed mutants in which neuropeptide processing was disrupted due to the loss of function of: (i) the proprotein convertase required for preprocessing of many, but not all, neuropeptides, EGL-3 [162, 184], or (ii) the carboxypeptidase E (CPE) required to complete the processing of the majority of non insulin-like neuropeptides, EGL-21 [164, 168]. Consistent with previous reports [373], overall quiescence during lethargus was significantly reduced in *egl-3* mutants, individual quiescence bouts were very short, and (as expected) correlations between bout durations were abolished. Loss of function of EGL-21 resulted in an identical phenotype, demonstrating that the phenotype was caused by the mutations of interest (Figure 2.15 A, B). These results stood in contrast to the mild change in overall quiescence observed in *unc-31* mutants, and this apparent discrepancy is discussed below.

2.3.9 The FMRFamide-like peptide FLP-18 plays a role in micro-homeostasis

The FMRFamide-related neuropeptides FLP-18 and FLP-21 were shown to be ligands of NPR-1, as well as two additional receptors [76, 296]. In addition, FLP-18 (but not FLP-21) was shown to act synergistically, in an inhibitory fashion, in the homeostatic response to motoneuron imbalance (see discussion and [335]). In our hands, *flp-21* mutants did not exhibit defective micro-homeostasis. We used posture analysis to assay unperturbed *flp-18(gk3036)* mutants and the frame subtraction method to assay *flp-18(gk3036)* and *flp-18(db99)* mu-

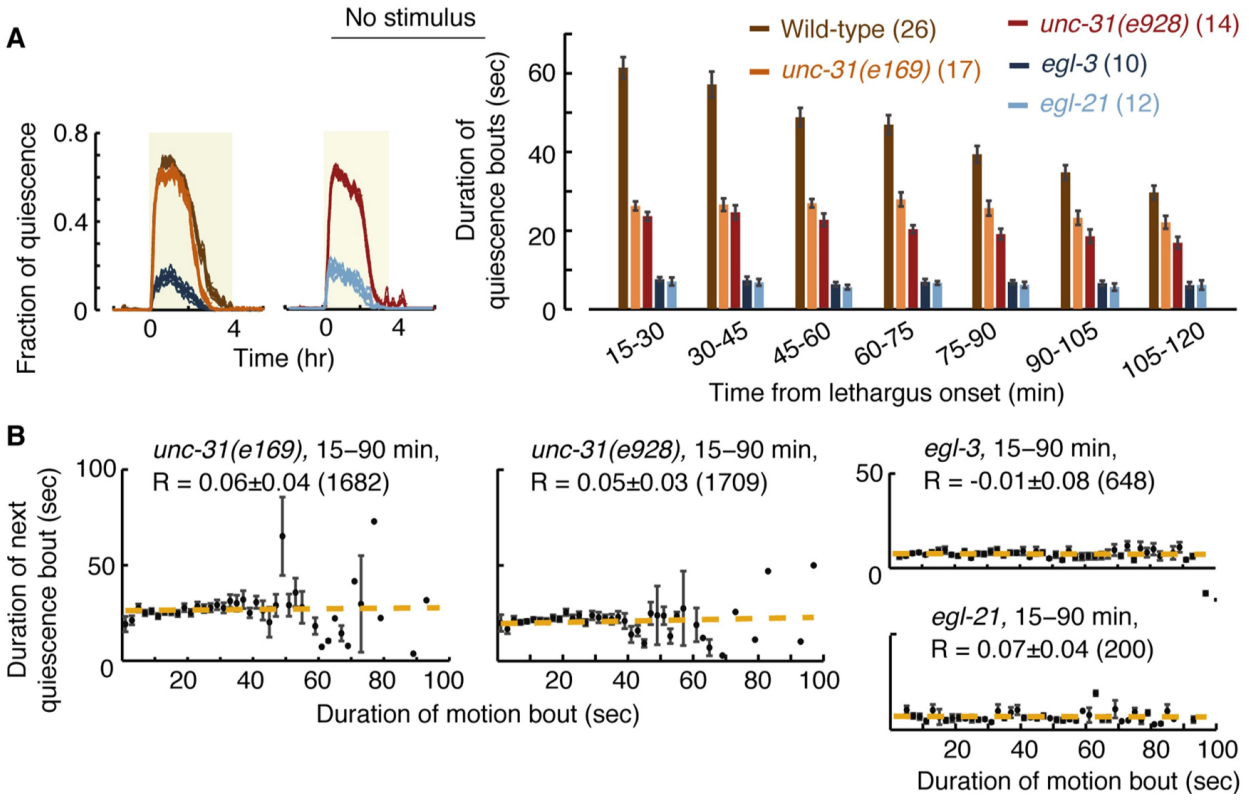

Figure 2.15: **UNC-31/CAPS is not required for establishing a high fraction of quiescence during lethargus but is necessary for establishing pairwise correlations in undisturbed animals.** (A) Left: the fraction of quiescence of wild type animals and *unc-31*, *egl-3*, and *egl-21* mutants during L4leth (shaded area). Quiescence was strongly reduced by the loss of function of EGL-3 or EGL-21, but not UNC-31. Right: the mean durations of bouts of quiescence of the same wild type and mutant animals during the 15-min period of L4leth. Plots and bars depict mean ± s.e.m, the numbers of animals assayed are denoted in parentheses. (B) Pairwise bout correlations and plots of binned bouts in undisturbed animals (see Figure 2.5 for details). Pairwise correlations were abolished in *unc-31*, *egl-3*, and *egl-21* mutants. All correlations are given with 95% confidence intervals ($p < 0.05$) and error bars depict ± s.e.m. The number of bouts in each case is denoted in parentheses.

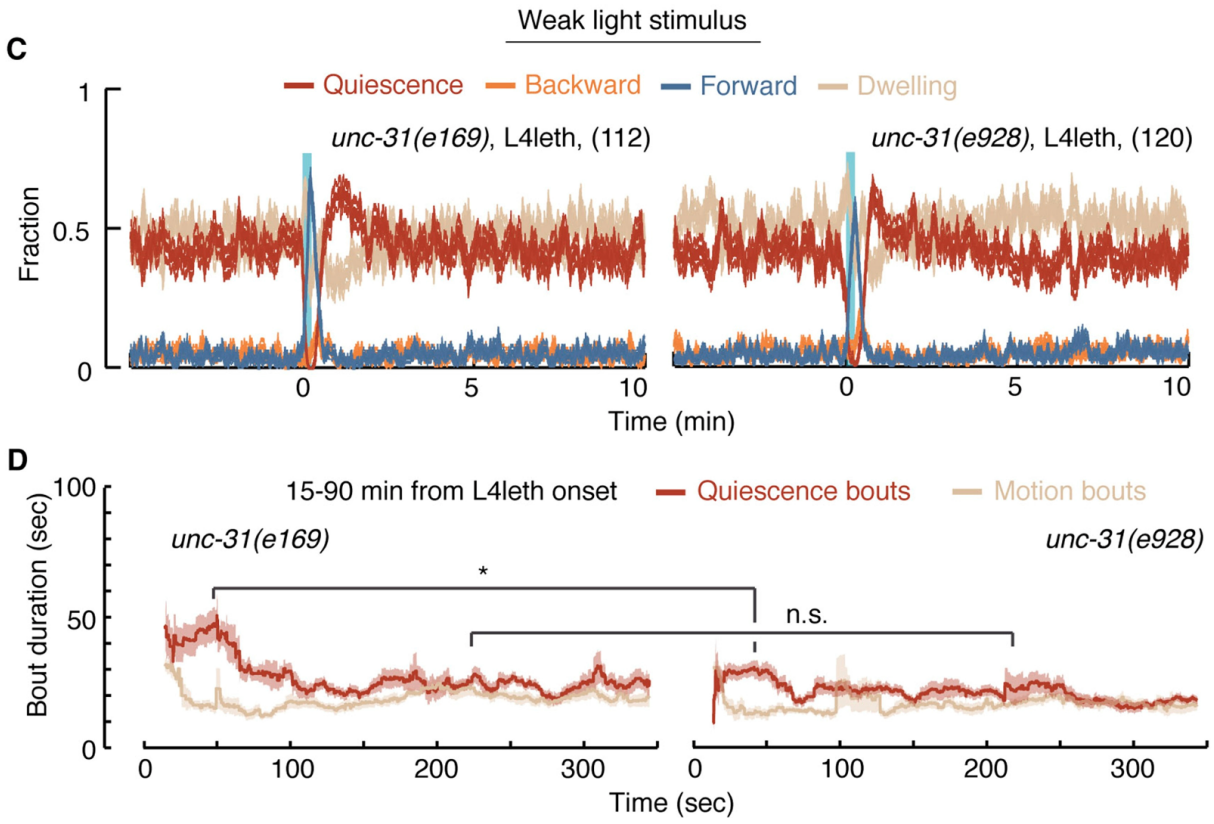

Figure 2.16: **UNC-31/CAPS is not required for establishing a high fraction of quiescence during lethargus but is required for micro-homeostasis.** (C) A posture-based analysis of behavior of L4leth *unc-31* mutants: the fraction of forward locomotion, backward locomotion, dwelling, and quiescence before, during, and after a weak (15 s, 20 mW/cm^2, blue light) stimulus. (D) A posture-based analysis of bout dynamics of *unc-31* mutants following a weak stimulus. The duration of the motion induced by the weak stimulus was shorter than that of wild type animals, and the compensatory enhancement of quiescence was weaker. N = 11 and 12 animals (*e169* and *e928*), plots depict mean ± s.e.m, smoothed using a 30 s running window.

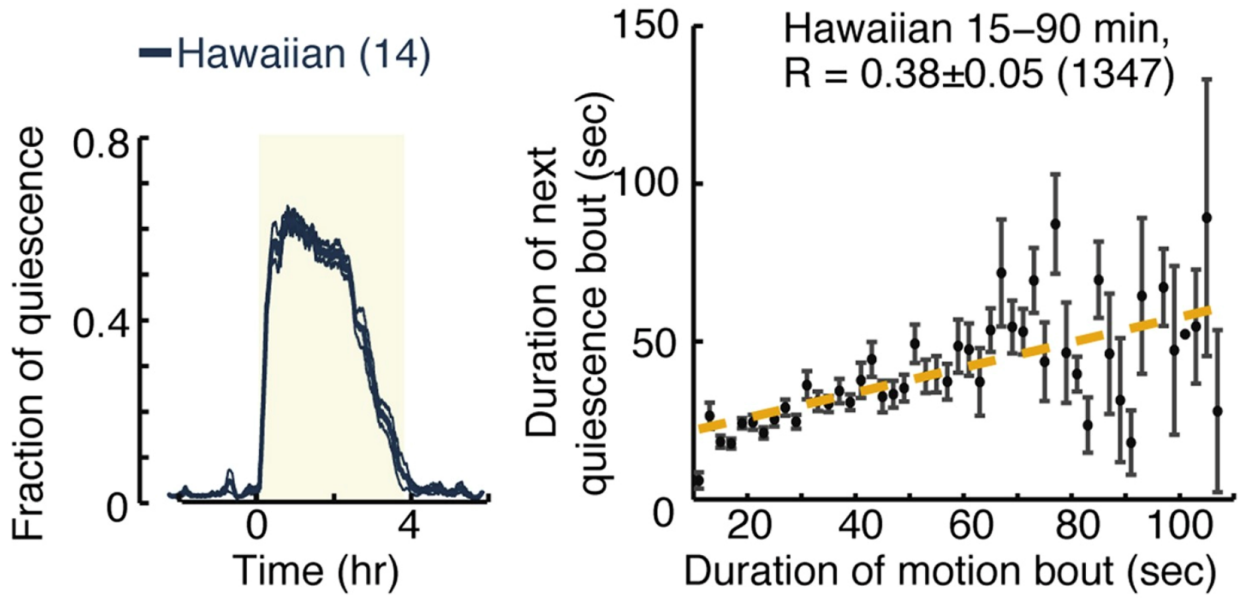

Figure 2.17: **Motion bouts and quiescence bouts are correlated in undisturbed Hawaiian wild-isolates.** Hawaiian animals display overall quiescence similar to those of *unc-31* mutants. However, while pairwise correlations between consecutive bouts are abolished in *unc-31* mutants, this is not the case in Hawaiian animals (see Figure 2.15 for details).

tants in the presence of weak perturbations [76]. The overall quiescence fraction and the durations of quiescence bouts of *flp-18* mutants were comparable to those of *npr-1* mutants (Figure 2.19 and data not shown). However, the correlations between subsequent bouts in undisturbed *flp-18(gk3036)* mutants were intermediate between the wild type and *npr-1* values: 0.33±0.06, 0.47±0.03, and 0.20±0.07, respectively ($p<0.05$, Figure 2.19 B). When stimulated with blue light, both *flp-18* alleles were associated with defective compensatory responses, and the defect was more pronounced in *flp-18(db99)* mutants (Figure 2.19 A, B).

If FLP-18 plays a role in micro-homeostasis then its production, secretion, or both may be temporally correlated with lethargus. To test this, we examined the temporal dynamics of expression during the L4int and L4leth stages of the *Pflp-18*::*flp-18*::SL2::GFP reporter, which contains the upstream promoter region and the entire genomic locus of *flp-18* [76, 335]. As previously reported, expression was observed in several head and ventral cord (VC) neurons. We measured the total GFP fluorescence in head or VC neurons separately. Expression

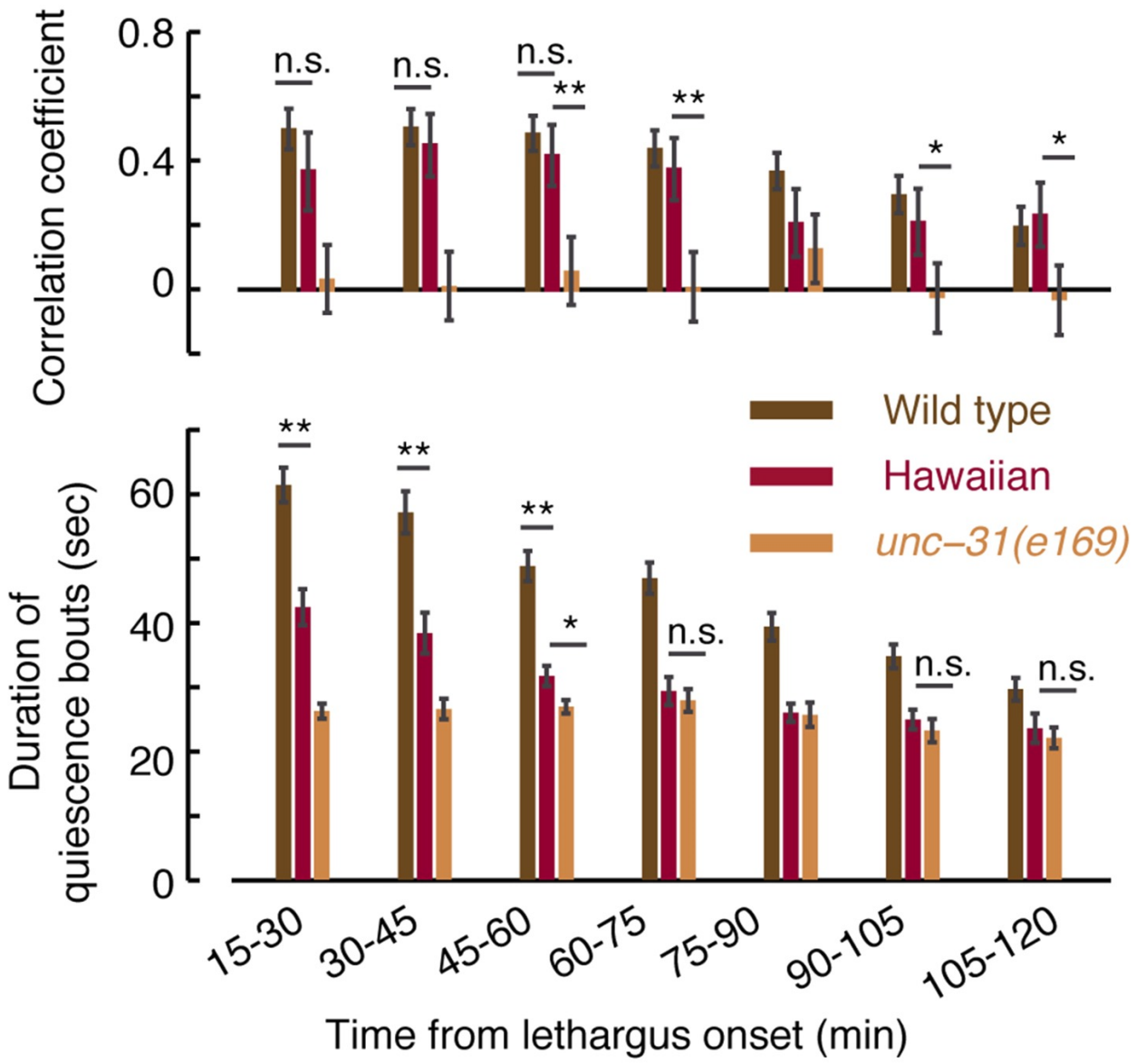

Figure 2.18: **Micro-homeostasis is evident in undisturbed Hawaiian wild-isolates despite having bouts comparable in duration to *unc-31*.** The durations of bouts of quiescence, as well as pairwise correlations, were measured for Hawaiian animals and *unc-31* mutants as a function of time during L4 lethargus. Hawaiian animals display overall quiescence similar to those of *unc-31* mutants. However, while pairwise correlations between consecutive bouts are abolished in *unc-31* mutants, this is not the case in Hawaiian animals The detailed comparison reveals that the duration of bouts cannot in and of themselves account for the strength of the pairwise correlations (see Figure 2.15 for details).

in head neurons was constant prior to the onset of and during L4leth (Figure 2.20). Surprisingly, the reporter expression in VC neurons differed between two sub-populations of animals. When low levels of fluorescence were initially detected during L4int, reporter fluorescence was enhanced more than 2-fold during the first half of L4leth. In contrast, initially high fluorescence levels were not further enhanced. Expression levels of the reporter in the VC neurons of the two sub-populations were similar during the second half of L4leth (Figure 2.20). The absence of a peak in fluorescence during lethargus in the initially strongly fluorescent sub-population may have resulted from non-physiological effects of overexpression. Alternatively, it may be the case that the shift in *flp-18* expression or secretion can precede the onset of lethargus or be conditioned on ambient levels during late L4int.

Since *Pflp-18*::*flp-18*::SL2::gfp expression peaked during the first half of lethargus, we examined the timing of the defect in bout correlations in *flp-18* mutants with respect to the onset of lethargus. The positive pairwise bout correlations in *flp-18* mutants were found to be smaller than wild type during the first hour of lethargus, but not during the second hour, corresponding to the observed period of upregulation in expression of the reporter (Figure 2.21). Collectively, these findings suggest that both FLP-18 and its known receptor, NPR-1, regulate micro-homeostasis during lethargus.

2.3.10 Homeostatic responses to strong stimuli and micro-homeostasis are differentially regulated

Prolonged and stressful deprivation of quiescence during lethargus causes the translocation of DAF-16, a FOXO transcription factor that activates stress responses, into the nucleus. Moreover, *daf-16* mutants were shown to be defective in their behavioral response to prolonged deprivation [91, 146, 223]. Although micro-homeostasis responses occur on a timescale that is too short to be regulated by changes in transcription, repeated weak stimuli may still be stressful. To test the roles of DAF-16 in regulating homeostasis during lethargus we assayed null *daf-16(mu86)* deletion [222] mutants under no-, weak-, and strong-stimulus conditions.

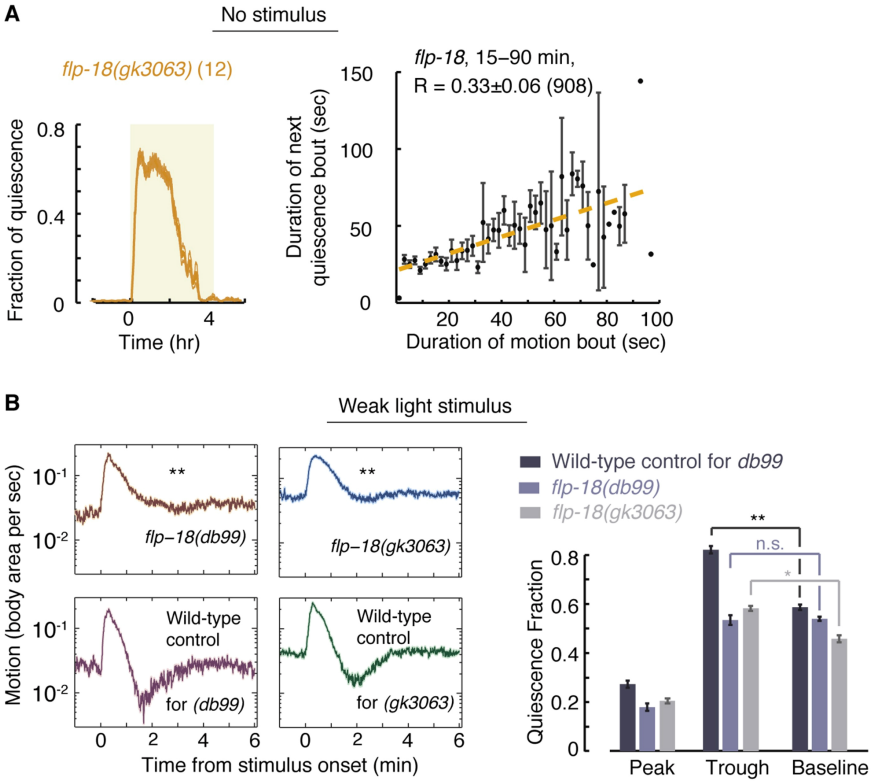

Figure 2.19: **FLP-18 plays a role in modulating bout durations in the presence of weak disturbances.** (A) Posture analysis of undisturbed *flp-18(gk3063)* mutants revealed wild type-like overall quiescence but reduced correlations between subsequent bouts. R = 0.33 ± 0.06, N = 12 animals. These correlations were significantly different ($p < 0.05$) from those of wild type and npr-1 mutants shown in Figures 2.5 and 2.10, respectively. (B) Frame subtraction analysis of *flp-18* mutants during L4leth in the presence of weak blue light stimuli (15 s, 20 mW/cm^2). All stimuli were initiated at t = 0. The dynamics of locomotion revealed defects in the ability of *flp-18* mutants to compensate for the motion induced by the stimulus with enhanced quiescence. Left: the locomotion responses during lethargus of each of the two alleles tested and its wild type control group shown on a semi-log scale. Shaded area denotes mean ± s.e.m. Asterisks denote that during the trough in locomotion, the fraction of quiescence of the mutant allele was significantly lower than that of its respective wild type control ($p < 0.01$). Right: for each strain, the quiescence fraction was calculated during 1 min intervals centered at the times of the peak and trough of the L4leth responses, as well as for their respective pre-stimulus baselines. Plots and bars depict mean ± s.e.m obtained from datasets of N = 4050 animals per condition. Asterisks and double asterisks denote $p < 0.05$ and $p < 0.01$, respectively.

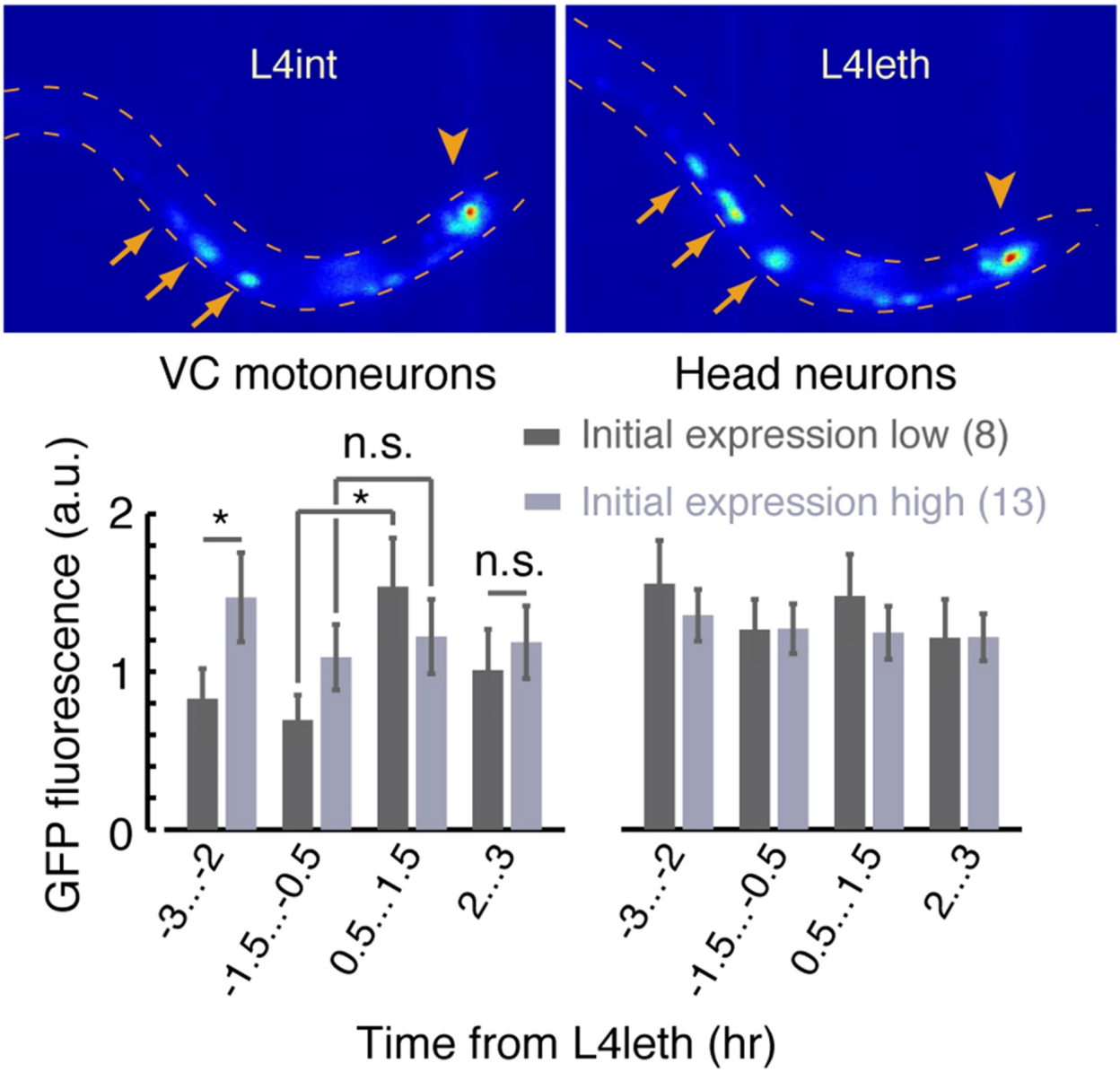

Figure 2.20: **A fluorescent reporter of FLP-18 in VC motor neurons and head neurons.** Top: sample images of the same animal during the late L4int stage (left) and the first half of L4leth (right). Arrows point to VC motoneurons in which expression of the *Pflp-18::flp-18::SL2::gfp* reporter was visibly upregulated. Arrowheads point to head neurons in which changes in expression were not detected. Bottom: the mean fluorescence, before and during lethargus, from reporter expressed in VC motoneurons (left) and head neurons (right). When VC neurons fluorescence prior to lethargus was low (dark grey), it increased more than twofold during the first half of lethargus ($p < 0.05$). When VC neurons' fluorescence prior to lethargus was high (light grey), it did not change significantly afterward. The number of animals assayed is denoted in parentheses, error bars depict ± s.e.m, and asterisks denote $p < 0.05$.

