The evolutionary construction of sleep

Thesis by

Ravi David Nath

In Partial Fulfillment of the Requirements for

the degree of

Doctor of Philosophy in Biology

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2018

Defended May 4th, 2018

Ravi David Nath
ORCID: 0000-0003-0905-2707

ACKNOWLEDGEMENTS

This work is dedicated to the special, smart, and creative spirits that have influenced my development as a person and scientist across the years. To my mother, Monica Savasta, whose creativity, energy, intelligence, and dedication to learning are without a doubt the most paramount factor in my academic pursuits. To my father, Rajinder Nath, the adventurer, who with a childlike sense of curiosity, introduced me to the wonders of exploration and the natural world. My parents provided a strong, supportive environment and demonstrated resilience. I also thank my sister and brother-in-law, Michelle Murray and Adrian Murray, for their support and helping me advance as a professional.

I want to thank the California Institute of Technology. Caltech is an amazing community of people where I had the luxury of great conversations and discussions with emerging and preeminent biologists, chemists, engineers, mathematicians, astronomers, and physicists. I enjoyed every minute of work within the Kerckhoff Laboratories of the Biological Sciences, and I treasured working within the same walls of the founding father of modern genetics, Thomas Hunt Morgan, as well as many other famed scientists. In that spirit of great minds, I want to thank Professor Paul Sternberg for giving me the freedom and opportunity to experiment. Professor Sternberg embodies the academic spirit, encouraging students to pursue fun and creative ideas. Professor Sternberg provided an inspirational educational experience; he is a fantastic mentor and great friend.

I had an amazing thesis committee composed of several great scientists. The collaborations I had with Professors David Prober and Viviana Gradinaru were exciting, fun, and thought provoking. Professor Prober provided important advice and guidance on the biology of sleep. His mastery of the subject is truly amazing and his technical guidance on genetics was always helpful. Professor Gradinaru pursues bold hypotheses and pushes technology which makes the once impossible now possible. Her advice and judgment were always focused, insightful, and essential. I also am grateful to have had discussions, feedback, and conversations with Professor David Anderson where I learned to be an advocate for elegant experimental design. I am inspired by his everlasting passion for science.

I am most indebted and grateful to the advice and guidance of Claire Bedbrook, who has helped me advance as a person and scientist in many ways. Claire's mind is special—she asks cutting questions, thinks creatively about the solutions, and designs the best experiments. Our conversations about science were endless, ranging from technical minutia to fun and absurd speculation.

ABSTRACT

To understand the biological basis of sleep we need to understand its neuronal and genetic regulation. In this thesis, I explore how individual behaviors serve as building blocks to construct the sleep state where a block is defined as a set of measurable behaviors. These behavioral blocks are shaped by evolutionary forces. From one animal to the next, blocks may remain or change. If a block remains across all the sleep states in the metazoan lineage then it must have an important and conserved role in sleep regulation. For example, reduced locomotion is a behavior that is often observed during sleep. There are two possible explanations for the changing of a block: either the block was vestigial or the block was easily replaceable with another block that fulfills the same function. Consider sleep duration: some animals may require five hours of sleep, while others only require one hour. The changing of a block is one way that the sleep state could evolve. Blocks may also be added during the evolution of the sleep state, increasing the dimensions and number of tasks that are accomplished during sleep. Here, I discuss the origin of sleep, as well as its conserved neuronal and genetic regulation. I report the following: the discovery of sleep in jellyfish which are among the first animals to evolve neurons and the identification of novel sleep regulators in the roundworm Nematode *Caenorhabiditis elegans* (*C. elegans*). The sleep regulators discovered in *C. elegans* may have conserved functions in vertebrates. These studies show that some sleep behaviors and various sleep molecules change or remain homologous across metazoans. The studies are united by our simple block hypothesis of sleep construction.

PUBLISHED CONTENT AND CONTRIBUTIONS

Nath RD, Bedbrook CN, Abrams MJ, Basinger T, Bois JS, Prober DA, Sternberg PW, Gradinaru V, Goentoro L. The Jellyfish Cassiopea Exhibits a Sleep-like State. Curr Biol. 2017 Oct 9;27(19):2984-2990.e3. doi: 10.1016/j.cub.2017.08.014.

R.D.N. participated in the conception of the project R.D.N. participated in *C. elegans* behavior experiments. R.D.N. participated in the generation of reagents. R.D.N. participated in analyzing the data and writing the paper.

Nath RD, Chow ES, Wang H, Schwarz EM, Sternberg PW. C. elegans Stress-Induced Sleep Emerges from the Collective Action of Multiple Neuropeptides. Curr Biol. 2016 Sep 26;26(18):2446-2455. doi: 10.1016/j.cub.2016.07.048.

R.D.N. participated in the conception of the project R.D.N. participated in *C. elegans* behavior experiments. R.D.N. participated in the generation of reagents. R.D.N. participated in analyzing the data and writing the paper.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

C h a p t e r I

THE CONSTRUCTION OF SLEEP

On the origin of sleep

The last common ancestor of jellyfish and humans may have had a single or many behaviors. Certainly those behaviors present in the last common ancestor and present amongst its evolutionary derivatives represent conserved and important behaviors for animal survival. One such conserved and important behavior is sleep [1-5].

To test if an animal sleeps it must display the three hallmarks of sleep [2]. First, the animal must exhibit rapidly reversible behavioral quiescence. The rapid reversibility differentiates the behavioral quiescence from an anesthetized or comatose state, as well as death. Second, the animal must exhibit a period of reduced responsiveness to stimuli which differentiates sleep from a period of restful wakefulness. Third, the sleep state must be under homeostatic regulation, meaning that if an animal is deprived of sleep there is a subsequent increased need for sleep (sleep drive). This behavioral definition attempts to capture the essence of sleep, and has been used to demonstrate novel and conserved sleep circuits and regulators across the animal kingdom. With this definition as a guide, sleep has been observed in all animals closely investigated [2, 6-10].

The fundamental and conserved role for sleep in animals may be discovered by investigation of sleep in early-branching metazoan lineages [11, 12]. One early-branching metazoan lineage is the phylum Cnidaria, comprising coral, hydra, and scyphozoa (the true jellyfish) [13]. Cnidarians are among the first animals to develop tissue level organization, including neurons [13-18]. Though the exact origin of neurons remains controversial [19], one can assume that the neurons of cnidarians are an early and distinct lineage of our ancestral nervous system [13-18]. Behavioral quiescence was characterized in coral and other types of jellyfish. A systematic investigation of jellyfish behavior revealed that these animals sleep [6].

The discovery of sleep in a cnidarian, such as in the upside-down jellyfish *Cassiopea* [6], pushes the known root of sleep back within the animal lineage, and these data suggest that sleep is required within even those animals with the most rudimentary of nervous systems. Superficially, it seems that humans have very little in common with jellyfish. The discovery of sleep in such a primitive animal underscores the importance of sleep in the animal kingdom. These results suggest that the presence of neurons themselves demands a fundamental requirement for sleep. Intriguingly, the work of the first neuron may have simply been to regulate behavior as a binary on / off switch.

The neuronal regulation of sleep

Sleep like other behaviors is driven by neurons [20]. Specifically, neuromodulators are generated within, released from, and act on neurons. The main neuronal regulators of sleep; i.e., "sleep-active neurons," have interesting conserved properties across diverse phyla. These neurons are usually peptidergic [20] and release neuropeptides that induce long-lasting modulation of the nervous system by changing the systems functional state. The neuropeptides fundamentally alter the state of target neurons to generate vastly different behavioral states, including sleep [21]. In fact, the system of peptidergic modulation of behavioral states may be an ancient strategy used by animals to respond to their environment. Peptidergic cells that control behavior may have set the stage for the emergence of *bona fide* neurosecretory cells that regulate behavior.

One hypothesis as to the origin of neurons within the animal lineage is the so called proto-neuron [22]. This cell-type looked and acted somewhat like a neuron. The cells integrated sensory input and sent peptidgeric signals to control animal output (e.g., state changes and behavior) [22, 23]. For more information on this hypothesis see work by Detlev and colleagues [15, 22-25]. The best window into the proto-neuron hypothesis is *Trichoplax*, a Placazoan animal that lacks neurons altogether ([26]; **Figure 1.1**). *Trichoplax* are thought to have lost neurons through secondary simplification but have retained their two cell-layers and the neuron-like crystal and gland cells. Recent studies have demonstrated that the gland cells are peptidergic, sense the environment, integrate information, and modulate

behavior [27]. The existence of an animal lacking neurons, which uses neuropeptides to alter its behavior, suggests that neuropeptides have a very important and fundamental role in animal survival. Perhaps the function of neuropeptides is more important to ancient animal survival than neurons themselves. In any case, these gland cells may represent one form of the proto-neuron that over evolutionary time began to specialize and adopt specific sets of genes that encode synaptic machinery, neurotransmitters, and voltage-gated ion channels.

The genetic regulation of sleep

Sleep is a genetically-encoded behavioral state requiring shut down or rate reductions of multiple behaviors [28-31]. Neurosecretory cells are responsible for the coordinate shut down of behavior. The messengers of these cells are specific gene cassettes which include neurotransmitters and neuropeptides. Neuropeptides are key regulators of the sleep state [21] and are thought to act broadly to change the physiology of entire brain circuits [32]. This type of neuromodulation increases the functional potential of hard-wired neural circuits and generates different brain states [33-38]. Here we look at how neuropeptides construct sleep in *C. elegans* by shutting down specific behaviors and the impact of evolutionary forces on neuropeptide signaling.

It is known that multiple neuropeptides act together to regulate sleep [21], but the pathway by which these neuropeptides work together is unknown. Because of its genetic tractability [39], mapped nervous system [40], and easily measured sleep state [5], *C. elegans* is a suitable model system to determine how multiple neuropeptides act together to regulate sleep. One of the neurons of *C. elegans'* 302 neurons is the master regulator of stress-induced sleep [41, 42]. This neurosecretory cell is called ALA and regulates sleep through secretion of three specific neuropeptides [43, 44]. Removing all of these neuropeptides results in an insomniac worm, suggesting that these neuropeptides work together to regulate sleep [43]. Each neuropeptide is sufficient to shut down a specific set of sleep-associated behaviors. One inhibits eating, another locomotion, and another defecation [43]. One interesting observed phenotype was an animal that did not move but continued to eat

and defecate during neuropeptide overexpression [43]. Individual neuropeptides inhibit either specific cells within the nervous system or act broadly throughout the worm to regulate behavior.

These data indicated that each neuropeptide has a specific role in regulating sleep by controlling specific behaviors. It is possible that these neuropeptides regulate specific behaviors outside of the sleep state as well. For example, it is likely that one of these neuropeptides regulates the speed of locomotion during *C. elegans'* exploratory behavior by acting on command interneurons that regulate locomotion. If this is true, the ALA neuron simply turns on the synthesis of this neuropeptide; this will slow down the speed of locomotion, or if over expressed enough, stop locomotion entirely. In this way, the ALA neuron turns on regulatory modules that shut down specific behaviors to construct sleep.

The method by which individual neuropeptides shut down specific behaviors to control the sleep state in *C. elegans* [43] could be conserved in other animals. The system relies on multiple lock and keys, where the neuropeptides are the key and their receptors the G-protein coupled receptors (GPCRs) are the lock. Evolutionary homology-based arguments are more easily made for GPCRs because they are transmembrane proteins with long sequences, while the neuropeptides are short proteins encoded within a large prepropeptide [45, 46]. These genes are thought to evolve quickly precluding a homology based evolutionary argument [45, 46]. However, the C terminal sequence of neuropeptides have been shown to be the important residues for receptor binding. The C terminal binding moiety is used to classify neuropeptides and we use this classification system to explore their functional conservation.

Neuropeptides have also been shown to have significant functional conservation in sleep regulation across phyla [21]. Based on their C terminal homology the sleep regulating neuropeptides characterized in *C. elegans* belong to the –RFamide and tachykinin neuropeptide families. Neuropeptides found within the –RFamide family are a well-studied example of cross phylum regulators of sleep. –RFamides have been shown to regulate *C. elegans* [43, 44, 47], *D. melanogaster* [48], and even (vertebrate) Zebrafish sleep [49]. The

implication of these peptide homologs in mammalian sleep has not yet been characterized, though it would be very interesting to test. We recently determined that tachykinin neuropeptides are also sufficient to induce sleep in zebrafish (Nath and Hill *et al.* 2018, unpublished results). Interestingly, there is evidence that ectopic tachykinin treatment to the ventrolateral preoptic nucleus induces REM sleep in mice [50]. The C terminal domain of neuropeptides may represent key binding moieties for the regulation of behavior. These moieties have four times the specificity of traditional neurotransmitters, which are roughly the size of one amino acid. Further, these neuropeptides have added regulatory capacity because they are encoded by prepropeptide which must be processed. The two families of neuropeptides we characterized in *C. elegans* sleep regulation [43] also regulate sleep in other animal phyla from flies, fish, and mice [48-50].

Hypocretin signaling represents a key neuropeptide signaling system among vertebrates, and has been demonstrated to regulate sleep in dogs, mice, humans, zebrafish, and the blind cavefish [7]. Studies of hypocretin signaling in the Teleost (fish) phylum have provided mechanistic examples of how neuropeptide signaling can be modified within a single phylum to regulate sleep. For example, blind cavefish have lost vision together with their need for prolonged sleep [51]. Amazingly, multiple blind cavefish populations have converged on a sleep state that requires them to sleep much less than their non-blind surface dwelling populations [51]. Jaggard *et al*. recently demonstrated that this reduction in sleep is a result of an increase in the wake promoting hypocretin system by an increase in the number of hypocretin neurons and a general increase in hypocretin transcription [52]. Future studies may illuminate precisely how an increase in hypocretin signaling modifies (if at all) other aspects of the blind cavefish's sleep. This is a clear example of how modification of a neuropeptide system through evolution can result in a unique manifestation of sleep.

The evolution of sleep

A single behavioral class, such as sleep, exists in a multitude of forms [2, 6-10]. The consistent aspects of the behavior highlight core and essential functionalities of the

behavior, while the changing aspects of this behavior across its different manifestations reflects the capabilities and possibilities of the behavior. We present a hypothesis whereby protoneurons had a switch-like mechanism to regulate behavior by the release of neuropeptides from glandular peptidergic cell-types. This strategy for behavioral regulation was successful and evolved over time to the development of *bona fide* neurons, and ultimately entire brain regions composed of many neurons that release neuropeptides. This hypothesis provides a general mechanism by which behaviors could evolve.

Genetic, biochemical, and neuronal interrogation of the behavioral blocks of sleep in multi-phyla studies will reveal the essence of sleep. These core blocks must provide a selective advantage, and thus will continue to exists in the animal lineage. As we study sleep in model systems, we are limited to those sleep strategies that survived, though it is thought-provoking to speculate about those sleep states that may have provided no advantage. Perhaps there was an animal that never woke up.

Evolutionary studies of neuropeptide signaling will further illuminate the mechanism by which sleep evolved. The important motif of the neuropeptide is the C terminus, which varies between two to five amino acids. We hypothesize that neuropeptides regulate specific behaviors to construct the sleep state. The details of molecular conservation will test our hypothesis that the underlying architecture of sleep regulation is conserved [43].

The genetic conservation of many sleep regulators from worm to man suggest that sleep is rooted in a common ancestor [1-3]. The data suggest that nature has employed the same tools to accomplish the same task. If the rooted argument of sleep evolution is taken to an extreme, then one would posit that sleep is absolutely conserved and resistant to evolution. This is unlikely as there are many manifestations of sleep observed in nature. At the same time, an extreme convergent argument posits that all of these distinct sleep states could have evolved separately and all arrived at similar genetic and neuronal answers. This too seems unlikely. We think that the actual answer is biased towards a rooted theory of sleep evolution, and a multi-phylum approach to experimentation is needed to untangle the evolutionary web of behavior, genes, and neural circuits that construct sleep.

Sleep has been observed in any animal closely investigated for this behavior [1-3, 6-10] implying that sleep is an ancestral behavioral state and that the sleep behavior must provide something essential to animal physiology and survival. While the original manifestation of sleep may have been for something as trivial as energy conservation [3], the sleep that humans experience may have been decorated with higher-order functions such as memory consolidation. Interrogating the neuronal and genetic structures of sleep across phyla will shed light on the function of sleep as we learn why animals started sleeping, and how this sleep state has evolved with time.