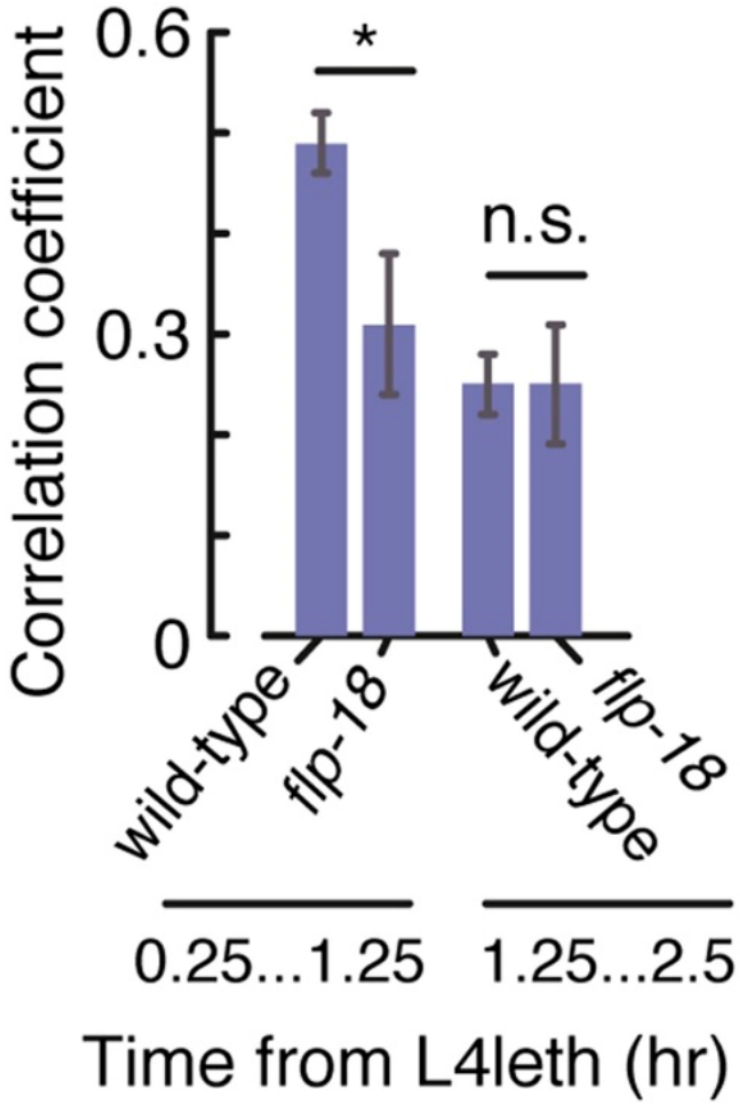

Figure 2.21: **Bout correlations in undisturbed *flp-18* mutants.** Pairwise bout correlations in wild type animals and *flp-18* mutants during the first and second halves of L4leth. The behavior of the mutants differed from wild type only during the first half of L4leth, corresponding to the period of upregulation of the expression reporter. Error bars depict 95% confidence intervals and asterisks denote $p < 0.05$.

These mutants were similar to wild type in their total fraction of quiescence, their initial responses to weak stimuli and subsequent compensation, their responses outside of lethargus to weak and to strong stimuli, and their initial responses during lethargus to strong stimuli. When not disturbed, the quiescence bouts of *daf-16* mutants were shorter than wild type (data not shown) and their pairwise correlations between subsequent bouts were smaller, but not abolished (Figure 2.22). A second mutant allele, *daf-16(mgDf50)* [270], exhibited similar behavior under unstimulated conditions (data not shown). Thus, micro-homeostasis during *C. elegans* lethargus was mostly independent of DAF-16/FOXO signaling.

In contrast, the homeostatic compensation in our strong stimulus assay was completely abolished in both *daf-16* mutants (Figure 2.22). To test where the function of *daf-16* was required, the function of *daf-16* was rescued under the control of the *daf-16* native promoter (*Pdaf-16*), a pan-neuronal promoter (*Punc-119*), and a body-wall muscle promoter (*Pmyo-3*) [38]. Homeostatic compensation for strong disturbances was restored when daf-16 was expressed under its native promoter or in neurons, but not in muscles (Figure 2.22). These findings differ from the reported role of DAF-16 in sleep homeostasis, assayed using response latencies to a noxious chemical, where rescue in muscles but not in neurons restored wild type-like latencies [91]. However, adult locomotion quiescence in *daf-2* mutants (a insulin/IGF-1 receptor homolog), was dependent on the function of DAF-16 in neurons [109]. Thus, DAF-16 may act in multiple tissues to regulate different aspects of the homeostatic response in *C. elegans* sleep.

We noted that the baseline level of quiescence in undisturbed animals varied between the different transgenic strains (Figure 2.22). Broad expression of a rescue gene, or even a fluorescent reporter, often results in subtle changes in locomotion and quiescence that our assays are able to detect. Nevertheless, the data raised the possibility of a ceiling effect for quiescence in these experiments. Two observations suggest that, plausibly, this is not the case: (i) similar differences in undisturbed baseline quiescence were observed between the two *npr-1* mutant alleles, yet both strains exhibited compensation for strong stimuli; and (ii)

undisturbed baseline quiescence in all *daf-16* strains was similar to *npr-1(ky13)* mutants and lower than wild type. Therefore, we favor the interpretation that the function of DAF-16 was required in neurons in our assays. Importantly, the opposing phenotypes of *npr-1* and *daf-16* mutants show that micro-homeostasis, routinely used to stabilize bout architecture in weakly noisy environments, is genetically distinct from the homeostatic responses that strong and stressful disturbances invoke.

2.4 Discussion

Using tunable photo- and mechano-stimulation, we have identified two distinct categories of disruptions to *C. elegans* sleep and characterized the corresponding responses. We have shown that during lethargus, motion plays a causal role in modulating the duration of subsequent quiescence. Under low noise conditions, micro-homeostasis manifested as a dependence of the duration of quiescence bouts on the duration and nature of recently preceding motion. The dynamic extension of quiescence bouts depended on the function of an NPY receptor-like protein (NPR-1). However, this did not require DAF-16/FOXO, perhaps because transcriptional level control is typically too slow to respond dynamically on timescales of 10s of seconds [410]. Since biological mechanisms naturally function in a continuous range of conditions, it was both expected and observed that similar mechanisms regulated the compensatory responses in the presence of weak or no stimuli. However, homeostasis in the presence of strong stimuli was behaviorally and mechanistically distinct. A strong disturbance resulted in the temporary disruption of normal bout dynamics followed by a compensatory upshift of baseline levels of quiescence. These responses did require the function of DAF-16/FOXO but not of NPR-1.

Neuropeptides have been proposed to regulate quiescence during lethargus, but their roles were not examined in detail [264, 373, 376, 377]. The apparent discrepancy between the quiescence phenotypes of *egl-3/egl-21* and *unc-31* mutants resembles their seemingly contradicting roles in homeostatically ameliorating convulsions caused by cholinergic overexcitation,

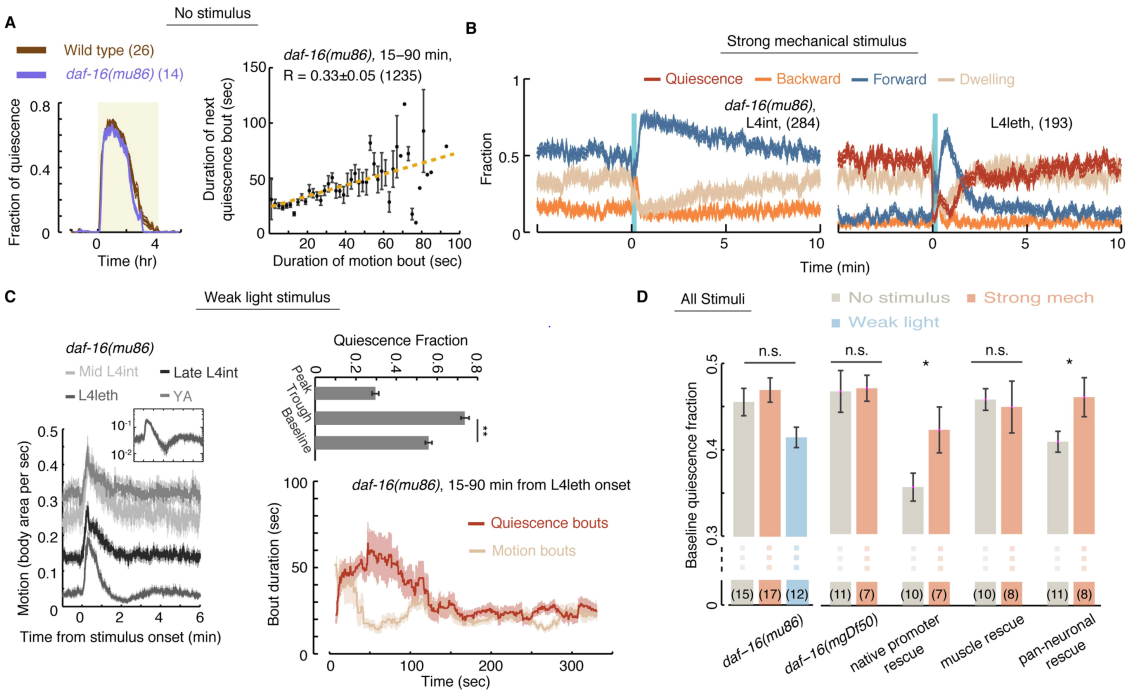

Figure 2.22: **Homeostatic responses to strong stimuli, but not micro-homeostasis, require DAF-16.** (A) Left: the fraction of quiescence of wild type animals and *daf-16* mutants during L4leth (shaded area). Plots depict mean ± s.e.m, the numbers of animals assayed are denoted in parentheses. Right: pairwise bout correlations shown with a plot of binned bouts (see Figure 2.5 for details). Pairwise correlations were reduced in the mutant, although less so than in *npr-1* mutants ($p < 0.05$). All correlations are given with 95% confidence intervals and error bars depict ±s.e.m. The number of bouts in each case is denoted in parentheses. (B) A posture-based analysis of responses of L4int and L4leth *daf-16* mutants to strong stimuli (15 s, 1 kHz vibrations): the fraction of forward locomotion, backward locomotion, dwelling, and quiescence before, during, and after the stimulus. (C) Left: frame subtraction based analyses of responses of L4leth *daf-16* mutants to weak stimuli (15 s, 20 mW/cm^2, blue light). Inset: the response of *daf-16* mutants during L4leth on a semi-log scale. Middle: the fraction of quiescence during 1 min intervals centered at the times of the peak and trough of the L4leth responses, as well as for their respective pre-stimulus baselines. All stimuli were initiated at t = 0. N = 5060 animals. Plots and bars depict mean ± s.e.m, asterisks denote $p < 0.001$. Right: a posture-based analysis of bout dynamics of *daf-16* mutants following a weak stimulus. Plots depict mean ± s.e.m, smoothed using a 30 s running window average. N = 12 animals. The compensatory enhancement of quiescence bouts shortly after the stimulus, as assayed by both methods, was similar to wild type. (D) The mean baseline fractions of quiescence of *daf-16* mutants in undisturbed animals and in the presence of weak and strong stimuli. In contrast to wild type, baseline quiescence fraction was indistinguishable between the different conditions. Expression of *daf-16* in neurons, but not in body-wall muscles, restored the homeostatic response of *daf-16* mutants to strong mechanical stimuli. Error bar depicts ±s.e.m. The number of stimuli assayed is noted in parentheses for each condition.

and can be similarly rationalized [335]. The mature neuropeptides processed by EGL-3 and EGL-21 are but a subset of the components of dense core vesicles, such that excitatory and inhibitory neuropeptides could act in a combinatorial manner to affect quiescence.

Resuming sleep after a strong or a mild disruption are both common experiences. Subjectively, the two are easily distinguishable, and in both cases the resulting changes to the architecture of sleep reflect homeostatic regulation. Broadly, homeostatic control ensuring adequate sleep amount and quality is a key criterion for sleep-like states [44, 263, 316, 357, 359]. Homeostasis in mammalian sleep can be readily observed under disturbed or undisturbed conditions. For instance, the spectral power density associated with slow wave sleep (in the 0.754.0 Hz range) decays exponentially during an undisturbed sleep period, while extending the duration of wakefulness enhances it [108, 121]. Nevertheless, the sleep literature generally regards sleep homeostasis as a single mechanism [11, 34, 37, 68, 80, 82, 147, 231, 263, 280, 302, 389]. To our knowledge, responses to weak disturbances to sleep were not previously carefully analyzed, and the distinction between routine stabilization and compensation for stressful agitation was not examined in detail.

Despite recent findings in genetically tractable invertebrate models, the understanding of mechanisms that regulate sleep homeostasis remains incomplete [11, 68, 91, 316, 321]. NPY was implicated in the regulation of sleep in humans, rats, fruit flies, and nematodes [11, 63, 94, 143, 253, 361, 378]. In *C. elegans*, the NPY receptor homolog NPR-1 affects a range of responses to external stimuli, as well as innate behaviors such as social feeding and quiescence [54, 63, 84, 85, 86, 232, 242, 253]. Interestingly, NPR-1 was found to play a major role in both lethargus micro-homeostasis (this study) and the homeostatic response to a motoneuron imbalance. In the latter case, NPR-1 was required to compensate for cholinergic overexcitation and GABAergic inhibition that were caused by a gain-of-function in a neuronal nicotinic acetylcholine receptor [335]. We hypothesize that these two types of homeostatic responses are closely linked, and further studies will be required to conclusively determine if this is the case.

Recent years have seen a rise in the appreciation of the importance and abundance of peptidergic modulation of neuronal function [23, 156, 219, 233, 345]. In *C. elegans*, peptidergic regulation was shown to affect quiescence during lethargus [264, 373]. The apparent discrepancy between the phenotypes of *egl-3/egl-21* and *unc-31* mutants suggests that quiescence may be regulated by the combinatorial action of excitatory and inhibitory neuropeptides and that this combinatorial regulation promotes responsive bout dynamics. Our findings are consistent with a model in which activity during lethargus generates a pressure which is ameliorated during periods of quiescence. A particular balance of inhibitory and excitatory neuropeptides may be required for keeping a record of and/or for the process of alleviating this pressure.

Finally, responses to external stimuli during lethargus were different from responses during the L4int and YA stages. In contrast, responses were similar whether the onset of the stimulus coincided with quiescence or motion during lethargus. This suggests that bouts of motion are not analogous to brief intervals of wakefulness. Rather, *C. elegans* sleep may progress through two alternating micro-states.

2.5 Materials and Methods

2.5.1 Strains

C. elegans strains were maintained and grown according to standard protocols [35]. The following strains were used: wild type strain N2, Hawaiian CB4856, CB169 *unc-31(e169)*, CB928 *unc-31(e928)*, CX4148 *npr-1(ky13)*, DA609 *npr-1(ad609)*, MT1541 *egl-3(n729)*, MT1241 *egl-21(n611)*, CF1038 *daf-16(mu86)*, GR1307 *daf-16(mgDf50)*, NQ440 *daf-16(mgDf50)*; qnIs42[*Punc-119::GFP::daf-16*; *Pmyo-2::mCherry*], NQ441 *daf-16(mgDf50)*; qnIs45[*Pdaf-16::GFP::daf-16; Pmyo-2::mCherry*], NQ145 *daf-16(mgDf50)*; qnEx38[*Pmyo-3::GFP::daf-16; Pmyo-2:mCherry*], VC2016 *flp-18(gk3063)*, AX1410 *flp-18(db99)*, AX1444 dbIs[*Pflp-18::flp-18::sl2::gfp*], BOL171 *npr1(ad609); lin15[ncs1p::Cre*

flp21p::loxPstoploxP::npr1 SL2 gfp], lin15(+) .

2.5.2 Behavioral Assays

Motion and quiescence were identified using previously described methods [253]. Briefly, animals were grown at 20 degrees C on standard NGM plates seeded with E. coli OP50 bacteria. Mid to late L4 individuals were sealed into individual artificial dirt chambers filled with an overnight OP50 culture concentrated tenfold and resuspended in NGM medium [326]. Animals were imaged at 2 frames per second at a 1.2x magnification for frame subtraction experiments or 10 frames per second at a 4.2x magnification for posture-based analysis using a CCD camera (Prosilica GC2450, Allied Vision Technologies, Stadtroda, Germany). Motion and quiescence were determined as previously described [167, 253]. Frame subtraction data was obtained from the raw images using custom Matlab script (Mathworks Inc., Natick MA) and quiescence was scored when no pixel changed its greyscale value beyond a threshold value [164] between consecutive frames.

2.5.3 Posture based behavioral analysis

The precise analysis of animal behavior, based on the identification of the body posture, required high spatial and temporal resolution data. Image analysis and secondary data analysis were performed as previously described using a custom suite of machine vision tools, called PyCelegans, and custom Matlab scripts, respectively [253, 255]. In brief, we identified the body midline in each frame, as well as the positions of the head and the tail. Each midline was divided into 20 equal intervals and the dynamics of the angles between these intervals were used to identify quiescence and directed locomotion states. The onset of lethargus was identified by visual inspection of quiescence data. We note that typical *C. elegans* behavioral assays provide a throughput of 100-1000 animals per day. In contrast, the detailed and computationally intensive posture-based analysis produced a detailed and accurate account of behavior over 10 hours at a throughput of 3-5 animals per day.

2.5.4 External stimuli

Blue light ($\lambda = 475 \pm 15$nm) was supplied by a Luxeon Star 7-LED assembly with a diffused optic array driven by a 700mA FlexBlock driver. The LED assembly was mounted to the scopes approximately 7cm from the sample location. Light intensity was measured at the location of the animals. The timing of light stimuli was controlled using LabView (National Instruments Inc., Austin TX). Mechanical stimuli were generated using piezo buzzer elements (Digikey part no. 668-1190-ND) as previously described [253]. The timing and duration of the stimuli were controlled using a custom Matlab script. An external stimulus was provided every 15 min throughout the course of each experiment. Animals resumed baseline behavior dynamics after no more than 5 minutes after each individual stimuli and no habituation was observed in the responses to the repeated stimuli.

2.5.5 Fluorescent reporter expression

The *Pflp-18::flp-18::SL2::gfp* reporter strain was a kind gift from the de Bono lab [76]. Late L4int larvae were placed in an artificial dirt microfluidic device filled with an overnight OP50 culture concentrated tenfold and resuspended in NGM medium [226]. Epi-fluorescence images of the freely behaving animals were acquired for 5 sec every 15 min, for 8-9 hours, at a magnification of 20X and a frame rate of 4 frames per second. Regions of interest containing the neurons were identified by visual inspection. Fluorescence was quantified as the sum of pixel intensities that were higher than one standard deviation above the mean of the background pixel intensity. The background was calculated from a region of the body of the animal that was proximal to the neuron of interest but did not contain it. Under these conditions, no photo-bleaching was detected.

2.5.6 Statistical and Numerical analysis

Data analysis was performed using custom Matlab (Mathworks Inc., Natick, MA) scripts. For comparisons in summary statistics panels, significance was calculated using a one-way ANOVA test. Post-hoc correction for multiple comparisons was performed using the Bonferroni adjustment. Correlation coefficients are presented with 95% confidence intervals (Matlab statistical toolbox). Corresponding p-value are the probabilities of obtaining the observed correlation by chance, when the true correlation is zero. To graphically demonstrate pairwise correlations between durations of bouts in Figures 2.1, 2.9, 2.15, 2.19, we grouped all bouts of motion in order of ascending duration in bins of 2 sec and used a linear fit as a guide for the eye. The correlation coefficients were calculated using the original pairs of bout durations (as opposed to the binned data). Bout correlations of wild type animals, *flp-18* mutants, and *npr-1* mutants during the period 15-90 min from the onset of L4leth were compared by applying Fishers z-transformation and calculating the 95% confidence interval for the for the difference of the correlation coefficients as described in [423].

CHAPTER 3

DISTINCT UNFOLDED PROTEIN RESPONSES MITIGATE EFFECTS OF NONLETHAL DEPRIVATION OF *C. ELEGANS* SLEEP IN DIFFERENT TISSUES

JarredSanders[*1], Monika Scholz[*2], Ilaria Merutka[2], David Biron[2,3]

Submitted for publication. JS contributed to the conception and design of the study, performed, analyzed, and interpreted egg-laying and egg-laying circuit experiments, and performed the fluorescent reporter expression experiments and *daf-16(mgDf50)* 5-HT EPGs (Figures 3.1, 3.5 AB, 3.6, 3.8B, 3.9A, 3.10, 3.11, 3.12, 3.13, 3.14, 3.15, 3.16 AB, 3.17, 3.18). MS contributed to the conception and design of the study and performed, analyzed, and interpreted pharyngeal pumping assays (both optical measurements and EPGs) as well as contributing to software for analyzing vm twitches (Figures 3.2, 3.3, 3.4, 3.7, 3.8 ACD, 3.9 BC, 3.16 C). IM performed and analyzed egg-laying and qPCR experiments (Figures 3.5 C, 3.10, 3.15 C). DB contributed to the conception and design of the study and helped analyze and interpret the data. All authors contributed to writing and editing of drafts, and approved the final manuscript. An asterisk (*) denotes equal contribution.

3.1 Abstract

Disruptions to sleep during development can lead to lasting deficits in chordates and arthropods. Using controlled mechano-stimulation, we established an automated protocol for depriving *C. elegans* of developmentally timed sleep in a severe yet nonlethal manner. We

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then characterized three lasting impacts of nonlethal deprivation: a deficiency in pharyngeal pumping, a reduction in brood size and sperm count, and excess twitching in vulval muscles. The mitochondrial unfolded protein response (UPR^{MT}), not commonly associated with consequences of sleep deprivation, mitigated the defect in pharyngeal neurons that underlay the post-deprivation deficiency. In contrast, the fecundity defect was mitigated by the endoplasmic reticulum unfolded protein response (UPR^{ER}), known to be triggered by sleep deprivation in rodents and insects. The reduction in brood size was mirrored by deprivation-induced apoptotic loss of germ cells. However, the egg-laying circuit exhibited an independent phenotype: excess twitching in the vulval muscles, also observed when an egg-laying command neuron is genetically ablated. While the pharyngeal circuit was primarily protected from impacts of deprivation by the UPR^{MT}, the egg-laying circuit exhibited the complementing set of phenotypes and specifically required the function of the UPR^{ER}. Thus, nonlethal deprivation of C. elegans sleep can result in proteotoxic stress which, if gone unmitigated, can cause lasting defects. The defects are anatomically, functionally, and genetically distinct. Correspondingly, the underlying proteotoxic stress responses, which are deeply conserved, can vary between circuits and tissues.

3.2 Introduction

Disrupting mammalian sleep during development correlates with negative effects on physical, cognitive, and social health, thereby supporting the widely accepted notion that sleep is important for appropriate development [128, 170, 273]. Nonlethal sleep deprivation was also shown to cause lasting neurological and behavioral deficits in *D. melanogaster* [189]. However, a mechanistic grasp of why inadequate sleep during development is particularly deleterious is lacking.

Caenorhabditis elegans exhibit developmentally timed sleep during lethargus, a 2-3 hour long period at the termination of each larval stage [40, 283, 328]. Similar to mammalian sleep, lethargus is characterized by locomotion and feeding quiescence, sensory gating, a typical

posture, rebound sleep, and deeply conserved regulation [62, 91, 167, 254, 283, 314, 326, 327, 362]. Disruption of developmentally timed worm sleep promotes transcriptional activity of DAF-16/FoxO, the C. elegans Forkhead box O (FoxO) stress activated transcription factor, whose nuclear translocation is inhibited by the Insulin/Insulin-like growth factor signaling (IIS) pathway [214, 224]. In response to prolonged and continuous deprivation, DAF-16 translocates to the nucleus to mitigate or delay lethality [91] and in the presence of much weaker disruptions DAF-16 is required for rebound sleep [254].

Sleep deprivation is broadly known to be a stressor. In all species examined, including fruit flies and rodents, expression of immunoglobulin binding protein (BiP), an endoplasmic reticulum (ER) chaperone, is upregulated upon sleep deprivation [67, 68, 256, 257, 318, 319]. BiP upregulation requires the action of the ribonuclease inositol requiring protein-1 (IRE-1): a key receptor for sensing ER proteotoxic stress and triggering the ER unfolded protein response (UPR^{ER}) [42, 250, 320, 349, 375, 415]. Upon sensing proteotoxicity, IRE-1 signaling activates the XBP-1 transcription factor which upregulates BiP, a member of the heat shock 70 (Hsp70) protein family, and other UPR^{ER} genes. Thus, the activation of the UPR^{ER} by sleep deprivation is conserved.

In contrast, the connection between sleep deprivation and the mitochondrial UPR (UPR^{MT}) is less clear [142]. Prolonged wakefulness increases daily energy expenditure [93, 98, 179]. For instance, increased neural activity during sleep deprivation increases the energy consumption of the brain [337, 356]. Consequently, energy production by the mitochondrial oxidative phosphorylation system is upregulated [68, 134, 175, 267, 303, 318]. Upon proteotoxic stress, expression of *ubl-5*, encoding a ubiquitin-like protein, is upregulated and UBL-5 plays a key role in activating dedicated chaperones and proteases of the UPR^{MT} [30, 141, 142, 409]. One study found that the mitochondrial chaperones Hsp60 and Grp75 were induced (to a lesser degree than BiP) by sleep deprivation in rat cerebral cortexes [68]. However, the impact of mitochondrial stress during sleep deprivation is unclear.

Tractable model organisms have been prominently used to study responses to environ-

mental stressors, such as oxidation or heat. Protective signaling triggered by such factors is complex and highly conserved [46, 235]. In contrast, the stress inflicted by deprivation of *C. elegans* developmentally timed sleep was minimally explored and deprivation-induced deficits, other than lethality, were never characterized. Here we establish an automated approach to inflicting severe yet nonlethal deprivation of developmentally timed sleep in *C. elegans*. Next, we characterize lasting impacts of deprivation on behavior and physiology and identify distinct UPRs that mitigate these ill effects in different tissues.

Our automated deprivation protocol resulted in a 50% reduction in quiescence during worm sleep while never forcing locomotion for continuous periods longer than a few minutes. Under these conditions, deprivation was nonlethal but negatively impacted feeding, fecundity, and egg-laying physiology. Deprivation upregulated the expression of *ubl-5* and the mitochondrial chaperone gene *hsp-6*, and loss of UBL-5 function specifically affected feeding. Two lines of evidence suggested that the feeding deficiency is likely caused by the impact of deprivation on pharyngeal neurons. Combined, these results suggest that the UPR^{MT} is triggered specifically in the context of the pharynx at severity of sleep deprivation achieved by our assays.

Disrupting worm sleep also triggered the UPR^{ER}, as evident from the upregulation of *hsp-4/BiP* expression. The deprivation-induced reduction in brood size required the UPR^{ER} and resulted from germ cell apoptosis: it required the core apoptosis machinery, activated a germ cell apoptosis reporter, and mirrored a reduction in sperm count [1, 96, 412]. The egg-laying circuit exhibited an independent phenotype, excess twitching in vulval muscles, which is also observed when an egg-laying command neuron is genetically ablated. Opposite to the case of the feeding circuit, the UPR^{ER} (but not the UPR^{MT}) mitigated the impact of deprivation in the egg-laying circuit. In all cases, loss of DAF-16/FoxO function exacerbated the impacts of deprivation.

Collectively, these findings implicate two UPRs and IIS in mitigating the impacts of disrupting worm sleep. They show that developmentally timed sleep is a vulnerable period:

external stimuli that are benign outside of lethargus inflict lasting deficits when administered during lethargus. Appropriate quiescence during this period promotes normal functions in tissues differing in developmental dynamics and physiological activity. Different protective responses mitigate distinct effects of deprivation, depending on the function of the tissue in question and perhaps on its developmental state.

3.3 Results

3.3.1 Substantial deprivation of lethargus quiescence can be automatically inflicted.

Manual poking with a platinum wire or immersion in liquid were previously used to deprive *C. elegans* of quiescence with lethal outcomes [91, 283]. Considerably weaker and nonlethal stimuli, e.g., gently vibrating the cultivation plate once every 15 minutes, transiently forced motion followed by homeostatic compensation [254]. We found that *C. elegans* can desensitize to such stimuli if they were delivered too frequently. However, a rectangular pulse train with a period of 6 minutes and duty-ratio of 50% reduced the average quiescence by 50%. The effects of the stimuli on the quiescence of wild type animals and *daf-16* mutants were similar, although undisturbed *daf-16(mgDf50)* mutants exhibited the highest amount of quiescence (Figure 3.1 A,B).

Forkhead box O (FoxO) transcription factors have highly conserved structures and function in regulating protective responses, metabolism, and lifespan [110, 180, 270, 347]. Following various forms of stress, insulin/insulin-like signaling (IIS) causes translocation of the *C. elegans* DAF-16/FoxO to the nucleus to initiate protective transcriptional responses [112, 146, 214, 224, 247]. In particular, manually forced locomotion during worm sleep results in translocation of DAF-16 to nuclei of intestinal and body wall muscle cells [91]. We asked whether our periodic perturbation protocol would induce similar translocation of DAF-16. To address this, we applied our protocol for one hour starting during the first 30 minutes of

the fourth lethargus stage, L4 lethargus. In agreement with the manual (lethal) deprivation protocol, we observed nuclear localization in intestinal cells (Figure 3.1 C). Translocation was not observed following a mock perturbation protocol, where vibrations were not applied during an equivalent one-hour period. In our hands, a clear translocation response was not observed in body wall muscles.

In contrast to the consequences of continuous manual deprivation [91], we did not observe any molting defects or lethality following our deprivation conditions. Possibly, this was a consequence of not depriving the animals of quiescence for a continuous period that exceeded 3 minutes. Collectively, these results demonstrate the ability to automatically and severely disrupt quiescence to a stressful yet nonlethal degree. In the assays described below, deprivation was achieved through applying our mechanical stimuli starting prior to the onset of lethargus and ending early in the young adult stage. Control animals were stimulated during a comparable period that excluded lethargus (see methods).

3.3.2 Nonlethal deprivation results in a DAF-16/FoxO dependent deficit in pharyngeal pumping.

The pharynx of *C. elegans* is a simple and self-contained neuromuscular organ with a well-defined function. Therefore, we asked whether sleep deprivation would affect its operation. The contraction and relaxation cycle of pharyngeal pumping takes in bacterial food, expels the surrounding liquid, and traps the food [16, 20]. Pumping rates depend on feeding history, quality of food, and endogenous serotonin levels [154, 309, 322, 333]. In the absence of food, pumping can be stimulated with exogenous serotonin [154, 332]. The pharyngeal nervous system consists of 20 neurons, it is isolated from the rest of the animal by the basal lamina, and it can operate independently [3, 282]. To characterize the effects of deprivation on pharyngeal function, we measured pumping continuously for 60 minutes at a food concentration corresponding to an optical density $OD_{600} = 2.5$, where pumping activity was found to be intermediate (Figure 3.2). We then compared between deprived and control (stimulated for

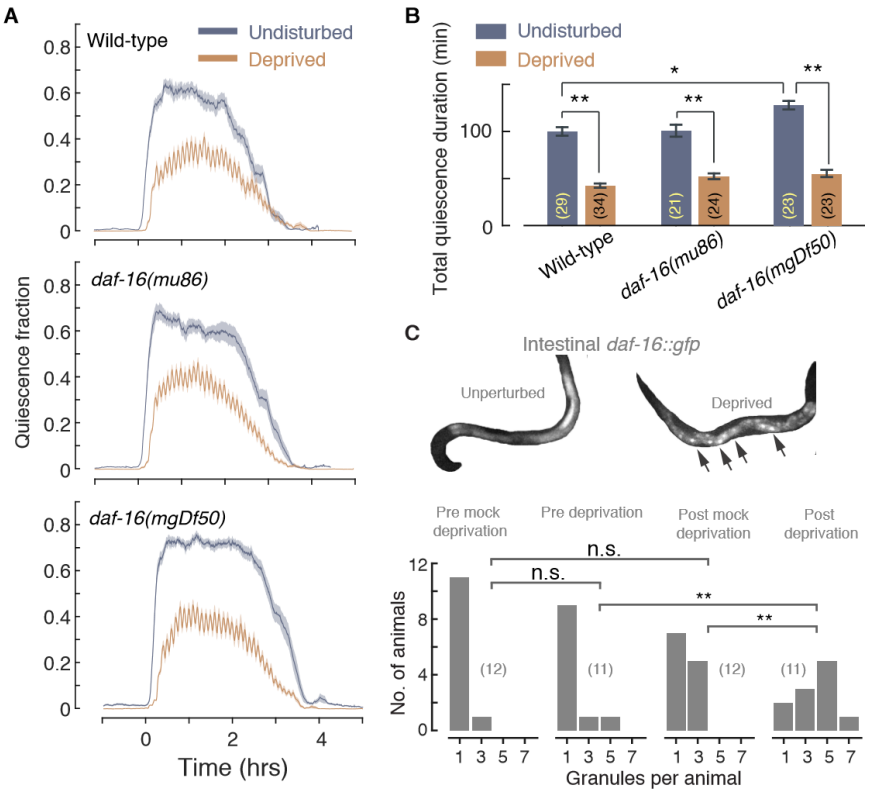

Figure 3.1: **A periodic mechanical stimulus can partially and nonlethally reduce lethargus quiescence and induce translocation of DAF-16.** (A) The fraction of quiescence measured during L4 lethargus of undisturbed (grey) and deprived (orange) wild type animals and *daf-16* mutants. Locomotion was forced using a square wave of mechanical vibrations with a 6 minute period and 50% duty cycle. Shaded areas depict mean±s.e.m. (B) The total time in which quiescence was observed integrated over the data presented in panel A. Error bars depict mean±s.e.m. (C) DAF-16::GFP fluorescence in the intestine after one hour of partial deprivation. Top: examples of GFP fluorescence in unperturbed and partially deprived animals. Arrowheads point to bright particles indicating concentration of DAF-16::GFP. Bottom: Histograms of the number of bright particles per animal identified under each set of conditions. Sample sizes are denoted in parentheses. Single and double asterisks denote significant differences with $p<0.05$ and $p<0.01$, respectively.

a comparable amount of time outside of lethargus) animals. For all remaining experiments, "undisrupted" means unstimulated and "control" are animals that have been stimulated outside of lethargus.

Pharyngeal pumping can be adequately described as a time series of discrete stereotypical events [20]. These time series are composed of bursts of rapid pumping interspersed by pauses [309]. To summarize the data, we used the mean instantaneous pumping rate and the duty ratio of continuous rapid pumping (i.e., the persistence of pumping). These summary statistics did not reveal significant differences between deprived and control wild type animals thus implying there was no effect. However, forced locomotion specifically during lethargus reduced pumping in *daf-16* mutants and the native promoter rescue of DAF-16 function restored the wild type phenotype (Figure 3.3 A). These results suggest that worm sleep deprivation can negatively impact feeding by shortening the persistence of rapid pharyngeal pumping and that DAF-16 mitigates this adverse effect.

Optical tracking of pumping motion enabled high-throughput measurements over prolonged periods, beneficial for statistical characterization. Complementarily, electropharyngeograms (EPGs) revealed the dynamics of contraction and relaxation of the pharyngeal corpus and terminal bulb, and enabled precise measurements of the duration of a single pump. We found that repeated mechanical stimuli extended the duration of individual pumps and could alter the shape of the contraction peak (Figure 3.3 B,C). However, these effects were not specifically associated with sleep deprivation: deprived animals and controls exposed to the mechanical stimuli before and after lethargus exhibited similar EPG characteristics (Figure 3.3 B,C). Nevertheless, EPGs did recapitulate the mean pumping rate defect in *daf-16* mutants (Figure 3.4). Thus, our assays differentiated between deprivation related and nonspecific impacts of mechanical stimuli.

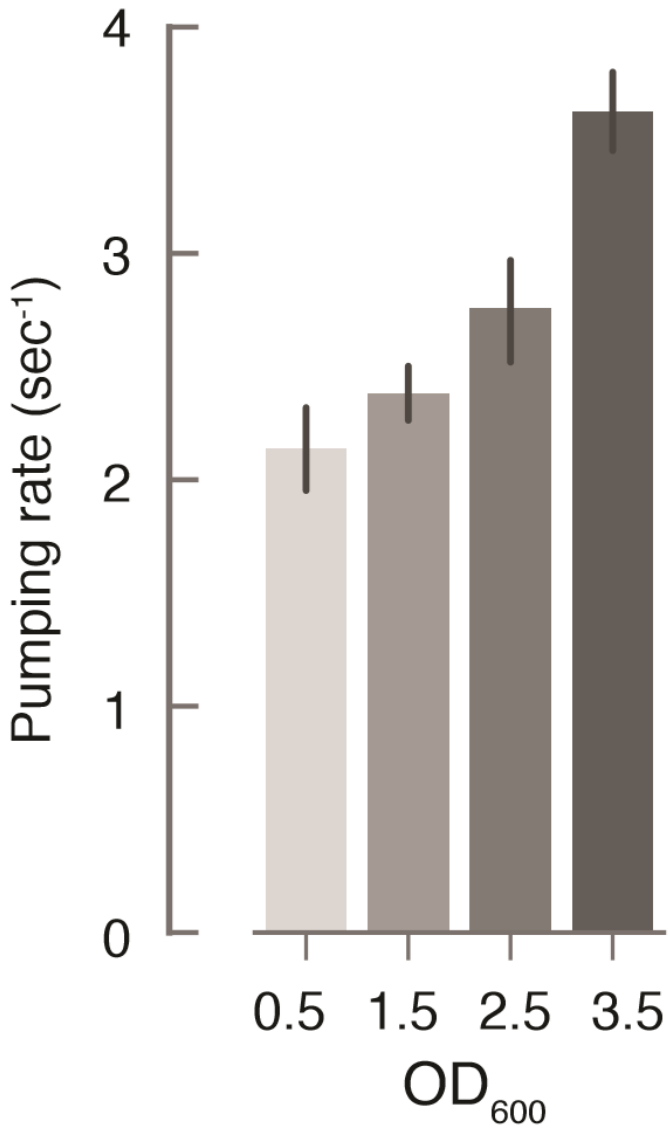

Figure 3.2: **Pumping rates increase as a function of food availability.** The average pumping rate of wild type animals at different concentrations of ambient bacterial food (as measured by optical density, OD_600). Error bars depict mean±s.e.m.

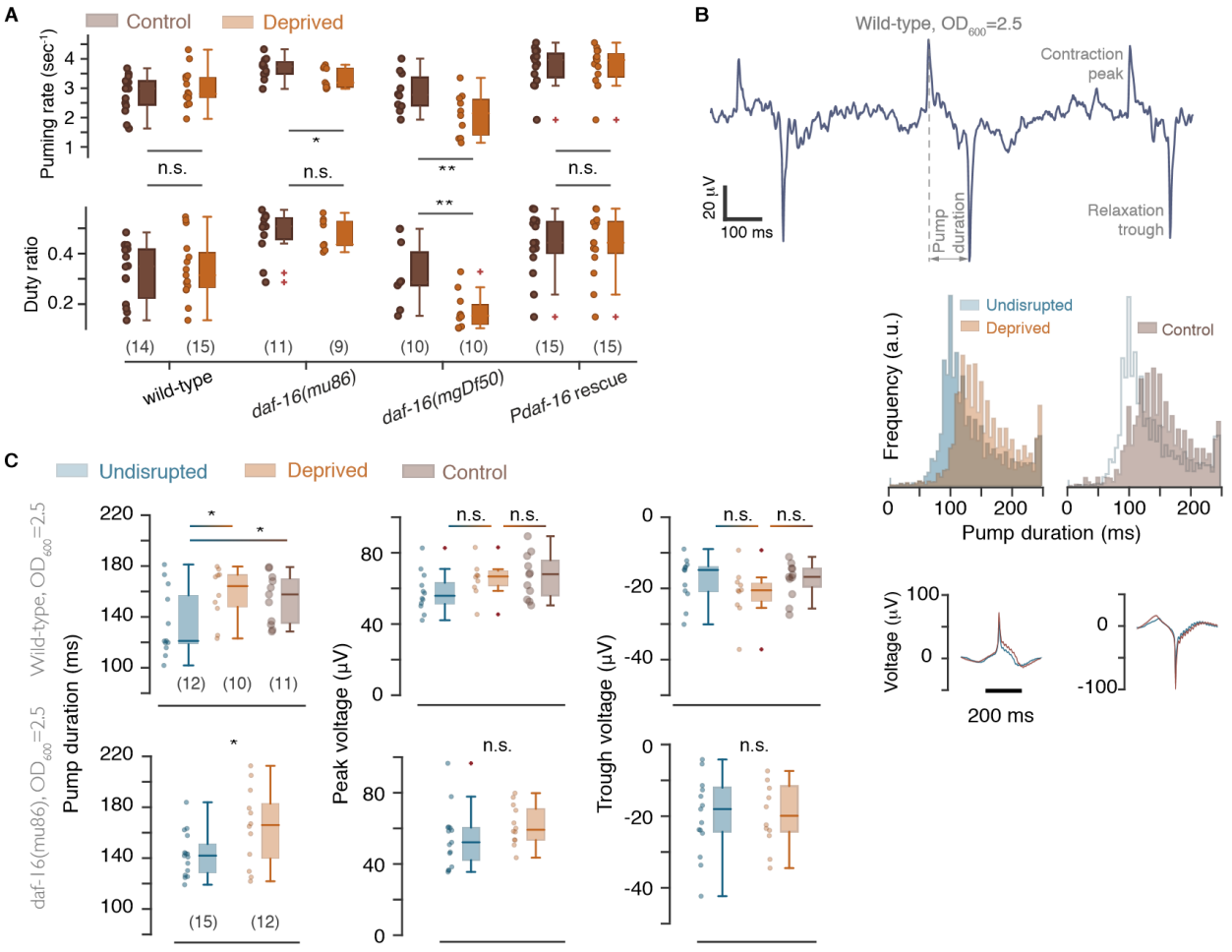

Figure 3.3: **Pharyngeal pumping is negatively impacted by nonlethal deprivation in a DAF-16 dependent manner.** (A) Box plots of average pumping rates and duty ratios for control and deprived animals. Continuous pumping was defined as a period in which the delay between pumps did not exceed 192 ms. (B) Top: a sample EPG trace of a wild type animal in the presence of food at OD_{600} = 2.5 concentration. Peaks correspond to corpus and terminal bulb contraction. Troughs correspond to corpus relaxation. Bottom: average contraction and relaxation EPG traces for undisturbed and deprived wild type animals. Distributions of pump durations are shown for undisrupted, deprived, and control (disturbed outside of lethargus) animals. The outline of the distribution for undisturbed animals was duplicated as a guide to the eye. (C) The mean (per animal) pump durations and amplitudes of EPG peaks and troughs for wild type animals (top) and *daf-16(mu86)* mutants (bottom). Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes are noted in parentheses, asterisks and double asterisks denote significant differences ($p<0.05$ and $p<0.01$, respectively).

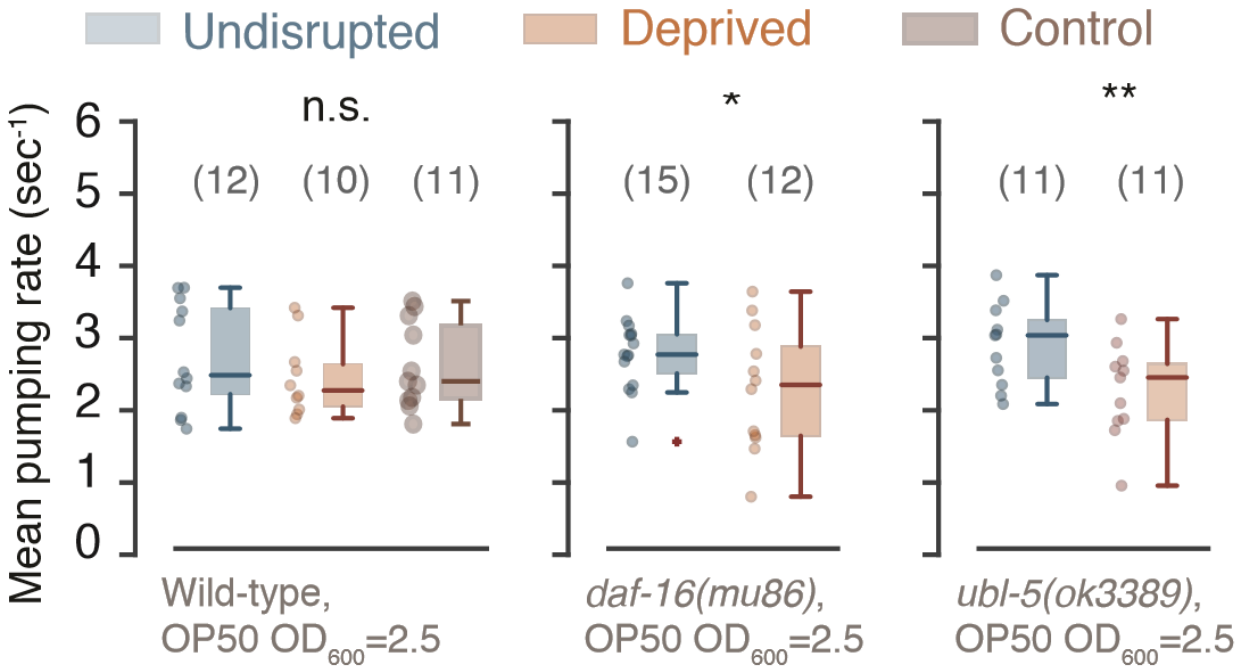

Figure 3.4: **Instantaneous pumping rates measured from EPG traces.** Instantaneous pumping rates were calculated as 1/(duration between consecutive contraction peaks). Mean (per animal) rates for of wild type animals, *daf-16(mu86)* mutants, and *ubl-5(ok3389)* mutants reproduced the phenotypes seen in the optical measurements.

3.3.3 The UPR^{MT} plays a role in mitigating effects of nonlethal deprivation on pumping.

The ubiquitin-like protein UBL-5 is required for UPR^{MT} promotion of expression of the mitochondrial chaperone genes. Expression of *ubl-5* was shown to be upregulated in response to mitochondrial stress [30, 141, 142, 409]. While mitochondrial turnover may be correlated with the sleep-wake cycle, as sleep is a period of fasting [123], a connection between lasting impacts of sleep loss and the mitochondrial unfolded protein response (UPR^{MT}) is not well established.

The *ubl-5* translational reporter expresses broadly at low levels and brightly in the posterior bulb of the pharynx, the posterior of the intestine, and the anterior edge of the intestine near the pharyngeal-intestinal valve [30]. After exposure to one hour of our sleep disrupting stimulus, we observed a small but significant upregulation of *Publ-5::ubl-5::gfp* expression in the intestine. No increase in reporter fluorescence was observed following the mock protocol or when vibrations were applied at the mid L4 larval stage (Figure 3.5 A). We similarly assayed two additional indicators of the UPR^{MT}: the *hsp-6* and *hsp-60* transcriptional reporters. HSP-6 and HSP-60 are the C. elegans mitochondrion-specific chaperones belonging to the Hsp70 and Hsp10/16 superfamilies, respectively. Both are upregulated by chemically induced mitochondrial stress [195, 409].

Expression of *hsp-6* was broad and most clearly visible in the intestine. Strong expression of *hsp-6* or an accumulation of the reporter brightly stained the posterior segment of the intestine and sleep deprivation did not affect this posterior bright patch. However, expression in the rest of the intestine was upregulated after our one-hour deprivation protocol while mock deprivation did not affect *hsp-6* expression (Figure 3.5 B). In contrast to the case of the pharmacologically induced UPR^{MT}, we could not detect a similar upregulation of *hsp-60* expression (Figure 3.6). However, we cannot rule out the possibility that upregulation of this chaperone in our hands was below the detection threshold, e.g., due to tissue specificity. We used real-time PCR to assay the relative expression of these genes after a four-hour period of

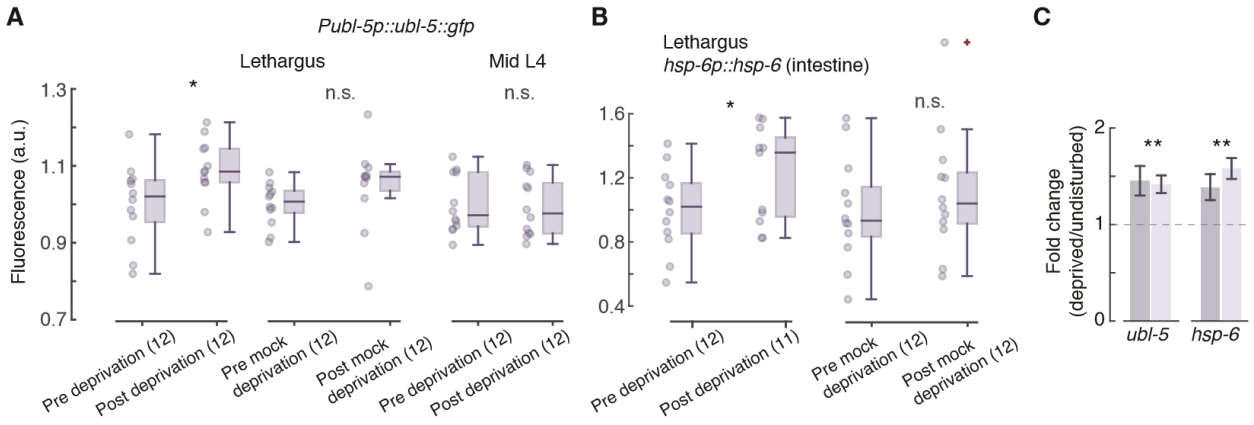

Figure 3.5: **The UPR^{MT} is triggered by nonlethal deprivation of worm sleep.** (A) Intestinal fluorescence of the *ubl-5p::ubl-5::gfp* UPR^{MT} reporter before and after deprivation, mock deprivation, and stimulating mid L4 larvae. (B) Intestinal fluorescence of the mitochondrial chaperone *hsp-6::gfp* reporter after deprivation and mock deprivation. Sample sizes are noted in parentheses, asterisks and double asterisks denote significant differences ($p<0.05$ and $p<0.01$, respectively). (C) Relative expression of *ubl-5* and *hsp-6* in deprived as compared to undisrupted wild type animals (2 biological replicates). The mechanical stimulus was applied for 4 hours (which included L4 lethargus) and RNA was prepared immediately after this period. Error bars depict mean±s.e.m. from 6 technical replicates and asterisks denote significant differences from a ratio of 1.0 ($p<0.05$).

administering the disruptive stimuli that included L4 lethargus. Consistently, we observed elevated expression of *ubl-5* and *hsp-6* in deprived animals (Figure 3.5 C).

To test whether the C. elegans UPR^{MT} plays a role in mitigating consequences of nonlethal sleep deprivation we examined feeding in *ubl-5* null mutants. Deprived *ubl-5* mutants exhibited a lower pumping rate as compared to the control group. This pumping defect was rescued by expressing the *Publ-5::ubl-5::gfp* translational reporter (Figure 3.7 A, Figure 3.4). The elongation of durations of individual pumps in *ubl-5* mutants was comparable to that of wild type animals and *daf-16* mutants (Figure 3.7 B). Thus, we did not identify a role for the UPR^{MT} in mitigating this non-specific effect of mechanical stimulation. Taken together, our results indicate that the UPR^{MT} is triggered by deprivation of *C. elegans* sleep and plays a role in mitigating the effects of deprivation on pumping but not on germ cell apoptosis.

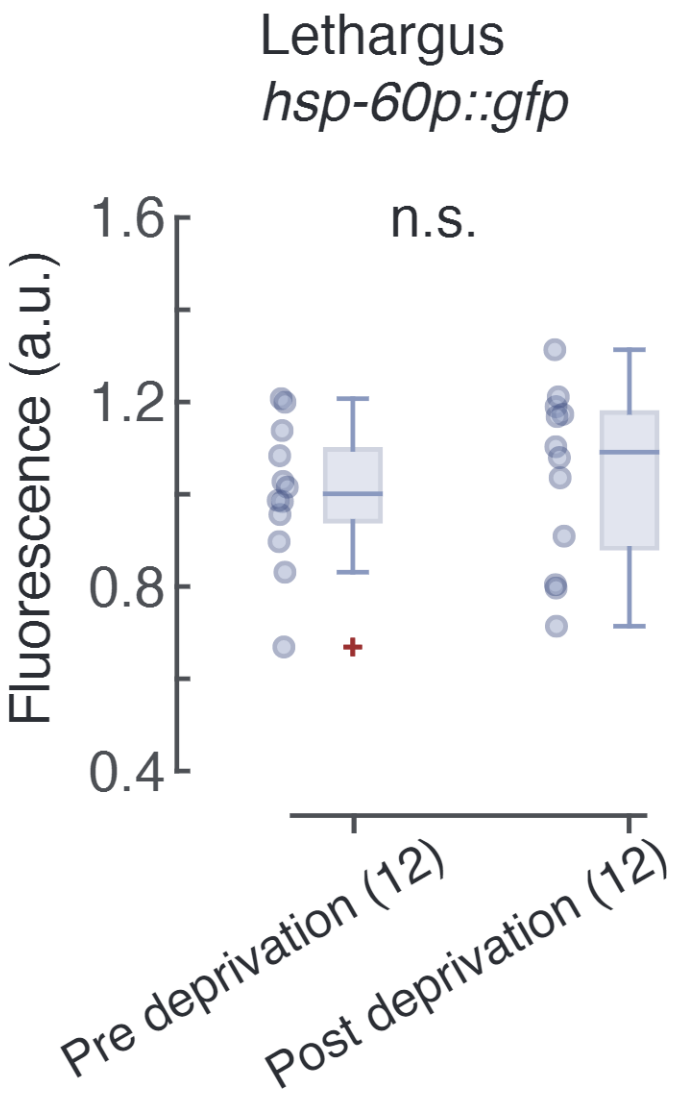

Figure 3.6: **Intestinal fluorescence of the *hsp-60p::GFP* fluorescent reporter before and after deprivation.** Elevated expression of the reporter was not observed after one hour of disrupting worm sleep with periodic vibration stimuli.

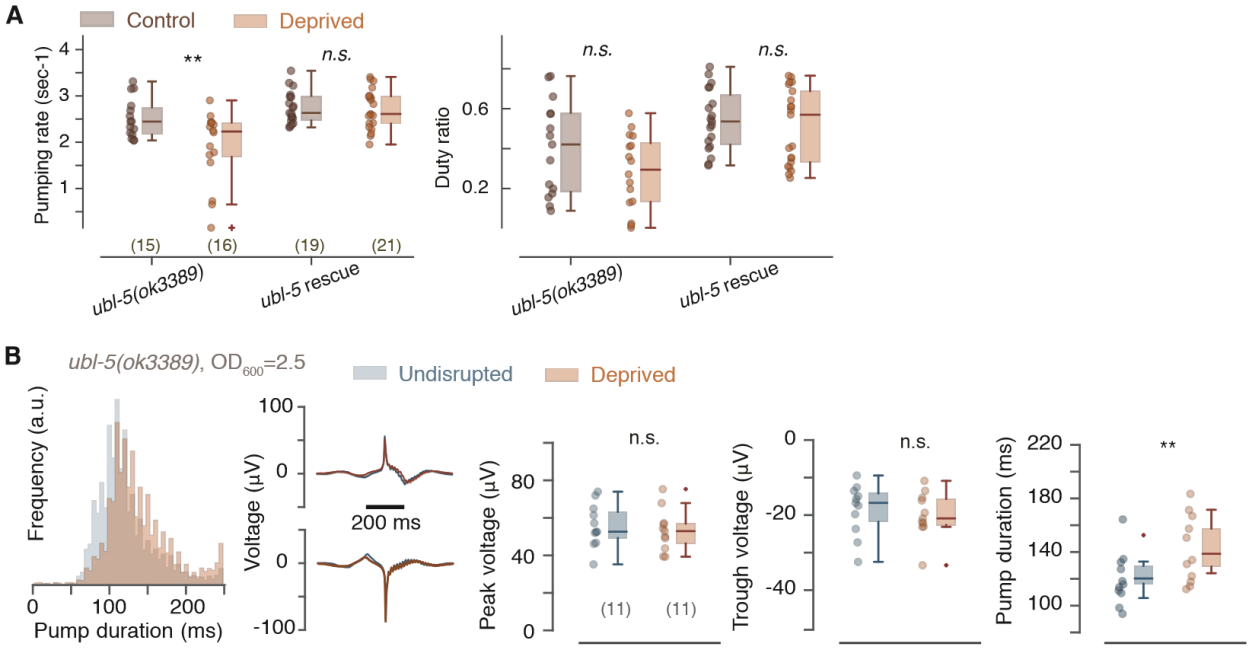

Figure 3.7: **Post-deprivation feeding is protected by the UPR^{MT}.** (A) Average pumping rates and duty ratios for control and deprived animals. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. (B) Distributions of pump durations, average contraction and relaxation EPG traces, mean (per animals) EPG peak and trough amplitudes, and mean (per animal) duration of individual pump for *ubl-5* mutants. Sample sizes are noted in parentheses and double asterisks denote significant differences ($p<0.01$).