Figure

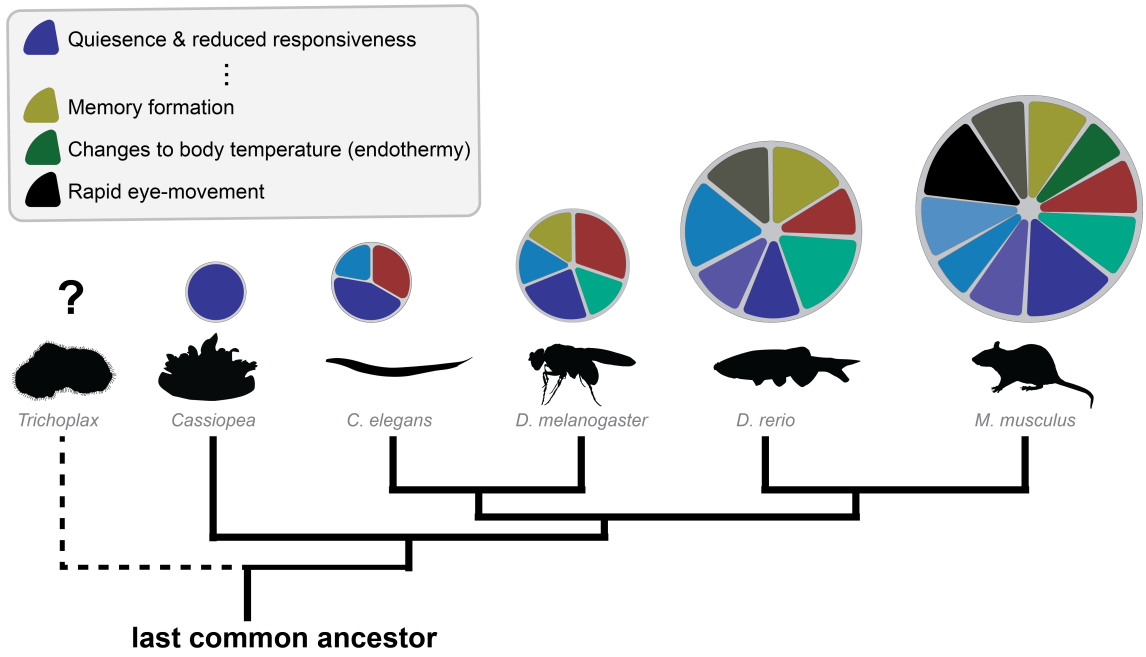
Quiesence & reduced responsiveness
Memory formation
Changes to body temperature (endothermy)
Rapid eye-movement
?
Trichoplax
Cassiopea
C. elegans
D. melanogaster
D. rerio
M. musculus
last common ancestor

Figure 1.1: The construction of sleep. Sleep has been observed in any animal closely investigated from the phyla cnidaria (*Cassiopea*) to chordata (*M. musculus* and humans) [1-6]. Each of these manifestations of sleep represent a distinct sleep state that is rooted at the branch point between radially and bilaterally symmetric animals. The colored wedges within each circle (referred to as blocks in the text) represent distinct aspects of the sleep state. Some blocks appear (e.g., memory formation) or change between the different manifestations of sleep, while other important aspects of sleep remain unchanged (e.g., quiescence). As illustrated by the increasing complexity of the "behavior circle" (e.g., more colored blocks), we hypothesize that sleep has grown increasingly complex from a behavioral perspective over evolutionary time. The dashed line indicates that sleep has not been tested in *Trichoplax*, an animal that does not have neurons [26].

Chapter II

THE JELLYFISH *CASSIOPEA* EXHIBITS A SLEEP-LIKE STATE

Nath RD, Bedbrook CN, Abrams MJ, Basinger T, Bois JS, Prober DA, Sternberg PW, Gradinaru V, Goentoro L. The Jellyfish Cassiopea Exhibits a Sleep-like State. Curr Biol. 2017 Oct 9;27(19):2984-2990.e3. doi: 10.1016/j.cub.2017.08.014.

Introduction

Do all animals sleep? Sleep has been observed in many vertebrates, and there is a growing body of evidence for sleep-like states in arthropods and nematodes [5, 8-10, 41]. Here we show that sleep is also present in Cnidaria [1, 3, 55], an earlier branching metazoan lineage. Cnidaria, along with Ctenophora, are the first metazoan phyla to evolve tissue-level organization and differentiated cell types, such as neurons and muscle [11, 13-15, 17, 18, 45]. In Cnidaria, neurons are organized into a non-centralized radially symmetric nerve net [13, 17, 18, 56, 57] that nevertheless shares fundamental properties with the vertebrate nervous system: action potentials, synaptic transmission, neuropeptides, and neurotransmitters [13, 16, 56-59]. It was reported that cnidarian soft corals {Kremien:2013ir} and box jellyfish [60, 61] exhibit periods of quiescence, a pre-requisite for sleep-like states, prompting us to ask if sleep is present in Cnidaria. Within Cnidaria, the upside-down jellyfish *Cassiopea spp.* displays a quantifiable pulsing behavior, allowing us to perform long-term behavioral tracking. Monitoring *Cassiopea* pulsing activity for consecutive days and nights revealed behavioral quiescence at night that is rapidly reversible, and a delayed response to stimulation in the quiescent state. When deprived of nighttime quiescence, *Cassiopea* exhibited decreased activity and reduced responsiveness to a sensory stimulus during the subsequent day, consistent with homeostatic regulation of the quiescent state. Together these results indicate that *Cassiopea* has a sleep-like state, supporting the hypothesis that sleep arose early in the metazoan lineage, prior to the emergence of a centralized nervous system.

Three behavioral characteristics define a sleep state [1-3]: (1) behavioral quiescence, a period of decreased activity; (2) reduced responsiveness to stimuli during the quiescent state; and (3) homeostatic regulation of the quiescent state. Both behavioral quiescence and reduced responsiveness must be rapidly reversible to differentiate sleep-like states from other immobile states (e.g., paralysis or coma) and reduced responsiveness distinguishes sleep from quiet wakefulness. Homeostatic regulation results in a rebound response, i.e., a compensatory period of increased sleep following sleep deprivation. Here we asked whether the cnidarian jellyfish *Cassiopea* exhibits these behavioral characteristics.

Results

Cassiopea are found throughout the tropics in shallow ocean waters and mudflats (**Figure 2.1**; [62, 63]). They rarely swim and rather remain stationary with their bell on a surface, hence their name, the upside-down jellyfish (**Figure 2.1B**; **Figure 2.5A**; [62, 63]). *Cassiopea*, like coral and sea anemones, have a photosynthetic obligate endosymbiote, *Symbiodinium* (**Figure 2.1C**). *Cassiopea* continuously pulse by relaxing and contracting their bell at a rate of about 1 pulse/second (**Figure 2.1D**). This pulsing behavior generates fluid currents that facilitate vital processes such as filter feeding, circulation of metabolites, expulsion of byproducts, and gamete dispersion [63, 64]. The pulsing behavior is controlled by light and gravity sensing organs called rhopalia (**Figure 2.1C**; [18]). This stationary pulsing behavior makes *Cassiopea* a suitable jellyfish for behavioral tracking.

To track behavior in *Cassiopea*, we designed an imaging system (**Figure 2.5C-F**) for counting pulses of individual jellyfish over successive cycles of day and night, defined as a 12-hour period when the light is on or off, respectively. As *Cassiopea* pulse, the relaxation and contraction of the bell causes a corresponding change in average pixel intensity, which was measured for each frame of the recording, producing a pulse-trace (**Figure 2.1D**). Pulse events were counted using the peak of the pulse-trace, and the inter-pulse interval (IPI) was calculated as the time between the peaks (**Figure 2.1D**; **Figure 2.6**).

We observed that *Cassiopea* pulse less at night than during the day (**Figure 2.2**). To quantify this difference in pulsing frequency, we tracked the pulsing behavior of 23 jellyfish over 6 consecutive days and nights (**Figure 2.2C**). We define *activity* as the total number of pulses in the first 20 minutes of each hour. While individual jellyfish showed different basal activity levels (**Figure 2.2C**), all showed a large decrease in mean activity (~32%) at night (781 ± 199 pulses/20 min, mean ± s.d.) compared to the day (1155 ± 315 pulses/20 min, mean ± s.d.; **Figure 2.2C,E**). To determine if fast and slow pulsing jellyfish change their activity to a similar degree, we normalized activity of individual jellyfish by their mean day activity. Despite variations in basal activity, the relative change from day to night was similar between jellyfish (**Figure 2.2D**). Jellyfish activity decreased throughout the first 3-6 hours of the night, with the lowest activity occurring 6-12 hours after the day to night transition. Pulsing activity peaked upon feeding, occurring on the 4^{th} hour of each day (**Figure 2.2C,D**). To ensure that day feeding does not cause the day-night behavioral difference, we tracked the activity of 16 jellyfish over three consecutive days and nights without feeding and observed results consistent with those including feeding (**Figure 2.2F,G**; **Figure 2.7D**). These results demonstrate that *Cassiopea* have a quiescent state during the night. To test the reversibility of this nighttime quiescent state we introduced a food stimulus at night, which transiently increased activity to daytime levels (**Figure 2.7E**). The nighttime quiescent state in *Cassiopea* is thus rapidly reversible, consistent with a sleep-like behavior.

To better understand the nighttime quiescence, we compared day and night pulse-traces of individual jellyfish. The day and night pulse-traces of one representative jellyfish are shown in **Figure 2.2A**. During the night, the IPI is typically longer than during the day (**Figure 2.2A,B**; **Figure 2.7A**). Two features contribute to this lengthening of the IPI: (1) the mode of the IPI distribution is longer at night than during the day, and (2) night pulsing is more often interrupted by pauses of variable length. These pauses are seen as a tail in the IPI frequency distribution (**Figure 2.2B**: 95^{th} percentile of night IPI frequency distribution (gray) is 13.9 s). Such long pauses are rarely seen during the day (**Figure 2.2B**: 95^{th} percentile of day IPI frequency distribution (yellow) is 2.5 s). This pause behavior may be

analogous to long rest bouts observed in *Drosophila* and zebrafish, which are suggested to be periods of deep quiescence with reduced responsiveness to stimuli [8] [65].

To test whether *Cassiopea* exhibit reduced responsiveness to stimuli during their nighttime-quiescent state, we designed an experiment to deliver a consistent arousing stimulus to the jellyfish. We observed in our nursery that *Cassiopea* prefer staying on solid surfaces as is found in nature. If *Cassiopea* are released into the water column, they quickly reorient and move to the bottom of the tank. We used placement into the water column as a stimulus to compare responsiveness during the night versus the day. *Cassiopea* were put inside a short PVC pipe with a screen bottom (**Figure 2.3A**). This was lifted to a fixed height, held for 5 min to allow the jellyfish to acclimate, and then rapidly lowered, which placed the jellyfish free-floating into the water column. We then scored the time it took for the jellyfish to first pulse and the time to reach the screen bottom (**Figure 2.3A**; **Methods**). At night, the jellyfish showed an increase in the time to first pulse and the time to reach bottom, compared to day (time to first pulse day: 2.1 ± 0.9 s versus night: 5.9 ± 4.0 s, and the time to reach bottom day: 8.6 ± 2.9 s versus night: 12.0 ± 3.2 s, mean ± s.d.; n = 23 animals) (**Figure 2.3B,C**). This increased latency in response to stimulus indicates that *Cassiopea* have reduced responsiveness to stimulus during the night.

To determine if the increased latency at night is rapidly reversible, a second drop was initiated within 30 s of the first drop, that is, after the jellyfish have been aroused. Reversibility was tested during both the day and night for 23 jellyfish. During the night, there is a large decrease in the time to first pulse and time to reach the bottom, after the second drop when compared to the first drop (**Figure 2.3D,E**). During the day and night, the time to first pulse and time to bottom after the second drop were indistinguishable, demonstrating that after perturbation, animals have similar arousal levels during the day and night. These results indicate that *Cassiopea* have rapidly reversible reduced responsiveness to a stimulus during the night.

To test whether *Cassiopea* nighttime quiescence is homeostatically regulated, we deprived jellyfish of behavioral quiescence for either 6 or 12 hours using a mechanical stimulus

(**Figure 2.4**). The stimulus consisted of a brief (10 s) pulse of water every 20 min, which caused a transient increase in pulsing activity. This increase in pulsing activity lasts for approximately 5 min after the 10 s pulse of water. Thus, the perturbation disrupts quiescence for approximately 25% of the perturbation period (either 6 hours or 12 hours). When the perturbation was performed during the last 6 hours of the night (**Figure 2.4A**), we observed a significant decrease in activity (~12%) during the first 4 hours of the following day relative to the pre-perturbation day (mean of first 4 hours of pre-perturbation day: 1146 ± 232 pulses/20 min compared to post-perturbation day: 1008 ± 210 pulses/20 min, mean ± s.d.; n = 30 animals; **Figure 2.4C**). This period of decreased activity is due to both decreased pulsing frequency (increased mode of IPI-length) and increased pause length (increase in the IPI-length 95th percentile) (**Figure 2.8B,C**). This result is consistent with an increased sleep-drive after sleep deprivation. After a single day of decreased activity, the jellyfish return to baseline levels of day and night activity. Similar results were observed after an entire night of perturbation (12 hours; **Figure 2.4D**), with a large decrease in activity (~17%) throughout the following day (mean of 12 hours of pre-perturbation day: 1361 ± 254 pulses/20 min compared to post-perturbation day: 1132 ± 263 pulses/20 min, mean ± s.d.; n = 16 animals; **Figure 2.4F**). The decrease in activity caused by the 12-hour perturbation was larger than that of the 6-hour perturbation, indicating that the amount of sleep rebound is dependent on the level of sleep deprivation. During periods of decreased activity after either the 6-hour or 12-hour perturbation, we also observed increased response latency to a sensory stimulus (**Figure 2.8A**), indicating a sleep-like state.

If the reduced activity following nighttime perturbation is due to sleep deprivation rather than muscle fatigue, applying the perturbation during the day, when *Cassiopea* are much less quiescent, should not result in reduced activity. To distinguish between sleep deprivation and muscle fatigue, we performed the 6- or 12-hour mechanical stimulus experiments during the day (**Figure 2.4B,E**). We observed no significant difference between pre- and post- perturbation activity levels (**Figure 2.4C,F**), indicating that the rebound response is specific to deprivation of nighttime quiescence. Taken together, these

results demonstrate that *Cassiopea* have a nighttime-quiescent state that is homeostatically controlled.

In many animals sleep is regulated by both homeostatic and circadian systems [66], but this is not always the case [1, 3, 5, 41, 67]. For instance, the nematode *C. elegans* exhibits a developmentally regulated sleep state, and adult *C. elegans* show a non-circadian stress-induced-sleep state [5, 41, 43]. A fully functioning circadian system is also not essential for sleep to occur; animals with null mutations of circadian rhythm genes still sleep, though sleep timing is altered [67]. To test if nighttime quiescence in *Cassiopea* is regulated by a circadian rhythm, we first entrained the jellyfish for one week in a normal 12:12-hour light/dark cycle, and then shifted them to constant lighting conditions for 36 hours. We tested low- (~0.5 Photosynthetic Photon Flux [PPF]), mid- (~100 PPF), and full-intensity (~200 PPF) light, as well as dark (**Figure 2.8D,E**). If jellyfish activity is regulated by a circadian rhythm, cycling activity should persist in the absence of entraining stimuli, such as light. We observed no circadian oscillation of jellyfish activity under any of the constant light conditions (**Figure 2.8D**). However, we do observe circadian oscillation of activity in constant dark conditions (**Figure 2.8E**). This result suggests that the quiescent state may be under circadian regulation.

Cassiopea display the key behavioral characteristics of a sleep-like state: a reversible quiescent state with reduced responsiveness to stimuli and both homeostatic and possibly circadian regulation. To our knowledge, our finding is the first example of a sleep-like state in an organism with a diffuse nerve net [3, 55], suggesting that this behavioral state arose prior to the evolution of a centralized nervous system. Though at least 600 million years of evolution separate cnidarians from bilaterians [11-15, 17, 18, 56], many aspects of the nervous system are conserved, including neuropeptides and neurotransmitters [13, 16, 56-59]. One such conserved molecule, melatonin [68], promotes sleep in diurnal vertebrates, including zebrafish [69] and humans [70], and induces quiescence in invertebrates [71]. We observed that melatonin induces a reversible decrease in activity in *Cassiopea* during the day in a concentration-dependent manner (**Figure 2.8F-H**), suggesting that melatonin

has a conserved quiescence-inducing effect in *Cassiopea*. Pyrilamine, a histamine H1 receptor antagonist that induces sleep in vertebrates [72], also induces concentration-dependent quiescence in *Cassiopea* (**Figure 2.8F**). These results suggest that at least some mechanisms involved in vertebrate sleep may be conserved in *Cassiopea*.

Discussion

Although future studies are required to test whether other cnidarians sleep, field studies showing behavioral quiescence, diel vertical migration, and swimming speeds that vary with diel period [60, 61] suggest that a sleep-like state may not be specific to *Cassiopea*. A cnidarian sleep-like state could result from either divergent or convergent evolution. The observation of behaviorally and mechanistically conserved sleep-like states across the animal kingdom [1, 3] strongly supports the possibility for an early rooted sleep state rather than many instances of convergent evolution. It has been hypothesized that sleep has multiple functions, including synaptic homeostasis, regulation of neurotransmitters, repair of cellular damage, removal of toxins, memory consolidation and energy conservation [3], although the ancestral role and selective advantage of sleep remains elusive. Our discovery of a sleep-like state in an ancient metazoan phylum suggests that the ancestral role of sleep is rooted in basic requirements that are conserved across the animal kingdom. The ancestral function of sleep may be revealed by further study of early branching metazoa.