3.3.4 Deprivation impacts pharyngeal pumping likely through affecting regulatory neurons.

The pharynx is isolated from the rest of the animal and can exhibit pumping tens of minutes after it has been dissected out [3, 282]. Pumping defects induced by sleep deprivation can thus originate from pharyngeal regulatory neurons or pharyngeal muscles. We note that our mechanical stimuli do not noticeably affect the buccal plug, a cap of extracellular material that prevents food from entering the pharynx during lethargus [328], and they do not induce pumping. Consequently, the stimuli do not activate the pharyngeal muscles during lethargus and wear and tear damage caused by anachronistic muscle activation is unlikely.

To address whether pumping deficit was a result of damage to the neurons or muscles, we induced pharyngeal pumping in young adults using 10mM serotonin (5-HT) instead of food. Serotonin is known to robustly activate rapid pumping through the action of the neuronally expressed SER-7/ 5-HT7 receptor [154, 154, 332]. We found that pumping rates in the presence of 5-HT were high in undisrupted and deprived *daf-16(mgDf50)* and *ubl-5* mutants (Figure 3.8 B,C). The serotonin receptor SER-1/5-HT2 is expressed in pharyngeal muscles [369]. However, SER-1 was found to have a mild or no contribution to pharyngeal pumping rates, whether induced by food or by 5-HT [87, 154]. To assess the potential impact of activation of SER-1 we assayed mutants carrying the putative null allele *ser-1(ok345).* In our hands, loss of SER-1 did not significantly affect the shape of individual contractions and relaxations and did not affect the rate of 5-HT induced pumping (Figure 3.8 D). Moreover, we were able to rescue the pumping defect in *daf-16(mgDf50)* mutants post-deprivation by restoring DAF-16 function in neurons (Figure 3.8 A).

Rapid pumping in *ser-1* mutants suggests that 5-HT triggers pumping through activating pharyngeal neurons. The ability of deprived *daf-16* and *ubl-5* mutants to pump rapidly suggests that their deficits, exhibited in the presence of food, are the result of regulation rather than a biomechanical limit. Combined, these data indicate that rapid pumping is mechanically possible even in sleep deprived mutants upon activation of pharyngeal neu-

rons. Thus, sleep deprivation likely results in lasting deficits in the neural circuit regulating pumping.

3.3.5 Nonlethal deprivation results in DAF-16/FoxO dependent reduction in brood size.

Sleep deprivation was recently shown to adversely affect fertility in rodents [8, 360]. To address whether nonlethal deprivation impacts *C. elegans* fecundity, we compared brood sizes of deprived and control animals. We found that brood size in the control group (where stimuli were delivered outside of lethargus) was indistinguishable from that of undisrupted animals. However, sleep deprivation reduced wild type brood size by 10% (Figure 3.10 A). The negative impact of nonlethal deprivation was exacerbated in *daf-16(mu86)* mutants, where brood size was reduced by 29% (Figure 3.10 A). In *daf-16(mgDf50)* null mutants, where brood size was markedly lower in the control group, we observed an 18% reduction (Figure 3.10 B).

To address the possibility of a floor effect, we increased the brood size of *daf-16(mgDf50)* mutants through male mating [393]. In our hands, the frequency of mating encounters was not affected by our deprivation protocol (data not shown). By the third day of adulthood, we observed a 21% increase in the brood size of the control group as compared to self-fertilization, and a 24% reduction in brood size following forced locomotion during lethargus of both hermaphrodites and males. Disrupting quiescence of either hermaphrodites or males (but not both) resulted in intermediate phenotypes (Figure 3.10 B). When the function of DAF-16 was restored through driving expression with the native promoter, brood size was not reduced following forced locomotion during lethargus (Figure 3.10 C).

In addition, when the degeneration-causing, constitutively active nicotinic acetylcholine receptor (nAChR) channel subunit, *deg-3(u662)*, was expressed in touch neurons [2, 160, 301, 363, 364], animals did not respond to vibrations. As expected, failure to respond abolished the reduction in brood size in the presence of the stimuli (Figure 3.10 D, Figure

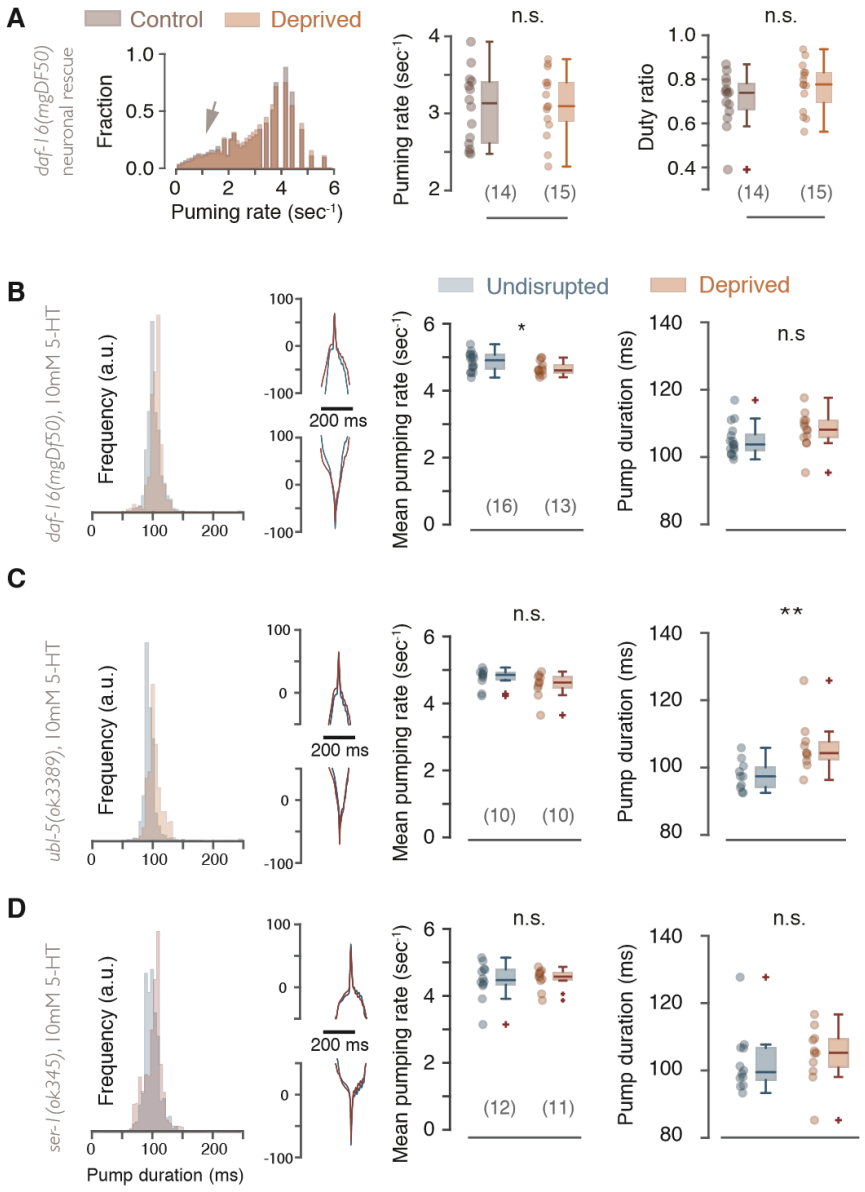

Figure 3.8: **A neuronal regulation deficiency underlies the post-deprivation slowdown of feeding.** (A) Left: distributions of pumping rates deprived and control *daf-16* mutants where neuronal function of DAF-16 was restored. Right: mean pumping rates and duty ratios of burst of rapid pumping under these conditions. (B) Distributions of pump durations, mean (per animal) contraction and relaxation EPG traces, mean pumping rates, and mean (per animal) durations of individual pumps for 5-HT triggered pumping in undisrupted and deprived *daf-16(mgDf50)* mutants. (C-D) Same as panel B for *ubl-5* and *ser-1* mutants. Shaded areas depict mean±s.e.m. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes are noted in parentheses, asterisks and double asterisks denote significant differences ($p<0.05$ and $p<0.01$, respectively).

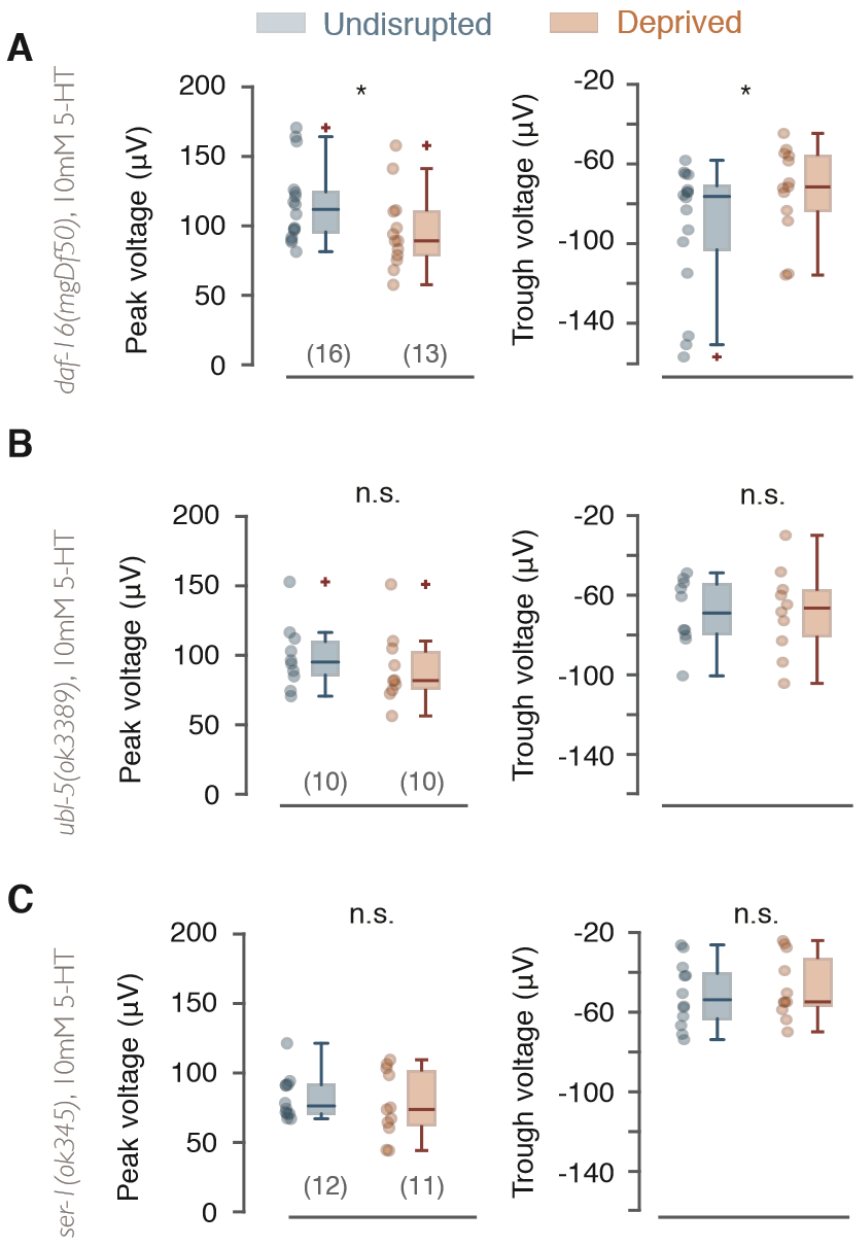

Figure 3.9: **Characteristics of 5-HT induced electropharyngeograms.** Mean (per animals) EPG peak and trough amplitudes for undisrupted *daf-16*, *ubl-5*, and *ser-1* mutants in the presence of 10 mM 5-HT. Sample sizes are noted in parentheses and asterisks denote significant differences ($p<0.05$).

3.12, Figure 3.11). In contrast to brood size, forced locomotion did not appreciably increase the number of retained eggs (Figure 3.13). Collectively, these results suggest that nonlethal sleep deprivation in *C. elegans* negatively impacts brood size but not through a pronounced egg-laying defect and that DAF-16/FoxO mitigates this adverse effect.

3.3.6 Germ cell apoptosis causes the DAF-16 dependent reduction in brood size following nonlethal deprivation

The key determinant of *C. elegans* brood size is the number of available sperm [393]. Germ cell apoptosis can be triggered to protect against DNA damage or environmental stressors that are not directly genotoxic, e.g., oxidative stress, heat shock, or pathogens. In both cases, core apoptotic genes are strictly required for the initiation of programmed cell death [1, 7, 113, 138, 298]. We reasoned that stress induced germ cell apoptosis may explain the effect of *C. elegans* sleep deprivation on fecundity. To address this, we explored three lines of evidence.

Developmental cell death in C. elegans requires a highly conserved pathway. This apoptotic core machinery includes CED-3, a cysteine-aspartate protease essential for execution of apoptosis [79, 96, 149, 217, 405, 412]. We therefore crossed *daf-16(mu86)* mutants, which exhibited the most pronounced effect on fecundity, with *ced-3(n1286)* mutants. On the *daf-16; ced-3* double mutant background, the post-deprivation reduction in brood size was completely eliminated (Figure 3.14 A). Moreover, the number of eggs counted in deprived, control, and undisrupted animals were indistinguishable from those of undisrupted *daf-16(mu86)* mutants. This suggested that the *ced-3* mutation did not affect fecundity independently of sleep deprivation.

CED-1 is a transmembrane receptor that mediates the engulfment of cell corpses [419]. It is found in sheath cells surrounding the germline, where it clusters around engulfed early apoptotic corpses of germ cells [311]. A translational *ced-1::gfp* reporter can be used for visualizing germ cell apoptosis when the number of cell deaths is not too large [113]. We

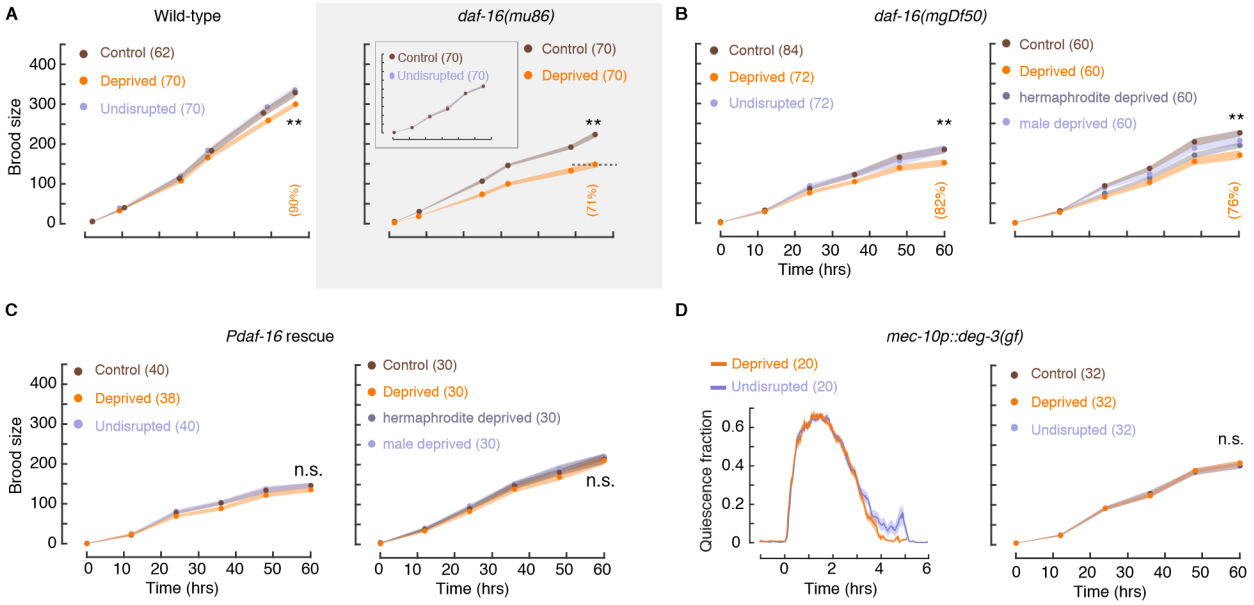

Figure 3.10: **Post-deprivation brood size is reduced in a DAF-16 dependent manner.** (A) Brood sizes of wild type animals and *daf-16(mu86)* mutants during the first three days after L4 lethargus (t=0 is 10-12 hours after the fourth molt). Deprived animals were exposed to the stimulus before, during, and after L4 lethargus. Control animals were exposed to the stimulus before and after L4 lethargus. Inset: undisrupted animals which were never exposed to the stimulus were indistinguishable from the control group. The dotted line depicts the brood size of deprived animals at the latest time point (B) The same as panel A for *daf-16(mgDf50)* mutants that either self-fertilized (left) or mated with males (right). In the latter case, either the males, the hermaphrodites, or both were deprived of quiescence. (C) The same as panel A for *daf-16(mgDf50)* mutants in which expression of *daf-16* cDNA was driven using the *daf-16* native promoter. (D) Left: quiescence fraction during L4 lethargus of animals deficient in touch sensation (*mec-10p::deg-3(gf)*), whose responses to vibrations were mostly or entirely abolished. Right: the same as panel A for transgenic animals deficient for touch sensation. Shaded areas depict mean±s.e.m., sample sizes are noted in parentheses, and double asterisks denote a significant difference in brood size at t=60 hours ($p<0.01$).

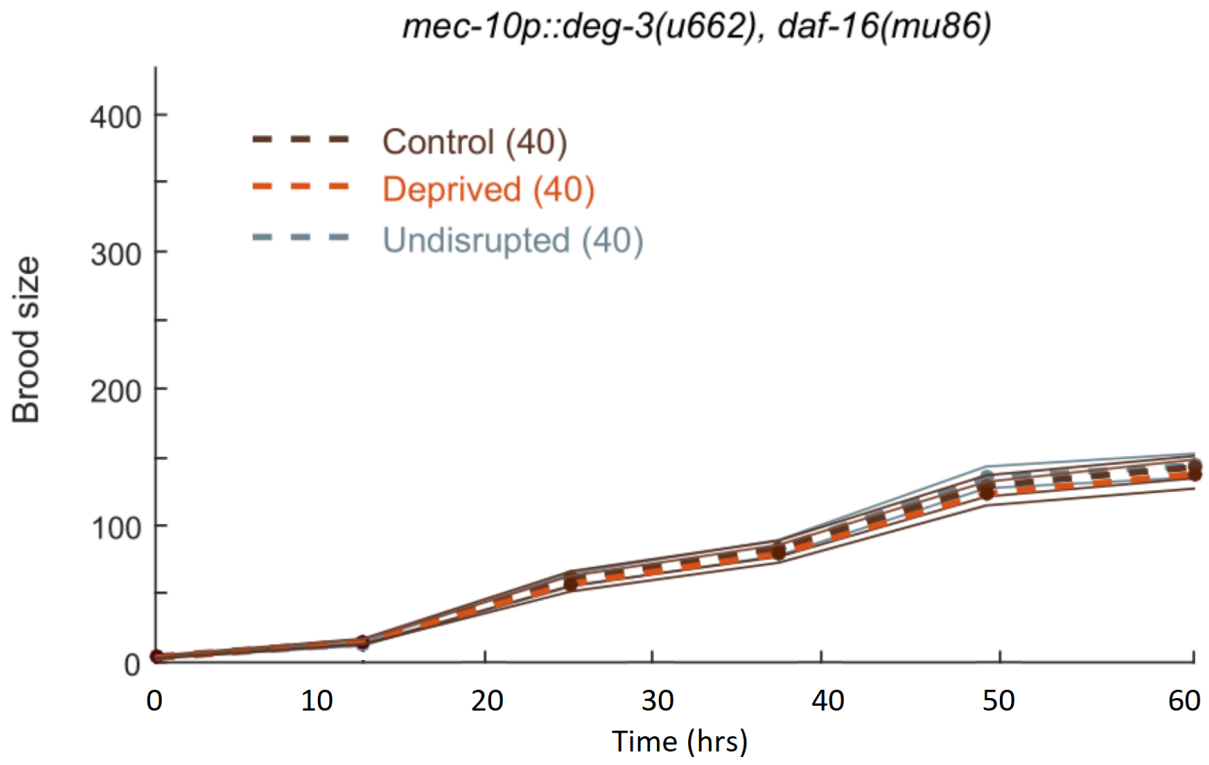

Figure 3.11: **Animals deficient for touch sensation do not exhibit brood size defects after deprivation.** *daf-16(mu86)* crossed to *mec-10p::deg-3(gf)* transgenic animals that are deficient for touch sensation do not have a decreased brood size following 1 kHz vibrations. N=40 animals; solid lines depict s.e.m. around the mean.

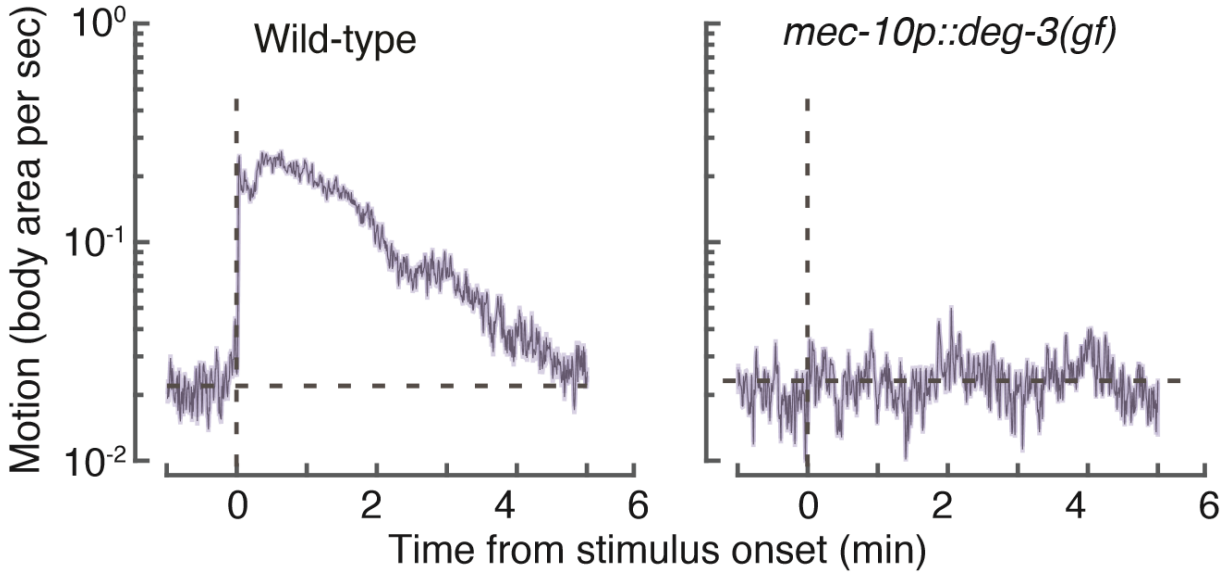

Figure 3.12: **Mean responses to the 3-minute vibration stimulus.** Locomotion in response to 1 kHz vibrations was quantified as the fraction of the body area the animal transversed per second. wild type animals (left) responded robustly to the stimulus while touch-insensitive *mec-10p::deg-3(gf)* transgenics (right) did not exhibit a detectable response. N=20 animals from each genotype were assayed and shaded areas depict mean±s.e.m.

imaged animals expressing this reporter and subjected to the one-hour deprivation protocol described above. Deprivation resulted in a significant increase in *ced-1::gfp* fluorescence. In contrast, the control group subjected to a mock deprivation protocol, i.e., where vibrations were not applied, did not exhibit a change in reporter fluorescence during the equivalent period (Figure 3.14 B).

Next, we counted fluorescently labeled sperm in self-fertilized deprived and control hermaphrodites as previously described [133, 414]. We observed reductions in sperm count following forced locomotion during L4 lethargus as compared to the control groups in all three strains assayed (Figure 3.14 C). The reduced sperm counts were sufficient to explain the corresponding reductions in brood sizes. However, the reduction in sperm count of *daf-16(mgDf50)* was as large as that of *daf-16(mu86)*. This may have been indicative of the variability of the measurement, of our 3-day assay having fallen short of revealing the full extent of the brood size deficiency in *daf-16(mgDf50)* hermaphrodites, or of further loss of

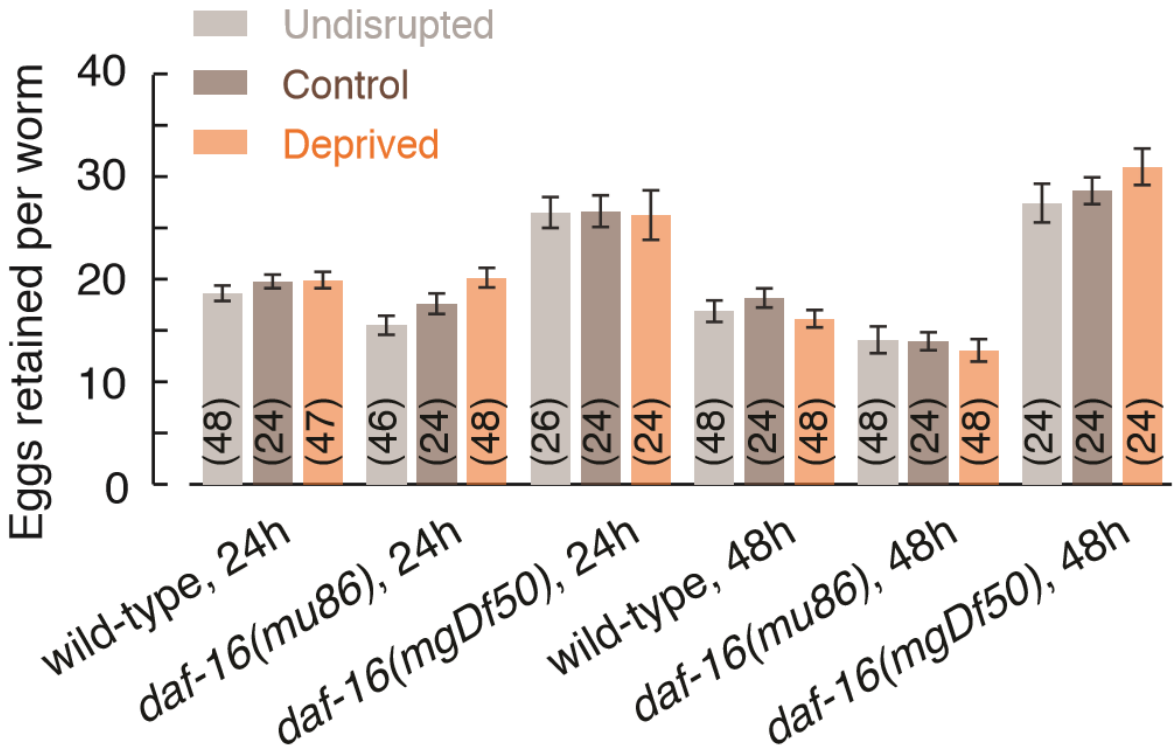

Figure 3.13: **Nonlethal deprivation does not increase egg retention.** The numbers of fertilized eggs retained in the uterus of wild type animals and *daf-16* mutants 24 hours and 48 hours after L4 lethargus. Deprived animals were exposed to the stimulus before, during, and after L4 lethargus. Control animals were exposed to the stimulus before and after L4 lethargus.