Figures

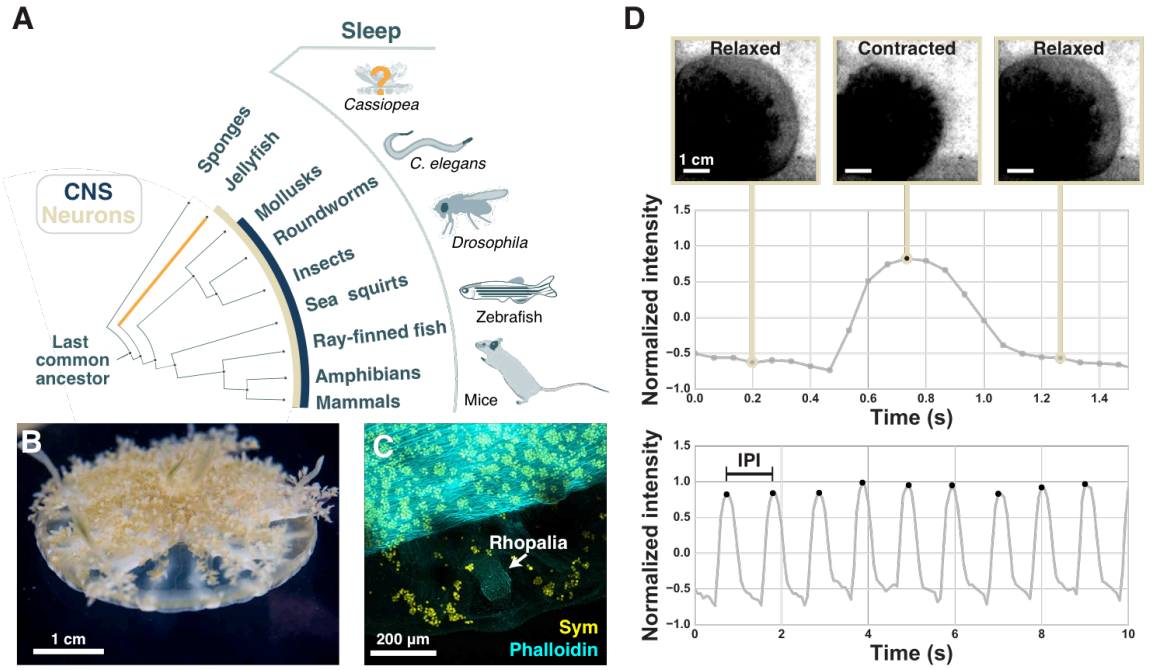

Figure 2.1: The pulsing behavior of the upside-down jellyfish, *Cassiopea spp.*, is trackable. (**A**) Phylogenetic tree schematic highlighting animals in which sleep behavior has been described, the presence of neurons (tan), and the emergence of a centralized nervous system (dark blue). See boxed key. (**B**) An image of *Cassiopea*. (**C**) Higher magnification view of *Cassiopea* with labeled actin-rich muscle (phalloidin stain; cyan), autofluorescent *Symbiodinium* (yellow), and a rhopalia, the sensory organ that controls pulsing, which is free of *Symbiodinium*. (**D**) As *Cassiopea* pulse the relaxation and contraction of the bell causes a corresponding change in average pixel intensity. Pulsing behavior was tracked by measuring this change in pixel intensity within the region of interest. (top) Representative frames and corresponding normalized pixel intensities for one pulse event. The local maxima in the pulse-trace was used to count pulse events. (bottom) A 10-second recording of one jellyfish shows multiple pulsing events. The inter-pulse interval (IPI) was calculated as the time between the maxima. See **Figure 2.5**, **Figure 2.6**.

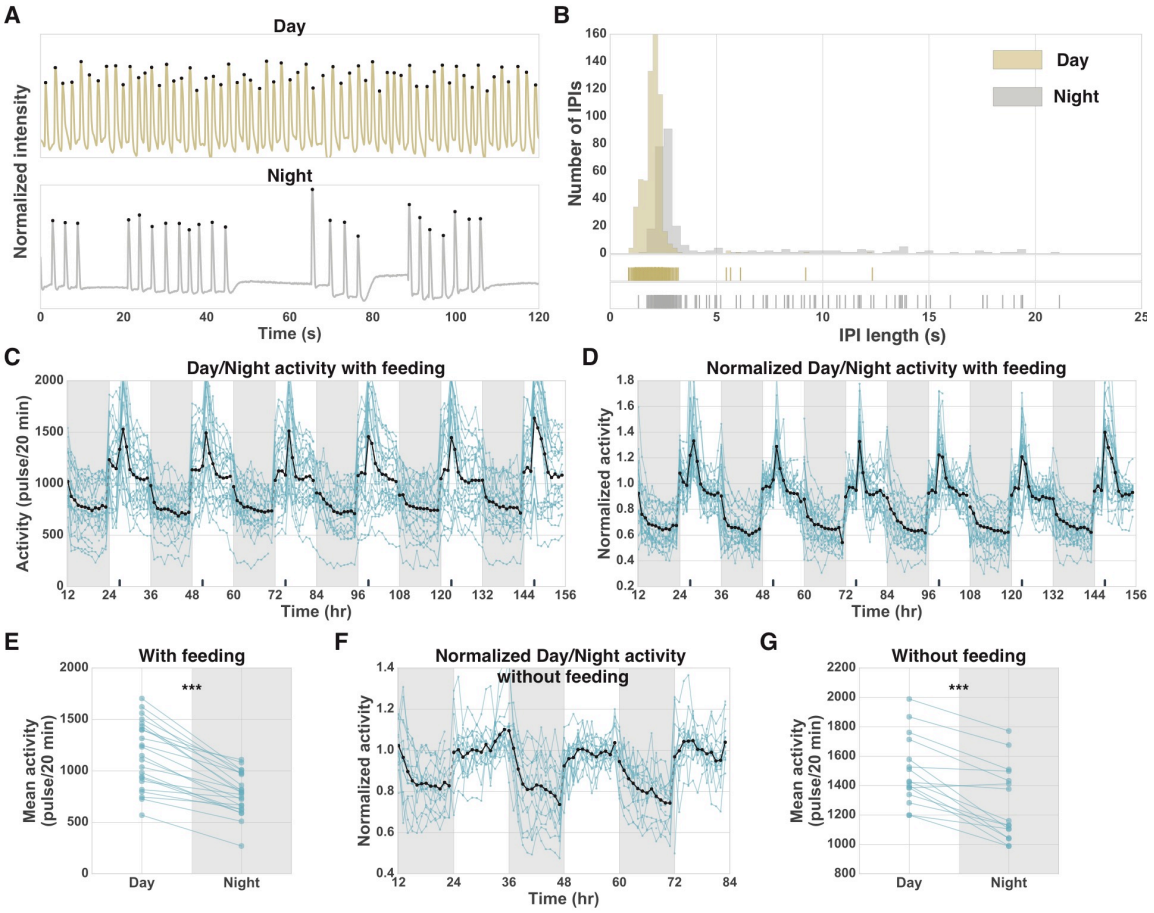

Figure 2.2: Continuous tracking of *Cassiopea* reveals pulsing quiescence at night. (**A**) Pulsing-traces for individual jellyfish during day and night over 120 s. (**B**) The distribution of IPI length for a 12-hour day and a 12-hour night for the same jellyfish shown in **A**. Tick marks below the distribution show each IPI length during the day and night. This highlights the long-pause events, which are more common at night (**Figure 2.7A**). (**C-G**) Each blue line corresponds to a single jellyfish. The black line indicates the mean activity of all jellyfish. Dark gray shading indicates night periods. Dark tick marks on the x-axis indicate time of feeding. (**C**) Baseline activity (pulses/20 min) of 23 jellyfish tracked for six days from four laboratory replicates. (**D**) Normalized baseline activity for jellyfish shown in **C**, where each jellyfish is normalized by their mean day activity. (**E**) Mean day activity versus mean night activity for each jellyfish over the six-day experiment shown in **C**. Two-sided paired *t*-test, day versus night, $P = 6\text{x}10^{-9}$. (**F**) Normalized baseline activity without feeding of 16 jellyfish tracked over three days from two laboratory replicates, where each jellyfish is normalized by its mean day activity. (**G**) Mean day

activity versus mean night activity for each jellyfish over the three-day experiment shown in **F**. Two-sided paired *t*-test, day versus night, $P = 10^{-5}$. ***$P < 10^{-3}$. See **Figure 2.7**.

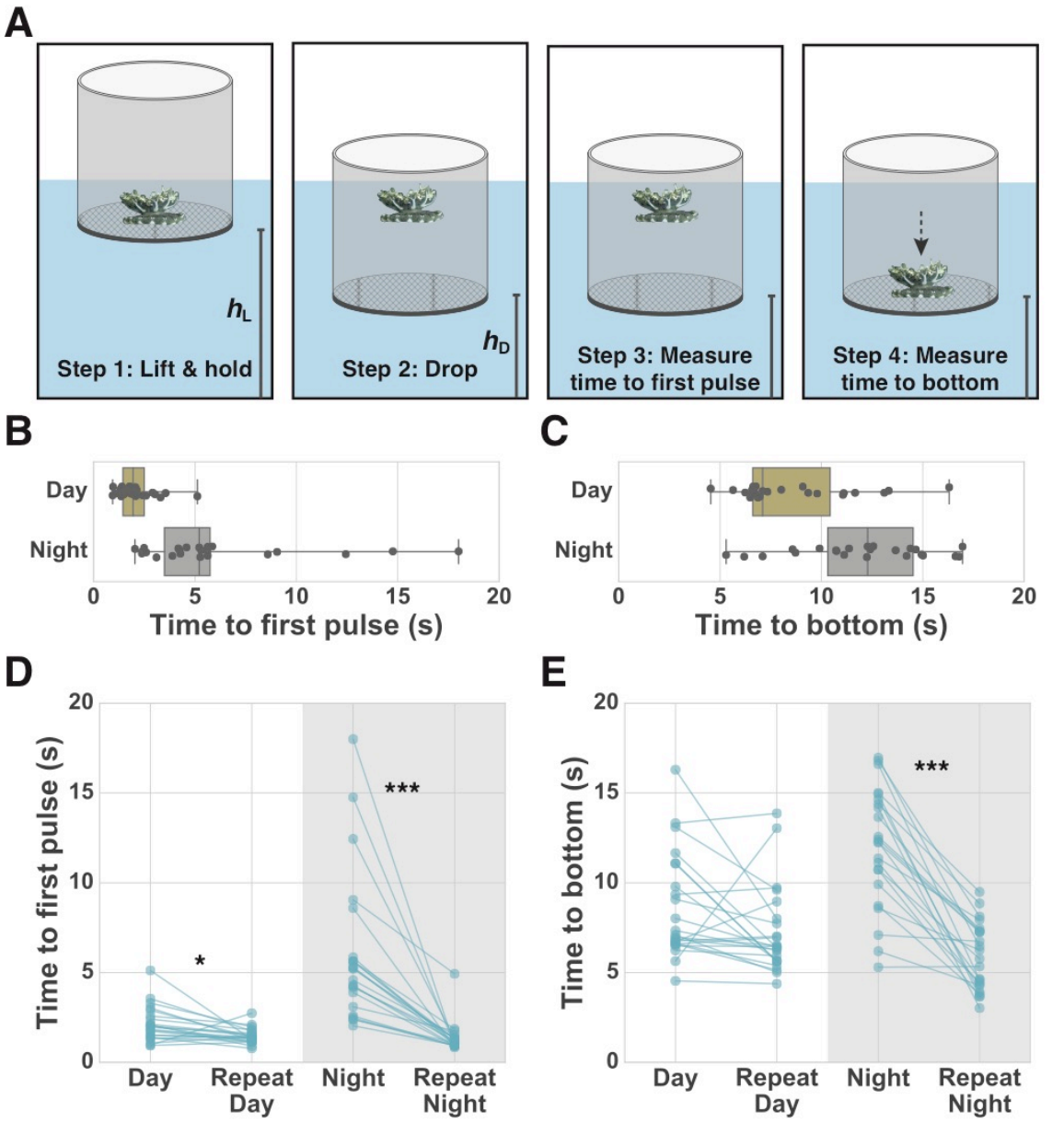

Figure 2.3: *Cassiopea* show reduced responsiveness to a sensory stimulus at night. (**A**) Schematic of experiment to test sensory responsiveness. Jellyfish were lifted and held at a fixed height (h_L) and then dropped to a fixed height (h_D). h_L and h_D were kept constant throughout experiments. Boxplots of time to first pulse after drop (**B**) for 23 jellyfish and time to reach bottom after drop (**C**) for 23 jellyfish during the day and night. Dots represent individual jellyfish collected from two laboratory replicates. Two-sided unpaired *t*-test, day versus night, (**B**) $P < 10^{-4}$ and (**C**) $P = 5\text{x}10^{-4}$. (**D**) Time to first pulse after initial drop and after perturbation for both day and night for 23 jellyfish. (**E**) Time to reach bottom after

initial drop and after perturbation for both day and night for 23 jellyfish. Two-way analysis of variance (ANOVA) for data shown in **D** and **E**, followed by post-hoc comparisons between experimental groups using B2onferroni posttest (*$P<5x10^{-2}$, ***$P<10^{-3}$). For the time to first pulse, two-sided unpaired *t*-test (**B**) and two-way ANOVA (**D**) were performed after log-transformation (**Methods**).

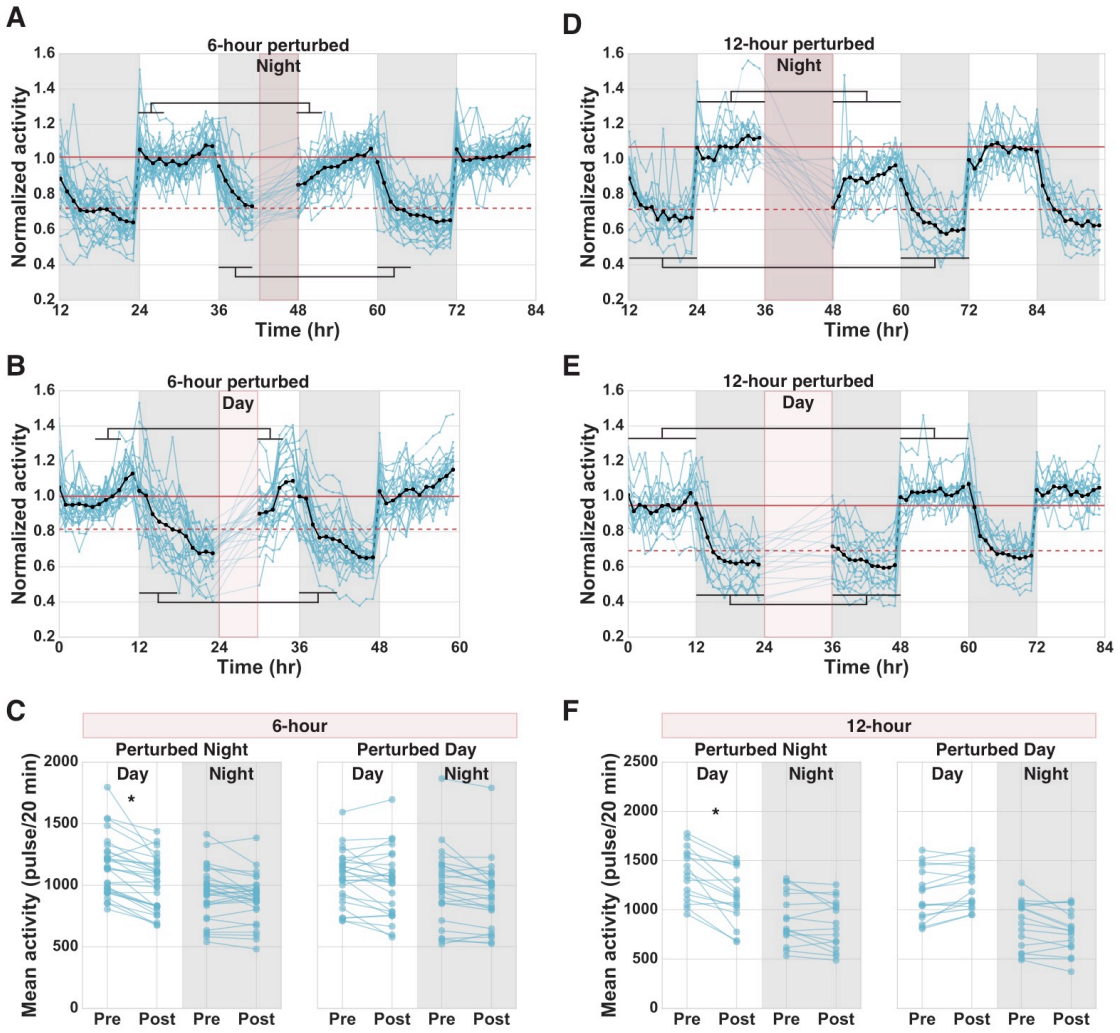

Figure 2.4: Homeostatic rebound in *Cassiopea*. Each blue line corresponds to a single jellyfish. The black line indicates the mean activity of all jellyfish. Dark gray shading indicates night periods. Maroon shading indicates perturbation periods with 10 s water pulses every 20 min. Jellyfish were exposed to different perturbation lengths (6 or 12 hours) at different times (day or night). The normalized activity of all jellyfish tracked over multiple days is plotted. Maroon horizontal lines show the mean activity of pre-perturbation day (solid) and pre-perturbation night (dashed). (**A**) Perturbation of 30 jellyfish for the last 6 hours of the night. (**B**) Perturbation of 26 jellyfish for the first 6 hours of the day. (**C**) Mean day and night activity pre- and post-perturbation for experiments shown in **A** and **B**. (**D**) Perturbation of 16 jellyfish for an entire 12-hour night. (**E**) Perturbation of 16 jellyfish for an entire 12-hour day. (**F**) Mean day and night activity pre-

and post-perturbation for experiments shown in **D** and **E**. Black-horizontal lines in **A**, **B**, **D**, and **E** indicate the windows of time used for calculating pre- and post-perturbation means shown in **C** and **F** for both the night (bottom lines) and day (top lines). For the 6-hour experiments we compared the first 4 hours of the post-perturbation day to the equivalent time pre-perturbation, and also compared the first 6 hours of post-perturbation night to the equivalent time pre-perturbation. For the 12-hour experiments we compared the full 12-hour days and nights pre- and post-perturbation. Two-way ANOVA followed by post-hoc comparisons between experimental groups using Bonferroni posttest ($*P<5x10^{-2}$). Both day and night 6-hour perturbation experiments include data from four laboratory replicates. Both day and night 12-hour perturbation experiments include data from two laboratory replicates. See **Figure 2.8**.

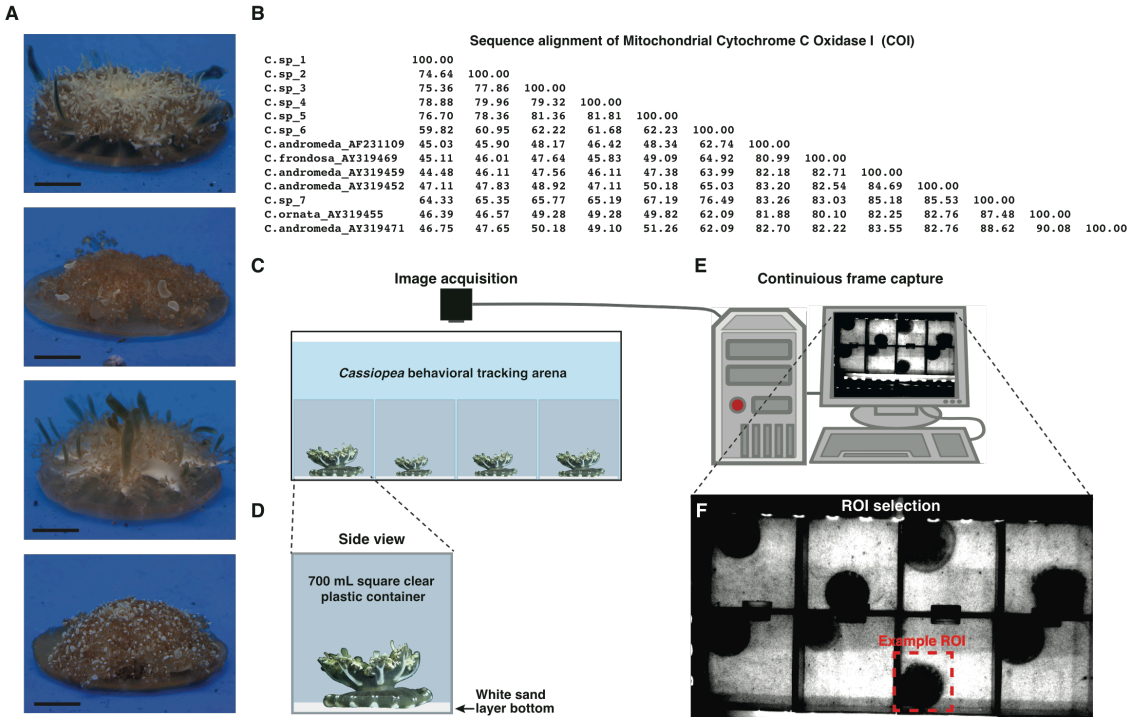

Figure 2.5: *Cassiopea spp.* diversity and behavioral tracking system. (**A**) Images of four *Cassiopea spp.* with different morphology (scale bar 1 cm). This is representative of the range of morphologies used in the experiments. (**B**) Percent amino acid identity matrix comparing mitochondrial cytochrome C oxidase I (COI) amino acid sequences of seven *Cassiopea spp*. used in this study (C.sp_1 – C. sp_7) with six previously described *Cassiopea spp.* (Taxon_GeneBank number). (**C**) For the behavioral tracking system jellyfish were placed in behavioral tracking arenas with cameras recording from above. (**D**) Each jellyfish was placed in a clear, plastic container with white sand layering the bottom. The white sand provides contrast, allowing better behavioral tracking. (**E**) Images were captured at a rate of 15 frames per second and saved directly onto solid-state hard drives. (**F**) A region of interest (ROI) around each jellyfish was selected for downstream processing.

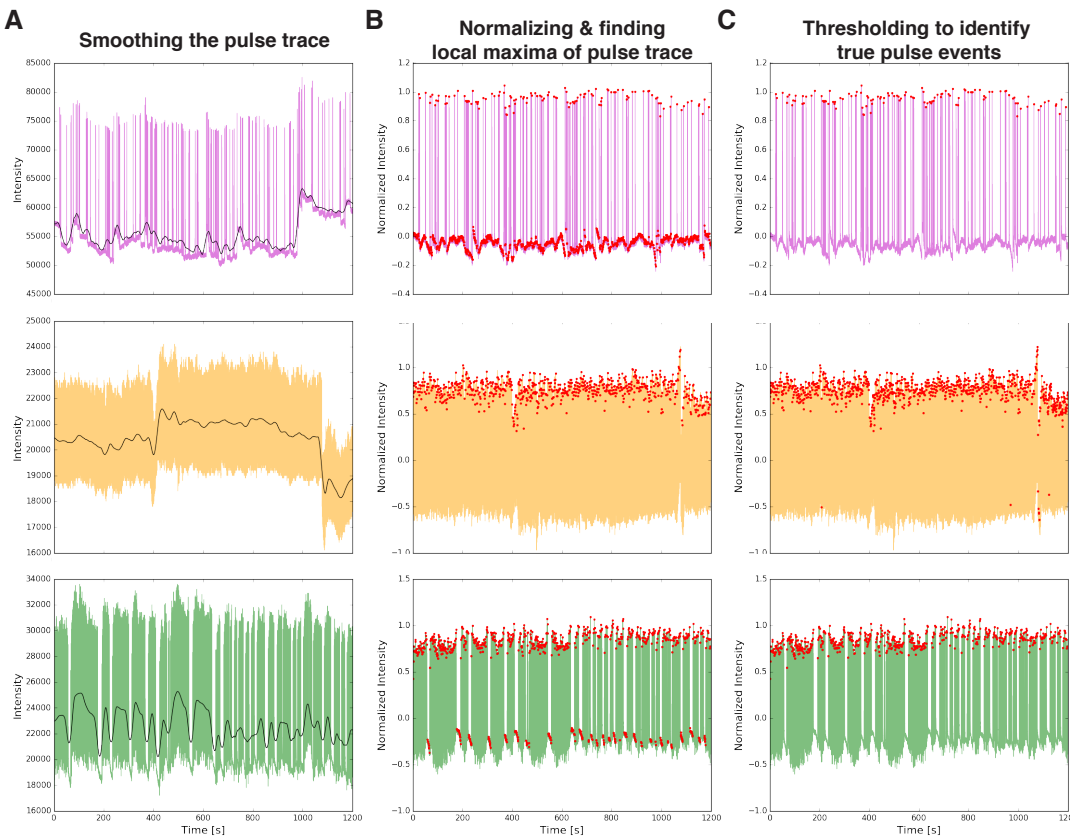

Figure 2.6: Processing the jellyfish pulse-trace data to count pulse events. Each color represents data from a different jellyfish (pink, orange, and green). (**A**) Smoothing the pulse-trace for normalization. Black line represents the smoothed trace for a 20 min recording. (**B**) Normalized pulsing traces for three different jellyfish with local maxima indicated by red dots. Many local maxima are detected within pauses in activity due to noise (small fluctuations in intensity), which are removed by thresholding. (**C**) Thresholding to identify local maxima at pulsing peaks. Pulsing peaks are indicated by red dots. For more details see the '*Cassiopea* behavioral tracking' section of the **Methods**.

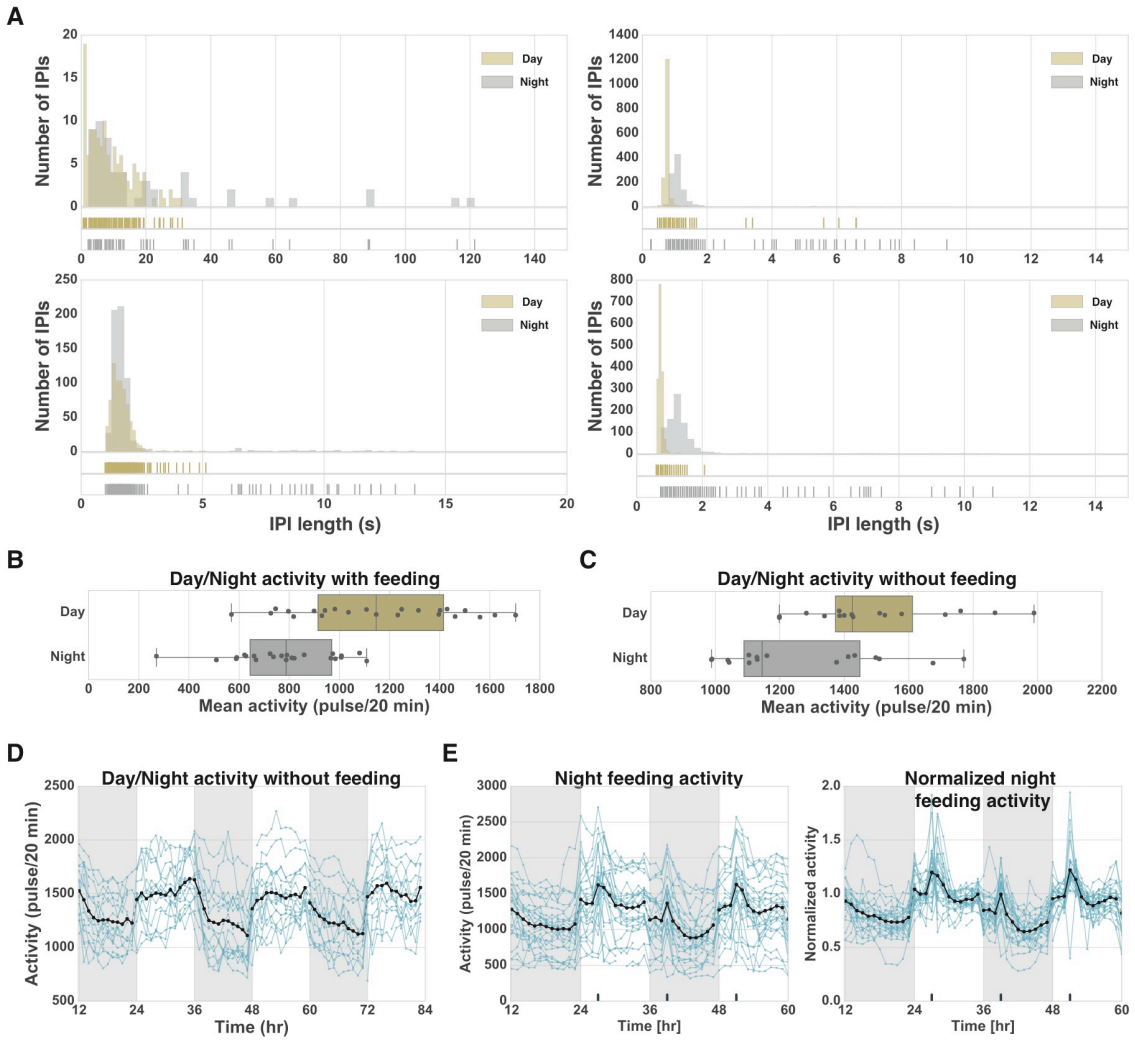

Figure 2.7: *Cassiopea* pulsing quiescence at night. (**A**) Distribution of IPI length for four *Cassiopea* during the day (yellow) and night (gray) showing each IPI event. Tick marks below the distributions show each IPI length during the day (yellow) and night (gray). The ticks highlight the long-pauses that are more common at night for all jellyfish. Box plot of *Cassiopea* day and night pulsing activity with feeding (**B**)**,** and without feeding (**C**). Each dot represents a single jellyfish, and mean activity is calculated over 6 (feeding, **B**) or 3 (without feeding, **C**) days and nights. For **D** and **E** each blue line corresponds to a single jellyfish. The black line indicates the mean activity of all jellyfish. Dark gray shading indicates night periods. (**D**) Day and night activity of *Cassiopea* without feeding. Baseline activity (pulses/20 min) without feeding of 16 jellyfish tracked over three days. (**E**) Feeding-induced arousal rapidly reverses the night quiescent state. Dark tick marks on x-

axis indicate time of feeding. Activity (pulses/20 min) and normalized activity of 30 jellyfish tracked over two day/nights from six laboratory replicates. Jellyfish were fed 4 hours into each day and 4 hours into the second night.

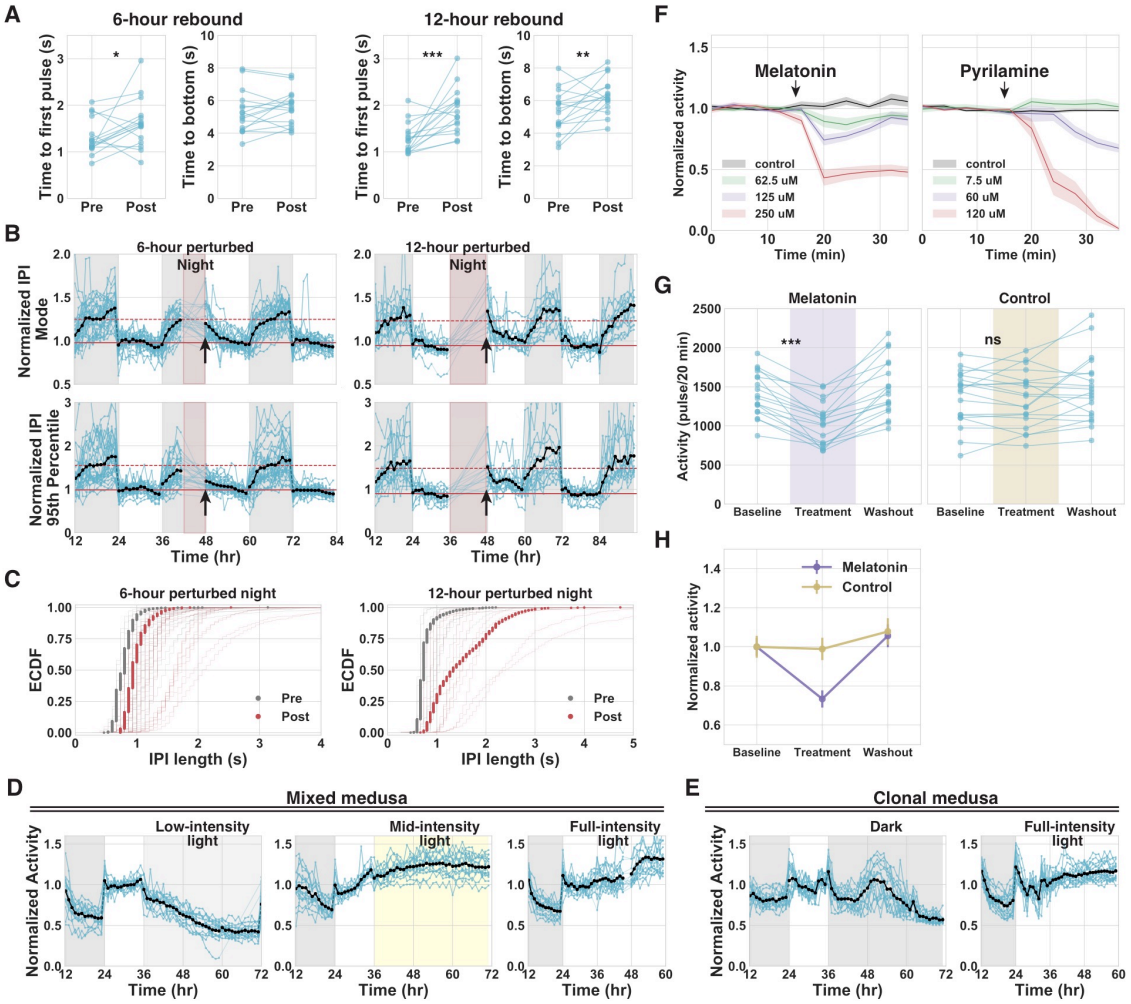

Figure 2.8: Regulation of quiescence in *Cassiopea*. Each blue line corresponds to a single jellyfish. The black line indicates the mean activity of all jellyfish. Dark gray shading indicates night periods. (**A**) Sensory responsiveness was tested during periods of decreased activity before (pre) and after (post) either the 6-hour or 12-hour perturbation periods (10 s water pulses every 20 min) using the assay described in **Figure 2.3**. Time to first pulse after drop and time to reach bottom after drop were measured during the day pre or post perturbation. After perturbation (post), an increased response latency was observed. Two-sided paired *t*-test, pre versus post, $*P<5x10^{-2}$, $**P<10^{-2}$, $***P<10^{-3}$. (**B**) Maroon horizontal lines show the mean activity of pre-perturbation day (solid) and pre-perturbation night (dashed). Maroon shading indicates perturbation periods with 10 s water pulses every 20 min. In these experiments jellyfish were exposed to different perturbation lengths (either