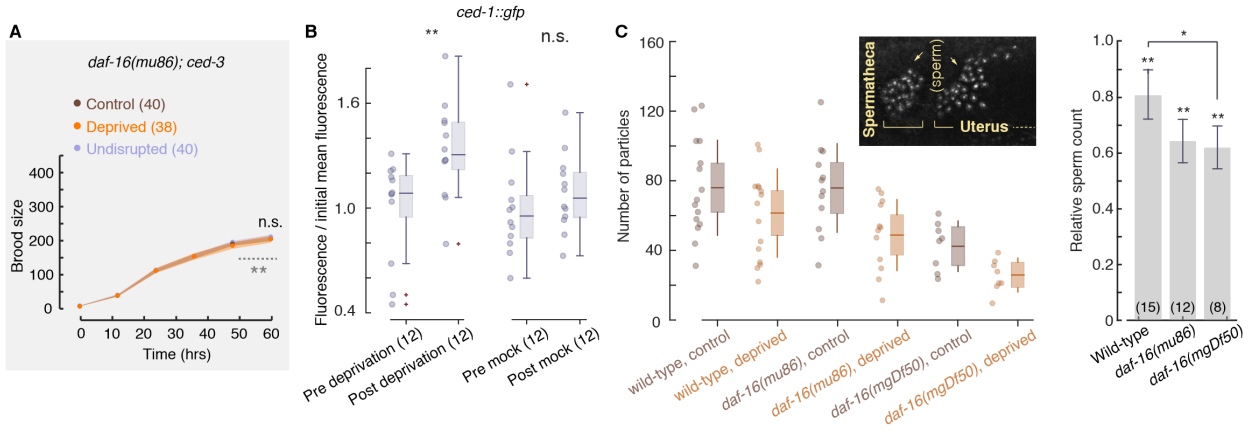

Figure 3.14: **Worm sleep deprivation causes germ cell apoptosis.** (A) Brood sizes of *daf-16(mu86); ced-3(n1286)* double mutants during the first three days after L4 lethargus (t=0 is 10-12 hours after the fourth molt). Deprivation failed to induce a reduction in brood size. The dotted line depicts the brood size of deprived *daf-16(mu86)* mutants at t=60 hours from Figure 3.10 A. (B) Box plots of the CED-1::GFP fluorescence after deprivation (left) or mock deprivation (right), normalized by the mean pre-treatment fluorescence. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes denoted in parentheses, double asterisks depict a significant difference ($p<0.01$). (C) Left: the number of sperm detected in a single gonad per animal. Horizontal lines, boxes, and bars depict means, 95% confidence intervals, and standard deviations, respectively. Inset: a confocal image of adult hermaphrodite sperm nuclei, specifically labeled by GFP-histone fusion driven by the *Pcomp-1* promoter. Right: the ratio between the sperm count of deprived and control animals. Error bars depict mean±s.e.m. Sample sizes are denoted in parentheses, double asterisks depict mean values significantly different than 1 ($p<0.01$) and the single asterisk depicts a significant difference between genotypes ($p<0.05$).

sperm during adulthood (e.g., in the *daf-16(mgDf50)* control group). These results suggest that forced locomotion specifically during L4 lethargus induces germ cell apoptosis and that this deleterious effect of sleep deprivation is mitigated by DAF-16.

3.3.7 The UPR^{ER} mitigates the effects of nonlethal deprivation on germ cells.

Germ cell apoptosis was shown to be enhanced by pharmacologically or genetically induced ER stress [218]. Moreover, nonlethal sleep deprivation triggers the UPR^{ER} in all animals previously examined [69, 259, 318, 351]. We therefore hypothesized that the UPR^{ER} may be triggered by nonlethal deprivation of worm sleep and that it may be required for the effect

of deprivation on fecundity.

HSP-4 is a *C. elegans* homolog chaperone of mammalian Grp78/BiP, upregulated in response to ER stress [42, 150, 320]. Expression of BiP is upregulated in fly and rodent brains in response to nonlethal sleep deprivation [68, 69, 256, 258, 259, 318, 351]. To address whether HSP-4/BiP is upregulated in sleep deprived worms, we subjected animals expressing the transcriptional reporter *Phsp-4::gfp (zcIs4)*, a key indicator of the UPR^{ER} [42], to the one hour deprivation protocol. An array of hypodermal stem cells, termed seam cells, regulate hypodermal/cuticle formation and transform to their adult fate at the time of the fourth molt [9, 225]. We observed that *hsp-4* was notably expressed in the epithelial seam of undisturbed animals during L4 lethargus (Figure 3.15 A). This temporal expression pattern coincides with the generation of the adult alae longitudinally oriented cuticular ridges by the seam cells [328, 352].

Animals subject to the one-hour sleep deprivation protocol significantly upregulated the expression of *hsp-4*. In contrast, mock deprivation or exposing the animals to one hour of vibrations during the mid L4 larval stage did not affect *hsp-4* expression (Figure 3.15 B). Consistently, *hsp-4* expression remained elevated after a 4-hour period of disruptions, as indicated by quantitative PCR (Figure 3.15 C). Upregulation of *hsp-4* requires essential components of the UPR^{ER} machinery: it is mediated non cell-autonomously by the transmembrane protein kinase and endoribonuclease IRE-1, which activates the bZIP transcription factor XBP-1 [42, 159, 375]. Consistent with pharmacological or genetic induction of ER stress, nonlethal sleep deprivation failed to upregulate *hsp-4* expression on *ire-1* or *xbp-1* mutant backgrounds (Figure 3.15 C). Combined, these results show that nonlethal sleep deprivation is proteotoxic and induces ER stress in *C. elegans*.

Next, we asked whether, as in pharmacologically-induced ER stress, IRE-1 function was essential for germ cell apoptosis in the case of sleep deprivation. To address this, we compared the brood sizes of deprived, control (exposed to vibrations outside of lethargus), and undisrupted *ire-1(ok799)* mutants. Loss of IRE-1 function abolished the reduction in brood

size (Figure 3.16 A). In contrast, *ubl-5* mutants, deficient in the UPR^{MT}, exhibited an identical reduction in brood size to wild type (Figure 3.16 B). Therefore, the UPR^{ER} was essential for germ cell apoptosis induced by sleep deprivation, but the UPR^{MT} did not affect fecundity under identical conditions.

Does the UPR^{ER} invoked by sleep deprivation affect pharyngeal pumping? To address this, we assayed two *ire-1* mutant alleles. Both mutants exhibited no significant change in the mean pumping rate post-deprivation. However, the duty ratio of bursts of rapid pumping was affected, indicating a potentially mild contribution of the UPR^{ER} to mitigating the deprivation-induced pumping deficiency (Figure 3.16 C). We could not detect a deprivation-induced pumping deficiency in *hsp-4* mutants, perhaps due to redundancy of downstream protective mechanisms. These results demonstrate that ER proteotoxic stress plays a secondary role in post-deprivation pumping deficiency as opposed to the prominent role played by mitochondrial stress. Combined, our data demonstrate that nonlethal sleep deprivation of worm sleep (at the severity enabled by our assays) induced distinct types of proteotoxic stress that trigger distinct UPRs in different tissues.

3.3.8 The UPR^{ER} (but not the UPR^{MT}) mitigates the effects of nonlethal deprivation on activity in the egg-laying circuit.

Germ cell apoptosis due to deprivation induced ER stress does not preclude an independent impact of deprivation on the egg-laying circuit. To address this, we examined animals carrying the *egl-1(n986dm)* allele, which lack the cell bodies of the HSN egg-laying neurons . The absence of HSN results in extended inactive egg-laying periods and a two-fold increase in calcium transients which correspond to twitching of the vulval muscles (vms) [77]. Therefore, defects in the egg-laying circuit can be assayed by measuring the calcium transients in vms of freely behaving animals. To do so, we sleep deprived worms co-expressing the genetically encoded calcium reporter GCaMP5 and the red fluorescent protein mCherry in their vulval muscles [77, 78] (Figure 3.17 A,B).

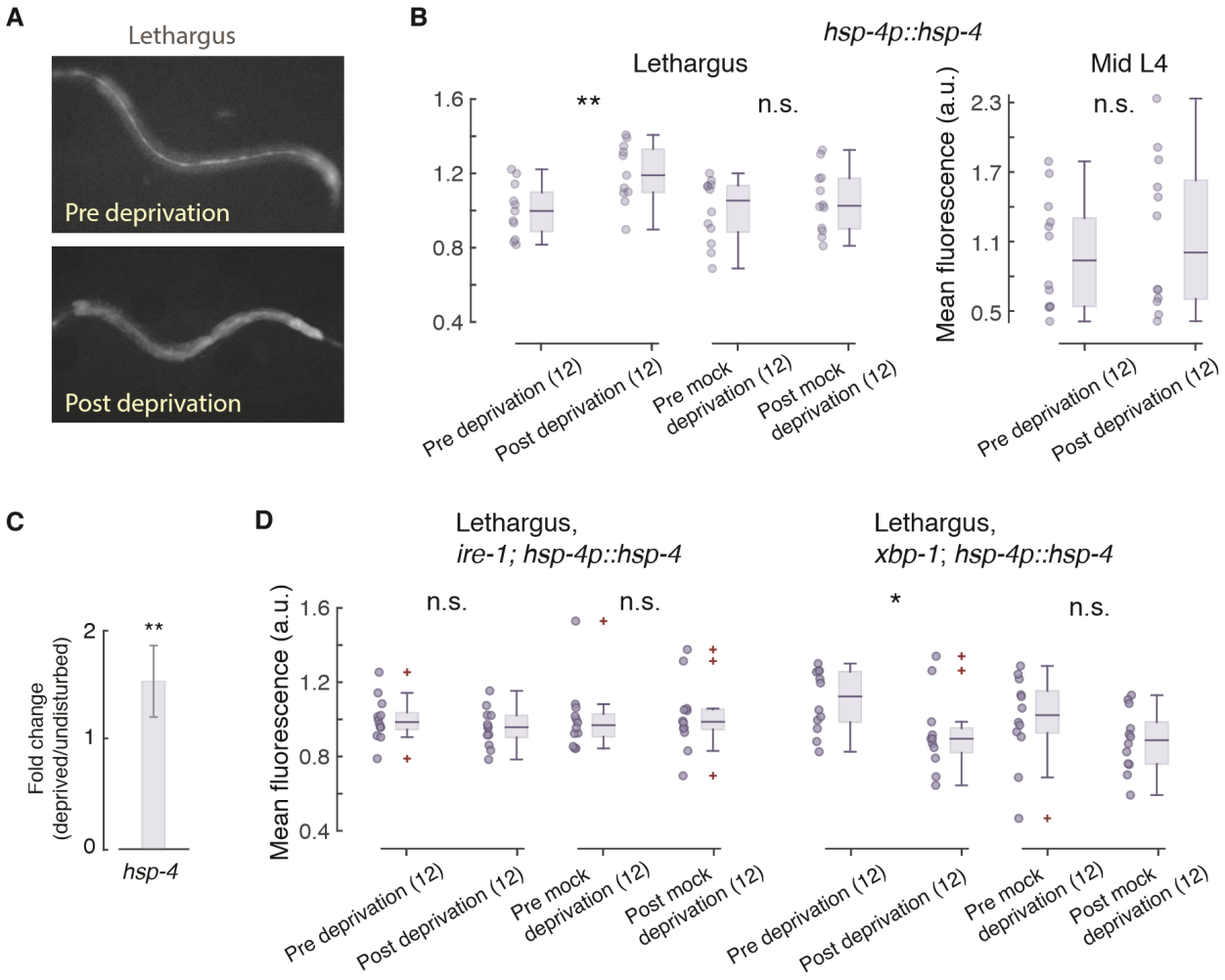

Figure 3.15: **The UPR^{ER} is triggered by nonlethal deprivation of worm sleep.** (A) Example of pre- (top) and post- (bottom) deprivation fluorescence of the *hsp-4p::GFP* reporter. Prior to deprivation the reporter was prominently observed in the seam cells. (B) Fluorescence of the *hsp-4p::GFP* reporter before and after deprivation, mock deprivation, and stimulating mid L4 larvae. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. (C) Quantification of *hsp-4* expression using real-time PCR. Error bars depict 99% confidence intervals. (D) Fluorescence of the *hsp-4p::GFP* reporter in mutants where the function of the UPR^{ER} genes *ire-1* and *xbp-1* was lost. On these mutant backgrounds sleep deprivation did not upregulate the expression of *hsp-4*. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes denoted in parentheses, asterisk and double asterisks depict a significant difference ($p<0.05$ and $p<0.01$).

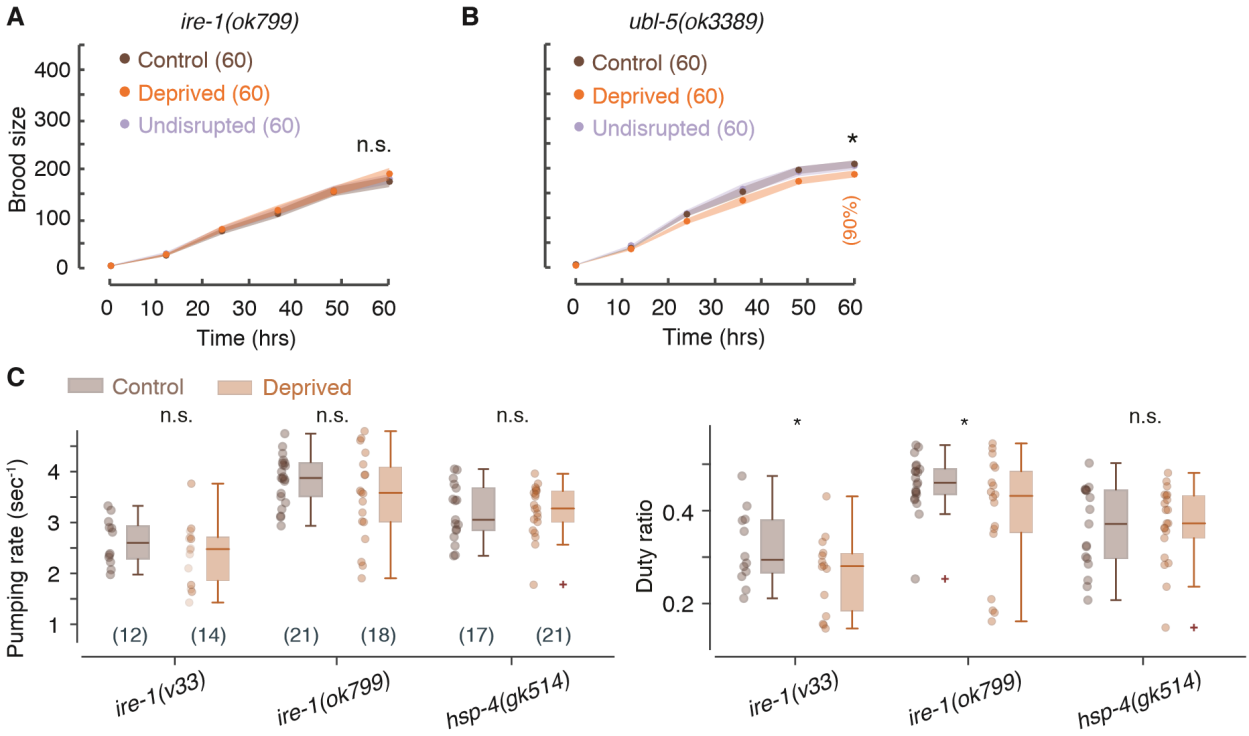

Figure 3.16: **The UPR^{ER} is required for post-deprivation germ cell apoptosis.** (A) Brood sizes of *ire-1(ok799)* mutants during the first three days after L4 lethargus (t=0 is 10-12 hours after the fourth molt). Fecundity was not reduced by sleep deprivation, similar to the requirement for IRE-1 for enhanced germ cell apoptosis when ER stress is induced pharmacologically. (B) Brood sizes of *ubl-5(ok3389)* mutants during the first three days after L4 lethargus (t=0 is 10-12 hours after the fourth molt). The effect of deprivation on fecundity was identical to wild type, indicting that the UPR^{MT} does not affect germ line apoptosis under these conditions. (C) Average pumping rates and duty ratios are not and mildly affected by deprivation, respectively. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes are noted in parentheses and asterisks denote significant differences ($p<0.05$).

Interestingly, in the egg-laying circuit, deprivation resulted in the complementing set of phenotypes to those found in the pharyngeal circuit. Neither wild type animals nor UPR^{MT} deficient *ubl-5* mutants exhibited abnormal post-deprivation twitching. However, UPR^{ER} deficient *ire-1* mutants, when deprived, exhibited a mean increase of 30% in the number of vm twitches (Figure 3.17 C). This elevation in vm twitching mirrored the trend exhibited by *egl-1(n986dm)* mutants.

Physiological activity in the egg-laying circuit is coupled with body posture and locomotion in the temporal vicinity of egg-laying events [77, 78, 388]. We therefore hypothesized that longer locomotion trends may also affect the egg-laying circuit. Specifically, we asked whether a potential effect of deprivation on locomotion during the 30 minutes of our assay may indirectly cause the vm twitching phenotype in *ire-1* mutants. To address this, we calculated the mean velocity of each animal throughout the duration of our assay, compared between mean velocities of undisrupted and deprived animals, and measured the correlation of the mean velocity to the number of vm twitches.

In all three genotypes assayed, the mean velocity did not vary significantly between undisrupted and deprived animals (Figure 3.18 A). While correlations between the mean velocity and vm twitching were found in all three strains, they were weakest in *ire-1* mutants. Even if the question of statistical significance was set aside, the differences between mean or median velocities was too small to plausibly account for the 30% increase in vm twitching. Thus, excess vm twitching was not an indirect consequence of elevated locomotion activity in our assays. These results are consistent with the conclusion that sleep deprivation upsets ER proteostasis and demonstrate that, if not mitigated, this stress also impacts the egg-laying circuit.

Interestingly, the correlations we found between activity in the egg-laying circuit and locomotion were stronger in deprived wild type animals and *ubl-5* mutants as compared to their respective undisrupted groups. This trend was reversed in *ire-1* mutants (Figure 3.18 B). This observation may indicate that elevating coordination between distinct behaviors

during stress may require secreted proteins, such as neuropeptides, whose function depends on processing in the endoplasmic reticulum [339].

3.4 Discussion

The cognitive, physiological, and behavioral changes resulting from deprivation of human sleep elude superficial phenotyping. Detecting them requires functional imaging and/or proper design of the task begin assayed, a clear definition of the sleep deprivation conditions, and careful measurements. Interpreting such results involves consideration of details such as differences in vulnerability to deprivation between individuals and whether the task was monotonous or complex [4, 60, 136, 277]. Similarly, wild type rodents and insects do not typically exhibit gross defects or substantial damage to brain cells following nonlethal deprivation protocols [71, 120, 189, 387]. Such findings suggest that the protective responses activated by deprivation have a capacity to effectively prevent or repair neuronal damage.

To address the lasting impact of sleep deprivation in *C. elegans*, we established an experimental paradigm enabling severe reduction in quiescence with no observed lethality or molting defects. Our periodic stimulus allowed for some quiescent behavior to take place throughout lethargus. Possibly, not forcing locomotion for extended continuous periods was key to avoiding a lethal outcome. In contrast, previous work assessed the impact of total sleep deprivation, i.e., consecutive forced movement for 30 minutes, which resulted in lethal molting defects. The impact on molting was interpreted to indicate a defect in metabolic regulation during lethargus as the loss of DAF-16 sensitized the animals to this effect [91]. Lasting defects in surviving animals were not previously assayed.

The complete lack of lethality despite a loss of 50% of quiescence during lethargus is consistent with the hypothesis that quiescence, in and of itself, is an imperfect measure of the quality and restorative benefits of developmentally timed sleep in *C. elegans*. If homeostatic compensation can affect the quality of sleep [326], periodically allowing for rebound could confer greater restoration per unit time as compared to uninterrupted sleep. Testing this

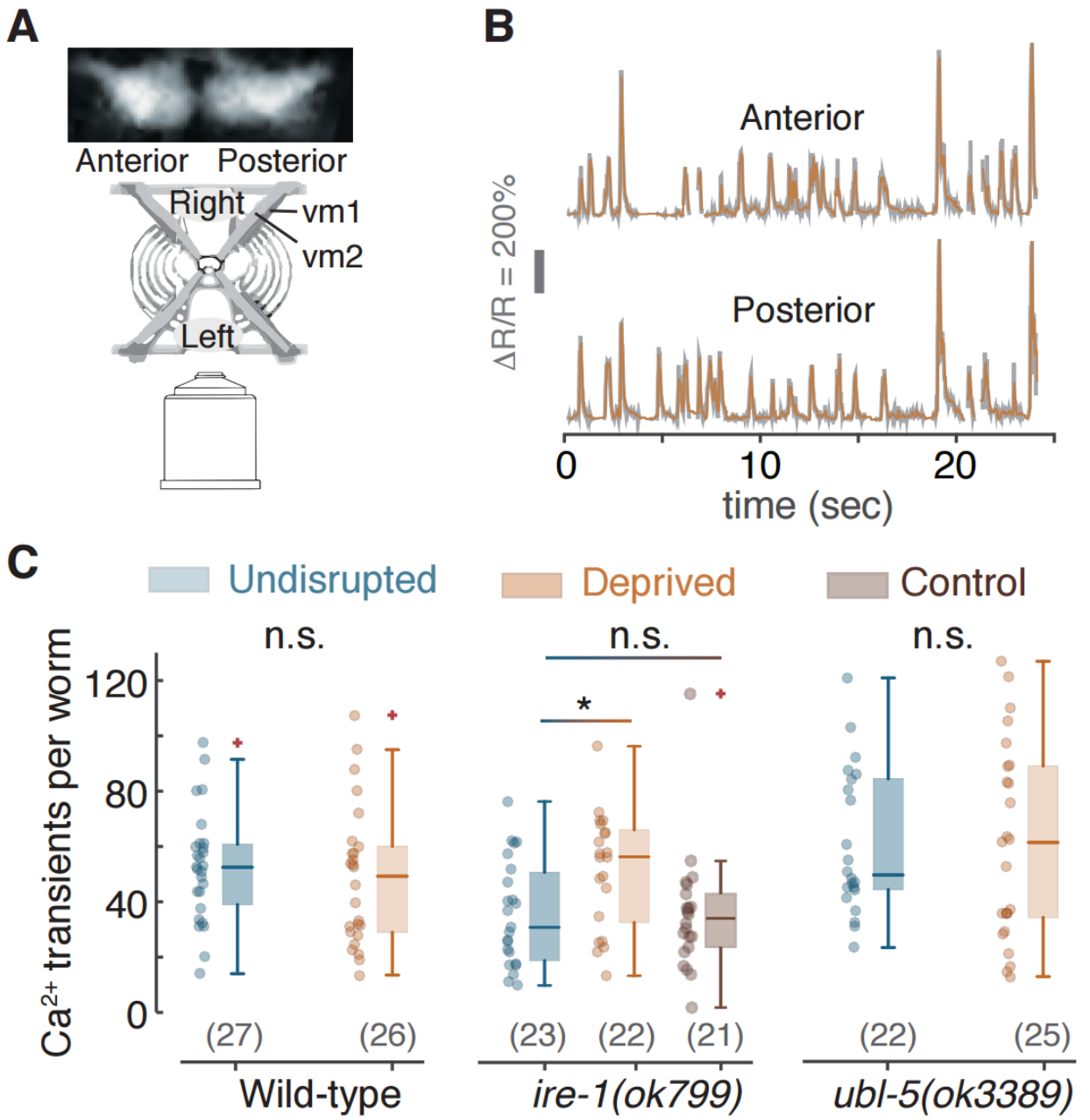

Figure 3.17: **Worm sleep deprivation results in excess twitching of vulval muscles.** (A) A schematic representation of the imaging setup. Top: fluorescently labeled anterior and posterior vulval muscle groups. Worms crawl on their left or right side such that their dorsoventral axis is parallel to the imaging plane. (B) Example traces of the ratio of GCaMP5 to mCherry fluorescence from anterior and posteriors vms. (C) The total number of calcium transients in vms of undisrupted and sleep deprived worms. An average increase of 30% in the number of vm twitches was detected in *ire-1* (UPR^{ER} deficient) mutants. No significant changes were detected in wild type animals and *ubl-5* (UPR^{MT} deficient) mutants. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes are noted in parentheses and double asterisks denote significant differences ($p<0.02$).

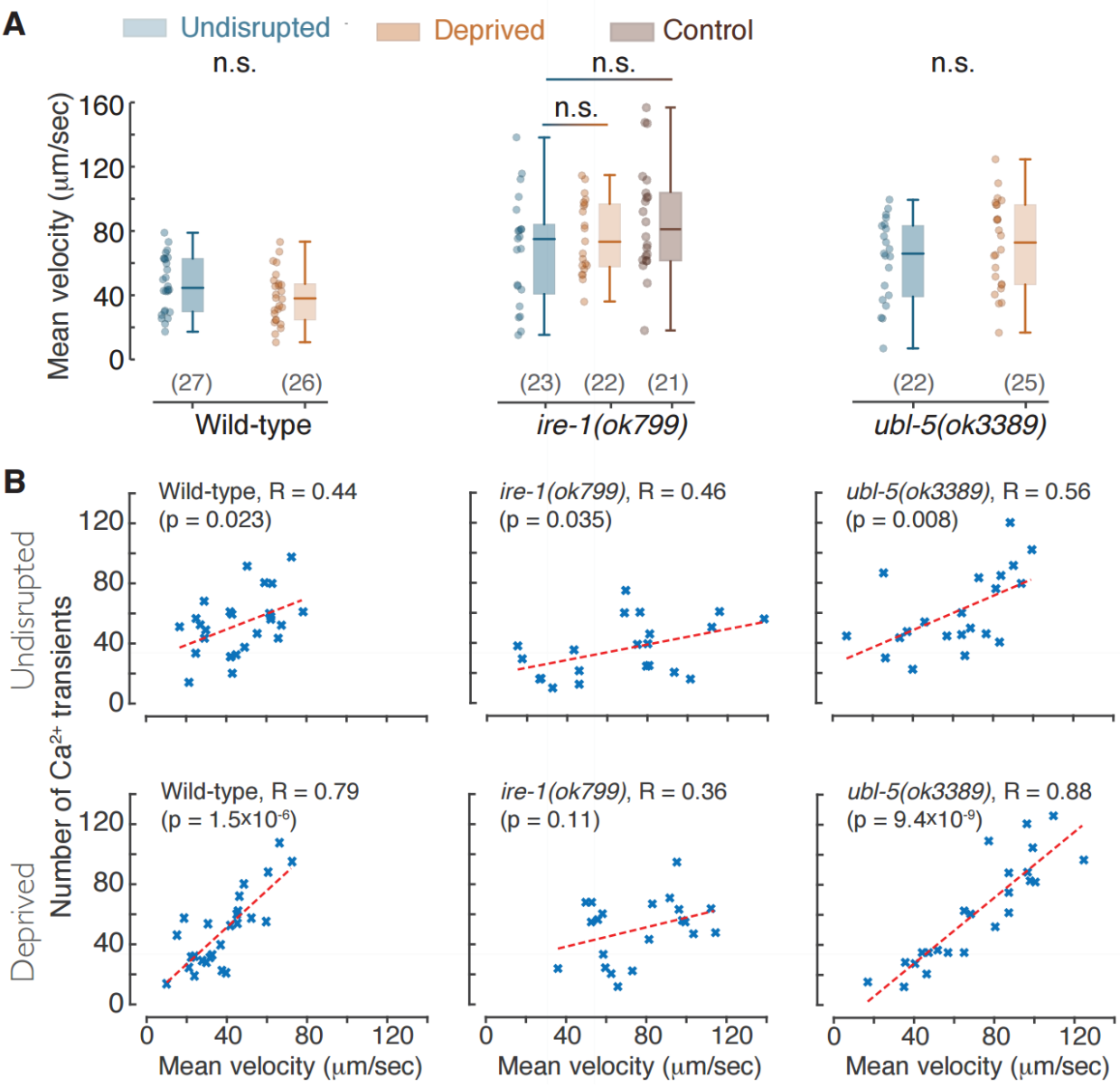

Figure 3.18: **Long-term mean velocities are not affected by deprivation but correlate with vm twitching.** (A) No significant differences were found between mean velocities (averaged over the 30 minutes of the assay) of undisrupted or deprived wild type animals, *ire-1* mutants, or *ubl-5* mutants. Horizontal lines, boxes, and bars depict medians, 1st and 3rd quartiles, and 5 and 95 percentiles, respectively. Sample sizes are noted in parentheses. (B) Mean velocities and vm twitching were significantly correlated in wild type animals, *ubl-5* mutants, and undisturbed *ire-1* mutants. Notably, these correlations were stronger in deprived wild type animals and *ubl-5* mutants as compared to undisturbed worms of the corresponding genotype. However, correlations did not increase in *ire-1* mutants, suggesting that secreted proteins may be required for deprivation-induced enhancement of coordination between vm activity and locomotion.

hypothesis will be key for understanding lethargus and may promote our understanding of additional quiescent states.