6 or 12 hours) during the night. Plotted here is the normalized mode and 95th percentile of the IPI length for all jellyfish tracked over multiple days. Perturbation of either 30 jellyfish for the last 6 hours of the night or 16 jellyfish for an entire 12-hour night. For both the 6-hour and 12-hour perturbation there is an increase in the mode and 95th percentile of the IPI length after perturbation (black arrowhead). (**C**) Empirical cumulative distribution function (ECDF) of daytime IPI length for all jellyfish pre (gray) and post (maroon) perturbation (thin lines, single jellyfish; dots, all jellyfish). Jellyfish exhibited increased IPI lengths after perturbation compared to before perturbation. These results suggest that the increased quiescence observed in **Figure 2.4** results from both a decreased frequency of pulsing and an increase in the length of pause events. (**D-E**) Monitoring activity with different light or dark conditions suggests that nighttime quiescence may be under circadian regulation. (**D**) Prolonged light exposure of *Cassiopea* shows no circadian cycling. 16 jellyfish were exposed to either 36-hours of continuous low-intensity light (light-gray shading) from hour 36 to hour 72, 36-hours of continuous mid-intensity light (yellow shading) from hour 36 to hour 72, or 36-hours of continuous full-intensity light from hour 24 to hour 60. Each experiment represents two laboratory replicates using a mixed population of *Cassiopea spp*. (**E**) Prolonged exposure to dark conditions of jellyfish shows circadian cycling when using a clonal population of medusa (*Cassiopea xamachana*), see **Methods**. 16 jellyfish were exposed to dark conditions from hour 36 to hour 72 or full-intensity light from hour 24 to hour 60. With this clonal population of jellyfish, circadian cycling of behavior is only observed for constant dark conditions and not constant full-intensity light conditions, consistent with results seen in the mixed population of *Cassiopea* shown in (**D**). (**F-H)** *Cassiopea* exhibit a decrease in activity in response to melatonin and pyrilamine exposure during the day. (**F**) Treatment with either pyrilamine or melatonin effects pulsing activity. The colored lines represent different concentrations of compounds tested. Activity was monitored before and after treatment. Time of treatment is indicated by a black arrow. Both melatonin and pyrilamine induce a concentration-dependent decrease in pulsing activity. (**G**) Activity of 18 *Cassiopea* exposed to 125 μM melatonin solubilized in ethanol compared to 19 *Cassiopea* treated with ethanol vehicle control from four laboratory replicates. *Cassiopea* were monitored for 20

min before (baseline), during (treatment), and after (washout) either melatonin or vehicle treatment. Two-sided paired *t*-test, before/during melatonin treatment: $P = 4x10^{-7}$, and before/during vehicle treatment: $P = 7x10^{-1}$. ***$P<10^{-3}$, ns not significant (ns) $P>5x10^{-2}$. (**H**) Comparison of the normalized mean activity between the melatonin and control treatment. Error-bars represent the standard error of the mean.

METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cassiopea spp. medusae used in this study were originally collected from the Florida Keys. For the majority of the experiments, a collection of multiple *Cassiopea* species were used (**Figure 2.5A,B**). For the experiments shown in **Figure 2.8A,E,F** a young (2-4 months old) clonal population of medusa were used (*Cassiopea xamachana*). This clonal polyp line was generated in Monica Medina's lab at Pennsylvania State University.

Cassiopea were reared in artificial seawater (ASW, Instant Ocean, 30-34 ppt) at pH 8.1-8.3, 26-28°C with a 12-hour day/night cycle. During the day, 450 and 250 W light sources were used to generate 200-300 PPF (Photosynthetic Photon Flux, a measurement of light power between 400 and 700 nm). To limit waste buildup, the *Cassiopea* aquarium was equipped with a refugium (*Chaetomorpha* algae aquaculture), a protein skimmer (Vertex Omega Skimmer), carbon dosing bio-pellets (Bulk Reef Supply), activated carbon in a media reactor (Bulk Reef Supply), and a UV sterilizer (Emperor Aquatics 25 W). Waste products were kept at or below the following levels: 0.1 ppm ammonia, 5 ppb phosphorus, 0 ppm nitrite, and 0 ppm nitrate.

Cassiopea were fed daily with brine shrimp (*Artremia nauplii*, Brine Shrimp Direct) enriched with *Nannochloropsis* algae (Reed Mariculture), and they were fed oyster roe once per week (Reed Mariculture). *Cassiopea* were group housed in a 60 gallon holding tank. Animals were randomly assigned to experimental groups. Medusae between 3-6 cm in diameter were used for experiments.

Cassiopea Genotyping

Cassiopea is a genus with many species that have not been classified. All of our experiments were performed with *Cassiopea spp.* of a range of sizes, ages, sex and morphologies (**Figure 2.5A,B**). To assess the diversity of *Cassiopea spp.* within our population we genotyped several animals by amplification and sequencing of the Mitochondrial cytochrome *c* oxidase I (COI). Genomic DNA extractions were performed as described [73]. Jellyfish fragments, about 2 mm of tissue from the tentacles, were placed

in 400 μL DNA extraction buffer (50% w/v guanidinium isothiocyanate; 50 mM Tris pH 7.6; 10 μM EDTA; 4.2% w/v sarkosyl; 2.1% v/v β-mercaptoethanol). Samples were incubated at 72°C for 10 min, centrifuged at 16,000 g for 5 min, and the resulting supernatant mixed with an equal volume of isopropanol and incubated at –20°C overnight. The DNA was precipitated by centrifugation at 16,000 g for 15 min and the DNA pellet washed in 70% ethanol and resuspended and stored in water.

Amplification of COI was performed using primers designed by Folmer *et al.* [74], which amplify a ~710 base pair fragment of COI across the broadest array of invertebrates. COI primers:

LCO1490 forward primer: `5'-ggtcaacaaatcataaagatattgg-3'`

HC02198 reverse primer: `5'-taaacttcagggtgaccaaaaaatca-3'`

Amplifications were performed under the following PCR conditions: 2 min at 92°C, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final 72°C extension for 7 min. Amplification products were then TOPO-cloned using OneTaq (NEB) and sequenced.

Multiple sequence alignment of *Cassiopea spp.* COI sequences were generated using Clustal Omega software. Sequences were aligned with each other (see **Figure 2.5B**), and to the previously identified cryptic species *Cassiopea ornata*, *Cassiopea andromeda*, and *Cassiopea frondosa* [62]. The level of identity between these sequences is presented in **Figure 2.5B**. Of the 15 *Cassiopea spp.* sequenced there were 8 identical COI sequences and 7 COI sequences with 45-90% identity.

METHODS DETAILS

Cassiopea behavioral tracking.

Individual jellyfish were placed into 700 mL square clear plastic containers (cubbies), with white sand bottoms, in 35 L (10 gallon) glass tanks (**Figure 2.5C-F**). Eight containers can fit in each tank, so eight jellyfish can be simultaneously recorded per tank. Tanks were housed inside Sterilite utility cabinets (65 cm W x 48 cm L x 176 cm H) with a door to

eliminate ambient light in the recording setup. During the 12-hour day (lights on) tanks were illuminated with 24-inch florescent lamps, each containing four florescent bulbs that provide a combination of wavelengths optimized for photosynthesis in water: two 24 W, 6000 K Mid-day lights, and two 24 W Actinic lights (Giesemann), which combined provided 200-300 PPF. During the 12-hour night (lights off) low-intensity red-LEDs were used to illuminate jellyfish to enable visualization. For all jellyfish recordings we used Unibrain 501b cameras above the tank running Firei software capturing at 15 frames per second. Camera aperture and Firei settings were adjusted to increase the contrast between jellyfish and background. Recordings were saved directly onto hard drives.

Jellyfish were acclimated in the recording tank in their cubbies for 2-3 days before starting recordings. 24-hour recordings were taken for successive days (7 am – 7 pm) and nights (7 pm – 7 am), unless otherwise indicated. *Cassiopea* were fed each day at 10:30 am, 3.5 hours after the lights turn on. Each jellyfish received 5 mL of 16 g/L brine shrimp. For each circadian rhythm experiment a different light condition was left on for 36-hours: dark conditions, low-intensity light conditions (an array of white-LED lights, 0-0.5 PPF), mid-intensity light conditions (two 24 W, 6000 K Mid-day lights, 75-150 PPF), or full light conditions (two 24 W, 6000 K Mid-day lights, and two 24 W Actinic lights, 200-300 PPF). For 6-hour and 12-hour rebound experiments the mechanical stimulus was applied for 10 s every 20 min.

All analysis was done using open-source packages in the SciPy ecosystem [75-77]. To monitor jellyfish activity, pulsing information was extracted from the individual frames of each recording. Approximately 648,000 frames were collected every 12 hours. To quantify pulsing activity, we processed the first 18,000 frames of every hour (20 min). As *Cassiopea* pulse, the relaxation and contraction of the bell causes a corresponding change in average pixel intensity. To measure this change in average pixel intensity we drew a rectangular region of interest (ROI) around each jellyfish (**Figure 2.1D**; **Figure 2.5F**). A user manually selected a ROI around each of the eight jellyfish in the first and last of the 18,000 frames. This was done so that the selected ROI accounts for any movement of the jellyfish. To

control for noise from oscillations in ambient lighting, we perform background subtraction using a similarly sized ROI containing no jellyfish.

We analyzed pixel intensity data, and identified pulse events and inter-pulse intervals (IPI) in a four-step process. Step 1: Gaussian smoothing of the mean intensity over time to eliminate high frequency oscillations (**Figure 2.6A**). This smoothed trace was used to account for large movements in the mean intensity due to jellyfish translational movement within the selected ROI. Step 2: Normalization of the mean intensity values with the max mean intensity and the smoothed mean intensity:

$$T^n = \frac{T^n_{raw} - T^n_{smooth}}{T_{max} - T^n_{smooth}}$$

where T_{raw} is the raw intensity trace, T_{smooth} is the smoothed trace generated in Step 1, T_{max} is maximum intensity across the raw trace, and n is the index of each frame of the recording. Step 3: find the indices (time) of local maxima and minima in the normalized trace. Because of noise in the pulsing trace there is a high rate of false positives when finding local maxima and minima (**Figure 2.6B**). We have used a set of criteria to identify a true pulse event from the local maxima and local minima. Step 4: identifying pulses from local maxima and minima (**Figure 2.6C**). A local maximum can be defined as a pulse peak if it meets two criteria. First, it must be above a set threshold (to eliminate local maxima due to noise in pause regions of the pulse trace). Second, it must be above a set distance from the next local maxima (to prevent double counting of a single pulse). The standard deviation of the Gaussian smoothing, the threshold level, and the minimum distance between pulses can all be changed from one jellyfish to another. For all data analysis these parameter values were optimized to quantify pulsing events for each animal.

We calculated the total number of pulses and the IPI for each 20-min time bin. With some jellyfish the difference in pixel intensity from the contracted to non-contracted state was not big enough to easily identify pulsing above the noise. These jellyfish were excluded from analysis. During the 20-min recordings jellyfish would occasionally move out of the

selected ROI. We would then exclude that 20-min recording for that jellyfish from the analysis. In compiling data to generate activity versus time plots we excluded jellyfish that we could not analyze for more than three 20-min recordings during a 12-hour day or night period.

For the arousal assay we designed an experiment to systematically test this sensory responsiveness. *Cassiopea* respond to being placed in the water column by rapidly orienting themselves and moving towards a stable surface. For the experimental system, *Cassiopea* were placed inside a 20 cm tall, 12 cm diameter, PVC pipe with a 53 μm filter screen bottom, called a *Cassiopea* dropper (CD). The experiment consists of four steps, as seen in the four panels in **Figure 2.3A**. Step 1, the jellyfish were placed on the screen bottom of the CD, which was positioned two cm below the water surface (h_L) and were acclimated for five min. At night jellyfish took less than five min to return to quiescence after being placed in the CD. Step 2, the CD was then "dropped" to a set depth (18 cm from the surface, h_D). This action leaves the jellyfish free-floating, two cm below the water surface. Step 3, the time to first pulse was measured. Step 4, the time to reach bottom was measured. To determine if the nighttime arousal latency is reversible, a second drop experiment was performed within 30 s of the initial drop. The CD was returned to two cm below the water surface, but instead of waiting for five min, steps 2 and 3 were performed immediately. Time to first pulse and time to bottom are not completely independent measures, though there is also not a perfect correlation. A jellyfish could pulse quickly but be delayed in reaching the bottom due to, for example, inactivity after the first pulse.

***Cassiopea* staining and imaging.**

Actin was stained using Alexa Flour 488-Phalloidin (ThermoFisher A12379). Jellyfish were anesthetized in ice-cold 0.8 mM menthol/ASW, and then fixed in 4% formaldehyde on ice for 45 min. Fixed jellyfish were permeabilized in 0.5% Triton/PBS for 2 hours and blocked using 3% BSA for 1 hour. They were then incubated in 1:100 Phalloidin solution in 0.5% Triton/PBS, for 18-24 hours in the dark at 4°C [78]. Stained jellyfish were

mounted in refractive index matching solution [79] and imaged using a LSM 780 confocal microscope (Zeiss).

QUANTIFICATION AND STATISTICAL ANALYSIS

The following statistical tests were used: two-sided paired Student's *t*-tests, two-sided unpaired Student's *t*-tests, and two-way ANOVA with Bonferroni posttest. We performed D'Agostino's omnibus K^2 normality test on all data sets to assess whether or not to reject the null hypothesis that all values were sampled from a population that follows a Gaussian distribution. For paired values, we tested if the pairs were sampled from a population where the difference between pairs follows a Gaussian distribution. Experimental groups that were statistically compared were tested for equal variance. The normality tests showed that all datasets were approximately Gaussian distributed with the exception of the time to first pulse arousal data. The time to first pulse data also showed grounds for rejecting the null hypothesis that there was equal variance between experimental groups. Tests of the log transformed time to first pulse data showed that the transformed data was approximately Gaussian distributed with equal variance between experimental groups, validating the use of standard two-way ANOVA and unpaired t-tests on the transformed data. Statistical tests were performed using either statistical functions from the SciPy ecosystem or GraphPad Prism (version 6.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). No statistical methods were used to predetermine sample size. For these experiments we performed at least two laboratory replicates within our recording setup, which is limited to 8 jellyfish. Investigators were not blinded to allocation during experiments and outcome assessment. No specific method for randomization was used.

DATA AND SOFTWARE AVAILABILITY

Code used for tracking jellyfish activity and analysis are available at https://github.com/GradinaruLab/Jellyfish.

C h a p t e r I I I

C. ELEGANS SLEEP EMERGES FROM THE ACTION OF NEUROPEPTIDES

Nath RD, Chow ES, Wang H, Schwarz EM, Sternberg PW. C. elegans Stress-Induced Sleep Emerges from the Collective Action of Multiple Neuropeptides. Curr Biol. 2016 Sep 26;26(18):2446-2455. doi: 10.1016/j.cub.2016.07.048.

Abstract

The genetic basis of sleep regulation remains poorly understood. In *C. elegans*, cellular stress induces sleep through Epidermal Growth Factor (EGF)-dependent activation of the EGF receptor in the ALA neuron. The downstream mechanism by which this neuron promotes sleep is unknown. Single-cell RNA-seq of ALA reveals that the most highly expressed, ALA-enriched genes encode neuropeptides. Here we have systematically investigated the four most highly enriched neuropeptides: *flp-7, nlp-8, flp-24*, and *flp-13*. When individually removed by null mutation, these peptides had little or no effect on stress-induced sleep. However, stress-induced sleep was abolished in the *nlp-8; flp-24*; *flp-13* triple mutant animals, indicating that these neuropeptides work collectively in controlling stress-induced sleep. We tested the effect of overexpression of these neuropeptide genes on five behaviors modulated during sleep—pharyngeal pumping, defecation, locomotion, head movement, and avoidance response to an aversive stimulus –and found that if individually overexpressed, each of three neuropeptides (*nlp-8, flp-24*, or *flp-13*) induced a different suite of sleep-associated behaviors. These overexpression results raise the possibility that individual components of sleep might be specified by individual or combinations of neuropeptides.

Introduction

Sleep is a complex behavioral state that requires the coordinated regulation of multiple behaviors and physiological processes. Sleep is defined as a state of reversible behavioral

quiescence, increased arousal threshold, and homeostatic regulation [1, 2]. This physiological state has been observed both in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, as well as in vertebrates such as *Danio rerio*, *Mus musculus*, and *Homo sapiens* [1, 2]. Sleep is a genetically encoded state, and key sleep genes are conserved from nematodes to mammals [31, 67, 80, 81]. One such sleep regulator is Epidermal Growth Factor Receptor (EGFR), whose activation promotes sleep in both *C. elegans* and *D. melanogaster* [42, 82], and inhibits locomotion in mammals [83-85].

C. elegans sleep has been observed during developmental molting (lethargus), satiety, and Epidermal Growth Factor (EGF)/EGFR signaling [5, 10, 41, 42, 44, 86-88]. Here we investigated the *C. elegans* EGF-induced sleep pathway, thought to represent a distinct molecular pathway from developmentally linked sleep (**Figure 3.1A**; [87]). The EGF-induced sleep state occurs in two contexts: by overexpressing the EGF ortholog (LIN-3C; [42]), or by EGF-signaling after stress (such as temperature elevation) in wild-type animals [41]. The EGF receptor ortholog (LET-23) is necessary for EGF-induced sleep and expressed in the ALA neuron [42]. Ablation of ALA demonstrated that it is necessary for EGF-induced sleep [42]. EGF-induced sleep is suppressed by genetic inactivation of the ALA neuron with null mutations of *ceh-14* or *ceh-17*, genes that respectively encode LIM-class and Paired-like homeodomain transcription factors [41, 42, 89]. These transcription factors control expression of genes in ALA shown to be required for EGF-induced sleep including EGFR [41, 42, 89]. The mechanism by which the ALA neuron controls animal behavior to induce the sleep phenotype is unknown. Henceforth, we refer to EGF-induced sleep as stress induced-sleep [5].

Little is known about the sleep-promoting molecules downstream of ALA; but they may include neuropeptides, which have been implicated in regulating a wide range of behavioral states, including sleep [21, 33, 37, 38, 90]. We hypothesized that ALA serves as a neurosecretory cell that releases neuropeptides to modulate sleep-associated behaviors based on two experimental results. First, mutation of *unc-31*, which encodes a

protein important for dense core vesicle (DCV) fusion [91], inhibits stress-induced sleep [42], suggesting that neuropeptide release is necessary for this state. Second, genetic axotomy of ALA does not inhibit stress-induced sleep [42], indicating that the axon of ALA, and thus neurotransmission, is dispensable for this state, and providing additional support for the hypothesis that neuropeptides mediate stress-induced sleep.

Only a few neuropeptide-encoding genes are known to be expressed in ALA, and little is known about their physiological roles. One such gene, *flp-7*, encodes a FMRFamide-like peptide not required for stress-induced sleep [89], whereas another, *flp-13*, was previously shown to be partially required for stress-induced sleep [44]. To identify novel genes that regulate sleep, we performed single-neuron RNA-seq of ALA, and observed that this neuron transcribed several genes encoding neuropeptides. We systematically characterized the four most highly expressed, ALA-enriched neuropeptides: *flp-7, nlp-8, flp-24*, and *flp-13*. Null mutation of each individual neuropeptide had little or no effect on stress-induced sleep, while a triple knockout (*nlp-8; flp-24*; *flp-13*) was fully defective. Overexpression experiments showed that these three neuropeptide genes had an effect on the five behaviors that are modulated during sleep: pharyngeal pumping, defecation, locomotion, head movement, and avoidance response. Each neuropeptide (*nlp-8, flp-24*, or *flp-13*) induced a different suite of sleep-associated behaviors. Taken together, these results demonstrate that the collective action of three neuropeptide genes results in stress-induced sleep.

Results

Identification of neuropeptide-coding genes enriched in the ALA neuron

To identify sleep-promoting neuropeptides expressed in ALA, we used microdissection-based single-cell RNA-seq [92] for transcriptomic analysis. We dissected individual ALA neurons from transgenic fourth-stage larval worms (L4) expressing GFP in the ALA neuron, reverse-transcribed mRNA to cDNA, and amplified the cDNA using PCR (**Figure 3.8**). Using this procedure we made one pool from four cells and another pool

from five cells, and performed deep sequencing. We mapped 17.8 million reads to 8,133 expressed protein-coding genes (**Figure 3.1B**). Four genes encoding neuropeptides (*flp-24*, *flp-7*, *flp-13*, and *nlp-8*) were among the most highly expressed and enriched in ALA compared to whole larvae (**Figure 3.1B**). *flp-24* and *flp-13* were previously found in the ALA neuron of *Ascaris suum* by single neuron mass spectrometry [93]. The *C. elegans* genome contains 122 neuropeptide genes whose mature products contain over 250 distinct neuropeptides [94]. RNA-seq analysis indicated that ALA expresses 23 of the 31 *C. elegans* FMRFamide-like neuropeptide encoding genes *(flp)*, five of which were expressed at least 10-fold more abundantly in ALA than in whole larvae. ALA also expressed 25 of the 51 *C. elegans* neuropeptide-like-coding genes (*nlp*), of which five were expressed over 10-fold more abundantly in ALA than in whole larvae. These data support our hypothesis that ALA is a neurosecretory cell. The three most ALA-enriched *flp* genes were *flp-24, flp-7,* and *flp-13* (in descending order of enrichment), and the most enriched *nlp* gene was *nlp-8*. Of these, only *flp-7* and *flp-13* were previously known to be expressed in ALA [44, 89, 95]. We verified expression of *flp-24* and *nlp-8* using GFP reporter constructs (**Figure 3.8E-H**). Previous analysis showed that each of these genes encodes a prepropeptide containing one or more mature neuropeptides ([96, 97]; **Figure 3.9**; **Figure 3.10**).

Loss-of-function of three ALA-enriched neuropeptides suppresses stress-induced sleep

C. elegans sleep has been associated with three behavioral phenotypes: suppression of pharyngeal pumping (a necessary component of eating), suppression of locomotion, and an increased response latency to arousing stimuli [5, 10, 41, 98]. We found that suppression of head movement and defecation are additional sleep-associated behavioral phenotypes. Stress, by heat shock, is sufficient to induce all of these phenotypes (**Figure 3.2**; **Figure 3.3**; [41, 42]). To determine whether ALA-enriched neuropeptides are necessary for stress-induced sleep, we assayed locomotion, head movement, pharyngeal pumping, avoidance response, and defecation before and 30 minutes after heat shock in *flp-24*, *flp-7*, *flp-13*, and *nlp-8* single-null mutants (**Figure 3.2**; **Figure 3.3**; **Figure 3.10**;

Figure 3.11; **Table 3.1; Table 3.2; Table 3.3**). Pumping, locomotion, and head movement were repeated in three independent experiments with 10 or more individuals per trial. To score movement we distinguished locomotion, defined as movement of the animal's centroid in the forward or reverse directions within a 10-second interval, and head movement, defined as dorsal-ventral displacement of the animal's head from the posterior of the second pharyngeal bulb to the anterior tip.

flp-24, *flp-7*, and *nlp-8* single-null mutants were indistinguishable from wild type with respect to pumping, locomotion, and head movement after heat shock ($p>0.5$; **Figure 3.2**). *flp-13* mutants were slightly resistant to pumping quiescence after heat shock (*flp-13*: 79±3% pumping quiescent, compared to N2: 100±0%; $p<0.05$; **Figure 3.2A**), confirming the results of Nelson et al. [44]. Resistance to pumping quiescence after heat shock in *flp-13* mutants was much weaker than the negative controls, *ceh-14* and *ceh-17*, suggesting that *flp-13* is not the only neuropeptide necessary for pumping quiescence during stress-induced sleep (*flp-13*: 79±3% pumping quiescent compared to *ceh-14*: 0±0% and compared to *ceh-17*: 5±3%; $p<0.001$; **Figure 3.2A**).

flp-13 mutants were partially resistant to head movement quiescence after heat shock (*flp-13*: 76±4% head movement quiescent, compared to N2: 100±0% head movement quiescent; $p<0.05$; **Figure 3.2C**), but we did not observe statistically significant resistance to locomotion quiescence after heat shock in *flp-13* mutants, not fully consistent with results reported by Nelson et al. (*flp-13*: 85±4% locomotion quiescent compared to N2: 100±0% locomotion quiescent; $p=0.1$; **Figure 3.2B**; [44]). *ceh-14* and *ceh-17* mutants, previously shown to be strongly resistant to heat shock [41, 42], displayed locomotion quiescence after heat shock (*ceh-14*: 0±0% locomotion quiescent before heat shock compared to *ceh-14*: 36±4% locomotion quiescent after heat shock, and *ceh-17*: 0±0% locomotion quiescent before heat shock compared to *ceh-17*: 56±5% locomotion quiescent after heat shock; $p<0.001$; **Figure 3.2B**). The difference in our results could be due to differences in heat shock protocol or scoring (**Methods**). Our data indicate that *flp-13* mutants are partly defective for pumping and head movement

quiescence after heat shock, but are not defective for locomotion quiescence after heat shock.

The co-expression of several neuropeptide genes in ALA (**Figure 3.1**; **Figure 3.8**) and the partial requirement for *flp-13* in stress-induced sleep suggested that these genes might be functionally redundant. We therefore constructed double- and triple-null mutants (**Methods**) and found that *nlp-8; flp-13* double mutants, *flp-24; flp-13* double mutants, and *nlp-8*; *flp-24*; *flp-13* triple mutants were more resistant to heat shock-induced pumping quiescence than the *flp-13* single mutant (*flp-24; flp-13*: 51±5% pumping quiescent; *nlp-8; flp-13*: 18±2% *nlp-8; flp-24; flp-13:* 24±4% compared to *flp-13*: 84±0%; $p<0.05$; **Figure 3.2D**), suggesting that *flp-24* and *nlp-8* enhance the effect of *flp-13* and strongly induce pumping quiescence. We found that *nlp-8*; *flp-24* double mutants were not resistant to heat shock-induced pumping quiescence compared to wild type ($p=0.4$; **Figure 3.2D**), suggesting that *flp-13* is a key regulator of pumping quiescence for stress-induced sleep. However, not all ALA-enriched neuropeptides enhanced the effect of *flp-13* on stress-induced sleep, for instance, *flp-13; flp-7* double mutants were phenotypically indistinguishable from *flp-13* single mutants ($p>0.5$; **Figure 3.2D**).

Loss of either *nlp-8* or *flp-24* in the *flp-13* knockout background enhanced both head movement and pumping quiescence resistance after heat shock (**Figure 3.2F**). The *nlp-8*; *flp-13* double mutants and *nlp-8*; *flp-24*; *flp-13* triple mutants were resistant to locomotion quiescence after heat shock (*nlp-8; flp-13*: 58±7% locomotion quiescent; *nlp-8; flp-24; flp-13:* 72±4% locomotion quiescent; compared to N2: 100±0% locomotion quiescent; $p<0.05$; **Figure 3.2E**). The resistance of *nlp-8*; *flp-13* double mutants and *nlp-8*; *flp-24*; *flp-13* triple mutants was similar to *ceh-14* mutants in locomotion quiescence (*ceh-14*: 42±9% locomotion quiescent; $p=0.1$; **Figure 3.2E**).

A characteristic feature of sleep is an increased arousal threshold, observed as an increased latency to an aversive stimulus. For example, *C. elegans* typically respond to 30% 1-octanol by moving backward (a reversal) within 5 seconds, but when the animal is

asleep the avoidance response either takes longer or does not occur at all [10, 98, 99]. We defined avoidance response as backward locomotion for at least one pharynx length within one minute of stimulus delivery. No single mutant was resistant, but the *flp-24*; *flp-13* double mutant, *nlp-8; flp-13* double mutant, and *nlp-8; flp-24; flp-13* triple mutant were all resistant to the increased time required to avoid aversive stimuli (**Figure 3.3**; **Table 3.2**; n=10). Therefore, these neuropeptides work collectively to induce the increased latency to avoid aversive stimulus.

Another behavior that is suppressed during sleep in *C. elegans* is defecation (**Figure 3.3B**; **Table 3.3**). The defecation motor program comprises posterior body wall contraction, anterior body wall contraction, and expulsion [100]. We scored defecation events using five-minute video recordings before and 30 minutes after heat shock. Some *ceh-14* and *ceh-17* mutants did not defecate after heat shock (**Table 3.3**), suggesting that the *ceh-14* and *ceh-17* mutants do not completely rescue this aspect of quiescence. The background phenotype could result from either expression of these or other neuropeptides in cells other than ALA, or residual expression of these or other neuropeptides in mutant *ceh-14* or *ceh-17* ALA neurons. No single or double mutant was resistant to the suppression of defecation ($n \geq 10$; $p > 0.5$; **Figure 3.3B**). We found that the triple mutant was resistant to the suppression of defecation (*nlp-8*; *flp-24*; *flp-13*: 1.6±0.3 events per individual post-heat shock, n=30, compared to N2: 0.3±0.2 events, n=24; $p < 0.05$; **Figure 3.3B**). Resistance to the suppression of defecation in the triple mutant was indistinguishable from *ceh-14* and *ceh-17* animals ($p > 0.4$; **Figure 3.3B**). Taken together, our loss-of-function analyses indicate that *nlp-8* and *flp-24* enhance the effect of *flp-13*, and that the collective action of these neuropeptides results in stress-induced sleep.

Experimental design to test the sufficiency of neuropeptides in sleep-associated behaviors

The functions of these candidate sleep-promoting genes were tested using a new overexpression strategy (**Figure 3.4A**). To determine if each ALA-enriched neuropeptide was sufficient to induce a sleep-associated behavior, we used a heat shock-inducible promoter to conditionally overexpress each of the four neuropeptide genes (**Figure**

3.4A). It is unclear if results from these experiments are hypermorphic or neomorphic, as it is assumed that the neuropeptides are acting at the right targets in physiological concentrations. Such experiments, however, are confounded by the fact that heat shock *per se* leads to stress-induced sleep [41]. To avoid this possible artifact, all of our overexpression experiments were conducted in *ceh-14* mutants, which do not express EGFR (*let-23*) in the defective ALA neuron, and thus do not exhibit stress-induced sleep (**Figure 3.4A**; [41, 42, 89]).

flp-13 *overexpression inhibits pharyngeal pumping*

Following the heat shock protocol illustrated in **Figure 3.4A**, we tested the effects of *flp-24, flp-7, flp-13*, or *nlp-8* overexpression on pumping. Pumping was scored for 10 seconds per worm before and for three hours after heat shock at 30 minute intervals. Experiments were repeated three or more times with 10 or more individuals per trial. Among the four genes tested, only *flp-13* overexpression induced pumping quiescence (HS*::flp-13; ceh-14*: 73±1% pumping quiescent compared to *ceh-14*: 0±0% pumping quiescent, at one hour post-heat shock; $p<0.001$; **Figure 3.4B,C)**. We conclude that overexpression of *flp-13*, but neither *flp-24*, *flp-7*, nor *nlp-8*, is sufficient to inhibit pumping (**Figure 3.4D**).

flp-13 *or* nlp-8 *overexpression inhibits defecation*

We tested if overexpression of any of the ALA-enriched neuropeptides was sufficient to suppress defecation, we scored defecation using five-minute video recordings before and one hour after heat shock (**Figure 3.5**). Overexpression of *flp-13* or *nlp-8* suppressed the number of defecation events (HS*::flp-13; ceh-14*: 0.1±0.1 events per individual post-heat shock compared to 3.5±0.2 events per individual pre-heat shock, n=10; $p<0.001$; HS*::nlp-8; ceh-14*: 0.2±0.1 events per individual post-heat shock compared to 3.9±0.2 events per individual pre-heat shock, n=13; $p<0.001$; **Figure 3.5A**; **Figure 3.12A)**. One hour after heat shock, *ceh-14* mutants were defecating, and no difference was observed in the total number of defecation events pre- and post-heat shock in *ceh-14* controls, nor in

animals overexpressing *flp-24* or *flp-7* ($p>0.05$; **Figure 3.5A**; **Figure 3.12A**). However, we did observe a difference in the time between defecation events (i.e., the defecation interval). While *ceh-14* mutants defecated, they exhibited a significantly longer defecation interval ($p<0.05$; **Figure 3.12B**), suggesting that the *ceh-14* mutation does not completely eliminate this aspect of quiescence. We treated the effects of heat shock on the *ceh-14* defecation interval as a background phenotype. We observed no significant difference between the post-heat shock lengthening of the defecation interval in *ceh-14* mutants and those overexpressing *flp-24* or *flp-7* ($p\geq0.4$; **Figure 3.12B**).

Since arrested defecation might be a consequence of halted feeding [100, 101], we wanted to test if arrested defecation in animals overexpressing *flp-13* or *nlp-8* was independent of pumping inhibition. To address this question, we measured pumping rates before and after heat shock from the same individuals, including those that ceased defecation (**Figure 3.5B**). Consistent with **Figure 3.4**, we found that most *flp-13* overexpressing animals (7 of 10) did not pump after heat shock. The 3 animals that continued to pump did so at reduced rates 40.0±12.1 pumps per minute (**Figure 3.5B**). This experiment was not able to determine if pumping and defecation are controlled separately by *flp-13* overexpression. However, our *nlp-8* overexpression results indicate that inhibition of defecation does not itself inhibit pumping. These data suggest that defecation and pumping rates, two aspects of the *C. elegans* sleep state, can be controlled separately by different neuropeptides.

flp-24*,* flp-13*, or* nlp-8 *overexpression inhibits specific aspects of movement*

Locomotion quiescence is a canonical sleep-associated behavior [1, 2]. Locomotion was scored before heat shock and every 30 minutes for 3 hours after heat shock in a blinded manner. We repeated these experiments three or more times with 10 or more individuals per trial. After heat shock, *ceh-14* mutants had less frequent bouts of locomotion (*ceh-14*: 31±5% locomotion quiescent one hour post-heat shock compared to 4±4% locomotion quiescent pre-heat shock; $p<0.001$; **Figure 3.6B**). For the purposes of this study, we treated the effects of heat shock on *ceh-14* locomotion as a background phenotype. *flp-7*

overexpression did not increase locomotion quiescence compared to *ceh-14* (HS*::flp-7; ceh-14*: 41±6% locomotion quiescent one hour post-heat shock compared to 0±0% locomotion quiescent pre-heat shock; $p<0.001$; and compared to *ceh-14*: 31±5% locomotion quiescent one hour post-heat shock; $p=0.3$; **Figure 3.6A,B**). By contrast, worms overexpressing *flp-24* showed severe inhibition of locomotion (HS*::flp-24; ceh-14*: 68±9% locomotion quiescent one hour post-heat shock compared to 0±0% locomotion quiescent pre-heat shock; $p<0.001$; and compared to *ceh-14*: 31±5% one hour post-heat shock; $p<0.01$; **Figure 3.6A,B**). *flp-24* overexpression suppressed locomotion, but not defecation or pumping, whereas *nlp-8* overexpression suppressed locomotion and defecation but not pumping, and *flp-13* overexpression suppressed locomotion, defecation, and pumping.