Next, we characterized outcomes of nonlethal deprivation and identified protective responses that mitigate them. Forced locomotion during *C. elegans* sleep negatively impacted feeding, fecundity, and the egg-laying circuit.

Several lines of evidence indicated fecundity was reduced due to germ cell apoptosis: the dependence of the effect on CED-3 and IRE-1, and the upregulation of CED-1 in sheath cells engulfing early apoptotic corpses of germ cells [96, 113, 311, 412]. Interestingly, sperm quality was recently shown to be negatively impacted by disruptions to sleep in rodents [8, 360].

Consistent with previous findings, our deprivation protocol triggered translocation of DAF-16/FoxO into intestinal cell nuclei [91], and implicated it in mitigating lasting adverse effects of deprivation. More specifically, we have shown triggering of the UPR^{MT} by deprivation and implicated it in protecting feeding behavior in sleep deprived worms. In contrast to the UPR^{ER}, the UPR^{MT}) was not strongly associated with sleep deprivation previously. This may be partly due to a focus on sleep-related changes in gene expression in the brain [67, 69, 70, 177, 256, 259, 318, 351, 390, 422]. Nevertheless, one study found that the mitochondrial chaperones Hsp60 and Glucose-regulated protein 75 (Grp75, from the Hsp70 superfamily) were upregulated in the cerebral cortex of rats after sleep deprivation, although not as much as BiP [68].

Broad transcriptional responses to sleep deprivation were reported in mouse livers, lungs, and hearts [10, 236]. These studies demonstrate that the molecular consequences of disrupting sleep are not limited to the brain and that sleep contributes to function in a manner that may vary between different tissues or organs. The pharynx of *C. elegans* is a highly active organ, required to repeatedly generate powerful contractions. Neurons that regulate pumping may face a distinct cellular metabolic challenge and therefore may particularly benefit from the UPR^{MT}) post-deprivation.

Nonlethal sleep deprivation upregulates the expression of BiP in rodents and flies, thus indicating the activation of the UPR^{ER} [69, 259, 318, 351]. We have shown a similar upregulation of HSP-4/BiP in response to disrupting developmentally timed worm sleep. Furthermore, loss of function of the misfolded ER protein receptor IRE-1 affected both fecundity and the egg-laying circuit post-deprivation. Therefore, the activation of the UPR^{ER} is a deeply conserved response to sleep deprivation [327]. It remains to be determined whether the complementing set of phenotypes exhibited by the pharyngeal and the egg-laying circuits, whose function depended on the UPR^{MT} and the UPR^{ER} respectively, is associated with differences in their developmental states, energy expenditure, or additional factors.

Several hypotheses have been offered to explain the core functions of sleep, including the notion that its utility may differ across species [323]. The synaptic homeostasis hypothesis proposes that continuous learning during wakefulness is bound to saturate synaptic connections. Therefore, renormalization of net synaptic strength during sleep is required to restore homeostasis [358, 359]. Other suggestions focus on wear and tear in physiologically active neurons during wakefulness due to accumulation of protein fragments, unfolded proteins, or other molecular stressors [66, 181, 379, 387, 406]. On a larger scale, metabolite clearance from the brain can increase during mammalian sleep [403]. In addition, disrupting sleep is linked to abnormal glucose metabolism and appetite regulation. These findings suggest that sleep is key to normal metabolic and hormonal processes outside the brain [200, 289].

This work describes multiple pathways by which sleep deprivation can create unfavorable biochemical conditions in cells and perturb proteostasis. Specific characteristics of physiological activity and development could affect the balance between accumulation and relief of allostatic load. To the best of our knowledge, how this balance might scale with different types of metabolic loads has not been systematically studied, let alone connected to sleep. Protection of post-deprivation pharyngeal function by the UPR^{MT} is consistent with the notions that sleep reduces cellular metabolic stress and that highly active organs may invoke distinct responses in this context. Implicating the UPRs in mitigating consequences of worm

sleep loss indicates that these responses are deeply conserved.

3.5 Materials and Methods

3.5.1 Strains

wild type, transgenic, and mutant C. elegans strains were cultivated with OP50 bacteria according to standard protocols at 20 degrees C. The following strains were used: N2 Bristol (wild type), CF1038 *daf-16(mu86)*, GR1307 *daf-16(mgDf50)*, NQ116 *muIs211 [pNL213(ges-1p::GFP::daf-16) + rol-6(su1006)]* (Gift from Prof David Raizen), NQ441 *daf-16(mgDf50), qnIs45[Pdaf-16:GFP::daf-16; Pmyo-2:mCherry]* (Gift from Prof David Raizen at the University of Pennsylvania), SJ4151 *zcIs19 [ubl-5p::ubl-5::gfp]*, SJ4100 *zcIs13 [hsp-6::gfp]*, SJ4058 *zcIs9 [hsp-60::GFP + lin-15(+)]*, VC2564 *ubl-5(ok3389)*, SJ4200 *zcIs41 [ubl-5p::3xmyc-His tag::ubl-5 + myo-3p::gfp]*, SJ4151 *zcIs19 [ubl-5p::ubl-5::gfp]*; NQ128 *muEx169[unc-119p::GFP::daf-16 + rol-6(su1006)]* (Gift from Prof David Raizen at the University of Pennsylvania); DA184 *ser-1(ok345)*; *Is[Pmec-10::deg-3(u662)]* (Gift from Prof Millet Treinin at the Hebrew University of Jerusalem); MT3002 *ced-3(n1286)*, MD701 *bcIs39 [lim-7p::ced-1::GFP + lin-15(+)]*; SJ4005 *zcIs4 [hsp-4::gfp]*; RE666 *ire-1(v33)*; SJ17 *xbp-1(zc12)*; SJ30 *ire-1(zc14) II, zcIs4 V*; SJ17 *xbp-1(zc12) III, zcIs4 V* RB925 *ire-1(ok799)*; VC1099 *hsp-4(gk514)II*; UX564 *jnSi118[Pcomp-1::GFP::H2B::3comp-1, Cb-unc-119(+)], him-5(ok1896)* (Gift from Prof Gillian Stanfield at the University of Utah); LX1938 *egl-1(n986dm)V, vsIs164 X, lite-1(ce314) X, lin-15(n765ts) X* (Gift from Prof Kevin Collins at the University of Miami); LX1918 *(vsIs164) X, lite-1(ce314) X, lin-15(n765ts) X* (Gift from Prof Kevin Collins at the University of Miami).

3.5.2 Severe nonlethal deprivation protocol

Motion and quiescence were identified using the image difference method as described in [253]. To disrupt quiescence during lethargus, mid-L4 animals were transferred to 60 mm

NGM plates containing 10 mL of medium and seeded with 50 µl of saturated OP50 culture. Vibrations (1kHz) were delivered as described in [254]. A square wave stimulus with a period of 6 minutes and duty cycle of 50% was delivered for 12 hours. To stimulate animals outside of lethargus, we similarly mechanically stimulated mid-L4 animals for 5 hours followed by a 5-hour pause and an additional 5 hours of stimulation.

3.5.3 Imaging of GFP fluorescent reporters

The one-hour deprivation protocol consisted of 3-minute long vibration pulses interspersed with 3 minute long pauses, starting during the first 30 minutes of L4 lethargus and lasting a total of one hour. Animals expressing a fluorescent reporter were exposed to these mechanical stimuli in artificial dirt microfluidic devices placed inside a standard petri dish. They were imaged in the device immediately before and after the one-hour period of disruptions. Imaging was performed at a magnification of 20X (0.5 NA) using a Nikon Eclipse Ti microscope and an Andor iXon X3 EMCCD camera. Fluorescence intensity was determined using custom Python scripts.

3.5.4 Calcium imaging in freely behaving animals

To reduce background fluorescence, calcium imaging was performed in artificial dirt microfluidic devices [226]. Animals co-expressing GCaMP5 and mCherry in their vms were mounted in the presence of bacterial food on an epi-fluorescence Nikon Eclipse Ti inverted microscope (Nikon Inc., Melville, NY). Each worm was imaged at a magnification of 20X (0.5 NA) and a frame rate of 6 frames per second. Images were captured by an Andor iXon X3 EMCCD camera. A Dualview (DV2) two-channel system was used for simultaneous imaging of the red and green channels (Photometrix, Tucson, AZ). Each animal was tracked manually and continuously imaged for a total period of 30 minutes. Calcium transients were analyzed using custom Matlab scripts (Mathworks Inc., Natick, MA).

3.5.5 Brood size

Brood size was counted by visual inspection: ten hours after mechanical stimulation ended, animals were transferred to individual 60 mm NGM plates seeded with 50 µl drop of OP50 (two animals per plate). Plate were scored in the morning and evening of the following three days. To avoid the accumulation of hatched offspring, animals were transferred to new plates prior to the evening counts each day. For male mating, two males and two hermaphrodites were transferred to each plate.

3.5.6 Pharyngeal pumping

Post-stimulus (see above) young adults were picked into liquid NGM and loaded into a WormSpa microfluidic device 65,125. An *E. coli* OP50 overnight culture, concentration-adjusted in NGM to OD600 = 2.5 (an intermediate food concentration) was flown through the device at a constant rate throughout the assay. After an hour of acclimation in the device, the animals were imaged for one additional hour at a magnification of 10x and 62.5 frames per second using a Basler acA1920-25 µm cMOS camera mounted on Celestron 44104 microscopes. Pumping events were identified using a custom Python script which aligned and subtracted consecutive images and calculated the entropy of the difference image. A pumping event resulted in a characteristic spike in this entropy [174, 309].

3.5.7 Sperm counting

Adult *Pcomp-1::GFP::H2B::3comp-1* animals 24 hours post-L4 lethargus were sealed into individual artificial dirt chambers filled with NGM medium and 10 µM levamisole. Confocal images of fluorescently labeled sperm were obtained using a Zeiss LSM 880 microscope with a Plan-Apochromat 40x/1.4 oil DIC objective. Image stacks were analyzed using the FARSIGHT Nucleus Editor (http://www.farsight-toolkit.org/wiki/NucleusEditor). We note that when the fluorescent marker was crossed to a *daf-16* mutant background, it was not

confined to the nuclei. However individual sperm cells were still detectable. We observed this in all *daf-16(mgDf60)* mutants and 25% of the *daf-16(mu86)* mutants.

3.5.8 Real time PCR

The *lmn-1* gene, encoding the *C. elegans* nuclear laminin, was chosen as the endogenous control gene. Primers (except for *lmn-1*, used previously by the Shaham lab at Rockefeller University) were designed using Wormbase.org and the NCBI Primer BLAST software. They were tested for specificity using NCBI BLAST and by agarose gel electrophoresis (using gDNA) and purchased from Integrated DNA Technologies (IDT, Coralville, IA). The primer used for *lmn-1* and *hsp-4* were TCGAGGCGGAAAAGGCTC (Fwd), GCTCCAGCGAGTTCTCTCTC (Rev), GCCGACAAGGAAAAACTCGG (Fwd), and GTGGGGTTGGGTTGGGAAA (Rev), respectively. Real-time quantitative PCR amplifications were performed using 25 µl of QuantiTect SYBR Green Real-Time PCR master mix (QIAGEN, Hilden, Germany), 2 µl of diluted reverse transcription product (2 ng/reaction), 1.5 µl each of forward and reverse primer and 20 µl of DNase/RNase free water in a total volume of 50 µl. Amplification was carried out in a AB 7900 HT Real-Time PCR cycler (Applied Biosystems, Foster City, CA) with initial polymerase activation at 95 degrees C for 15 min, followed by cycles of: 94 degrees C for 15 sec denaturation, 57 degrees C for 30 sec for primer-specific annealing and 72 degrees C for 30 sec for extension. A melting curve analysis was carried out (60 degrees C to 95 degrees C) to verify the specificity of amplicons, i.e., the absence of primer dimers and nonspecific products. Each assay included 6 technical replicates and a no-template control for every primer pair.

3.5.9 Statistical analysis

Pairwise comparisons of data represented in bar or box plot was done using the student t-test. In the case of multiple comparisons, significance was calculated using a one-way ANOVA test and the Bonferroni post-hoc correction. Distributions represented by histograms were

compared using the k-sample Anderson-Darling test and the Bonferroni post-hoc correction for multiple comparisons (when applicable).

CHAPTER 4

CONCLUSIONS AND DISCUSSION

Sleep is an evolutionarily conserved biological process in essentially all animals with a nervous system, but the core function of sleep remains unknown. One of the most proven strategies for understanding complex phenomena is to perturb simple systems that model the complex phenomenon of interest. With this in mind, this dissertation uses lethargus, a 2-3 hour period of developmentally-timed sleep, in the nematode *Caenorhabditis elegans* to explore how quiescence is homeostatically regulated and how disturbances during this period affect the organism.

In this dissertation, I provide evidence that two behaviorally and genetically distinct mechanisms regulate behavioral quiescence during lethargus. Previous work from our lab identified a correlation between motion bouts and subsequent quiescence bouts. To prove causality, I show that weak stimuli evoke homeostasis in response to extended motion by extending the subsequent bout of quiescence. I find that neuropeptides are required for this homeostatic response in both stimulated and unstimulated animals. Specifically, I find that the neuropeptide-Y receptor homolog NPR-1 in the RMG neuron class and inhibitory FLP-18 peptides are necessary for this "weak" homeostatic response. In contrast, strong stimuli repeated throughout lethargus result in a distinct homeostatic response: an elevation of the overall time spent in quiescence during lethargus. This"strong" homeostatic response does not require NPR-1, but instead requires the function of DAF-16/FOXO insulin-signaling transcription factor in neurons. Conversely, the "weak" homeostatic response does not require DAF-16/FOXO.

I further show that lethargus is a specifically vulnerable period during *C. elegans* development. I report that nonlethal deprivation of quiescence during lethargus beyond the regime that evokes homeostasis results in proteotoxic stress, that if left unmitigated, can cause long-lasting deficits in the animal. These deficits are anatomically, functionally, and genetically distinct. Specifically, I describe an automated protocol for depriving *Caenorhabditis elegans*

of developmentally-timed sleep in a severe yet nonlethal manner. I then characterize three lasting effects of nonlethal deprivation of quiescence: a deficiency in pharyngeal pumping, a reduction in brood size as a result of germ cell apoptosis, and an excess of twitching in the vulval muscles of young adults. Importantly, our protocol for depriving quiescence when applied outside of lethargus does not result in deficits. I show that while both the pumping and fecundity defects are mitigated by DAF-16/FOXO, deprivation evokes distinct proteotoxic stress responses in the germline and pharynx. The UPR^{MT} mitigates the pumping defect while the UPR^{ER} mitigates the fecundity and vulval muscle defects.

Taken together, this work identifies a number of new insights about a primitive form of sleep. This dissertation shows that neuropeptides modulate quiescence during lethargus, but that strong perturbations evoke a distinct behavioral and genetic response to increase quiescence requiring a known stress-activated transcription factor. This overall increase in quiescence as a result of perturbations to lethargus implies that a process or processes coupled to quiescence are less efficient when perturbed. One would not expect an increase in quiescence if a certain level of quiescence itself were sufficient during lethargus. This dissertation also shows that stronger perturbations beyond the regime of homeostasis result in the activation of distinct stress responses in distinct tissues and circuits of the animal. Importantly, these same perturbations inside and outside of lethargus evoke stress responses only during lethargus. Therefore, I show that lethargus is a vulnerable period of *C. elegans* development.

In this chapter, I speculate about what this work and the work of others studying *C. elegans* lethargus implies about the phenomenon of sleep.

4.0.1 Where are the sleep-specific genes?

From twin and familial studies, estimates for heritability of sleep duration in humans are 40% and between 25 to 45% for insomnia [237, 399]. Nevertheless, few genetic factors to explain this heritability have been found. In humans and model organisms, the genes that

have been found are not specific to sleep [127, 211]. Rather, it seems that many of the genes identified that alter sleep also broadly influence either neuronal excitability, metabolism, or the immune system [316]. While notable genes that are specific to circadian rhythms exist, evidence suggests that despite mutations in these genes affecting sleep timing, the total amount of sleep is not affected. Conversely, genes that decrease sleep and affect sleep rebound such as *sleepless (sss)* in *D. melanogaster* have normal clock function [203]. Relatedly, the *npr-1* and *daf-16* genes identified in this dissertation that influence sleep homeostasis do not affect the timing of sleep. Therefore, the mechanisms that govern the sleep homeostat and the circadian rhythm are at least partially independent.

Despite sleep manifesting itself to different degrees across phylogeny, sleep behavior exists in essentially every animal with a nervous system. Why has a constant waking state that maximizes foraging, reproduction, and other adaptive behaviors not evolved? Given the lack of sleep-specific genes, it is naively possible that the genes that control sleep have negative pleiotropic effects that prevent them from being easily targeted for selection. However, evidence from conspecific Blind Mexican Cavefish argue that sleep is an evolutionarily labile phenotype: eyed surface populations sleep far more than blind cave populations [92]. Fascinatingly, different neural mechanisms regulated the evolution of sleep loss in independently-derived cavefish populations [169]. Nevertheless, the blind cavefish populations still sleep. It also remains to be seen whether these sleep differences still manifest themselves in early development. To the extent of our knowledge, this evidence argues that sleep behavior can rapidly evolve, but that sleep remains necessary. Thus, aside from the unresolved question of why sleep evolved in the first place, the lack of sleep-specific genes cannot explain why sleep has not evolved away.

Indeed, one should expect a priori that the genes that influence sleep have broad effects. Sleep is a behavior, and because of adaptive reasons, genes that are particularly important in behavioral evolution tend to affect multiple behaviors that together serve a common purpose [17]. The other functions that "sleep genes" affect may provide insight into the nature of

sleep, however. In the case of the work presented here, both *npr-1* and *daf-16* are key integrators of stress and metabolism in worms and mammals [43, 100, 145, 312, 313].

The tissues and cells that a gene is needed in for a particular behavior can often offer even more valuable insight than the genes themselves. Information increases as more possibilities are excluded. Genes are broadly expressed, but tissue types and even further down, specific cells, are limited. Particularly for *C. elegans* which have a simple anatomy and 302 neurons, pinning down where sleep genes are necessary is powerfully informative. In the case of *npr-1* and *daf-16* identified in this dissertation, both genes are broadly expressed: *npr-1* is found in roughly 20 neuron types whereas *daf-16* is found in essentially all cells of the animal.

4.0.2 NPR-1 and sleep

In this dissertation, I find that to evoke homeostasis in response to weak stimuli, *npr-1* is required specifically in the RMG neuron type. RMG is a hub-and-spoke neuron that forms extensive electrical gap junctions to sensory neurons in *C. elegans*. Knowing this fact, one can hypothesize that sensory neurons may regulate quiescence in the worm. Supporting this, a recent study from the Kaplan lab at Harvard found that at high levels of oxygen, NPR-1 promotes quiescence during lethargus by diminishing the sensitivity of multiple classes of sensory neurons [63, 64]. Sensory neurons then signal through glutamate and neuropeptides to arouse locomotion thereby implicating NPR-1 in sensory gating. Perhaps relatedly, the response we measured to stimuli was also stronger in the strong *npr-1* mutant allele (Figure 2.11) than wild type (Figure 2.7).

As a caveat, the Kaplan lab found that *npr-1* mutants are much less quiescent during lethargus than we found. This was later explained by oxygen levels: our experiments are performed in microfluidic chips which have low oxygen whereas the Kaplan lab performed experiments on agar plates exposed to high oxygen [253]. However, as aforementioned, *npr-1* is a key integrator of stress and metabolism. Others have shown neuropeptide-Y signaling inhibits escape responses to noxious stimuli in the absence of food (a stress) but not in

the presence of food [100]. In other words, stress changes how neuropeptide-Y signaling functions. In the case of the Kaplan lab experiments, high oxygen is a stress. Our weak perturbations result in excess motion during a normal period of quiescence. As a result, we may be triggering quiescence promoting NPR-1 signaling, which may explain why we see additional quiescence following extended motion.

The involvement of stress-signaling in sleep homeostasis is not specific to *C. elegans*. In mammals, glutamate and pituitary adenylate cyclase-activating polypeptides from the retinohypothalamic tract convey sensory photic information from the retina to the SCN, the master circadian clock [132]. NPY-signaling from the geniculohypothalamic tract convey non-photic stimuli to the SCN in the form of stresses such as sleep deprivation or exercise [135]. Moreover, injection of NPY onto the SCN of hamsters phase-advances the circadian rhythm during the subjective day [161]. Together, this suggests that neuropeptide-Y signaling is conserved in the homeostatic regulation of sleep, and that at least in the case of mammals, interacts with the circadian clock.

4.0.3 DAF-16 and sleep

In contrast to *npr-1*, the involvement of *daf-16* in homeostasis seems to involve both muscles and neurons depending on the context. Sleep homeostasis can manifest itself as an increase latency to stimuli after deprivation. The Raizen lab at the University of Pennsylvania found that sleep deprived *daf-16* worms have response latencies to 1-octanol similar to undeprived *daf-16* animals. When rescued in muscles but not neurons, response latencies in *daf-16* mutants became wild type. We find that function of DAF-16 is necessary in neurons but not muscles for a global elevation in lethargus quiescence as a result of repeated strong stimuli. It is not known if DAF-16 is required in specific types of neurons for this homeostatic response. As a result of this ambiguity, it is more difficult to say how DAF-16 is promoting quiescence solely from the work presented in Chapter II.

Nevertheless, I find three pieces of data from our combined work compelling. One, our

lab showed that DAF-16 is needed to increase overall quiescence during lethargus in response to repeated stimuli. Note that this is an increase in quiescence above unstimulated controls. Two, our lab further showed that a native promoter rescue of *daf-16*, which is essentially an overexpression, results in a lower baseline quiescence fraction in both unstimulated and stimulated worms (Figure 2.22). Three, the Raizen lab showed that the increased death rate of *daf-16* mutants as a result of sleep deprivation was the result of a higher rate of defects in ecdysis (i.e., worms lacking DAF-16 were unable to successfully molt). DAF-16 is an integrator of stress and metabolism. Taken together, it is possible that a certain level of quiescence itself is not necessary but rather a process or processes coupled to quiescence. Perturbations divert metabolic resources from those unknown processes and therefore additional quiescence is required. Thus, sufficient perturbations may divert too many metabolic resources resulting in unsuccessful molts. In a collaboration with Anne Hart's lab at Brown University, pending publication but not discussed in this dissertation, this idea is explored further. The title of that work is "Sleep bouts are not essential for C. elegans survival, but FOXO is important for compensatory sleep."

4.0.4 Stress-response pathways and sleep

Assuming that there are indeed specific processes important to the function of sleep, how can we identify them? From work performed in the Raizen lab, we know that deprivation of lethargus quiescence can be lethal. However, death prevents the detection of impacts on the nervous system and behavior. To identify the consequences of sleep deprivation in *C. elegans*, we developed a protocol to deprive animals of sleep beyond the regime of compensation but less than that of lethality.

In Chapter III of this dissertation, I addressed some of the lasting deficits that occur in "sensitized" worms missing components of key stress response pathways when sleep deprived. In summary, we show that sleep deprivation compromises pharyngeal neuron function leading to a decrease in the pumping rate of *daf-16* mutants. For the first time, we show that core

components of the UPR^{MT} pathway are activated by sleep deprivation and that UPR^{MT} mutants are hypersensitive to the effects of sleep deprivation on pumping. Independent of the pharynx, we further show that sleep deprivation leads to a brood size reduction by triggering an increase in *ced-3*-mediated cell death in the germline. Mutations in *daf-16* exacerbate this effect. We show that the UPR^{ER} pathway is essential for this brood size reduction, but that the UPR^{MT} is not. Lastly, we provide evidence that excess vulval muscle twitching occurs after sleep deprivation and that the UPR^{ER} mitigates this defect. Importantly, because our deprivation protocol triggered stress response pathways only during lethargus and not when applied to other periods, our results argue that lethargus is a particularly vulnerable period of *C. elegans* development.

Why does our regimen of vibrating animals trigger stress response pathways in particular circuits and tissues of the worm only during lethargus? One possibility is that the circuits and tissues affected undergo significant changes during lethargus and thereby require substantial metabolic resources during this period. By diverting resources away from these areas to respond to our stimulus, proper development may not be possible. Supporting this hypothesis, the grinder of the pharynx increases in size only during molts [116], synaptic material accumulates in the egg-laying circuit during L4 lethargus [61], and the final stages of spermatogenesis to form mature sperm occur during L4 lethargus [268]. One potential circuit that we did not test that also shows significant developmental remodeling during this period is defecation circuit in males. Adult males release GABA from the AVAL and DVB neurons to contracts the sphincter muscles during the expulsion step of the highly regular defecation cycle. However, GABA released in L4 larva has the opposite effect [288]. Because defecation in worms is a highly coordinated and regular process, the defecation circuit is perhaps an interesting candidate to study the effects of sleep deprivation.

Our identification of particular circuits and tissues affected by sleep deprivation during development is the most extensive study to date performed in model organisms. Furthermore, the identification of circuits affected by sleep deprivation is novel in *C. elegans*. The only

other study similar in nature was performed in *D. melanogaster* in 2014 [189]. In that work, sleep deprivation of flies specifically during early development led to a modest impairment in adult mating. In agreement with our study, development played a critical role in this defect: a region of the fly brain responsible for sensing pheromones and undergoing rapid development during the period that deprivation occurred was significantly smaller in adult flies.

4.0.5 ALA as a sensory neuron

Of all 302 neurons in the worm, the neurons that I am most fascinated by are the single neuron ALA and the paired neurons CANL/R. Surprisingly, they are connected in the literal sense. In the most current wiring diagram for these neurons, ALA forms a gap junction with CANR and synapses onto CANL (wormwiring.org). This is intriguing because it is the only connection to the CAN neurons known to exist in the worm. Moreover, the CAN neurons are the only neurons in *C. elegans* that when ablated lead to death. Worms that lack CAN whither and fail to grow, but the exact function of the CAN neurons remains unknown. It was previously reported that the feeding motor neuron M4 was also essential [19], but this was later shown to be specific to large and difficult-to-eat bacteria. When grown on smaller bacteria, worms that lack M4 can still eat and are viable (JT Chiang, M Steciuk, B Shtonda, and L Avery, unpublished). While frustratingly little is known about CAN, more is known about ALA. Here, I will discuss my findings on ALA, an interneuron thought at first to regulate developmental sleep, but recently found by others to be essential for stress-induced sleep.

ALA is a unilaterally-placed single interneuron with a pair of bilaterally-symmetric processes that extend from the cell body to the tail. Previously, ALA was shown to decrease locomotion in an EGF-dependent manner specifically during lethargus [376]. ALA-ablated animals were more active than wild type during lethargus, but the ALA-ablated animals were more sluggish than wild type outside of lethargus. We initially set out to perform

calcium imaging on ALA during L4 lethargus, but we did not observe calcium transients before or during lethargus (data not shown). However, we did observe calcium transients in response to transferring the animals. I characterize these responses in Appendix I. I show that ALA exhibits these physiological responses to anterior and posterior picking like touch but not to less intense touch stimuli. I further show that these responses were independent of neurotransmitter and neuropeptide release from potential upstream neurons. Instead, I find that severing the long bilaterally-symmetric processes abolishes ALA's physiological response to posterior touch. Moreover, I find that ALA is required for an inhibition of egg-laying in response to picking-like touch. Taken together, these findings suggest that ALA can autonomously respond to intense touch. But how ALA is responding remains a mystery.