While scoring locomotion in animals overexpressing *flp-24* or *flp-13*, we noticed a lack of head movement 90 minutes after heat shock, defined as any dorsal-ventral displacement of the worm's head from the posterior of the second pharyngeal bulb to the anterior tip. As with pumping, *ceh-14* mutants showed no background quiescence for head movement after heat shock (**Figure 3.6D**). Overexpression of either *nlp-8* or *flp-7* failed to suppress head movement (**Figure 3.6D,E**). However, overexpression of either *flp-24* or *flp-13* inhibited head movement (HS::*flp-24; ceh-14*: 47±10% head movement quiescent; HS::*flp-13; ceh-14*: 40±12% head movement quiescent compared to *ceh-14*: 0±0% head movement quiescent; $p<0.01$; **Figure 3.6D**). The movement state of animals overexpressing *nlp-8* was unusual; their bodies showed significantly more locomotion quiescence than *ceh-14* animals, but their heads continued to move (**Figure 3.6**). We conclude that overexpression of *flp-24*, *flp-13*, or *nlp-8* inhibits movement behaviors.

flp-24*,* flp-13, *or* nlp-8 *overexpression increases latency to avoid aversive stimulus*

We tested if overexpression of any of the ALA-enriched neuropeptides was sufficient to increase the latency to avoid 1-octanol. *ceh-14* mutants and animals overexpressing *flp-7* exhibited normal avoidance behavior one hour after heat shock (**Figure 3.6G**). In contrast, overexpression of either *flp-24*, *flp-13,* or *nlp-8* increased the response time

compared to pre-heat shock (HS::*flp-24; ceh-14*: 23.7±4.6 seconds latency to reversal post-heat shock compared to 1.8±0.3 sec latency to reversal in seconds pre-heat shock, n=9; HS::*flp-13; ceh-14*: 20.4±4.2 sec latency to reversal post-heat shock compared to 1.7±0.3 sec latency to reversal pre-heat shock, n=2; HS::*nlp-8; ceh-14*: 21.5±5.6 sec latency to reversal post-heat shock compared to 1.5±0.2 sec mean latency to reversal pre-heat shock, n=6; $p<0.001$; **Figure 3.6G**). Five, two, and nine of eleven young adults overexpressing *flp-24*, *flp-13*, and *nlp-8*, respectively, did not respond to stimulus after 60 seconds, and were classified as non-responsive (**Table 3.4**). Thus, overexpression of *flp-24*, *flp-13*, or *nlp-8*, but not of *flp-7*, inhibited the avoidance response (**Figure 3.6H**).

Discussion

Sleep requires the coordinated regulation of multiple aspects of behavior and physiology. However, it is not well understood how disparate processes are coordinately regulated to produce the sleep state. At one extreme, a key factor may affect different aspects of the sleep state, thus ensuring that these processes are coordinately regulated. Alternatively, different processes may be controlled in series, such that one process initiates only if prior steps occur. A third possibility is that different factors may act in parallel to control the sleep state. Our data using a simple model organism supports the latter hypothesis.

We investigated how the *C. elegans* ALA neuron coordinately promotes multiple sleep-associated behaviors. Previous studies suggested that neuropeptides may mediate the sleep-promoting effects of ALA [42]. Using single-cell RNA-seq data of ALA, we observed that 23 *flp* and 25 *nlp* neuropeptide genes were highly expressed and enriched in ALA compared to whole larvae. We focused on four neuropeptides (*flp-24*, *flp-7*, *flp-13*, and *nlp-8*) with the highest level of expression and enrichment in ALA. Given the enrichment of multiple neuropeptide genes in ALA, we considered it unlikely that loss of individual neuropeptides would result in resistance to stress-induced sleep. Indeed, no defects were observed in stress-induced sleep for most neuropeptide single-null mutants. However, we found strong resistance to stress-induced sleep when multiple neuropeptide

genes were deleted, indicating that sleep regulation downstream of ALA involves the collective action of multiple neuropeptides.

To determine how each of these neuropeptides induces sleep, we used an experimental paradigm that avoided confounding effects of neuropeptides released by ALA in response to stress. We found that three ALA-enriched neuropeptides, *flp-24*, *flp-13*, and *nlp-8*, were sufficient to induce distinct sleep-associated behaviors, while another, *flp-7*, showed no behavioral phenotype. For instance, only overexpression of *flp-13* inhibited pumping, while worms overexpressing *nlp-8* halted defecation even though they continued to eat, and moved their heads but not their bodies. *flp-24* overexpression inhibited locomotion and head movement, but eating and defecation continued. In contrast to this specificity, overexpression of *flp-24*, *flp-13*, or *nlp-8* inhibited locomotion and the avoidance response. The observation that some behaviors were affected by only one of these neuropeptides, while other behaviors were affected by several neuropeptides, has two main implications for sleep regulation: one for multilevel modulation of behavior and the other for the evolution of sleep states.

The behaviors studied here involve multiple cell types (**Figure 3.7**). For example, the avoidance response results from sensory neurons, command interneurons, and motor neurons working in series (**Figure 3.7**; [98])**.** Inhibition of any cell type within the neural circuit that regulates the avoidance response should suppress this behavior. Previously we showed that a sleeping worm has dampened sensory neuron activation and asynchronous command interneuron activities [98], both of which contribute to the observed delay in response to an aversive stimulus. Strong neuropeptide modulation (by overexpression of a neuropeptide) of either the sensory neurons or one or more of the command interneurons would lead to the absence of behavioral output, consistent with our observations. On the other hand, this hypothesis suggests that elimination of any one neuropeptide would have a small effect on behavior, consistent with our results. Furthermore, the site of action of these neuropeptides may be redundant at the receptor, cellular, and behavioral level (**Figure 3.7**). These results are consistent with the

hypothesis that stress-induced sleep is driven by a set of neuropeptides produced by the ALA neuron, each of which independently induced a different suite of sleep-associated behaviors: suppression of eating, defecation, locomotion, head movement, and the avoidance response. We propose that neuropeptides act in parallel to control sleep, and that sleep states could be built during evolution from recruitment of factors controlling pre-existing regulatory pathways.

All the neuropeptides studied here have been reported to be expressed in cells other than ALA [44, 89, 95, 97, 102-104]. It is possible that these neuropeptides act from neurons other than ALA in stress-induced sleep. Another possibility is that these neuropeptides might have functions outside of sleep. For example, *nlp-8* is expressed in specific male sensory neurons [104], and thus might play a role in inhibiting defecation during mating [105]. The multiple and apparently non-overlapping expression patterns of these genes is consistent with this hypothesis. With all the usual caveats of overexpression, the apparently distinct effects of sleep-promoting neuropeptides raises the possibility that the *C. elegans* sleep state is assembled from pre-existing regulatory pathways. This level of separate molecular control over distinct behaviors associated with sleep would provide evolutionary flexibility to sleep regulation, as unique but overlapping sleep states could be constructed by recruiting modules that regulate specific aspects of sleep. Diverse sleep states are found through out the animal kingdom [1, 2], and this diversity may be partially explained by the recruitment of species-specific sleep modules (i.e., a module that shuts down defecation in humans). During the sleep state certain species specific regulatory modules must exist, such as modules that inhibit the avoidance response, defecation, and eating. The mammalian genome contains almost 70 different neuropeptide-encoding genes, many of which have detectable expression in the brain [106], and at least 20 of which may have important functions in sleep-wake regulation [21]. This extensive regulatory capacity is consistent with our view of modular regulatory logic. Testing this hypothesis would require associating each peptide with specific sleep-associated behaviors.

Figures and tables

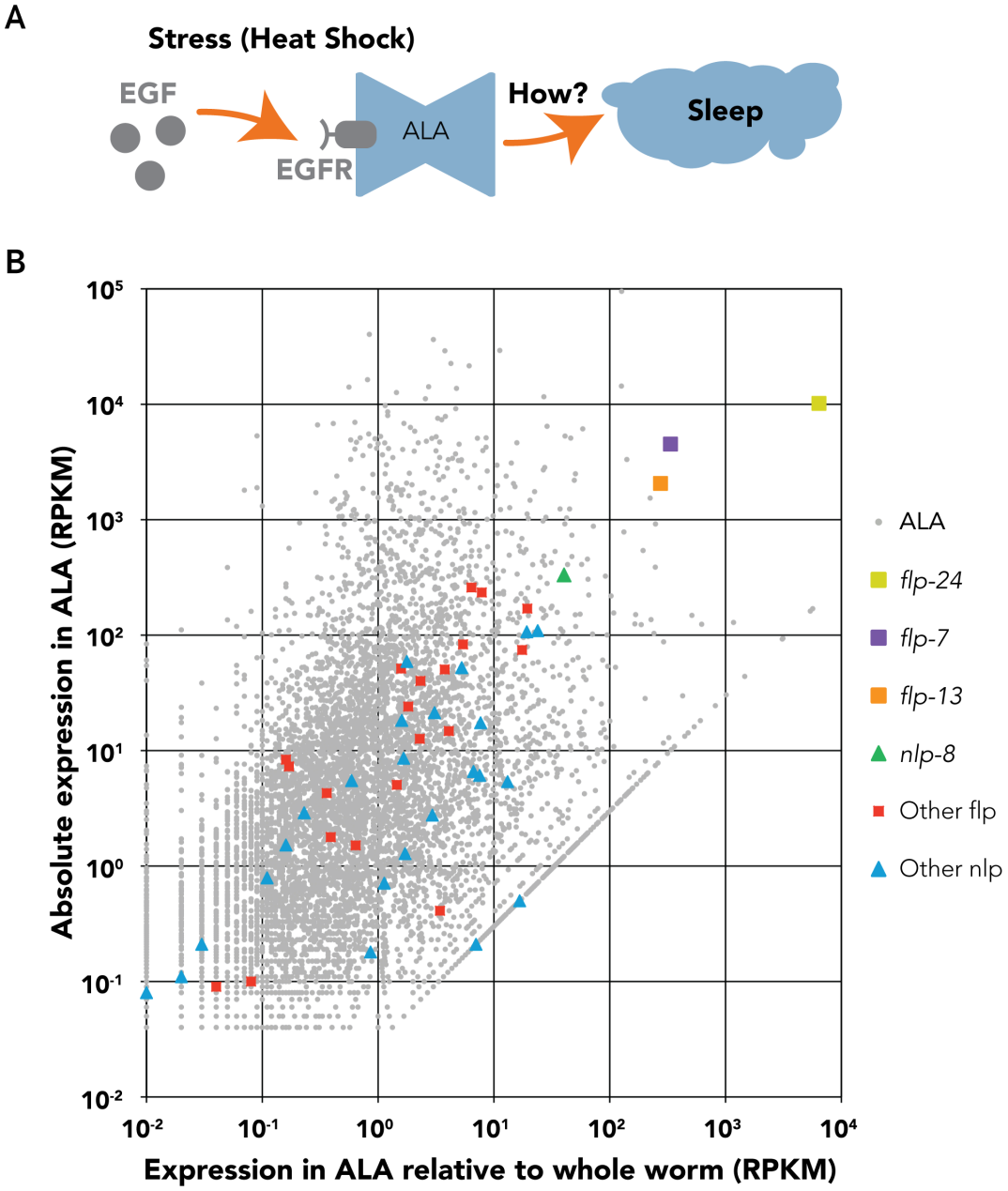

Figure 3.1: Single-cell RNA-seq of ALA, the neuron central to *C. elegans* stress-induced sleep. (**A**) Stress-induced sleep is regulated by LIN-3C (EGF) and LET-23 (EGFR) expressed on the surface of ALA. In this work, we study the mechanism of sleep induction downstream of ALA. (**B**) Single-cell RNA-seq expression data of 8,133 protein-coding genes (grey) collected from two pools of microdissected ALA neurons

(four and five cells; see also **Figure 3.8**) compared with mixed-stage whole larvae. The ratio of expression level of protein-coding genes from the ALA neuron versus whole larvae shows that four neuropeptide-coding genes have ≥10-fold higher expression in ALA than in whole larvae: *flp-24*, *flp-7*, *flp-13*, and *nlp-8* (highlighted with colored squares for *flp*s, and a green triangle for *nlp-8*; see also **Figure 3.9**). Expression levels of other *flp* and *nlp* coding genes are also highlighted by red squares and blue triangles respectively . RPKM unit: reads per kilobase of transcript per million mapped reads.

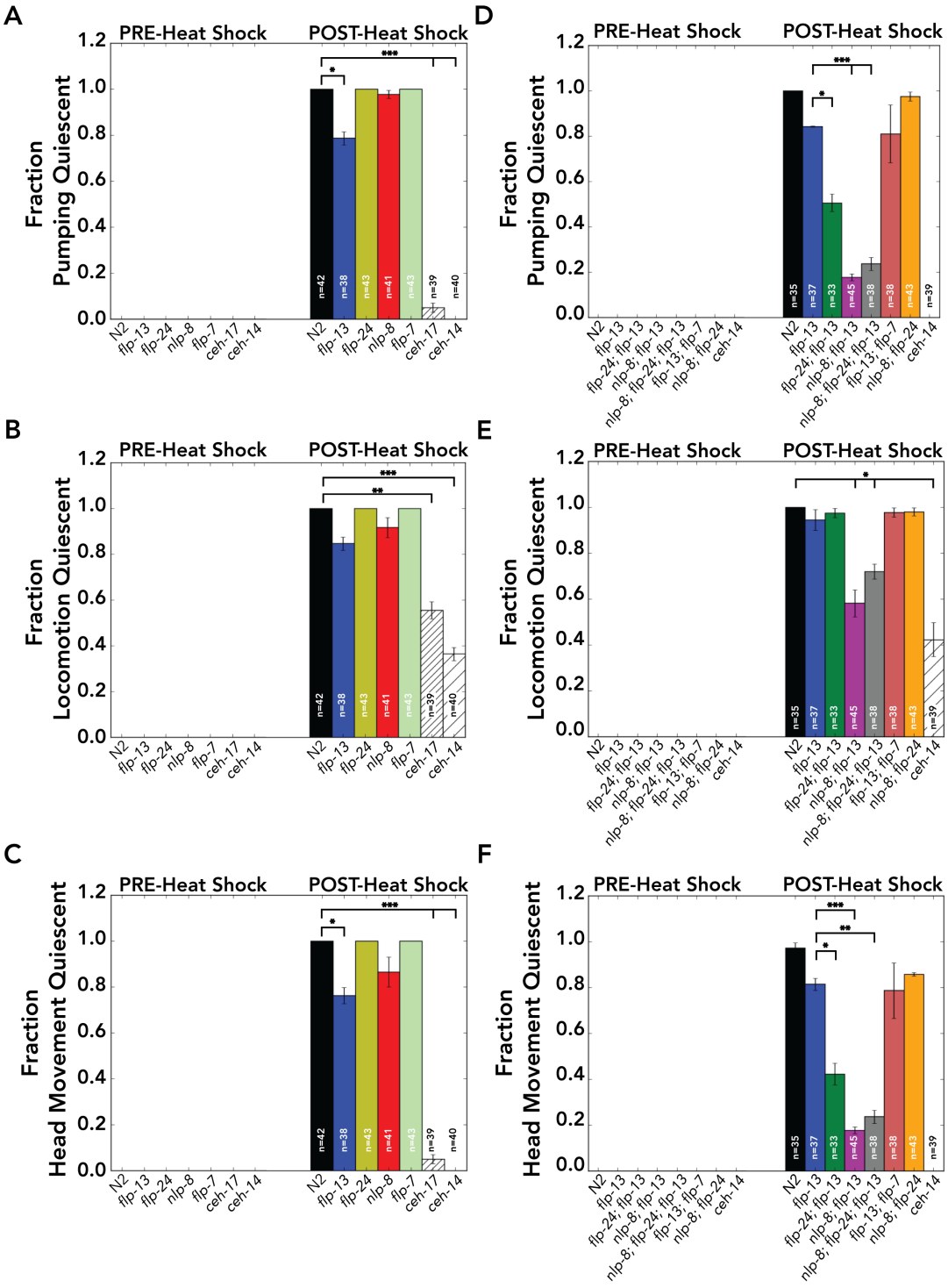

Figure 3.2: Double and triple mutants of ALA-enriched neuropeptides suppress pumping, head movement, and locomotion quiescence during stress-induced sleep. (**A-C**) The fraction of single-null mutants pumping, locomotion, and head movement

quiescent before (PRE) and 30 minutes after (POST) heat shock (a 35°C heat shock was used). N2 are wild-type animals, and *ceh-14* and *ceh-17* mutants serve as negative controls because they have defective ALA neurons which are deficient in EGF-signaling. (**D-F**) The fraction of double- or triple-null mutants pumping, locomotion, and head movement quiescent before (PRE) and 30 minutes after (POST) heat shock. (**A**) & (**D**) *C. elegans* were scored as quiescent for pumping if there was no pharyngeal grinder movement during 10 seconds of observation. Mutation of *flp-13* weakly suppressed pumping quiescence, which was enhanced by mutation of *flp-24* or *nlp-8*. (**B**) & (**E**) *C. elegans* were scored as quiescent for locomotion if there was no centroid movement during 10 seconds of observation. The negative controls *ceh-14* and *ceh-17* had background locomotion quiescence, and no suppression of locomotion quiescence was observed in single-null mutants of neuropeptides. (**C**) & (**F**) *C. elegans* were scored as quiescent for head movement if there was no head movement in the dorsal-ventral directions during 10 seconds of observation. Mutation of *flp-13* weakly suppressed head movement quiescence, which was enhanced by mutation of *flp-24* or *nlp-8.* Data represents the fraction of animals quiescent from three independent assays, where n=total number of *C. elegans*. Data shown as mean±SEM, *p<0.05; **p<0.01; ***p<0.001; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2 (**A-C**) & (**E**) and to *flp-13* (**D**) & (**F**). See also **Figure 3.10**, **Figure 3.11**, and **Table 3.1**.