Aside from my findings suggesting that ALA also acts as a sensory neuron, there is some correlation between genes expressed in ALA and those expressed in other classical sensory neurons. Polymodal nociceptors sense high-threshold mechanical and thermal stimuli. In mammals, acetylcholine acting through nicotinic acetylcholine receptors (nAChRs) sensitizes nociceptor neurons following injury or inflammation [32, 106, 118]. Similarly, PVD and FLP, the canonical polymodal nociceptors in *C. elegans*, express several nAChR subunits required for nociception [74]. Specifically, DEG-3, a nAChR alpha subunit that forms a heteromeric channel with DES-2, a second nAChR alpha subunit, is required for proper PVD function [74]. Expression of *deg-3* and *des-2* is confined to the IL2 chemosensory neurons, touch cells neurons, M1 head muscles, FLP and PVD sensory neurons, the PVC interneuron, and ALA. ACR-23, a betaine activated ligand-gated cation channel, that function in mechanosensory neurons to maintain basal levels of locomotion was also shown to be highly expressed in ALA [274]. Interestingly, ALA also expresses *tmc-1*, a putative sodium-sensitive channel [58] whose mammalian homolog is expressed in ear hair cells and is linked to deafness [187, 208, 386]. Identifying whether one of these proteins or a still unidentified protein influences ALA's sensory role remains to be seen.

4.0.6 Relationship between lethargus and sleep

There is abundant evidence that insufficient sleep results in a large economic and social cost for society [103, 158, 306, 374]. However, as shown in this dissertation, there are also biological costs for insufficient sleep. Perturbing sleep, at least in this work, seems to exert its costs mostly on energetically "expensive" processes such as development (Chapter III). Consequently, animals have evolved specific molecular and behavioral mechanisms to compensate for lost sleep (Chapter II). Here, I will broadly speculate on what I think my work suggests about the nature of sleep and its relation to biology.

With few exceptions, sleep research is predominately associated with the brain. Moreover, most sleep researchers study mammalian systems and are skeptical of the value of studying sleep in less complicated systems. This is evident in the following review of the homeostasis work performed in this dissertation:

> "I fail to see how this evidence strengthens the connnections between lethargus and sleep. Lethargus seems to be a molt-related response characterized by exhaustion of energy reserves. The authors admit (l. 63) that lethargus is "a developmental stage that precedes the termination of each larval stage." It seem [sic] to bear no more relationship to sleep than do the leaf movements of plants. I see no reason to think that the neurobiological basis resembles mammalian sleep or provides meaningful insight into it." - Reviewer 2

I believe that part of this bias exists because of historical reasons. The founders of sleep research were primarily medical professionals, and as such, the study of sleep is often thought to be synonymous with the study of human sleep. However, as interesting as I find this bias (and all biases) to be, I am more fascinated by the nearly universal association of sleep with the brain.

I think there are good reasons for why this association exists. One, the very definition of sleep inherently biases the study of sleep to the study of the nervous system. Reversible

quiescence can only occur if movement exists, and nervous systems control movement. And in mammals, patterns of activity in the brain define sleep. Two, the brain is the most energetically costly organ in the body. I mention in the introduction of this dissertation that I favor the energy allocation model of sleep. If the most important function of sleep is indeed to optimize energy utilization, then the largest effects of a lack of sleep will be seen in the most energetically expensive organs and processes. This is essentially the drunkard's search: a drunkard who lost his keys will search primarily beneath the streetlight at the neglect of other areas because there is light there. However, this focus on the brain may come at the cost of exploring other effects of sleep loss.

The work presented in this dissertation and that of others studying sleep in invertebrates lends some evidence to this hypothesis. Lethal sleep deprivation in *C. elegans* resulted from molting defects, a metabolically expensive process [91, 372]. I show in Chapter III that sleep deprivation evoke stress responses in anatomically distinct circuits and tissues of the worm. The common theme that unites the circuits and tissues affected by deprivation is developmental change during lethargus. In *D. melanogaster*, others have shown that early developmental sleep loss is correlated with the rapid growth of an olfactory region of the brain leads to mating deficiencies in adulthood [189].

An interesting property of *C. elegans* sleep is that it is primarily coupled to the molting cycle of larva and only occurs in adults after stress. Across phylogeny, there is a similar correlation between sleep need and age: sleep need is highest is early development and then tapers off during adulthood [178, 295, 318]. This original observation led to the ontogenetic hypothesis of sleep, which postulates that sleep is critical for brain patterning in early development.

However, if it is true that sleep evolved to optimize metabolic cost by partitioning energy expensive processes and coupling them to an inactive behavioral state, then one would expect that sleep manifests itself not just at an organismal level but also a cellular level. Thus, the core biological function of sleep may extend to even simpler organisms. Indeed, the one

unifying principle of life is that life must extract energy from the environment. If that energy is finite, then life would have evolved strategies to optimize the use of that energy with respect to its environment in order to maximize fitness.

At an organismal level, some progress has been made in *C. elegans* to identify specific cells and neuropeptides essential for quiescence during lethargus [151, 371, 373]. In the case of stress-induced sleep, feeding quiescence, locomotion quiescence, and defecation quiescence were found to be separable and controllable by different neuropeptides [260]. However, at the cellular level, little is known. Probing the consequences at the cellular level of animals that are not behaviorally quiescent may lead to new insight about what determines sleep need and how sleep is regulated.

CHAPTER 5

APPENDIX I: THE *CAENORHABDITIS ELEGANS* INTERNEURON ALA IS (ALSO) A HIGH-THRESHOLD MECHANOSENSOR

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This chapter is adapted from Sanders et al., BMC Neuroscience 2013. JS carried out the molecular, behavioral, and physiological studies and drafted the manuscript (Figures 5.1, 5.2, 5.3, 5.5, 5.6, 5.7, 5.8). GF performed egg-laying experiments (Figures 5.4). SN and MT participated in the molecular work. CR participated in the data analysis. DB conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors contributed to writing and editing of drafts, and approved the final manuscript.

5.1 Abstract

To survive dynamic environments, it is essential for all animals to appropriately modulate their behavior in response to various stimulus intensities. For instance, the nematode *Caenorhabditis elegans* suppresses the rate of egg-laying in response to intense mechanical stimuli, in a manner dependent on the mechanosensory neurons FLP and PVD. We have found that the unilaterally placed single interneuron ALA acted as a high-threshold mechanosensor, and that it was required for this protective behavioral response.

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ALA was required for the inhibition of egg-laying in response to a strong (picking-like) mechanical stimulus, characteristic of routine handling of the animals. Moreover, ALA did not respond physiologically to less intense touch stimuli, but exhibited distinct physiological responses to anterior and posterior picking-like touch, suggesting that it could distinguish between spatially separated stimuli. These responses required neither neurotransmitter nor neuropeptide release from potential upstream neurons. In contrast, the long, bilaterally symmetric processes of ALA itself were required for producing its physiological responses; when they were severed, responses to stimuli administered between the cut and the cell body were unaffected, while responses to stimuli administered posterior to the cut were abolished.

C. elegans neurons are typically classified into three major groups: sensory neurons with specialized sensory dendrites, interneurons, and motoneurons with neuromuscular junctions. Our findings suggest that ALA can autonomously sense intense touch and is thus a dual-function neuron, i.e., an interneuron as well as a novel high-threshold mechanosensor.

5.2 Introduction

To survive in dynamic or harsh environments, all animals must appropriately modulate their responses to various stimulus intensities. For instance, noxious stimuli are detected by nociceptors, an important class of high-threshold sensory neurons. These, in turn, lead to downstream immediate avoidance responses and enduring self-protective responses that are distinct from responses to milder stimuli [27, 36, 118, 248, 400]. Neurons similar to mammalian polymodal nociceptors in both function and molecular determinants have been found across the animal kingdom [2, 59, 329]. In the nematode *Caenorhabditis elegans*, the neuron types PVD and FLP have been shown to share many similarities with mammalian and *Drosophila melanogaster* nociceptors [27, 51, 119, 130, 160, 394], including a conservation of molecular mechanisms underlying the responses to noxious stimuli [2, 59, 129, 163, 248, 272, 329, 363, 364].

C. elegans neurons are typically classified into three major groups: sensory neurons with

specialized sensory dendrites, interneurons, and motoneurons with neuromuscular junctions. However, these groups are not strictly mutually exclusive. For instance, the DVA interneuron was found to be a stretch-sensitive sensory neuron [220]. ALA is a unilaterally-placed single interneuron (Figure5.1 A). It has a pair of bilaterally-symmetric processes that branch from the soma, and proceed along the left and right sides of the body to the tail region, adjacent to the excretory canals. A third, short process is sent from the soma to the dorsal cord [397]. ALA has been shown to be involved in reducing the velocity of animals, as well as their rate of pharyngeal pumping in an epidermal growth factor-dependent manner [376]. It has also been reported to decrease locomotion in a manner subject to regulation by the CEPsh sheath cells [252].

Here we show that ALA acted as a high-threshold mechanosensor, and that it played a role in a previously described response to intense mechanical stimuli [2]. ALA exhibited physiological responses to both anterior and posterior stimuli, and it was required for the inhibition of egg-laying in response to picking-touch (see Methods). The physiological responses of ALA to anterior and posterior touch were distinct, suggesting that it could distinguish between spatially separated stimuli. In addition, these responses did not require neurotransmitter or neuropeptide release from upstream neurons. However, the bilaterally symmetric processes of ALA itself were required for generating its physiological responses. These results suggest that ALA can autonomously sense picking-touch, but not lower intensity touch stimuli, and is thus a high-threshold mechanosensor.

5.3 Results

5.3.1 The ALA neuron responded to both anterior and posterior picking-touch stimuli

After serendipitously observing physiological responses to picking-touch (see Methods) in ALA neurons we sought to characterize these responses. In order to assay the physiological

responses of ALA, we expressed the genetically encoded calcium indicator GCaMP3 [354] under the control of the ALA-specific *ver-3* promoter [53, 102, 125, 186, 219, 276, 279, 368, 376, 398, 418]. Touch stimuli were applied to either the anterior or the posterior region of the animal, and the resulting fluorescence intensity of the cell soma was recorded. We did not observe a response either to gentle-touch or to harsh-touch in ALA neurons (see Methods). Therefore, ALA was not found to be a low-threshold mechanosensor. In contrast, both anterior and posterior picking-touch evoked calcium transients in ALA (Figure 5.1). In the case of anterior stimuli, GCaMP fluorescence exhibited a 10sec increase to 400% of the baseline followed by a decline to 200% of the baseline after 100sec (Figure 5.1 B). Posterior stimuli resulted in a gradual 50sec increase in fluorescence to 300% of the baseline (Figure 5.1 C). In both cases, GCaMP3 fluorescence did not return to baseline within the 5minutes of the measurement but sustained similar levels of 200% of the baseline (see also Figure 5.2). To test how long ALA remained active while minimizing photobleaching, we recorded it for 20sec every 5minutes. We found that 30minutes after an anterior stimulus had been given, GCaMP3 fluorescence was not significantly different than its pre-stimulus baseline (Figure 5.1 E).

To control for motion artifacts, the fluorescence of *Pver-3*::GFP was assayed for anterior picking-touch under identical conditions and did not deviate from its baseline level (Figure 5.1 D). A negative control (Figure 5.3 A) was provided by the absence of detectable physiological responses in the dopaminergic CEPD/L head neurons [305] despite the fact that the ALM neurons were previously shown to indirectly activate CEP in response to an appropriate anterior body touch [196]. As a positive control, we expressed GCaMP3 in the phasmid chemosensory PHB neurons. Although the physiological responses of PHB neurons to harsh touch have not been previously reported, they were implicated in posterior harsh touch in a laser ablation study [221]. In response to posterior picking-touch, we observed a 3-fold increase in GCaMP fluorescence in PHB, which returned to baseline level after 100sec (Figure 5.3 B). These results suggest that ALA either received input from an upstream high-

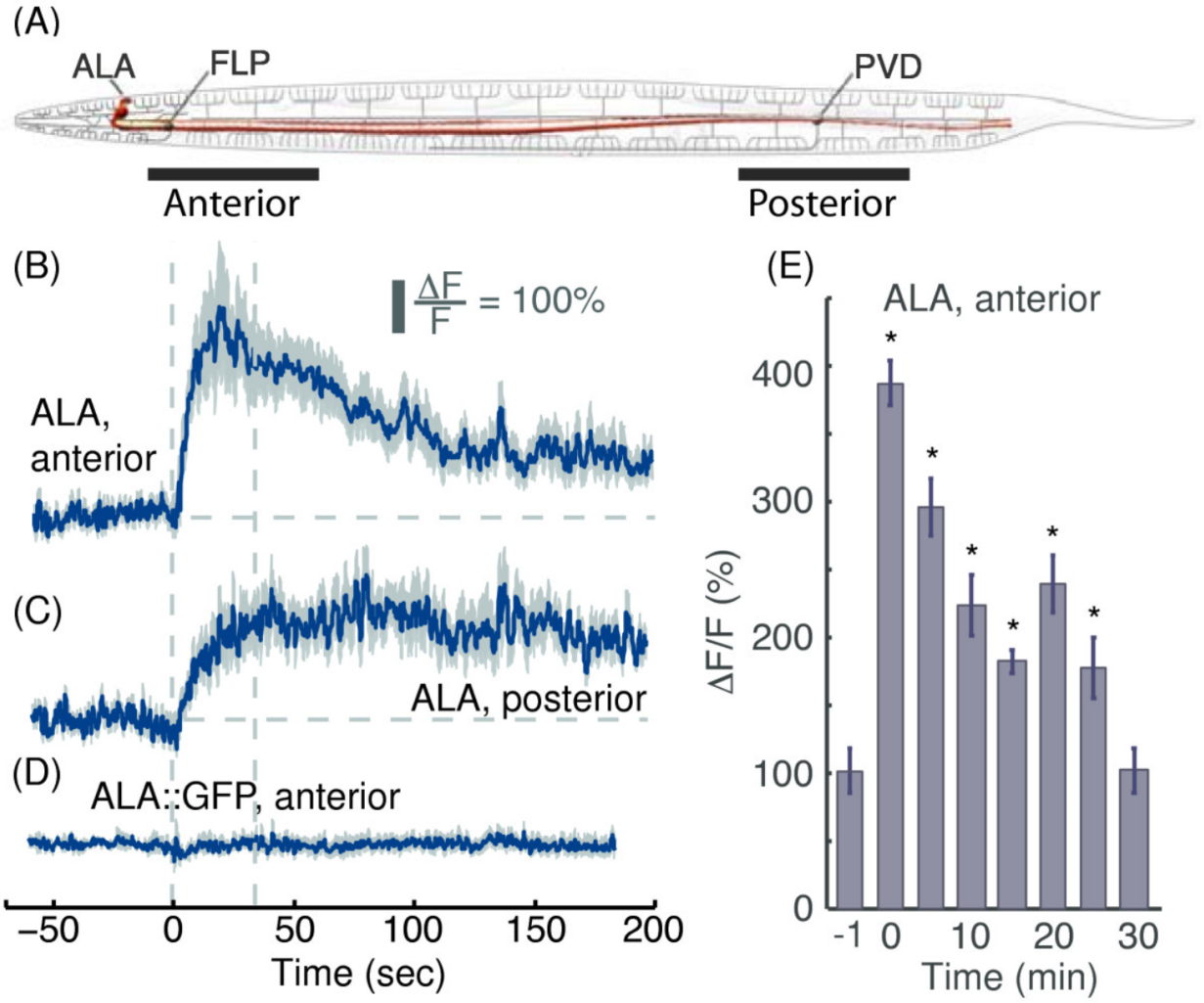

Figure 5.1: **ALA responds to anterior and posterior picking-touch stimuli.** (A) a schematic drawing of ALA (red), and the proprioceptor neurons PVD and FLP (grey). The locations where anterior and posterior mechanical stimuli were applied are denoted with black bars (schematic adapted from wormatlas.org). GCaMP3 fluorescence levels in ALA before and after administering anterior (B) or posterior (C) picking-touch stimuli at t=0 with a platinum wire pick. The scale bar represents a 100% deviation from the mean baseline fluorescence. Mean ± s.e.m, N=9-12 animals. (D) GFP fluorescence levels under the same conditions as in (B-C). Mean ± s.e.m, N=10 animals. Dashed lines in panels (B-D) are provided as a guide to the eye. (E) The mean GCaMP fluorescence levels during 20 sec intervals, recorded 1 minute prior to administering an anterior stimulus, immediately after the stimulus, and every 5 minutes thereafter. Mean ± s.e.m, N=10 animals. Asterisks denote a significant difference from the baseline mean fluorescence ($p < 0.05$).

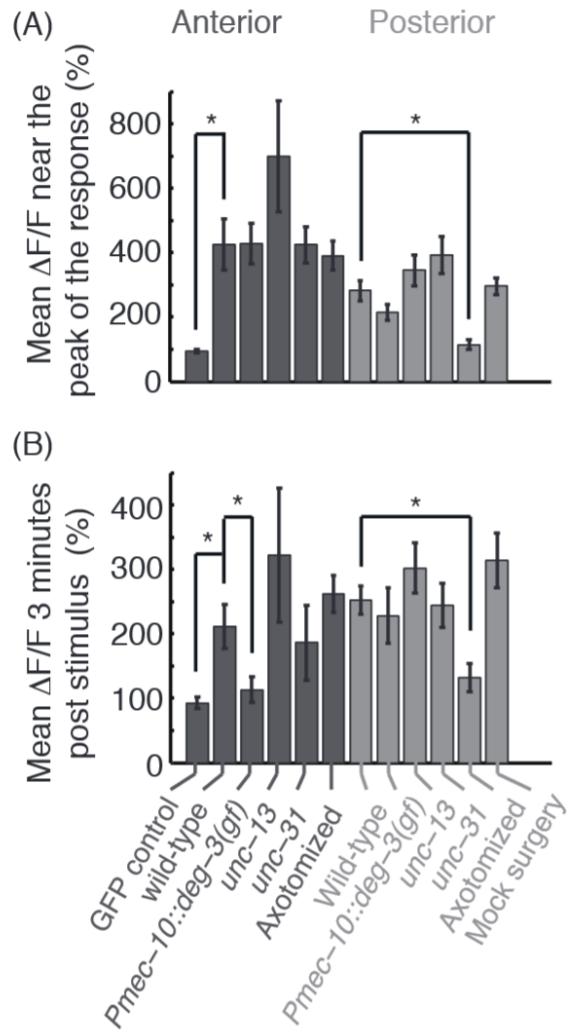

Figure 5.2: **A comparison of ALA physiological responses to picking-touch.** (A) The mean GCaMP fluorescence during the 20 seconds surrounding peak activity, t=5-25 sec and t = 4060 sec post-stimulus for anterior and posterior stimuli, respectively. Asterisks denote that the measurements of GFP (control) fluorescence and the post-surgery responses to posterior stimuli were significantly different from the corresponding wild type responses ($p < 0.05$). (B) The mean GCaMP fluorescence at t = 160180 sec. Asterisks denote that the measurements of GFP (control) fluorescence, the responses in *Pmec-10::deg-3(u662)* animals , and the post-surgery responses to posterior stimuli were both significantly different from the corresponding wild type responses ($p < 0.05$). All mean fluorescence levels were calculated from the datasets shown in Figures1, 5, 7, and 8.

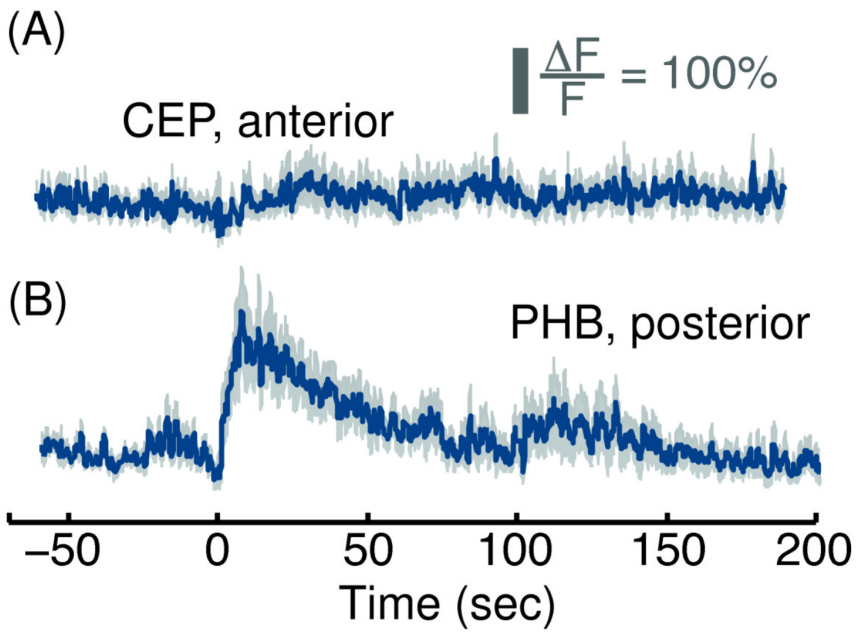

Figure 5.3: **CEP neurons did not exhibit a detectable response to anterior picking-touch, and PHB neurons responded to posterior picking-touch.** GCaMP3 fluorescence levels in CEPDL/R neurons before and after administering anterior stimulus (A) and in PHB neurons before and after administering a posterior stimulus (B) at t=0 with a platinum wire pick. The scale bar represents a 100% deviation from the mean baseline fluorescence. Mean ± s.e.m, N=10 animals.

threshold mechanosensor or sensed picking-touch autonomously. In addition, the differences in the temporal dynamics of the responses to anterior and posterior picking-touch suggested that the neuron sensing the stimulus was able to discriminate between different spatial positions of stimuli along the body of the animal.

5.3.2 A mutation that impairs ALA differentiation abolished the reduced egg-laying response to picking-touch

The commonly performed act of transferring a single animal to a new plate using a platinum wire pick inadvertently delivers an intense mechanical stimulus to the animal and evokes a characteristic behavioral response. It was previously shown that this mechanical stimulus leads to PVD- and FLP-mediated egg-laying inhibition lasting 30minutes [2]. The same

procedure was also shown to affect locomotion on a timescale of several minutes [75]. In addition, posterior gentle touch was shown to lead to increased intervals between calcium transients of the HSN egg-laying neurons [416]. We thus asked whether ALA might have a role in mediating these behavioral responses. To address this question, we compared the suppression of egg-laying in wild type animals to animals mutant for *ceh-17*, a gene encoding a paired-like homeodomain transcription factor.

CEH-17 is expressed in ALA and the cholinergic SIA head neurons and is involved in longitudinal axonal navigation and ALA differentiation [281, 376]. Specifically, CEH-17 was shown to regulate the expression of the tyrosine phosphatase-like receptor gene *ida-1*, which is required for ALA neuropeptide release, and *ceh-17* mutants exhibited shortened ALA processes [377, 416]. Since the ALA neuron has two bilaterally symmetrical processes that extend from the cell body to the tail of the animal [397], we assayed responses to anterior and posterior stimuli separately. In agreement with previous observation, in our hands both anterior and posterior picking-touch led to a 35% reduction in the number of eggs that were laid by wild type animals during a 3-hour period. In contrast, in *ceh-17(np1)* mutants the inhibition of egg-laying in response to both anterior and posterior stimuli was completely abolished (Figure 5.4). In agreement with previous findings [281], we did not observe differences between the locomotive responses of wild type animals and *ceh-17* mutants to picking-touch (data not shown).

5.3.3 Laser ablation of ALA abolished the reduced egg-laying response to picking-touch

To specifically test whether ALA was required for the egg-laying inhibition response to picking-touch, we assayed animals in which the ALA neuron was laser ablated during the L4 larval stage. As opposed to the mock-ablated control animals, ALA-ablated animals did not exhibit suppression of the egg-laying response (Figure 5.4). However, the egg-laying rate of unperturbed post-surgery animals was variable, with a lower mean (although not

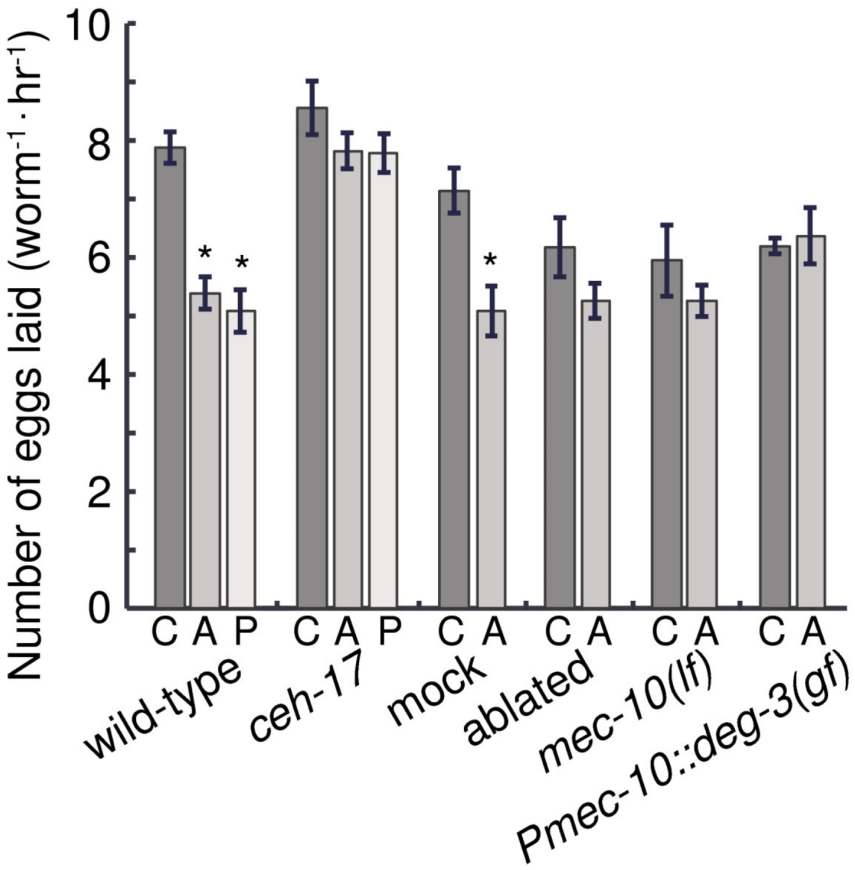

Figure 5.4: **ALA is required for the suppression of the egg-laying response to picking-touch.** The number of eggs laid per animal, per hour, in animals exposed to anterior or posterior picking-touch stimuli (A and P respectively) and in control animals (C). Mean ± s.e.m, N=4-13 assay plates (see Methods). Asterisks denote statistically significant comparisons ($p < 0.05$).

significantly: p=0.114 by one-way ANOVA) than the corresponding rate in mock-ablated controls. This variability may be indicative of unspecific damage of the surgery that could not be avoided. We obtained similar results (Figure 5.4) for two strains in which behavioral responses to harsh touch were abolished [2, 59]: mutants for the *mec-10* gene, encoding an amiloride-sensitive sodium channel protein of the DEG/ENaC family that is required for *C. elegans* touch sensation [27, 51, 119, 160, 394], and animals where a degeneration-causing, constitutively active nicotinic acetylcholine receptor (nAChR) channel subunit, *deg-3(u662)*, was expressed under a *Pmec-10* promoter [2, 363, 364], driving expression in and degeneration of PVD, FLP and the six touch receptor neurons [36, 160]. The suppression of egg-laying was not significant in *mec-10(tm1552)* mutants (p=0.193 by one-way ANOVA) or *Pmec-10::deg-3(u662)* animals (p=0.732 by one-way ANOVA) [15, 27, 59, 221, 400]. Taken together with the responses of *ceh-17* mutants, our results suggest that ALA is required for the inhibition of egg-laying in response to picking-touch, but not for immediate avoidance responses.

5.3.4 The harsh-touch sensory neurons were not required for the physiological response of ALA to picking-touch

The interneuron ALA has not been previously reported to act as a mechanosensory neuron, but the effects on egg-laying of ablating it were similar to those of ablating known mechanosensors. Moreover, although synapses between ALA and PVD have not been previously found, the elongated processes of ALA and the primary dendrites of PVD are in close proximity [27, 118, 397]. It was thus possible that ALA transduced signals from known mechanosensory neurons, so we asked whether the physiological responses that we observed were dependent on input from them. To test this, we crossed our Pver-3::GCaMP3 reporter into transgenic animals expressing the degeneration-causing nAChR channel subunit, *deg-3(u662)*, in PVD, FLP and the six harsh touch neurons [330, 363, 364]. In agreement with previous results [2, 130, 160], these animals failed to respond to standard harsh touch stimuli

(data not shown). In addition, the physiological responses of their ALA neurons to posterior picking-touch were unaffected by the genetic ablation (Figure 5.5 B, Figure 5.2). However, in response to anterior picking-touch, GCaMP fluorescence in ALA returned to its baseline level after 2minutes in these transgenics (Figure 5.5 A, Figure 5.2), in contrast to the wild type prolonged response to the same stimulus. We concluded that the responses of ALA did not require the function of PVD, FLP and the six touch receptor neurons, although a sub-group of these neurons may have a role in sustaining the responses to anterior stimuli.

5.3.5 The physiological response of ALA to picking-touch did not require neurotransmitter release

Although physiological responses in ALA did not require known mechanosensory neurons, it was still possible that the responses were dependent on input from other pre-synaptic partners of ALA. To answer this question we crossed our *Pver-3*:GCaMP3 reporter into an *unc-13(e51)* mutant background, where synaptic vesicle exocytosis is essentially eliminated [2, 59, 129, 163, 238, 272, 291, 329]. Anterior and posterior picking-touch evoked rapid and long-lasting calcium transients in the ALA neurons of *unc-13(e51)* mutants (Figure 5.5 C,D and Figure 5.2). The peaks of *unc-13(e51)* responses to anterior stimuli were highly variable, with a coefficient of variance of 0.78 (Figure 5.5), but were not significantly different from wild type ($p=0.103$ by one-way ANOVA). In contrast to wild type, the rise time of the responses to posterior stimuli was rapid (5sec) in the mutant animals, and their dynamics were similar to those of responses to anterior stimuli. These results suggested that synaptic input from neurotransmitter release was not necessary for producing the responses of ALA to picking-touch. However, such input may contribute to more subtle aspects of regulating these responses (similar to the case of the *Pmec-10::deg-3(u662)* transgenic background) such as the initial dampening of responses to posterior stimuli.

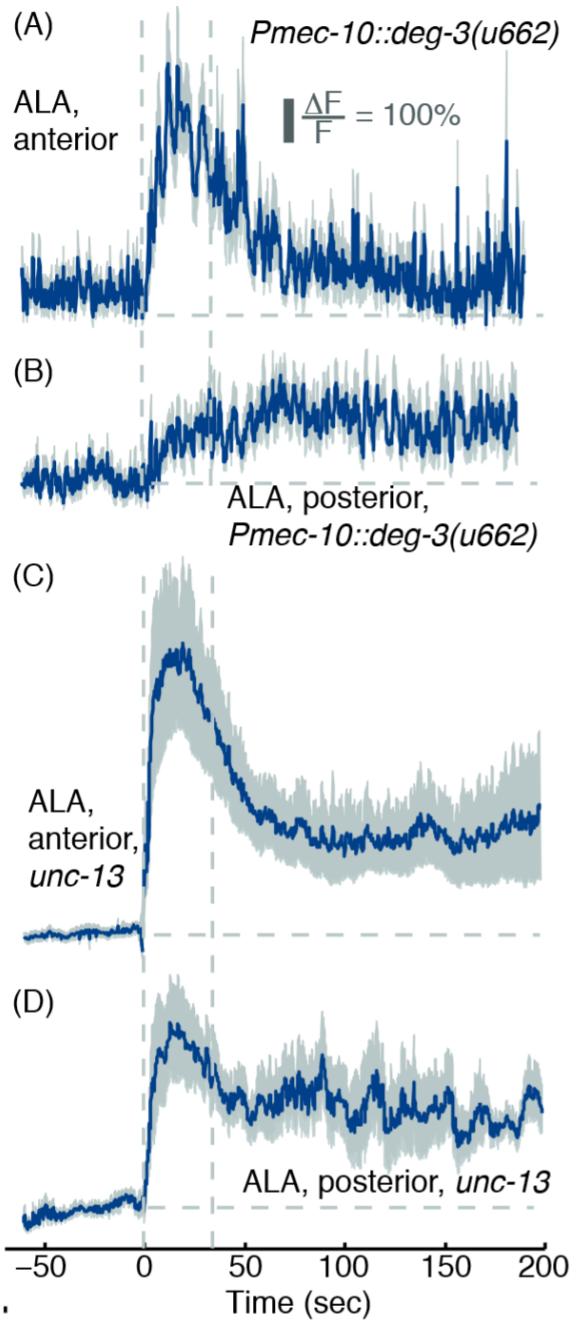

Figure 5.5: **PVD, FLP and the harsh touch response neurons, as well as neurotransmitter release, were not required for the responses of ALA to picking-touch.** GCaMP3 fluorescence levels in ALA neurons of animals lacking the PVD, FLP and six harsh touch neurons (A-B) and in *unc-13(e51)* mutants (C-D). The scale bar represents a 100% deviation from the mean baseline fluorescence. Mean ± s.e.m, N=10-11 animals. Dashed lines are provided as a guide to the eye.

5.3.6 Presynaptic partners of ALA responded to anterior picking-touch

Since blocking neurotransmitter release modulated the kinetics of the responses of ALA, we asked whether its presynaptic partners responded to picking-touch. Specifically, we focused on two presynaptic sensory neurons: ADE, previously implicated in anterior touch responses by laser ablation experiments [137, 152, 182, 196, 221], and ADL, a polymodal nociceptive neuron [114, 299, 366, 391]. We expressed GCaMP3 in ADE (and all of the dopaminergic neurons) and ADL using the *Pdat-1* [172, 397] and *Psrh-220* [240, 376] promoters, respectively. ADE neurons exhibited a slowly rising response to anterior but not posterior picking-touch, which peaked after 40sec and returned to baseline after 5min (Figure 5.6 A,B). However, abolishing vesicle exocytosis in all dopaminergic neurons by driving expression of the tetanus toxin light-chain with the *dat-1* promoter [232, 252, 307] did not significantly alter the responses of ALA to picking-touch (Figure 5.6 C,D). Consistent with previous reports [2, 391], ADL neurons responded to the onset of blue light. However, this response decayed after 4minutes of continuous illumination such that the animal could be assayed approximately 10minutes after initially being exposed to light. ADL neurons also responded to anterior, but not posterior picking-touch, and their response decayed to baseline after 70sec (Figure 5.6 E,F). Taken together with the responses observed in *unc-13* mutants, these results indicated that synaptic input from ADE and ADL did not contribute to the responses of ALA to picking-touch.

ADL, a presynaptic partner of ALA, has been shown to be a chemosensory neuron that plays a role in avoidance behavior in the presence of volatile repellents such as octanol [35, 366]. Ablating ADL has been shown to increase the latency of responses to octanol off food, but not on food [2, 56]. We thus sought to test whether ALA might be mediating octanol avoidance in addition to responses to picking-touch. To answer this question, we dipped an eyebrow hair in 30% octanol and presented it in front of the nose of *Pver-3*::GCaMP3 animals. We observed reversals characteristic of the avoidance response of *C. elegans* in both wild type animals and *ceh-17* mutants, with latencies of 4sec in both genetic backgrounds,

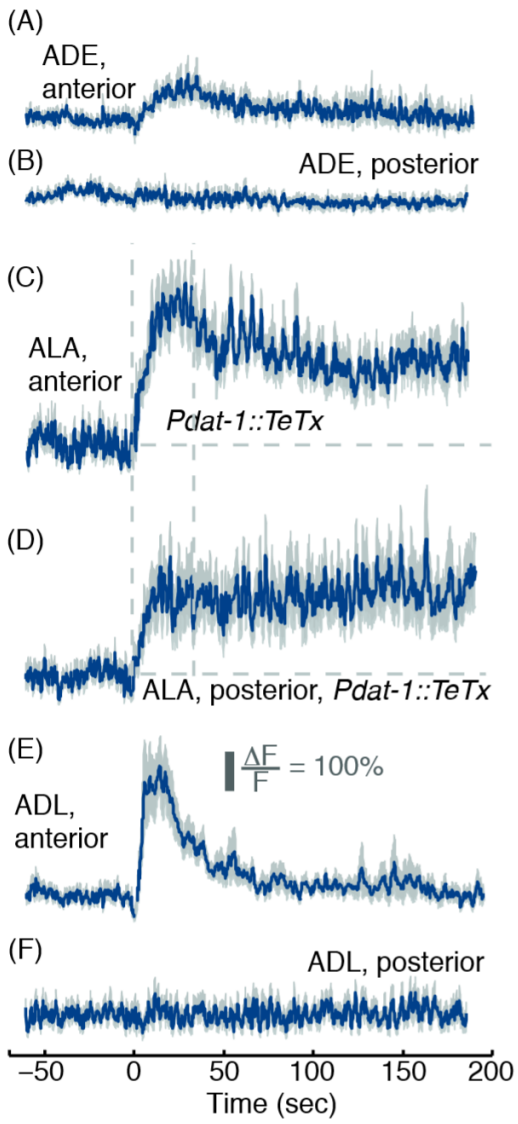

Figure 5.6: **The sensory neurons ADE and ADL, both presynaptic partners of ALA, responded to anterior but not to posterior picking-touch.** GCaMP3 fluorescence levels in ADE neurons (A-B), ALA neurons in animals expressing the tetanus toxin light chain in their dopaminergic neurons (C-D) and in ADL neurons (E-F). In all cases responses to both anterior and posterior stimuli are shown. Mean ± s.e.m, N=10 animals. Dashed lines in panels (C-D) are provided as a guide to the eye.

both on and off food (data not shown). Consistent with the behavioral assay, the repellent did not evoke detectable calcium transients in the ALA neuron (data not shown). Taken together, these results suggested that ALA did not mediate octanol avoidance.

5.3.7 The physiological responses of ALA to picking-touch were independent of neuropeptide release

Although input from neurotransmitter release of pre-synaptic partners of ALA was not required for producing its physiological responses to picking-touch, it could be the case that peptidergic signaling from an unknown mechanosensory neuron was required. The *unc-31* gene encodes an ortholog of the mammalian CAPS protein and is required for dense-core vesicle but not synaptic vesicle exocytosis of neuropeptides [221, 334, 365, 395]. Thus, we crossed our *Pver-3*::GCaMP3 reporter into an *unc-31(e169)* mutant background, where dense-core vesicle exocytosis is practically eliminated. Anterior picking-touch evoked wild type-like responses in the ALA neurons of *unc-31(e169)* mutants, while posterior touch evoked responses that appeared slightly enhanced during the first 50sec post-stimulus, but not significantly so (Figure5.7: p=0.998 and p=0.162 by one-way ANOVA for anterior and posterior stimuli, respectively). These results suggested that neuropeptide release was not required for evoking or sustaining the responses of ALA to picking-touch.

5.3.8 The elongated processes of the ALA neuron were required for its physiological response to picking-touch

We reasoned that if ALA was autonomously sensing picking-touch, then it would likely require its long, bilaterally symmetric processes to do so. If this is the case, severing the elongated processes between the nerve ring and the vulva should abolish ALA responses, at least to stimuli that are posterior to the cut. We used a femtosecond pulsed laser to sever ALA processes [52, 65, 221, 239, 395, 408] in wild type L4 larvae expressing our re-

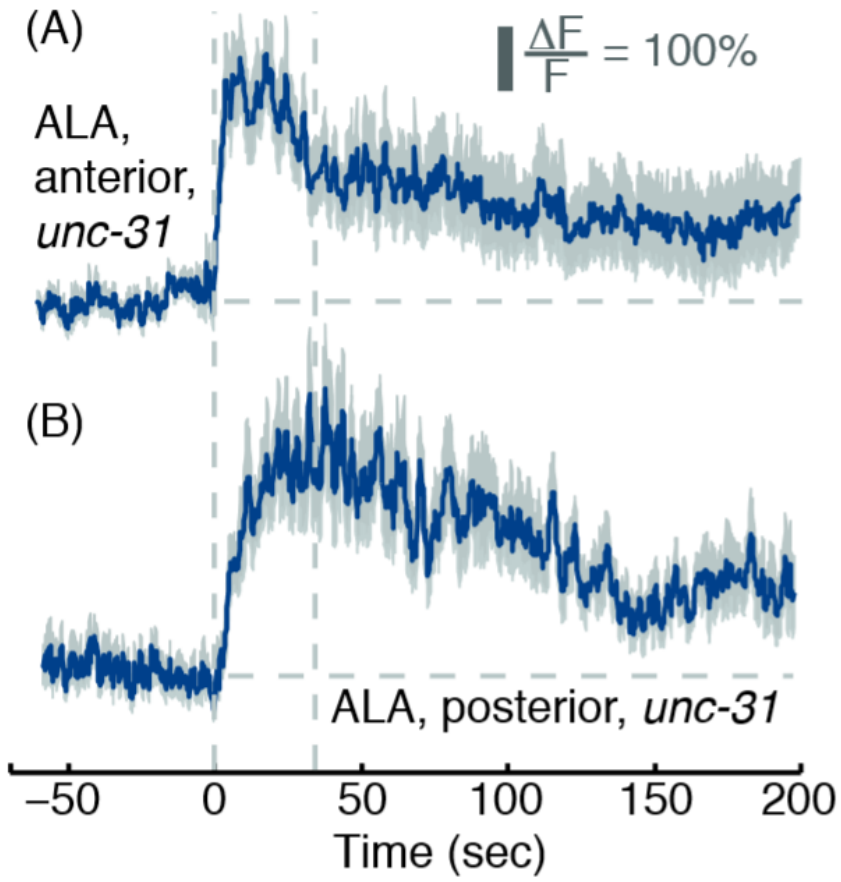

Figure 5.7: **Neuropeptide release was not required for the responses of ALA to picking-touch.** GCaMP3 fluorescence levels in ALA neurons of *unc-31(e169)* mutants responding to anterior (A) and posterior (B) picking-touch. Mean ± s.e.m, N=10 animals. Dashed lines are provided as a guide to the eye.

porter, *Pver-3*::GCaMP3, and assayed them as young adults 24hours post-surgery (Figure 5.8 A). We found that the physiological responses of ALA to posterior picking-touch downstream from the cut were completely abolished in axotomized animals and unaffected in the mock-axotomy controls (Figure 5.8 B). In contrast, ALA responses to anterior picking-touch, upstream from the cut, were unaffected by the surgery (Figure 5.8 D and Figure 5.2). The wild type like responses to anterior stimuli suggested that the part of the cell upstream from the cut, including the soma, was not damaged by the surgery an internal control that is typically difficult to obtain. These results supported the conclusion that ALA autonomously sensed picking-touch stimuli and that its long processes were required for this function. In addition, they suggested that ALA could discriminate between different spatial positions of stimuli along the body.

5.4 Discussion

In this study we show that the ALA interneuron of the nematode *C. elegans* exhibits physiological responses to picking-touch and that it is required for a stereotypical response to this stimulus, a suppression of egg-laying [2, 221, 416]. The physiological responses of ALA did not require input from presynaptic release of neurotransmitters, nor did they require neuropeptide release. When the elongated, bilaterally symmetric processes of ALA were axotomized, the physiological responses to stimuli that were on the anterior side (upstream) of the cut persisted, while the responses to stimuli on the posterior side (downstream) of the cut were eliminated. ALA is known to form several electrical synapses, all of which are located in the anterior sections of its processes, i.e., in vicinity of the cell soma [397, 404]. As a result, our axotomy experiments rule out the possibility that input from the known gap junctions of ALA was capable of producing the observed responses. Taken together, our findings suggest that ALA can sense picking-touch stimuli autonomously, i.e., that it is a high-threshold mechanosensor.

The ALA neuron could discriminate between spatially separated stimuli along the body

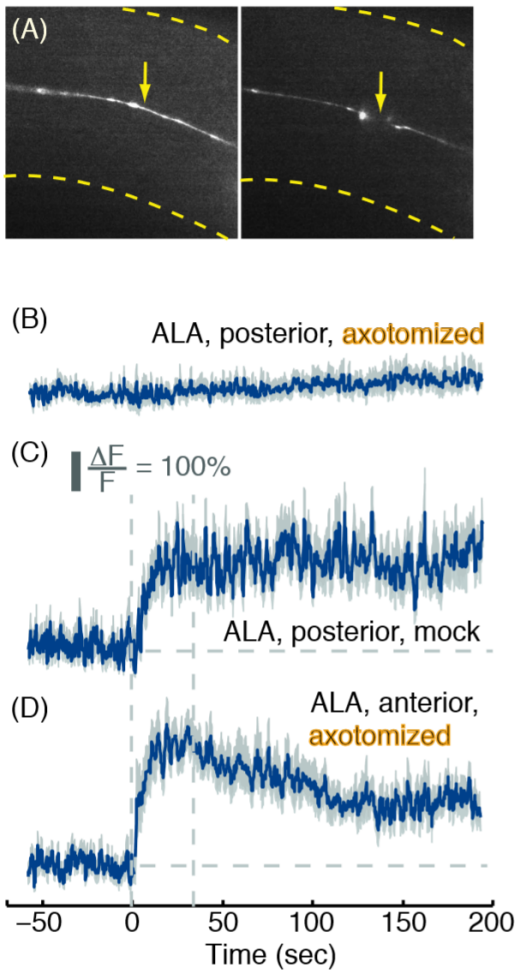

Figure 5.8: **Severing both of the elongated processes of ALA abolished its responses to picking-touch stimuli that were positioned posterior to the cut.** (A) A representative image of a single ALA process expressing GCaMP3 before (left) and after (right) it was severed. (B-C) GCaMP3 fluorescence levels in ALA neurons before and after a picking-touch stimulus was administered posterior to the cut in operated animals (B) and in mock-operated controls (C). Mean ± s.e.m, $N_{operated}$=8 and N_{mock}=10 animals. (D) Same as (B), but for stimuli that were administered anterior to the cut, i.e., between the cut and the ALA cell body. Mean ± s.e.m, $N_{operated}$=11 animals. Dashed lines in panels (C-D) are provided as a guide to the eye.

of the animal. Three observations are consistent with a model in which (as of yet unknown) molecular sensors are distributed along the processes of ALA: (1) the temporal dynamics of the responses to anterior and posterior stimuli were distinct, (2) neurotransmitter and neuropeptide release affected anterior and posterior responses differently, and, most importantly, (3) when the elongated processes of ALA were severed the responses to stimuli applied posterior but not anterior to the cut were abolished. Measuring the receptive field map of ALA using more accurately localized stimuli could provide insight into the spatial differentiation of this mechanosensor.

The physiological responses that we observed in ALA were unusually prolonged as compared to typical responses of *C. elegans* sensory neurons [33, 49, 65, 196, 207, 239, 343, 370, 420]. Application of menthol, a noxious stimulus, can produce similarly prolonged calcium transients in mammalian nociceptors [304]. However, axonal injury in mammalian and invertebrate preparation can also produce long-lasting calcium responses [193, 401]. Could the physiological and behavioral responses that we observed result from a compression or strain injury inflicted on ALA by picking-touch? Several considerations suggest that this is unlikely: (1) picking was used to transfer control animals to assay plates, as well as for their maintenance, such that a potential injury in ALA would be required to heal on the timescale of an hour in order to allow for the higher egg-laying rate in the absence of the recurring stimulus, (2) ALA is not at all unique in being located peripherally or having elongated processes; similar anatomical features are characteristic of many touch receptor, proprioceptor and motor neurons in *C. elegans* [397], (3) egg-laying was not suppressed in response to anterior picking-touch in *ceh-17* mutants, where the anterior half of the ALA process was typically present; moreover, the developmental defect in ALA neurons in *ceh-17* mutants did not suppress egg-laying, and (4) in *C. elegans*, axotomy induced calcium dynamics in the soma of the touch receptor neuron ALM exhibited a strong dependence on the lesion distance, effectively vanishing when the axon was injured merely 40 µm from the soma [193]. Thus, explaining the reported responses as a result of injury would require multiple unsup-

ported assumptions regarding the unique nature of ALA, such as an unexplained enhanced vulnerability to compression, an ability to heal rapidly, an effect on egg-laying in response to injury that is distinct from a non-specific loss of function, and post-injury calcium dynamics dissimilar from those of a *C. elegans* touch receptor neuron.

Our findings associate the responses of ALA to picking-touch with the enduring inhibition of egg-laying, but not with an immediate avoidance response. Since enduring behavioral responses have been shown to depend on neuropeptides in previous studies [2, 305], we hypothesize that this could also be the case here. Based on its anatomical features, it has been suggested that ALA may be a neurosecretory neuron [196, 263, 397]. It has been shown that ALA is required for the regulation by epidermal growth factor (EGF) signaling of feeding and locomotion patterns, and that feeding defects caused by overexpression of the EGF-like peptide LIN-3 are suppressed by a mutation in the gene encoding UNC-31/CAPS [221, 376]. In addition, the tyrosine phosphatase-like receptor gene *ida-1* was shown to be expressed in ALA [2, 377, 413]. IDA-1 was demonstrated to be important for dense-core vesicle cargo release; it acts genetically in the signaling pathway of *unc-31* (encoding the UNC-31/CAPS protein) suggesting that it has a role in the trafficking of dense core vesicles and/or in their cargo release [41, 75]. Importantly, CEH-17 was shown to regulate the expression of *ida-1* in ALA [377, 416], such that neuropeptide release from ALA would be expected to be impaired in *ceh-17* mutants. Taken together, these findings and the results presented here are consistent with the idea that ALA may use peptidergic signaling to communicate to its downstream targets.

Several *C. elegans* neurons, such as the proprioceptors DVA, AVG, and PVR, were originally classified as interneurons and later found to also function as sensory neurons. In addition, interneurons functioning also as sensory neurons have been described in other species, e.g. the B51 neuron in *Aplysia californica* [97, 278]. However, this study was the first to associate the role of a mechanosensor with the interneuron ALA, thus demonstrating that it is a dual-function neuron. The roles of neuropeptides in modulating the repertoire of

enduring responses, as well as the degree to which these roles may be either evolutionarily conserved, remain to be understood.

5.5 Materials and Methods

5.5.1 Strains

wild type, transgenic, and mutant *C. elegans* strains were maintained and cultivated with OP50 bacteria according to standard protocols [35]. The following strains were used: wild type strain N2, INV21001 N2;Ex[*Pver-3*::GCaMP3], INV21002 N2;Ex[*Pgpc-1*::GCaMP3], INV21003 N2;Ex[*Psrh-220*::GCaMP3], INV21004 N2;Ex[*Pdat-1*::GCaMP3], INV54001 N2; Ex[*Pdat-1*::TeTx; *Pver-3*::GCaMP3], INV54002 *egl-3(n150)V*; Ex[*Pver-3*::GCaMP3], INV54003 *unc-13(e51)*; Ex[*Pver-3*::GCaMP3], INV54004 *unc-31(e169)*; Ex[*Pver-3*::GCaMP3] and INV54005 N2 ;Is[*Pmec-10::deg-3(u662)*]; Ex[*Pver-3*::GCaMp3]. The OS5513 N2; Ex[*Pver-3*::GFP] and the IB16 *ceh-17(np1)I* strains were a gift from Menachem Katz of Shai Shahams laboratory (Rockefeller University).

5.5.2 Picking-, harsh- and gentle-touch stimuli

The routine maintenance procedure of picking animals entails applying pressure with a platinum wire covered with a thin sticky layer of bacteria, a procedure performed on the order of 10,000 times a year by a typical experimenter. Although it is difficult to obtain quantitative characterizations of hand-delivered mechanical stimuli, estimates of the pressure applied by a platinum wire pick were previously obtained using an NGM test plates placed on an analytical balance [221]. By this measure, in our hands, picking-touch was estimated to involve pressures that were 10-fold larger than those associated with the standard harsh-touch stimulus. Thus, *C. elegans* researchers commonly apply three distinct tiers of touch stimuli: (1) gentle touch the weakest intensity tier, e.g., brushing the animal with an eye-lash pick, (2) harsh touch an intermediate intensity tier, e.g., probing with a platinum wire or a glass rod,

and (3) picking touch the highest intensity tier in routine use [52, 221, 395]. In addition, picking-touch was previously shown to produce a behavioral response a suppression of the rate of egg-laying [2]. In our assays, a picking-touch stimulus was delivered in the same manner as in routine picking, but without the thin bacteria layer that would facilitate the lifting of the animal from the substrate. The duration of the stimulus was approximately one second. Thus applied, this common procedure was not previously reported to result in sustained tissue damage, and our data suggests that it did not injure the ALA neuron. To test responses to lower intensity mechanical stimuli, we delivered standard harsh-touch and gentle-touch stimuli using a platinum wire and an eyelash pick, respectively.

5.5.3 Octanol avoidance assay

Avoidance of 30% 1-octanol was assayed as previously described [56, 366]. In brief, an eyelash hair was attached to a Pasteur pipette, dipped in octanol, and presented in front of a forward moving animal without touching it. The amount of time it took the animal to initiate backward movement was determined by a handheld audible timer. Two well-fed animals were placed on fresh NGM plates (either with or without a lawn of bacterial food) 30minutes prior to each assay. Each animal was tested 710 times, with an interval of at least 2minutes between successive tests.

5.5.4 Egg-laying assay

Animals were synchronized and assayed at 70hours post-hatching unless noted otherwise. Animals were placed in triplets on a 3cm diameter NGM plates at 20 degrees C 20minutes prior to the beginning of the assay. Picking-touch stimuli were delivered manually to each individual animal every 20minutes during a 3hours period. The adults were then removed and the number of eggs per plate was counted. To simplify egg-counting, each assay plate was seeded with a small drop of OP50 bacteria at its center.

5.5.5 *Physiological imaging in freely behaving animals*

Animals were manually synchronized by transferring 10 gravid adults to fresh NGM plates and restricting the duration of egg-laying to two hours. The adults were then removed, and the embryos were grown at 20 degrees C until young adulthood. One hour prior to testing, young adult animals expressing the appropriate marker were transferred to a fresh standard NGM plate (6cm in diameter) spread with a thin layer of OP50 bacteria. Picking-touch stimuli were delivered manually to freely moving animals using a platinum-iridium wire (0.2mm diameter, 99.9% purity from Alfa Aesar) attached to a glass Pasteur pipette [221]. Anterior and posterior stimuli were applied to the farthest quartiles of the animal body, respectively. A single stimulus was delivered per animal. Calcium imaging of each worm was performed at a magnification of 11.5x for 1minute prior to the stimulus and 4minutes post-stimulus. Alternatively, in order to capture the slow decay of the signal, 20seconds of imaging were performed every 5minutes for 30minutes. Images were binned 4x, and captured at 5Hz using a cooled CCD camera (Photometrics CoolSNAP HQ2, Tucson, AZ), an Olympus SZX16 stereomicroscope equipped with an SDF PLAPO 1XPF objective (Olympus America Inc., Center Valley, PA), and Micro-manager [95]. Animals were tracked manually during the assay and image analysis was performed using custom MATLAB scripts (Mathworks, Inc. Natick, MA).

5.5.6 *Axotomy and ablations*

Ablations and axotomy were performed on L4 larvae, immobilized with 10 µm levamisole on a 2% agarose pad, using a Ti:sapphire femtosecond laser, as previously described [65, 239, 408]. The transgenes *Pver-3*::GFP and *Pver-3*::GCaMP3 were used to identify ALA neurons for ablations and axotomy, respectively.

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