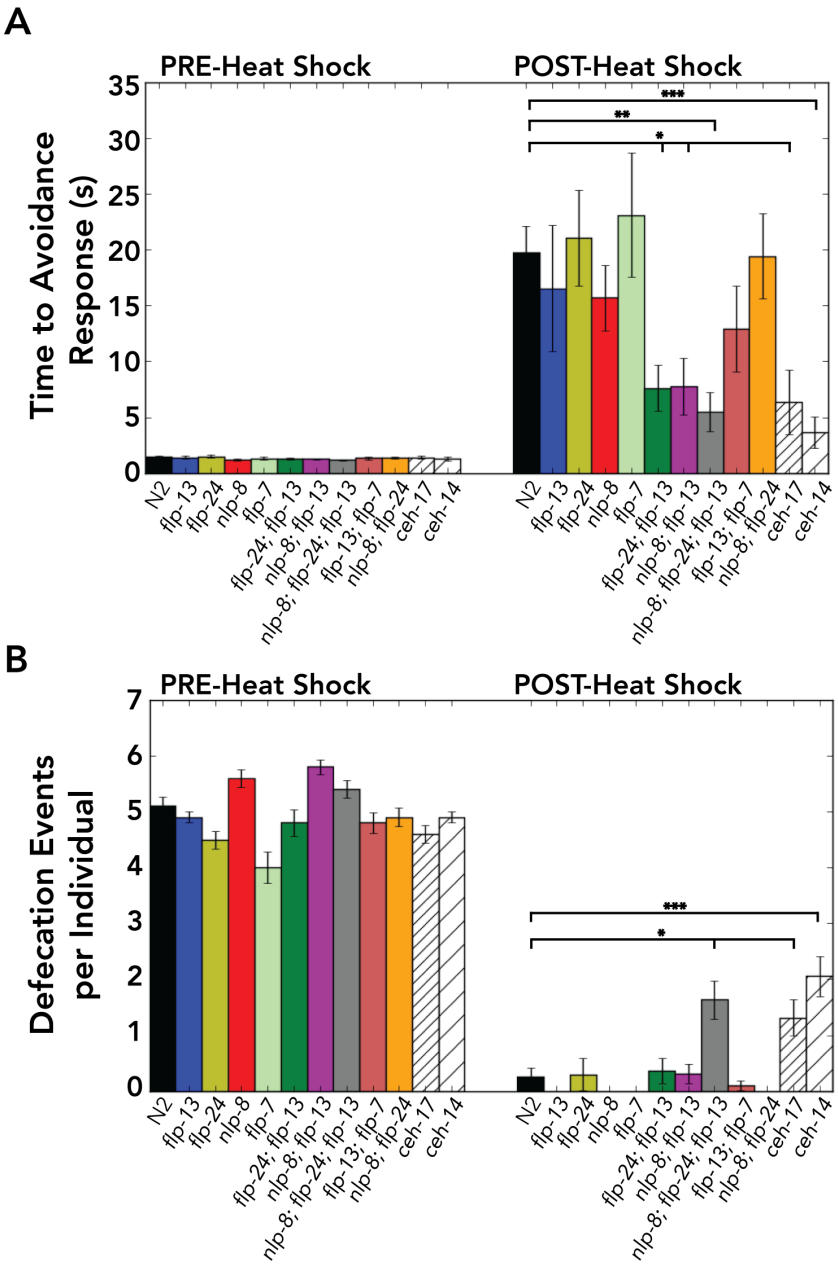

Figure 3.3: Double and triple mutants of ALA-enriched neuropeptides suppress the increased response latency to aversive stimuli, while only the triple mutant suppresses defecation quiescence during stress-induced sleep. (**A**) *C. elegans* were presented with 30% 1-octanol and avoidance behavior was scored by video recordings before (PRE) and 30 minutes after (POST) heat shock (a 35°C heat shock was used). If there was no response 60 seconds after stimulus delivery, the individuals were classified

as non-responsive (**Table 3.2**). The *nlp-8*; *flp-13* double mutant, *flp-24*; *flp-13* double mutant, and *nlp-8*; *flp-24*; *flp-13* triple mutant were resistant to the increased response latency observed during stress-induced sleep. (**B**) Average number of defecation events for individuals five minutes before (PRE) and 30 minutes after (POST) heat shock. A 33°C heat shock was used for more consistent results (**Methods**). We found that our negative controls *ceh-14* and *ceh-17* had background suppression of defecation at 30 minutes (**Table 3.3**). Only the triple mutant (*nlp-8*; *flp-24*; *flp-13*) was resistant to suppression of defecation during stress-induced sleep, and this was statistically indistinguishable from *ceh-14* and *ceh-17* ($p>0.4$). Data shown as mean±SEM; $n\geq10$ *C. elegans* for each strain (see also **Table 3.2; Table 3.3**); *$p<0.05$; **$p<0.01$; ***$p<0.001$; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2.

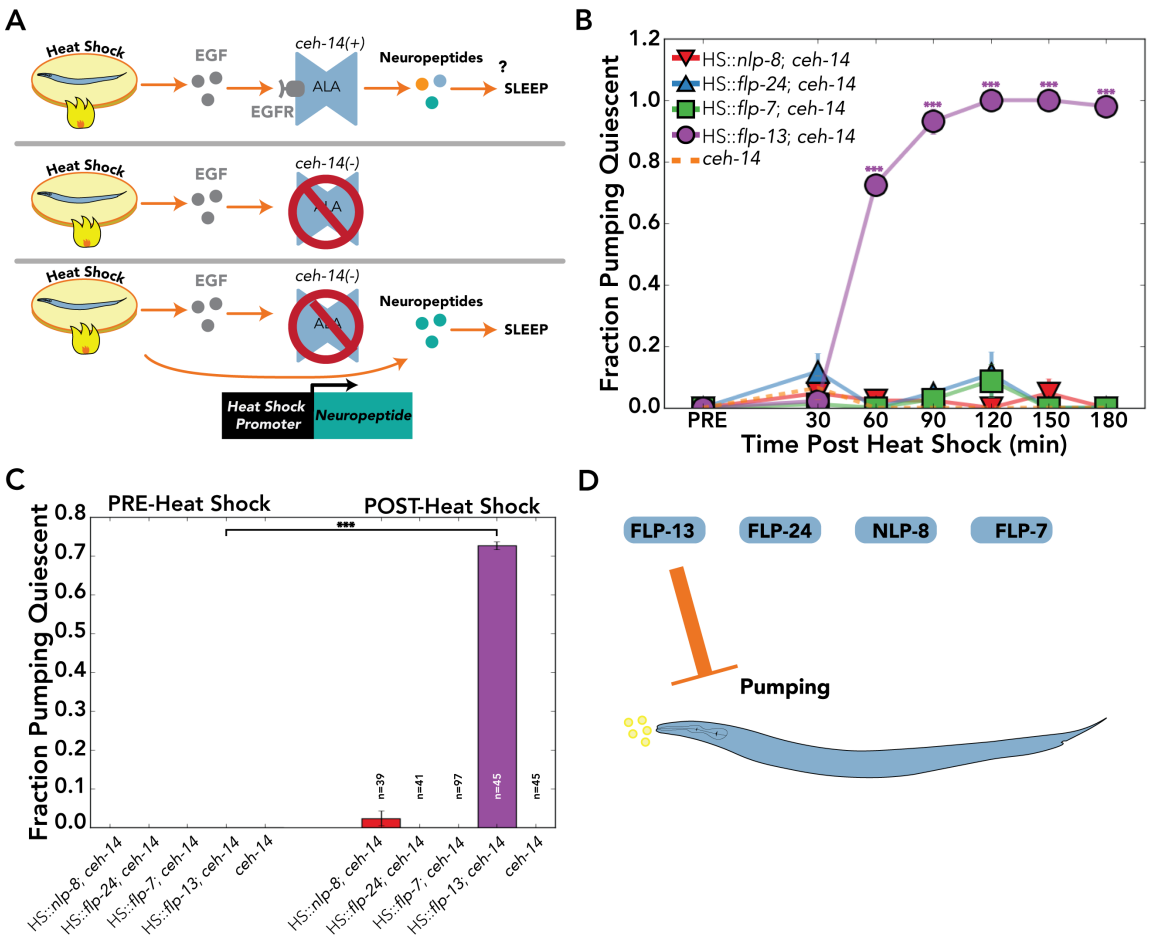

Figure 3.4: Overexpression of FLP-13 inhibits pumping in *C. elegans*. (**A**) (top) Heat shock induces sleep by EGF signaling. EGF binds to its receptor, EGFR, on ALA, which is thought to release neuropeptides that induce sleep. (middle) *ceh-14* mutants have defective ALA neurons that do not express EGFR and are resistant to heat shock induced sleep. (bottom) A conditional heat shock promoter (HS) driving neuropeptide expression in the *ceh-14* background can be induced upon heat shock without the confounding effects of EGF-induced sleep. This overexpression strategy assumes that the neuropeptides are acting at the right sites in physiological concentrations. It is unclear if results from these experiments are hypomorphic or neomorphic. (**B**) Time course monitoring the fraction of *C. elegans* pumping quiescent before heat shock (PRE) and up to three hours after heat shock induced neuropeptide overexpression (POST). (**C**) Fraction of *C. elegans* pumping quiescent before (PRE) and one hour after (POST) heat shock. (**D**) Overexpression of *flp-13*, but neither *flp-24*, *flp-7*, nor *nlp-8* inhibited

pumping. Data represents the fraction of animals quiescent from three or more independent assays, where n=total number of *C. elegans*. Data shown as mean±SEM, *p<0.05; **p<0.01; ****p*<0.001; for (**B**) two-way ANOVA comparing transgenic strains to *ceh-14* mutants with four post-hoc contrast using Bonferroni correction for multiple comparisons. (**C**) Fisher's exact test.

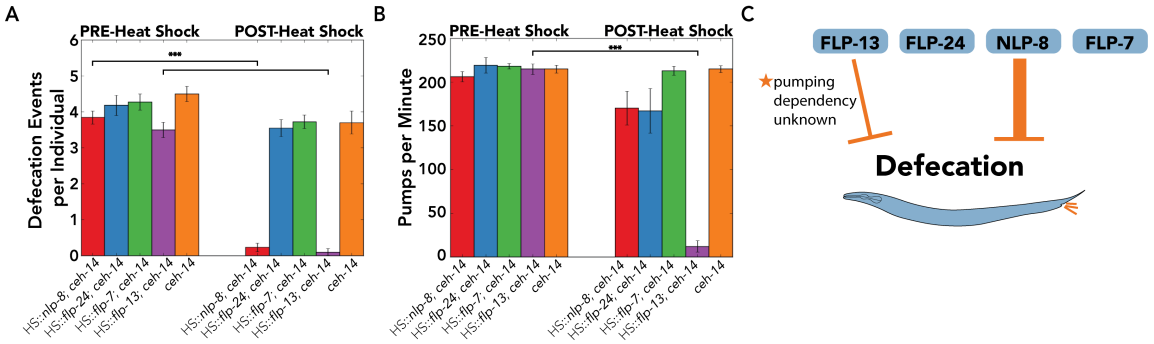

Figure 3.5: Overexpression of NLP-8 inhibits defecation while pumping continues. (**A**) Average number of defecation events for individuals five minutes before (PRE) and one hour after (POST) heat shock. Overexpressing *flp-13* or *nlp-8* inhibited defecation. (**B**) Pumping rate was scored from 10-second video recordings before and one hour after heat shock. (**C**) Overexpression of either *nlp-8* or *flp-13*, but neither *flp-24* nor *flp-7*, inhibited defecation. The thick line indicates strong and independent inhibition of defecation, while the thinner line indicates inhibition of defecation that may be a consequence of pumping quiescence. (**A**) & (**B**) Data shown as mean±SEM; n≥10 *C. elegans* for each strain (see also **Figure 3.12A**); ***$p<0.001$; paired t-test.

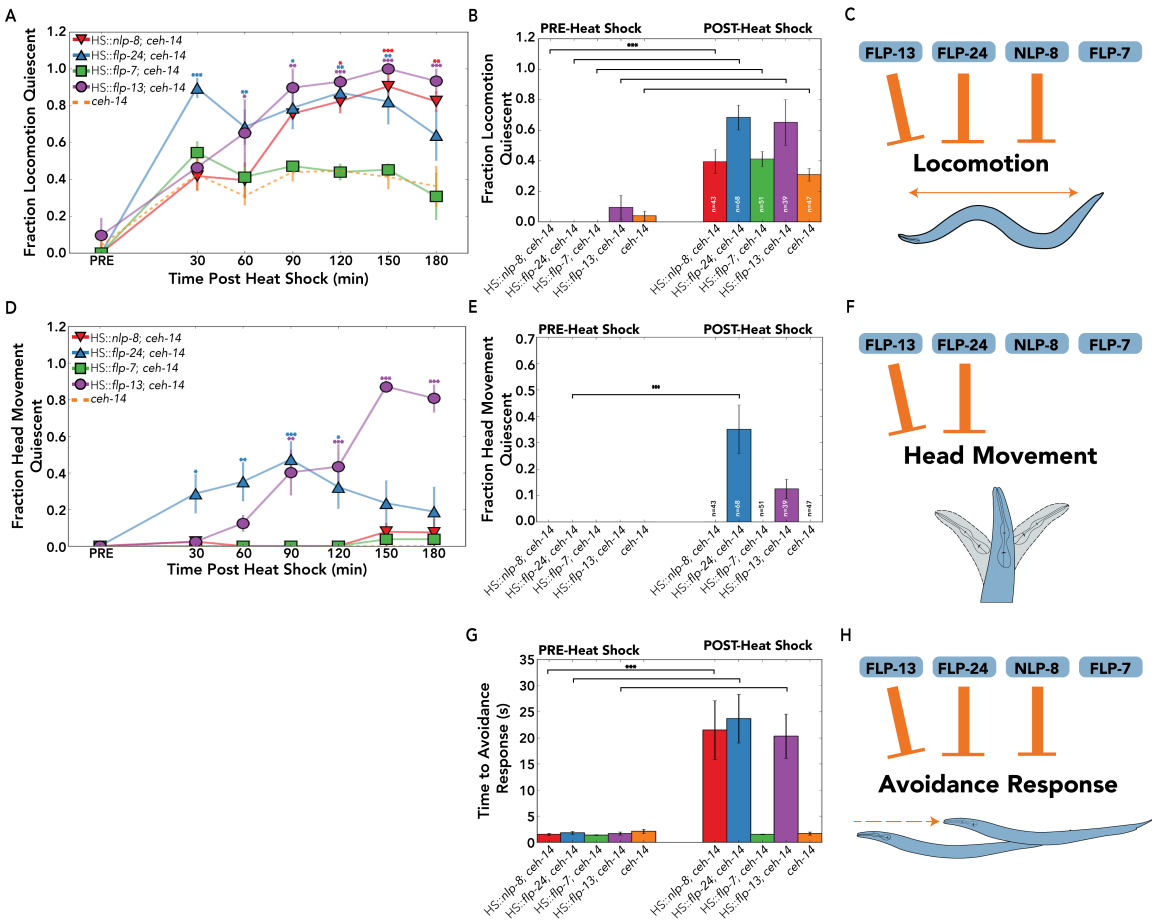

Figure 3.6: Overexpression of either FLP-13, FLP-24, or NLP-8 inhibit movement and the avoidance response. (**A**) & (**D**) Time-course monitoring the fraction of *C. elegans* that were locomotion and head-movement quiescent before (PRE) and up to three hours after (POST) heat shock induced neuropeptide overexpression. *C. elegans* were scored as locomotion quiescent if there was no centroid movement during 10 seconds of observation. *C. elegans* were scored as head movement quiescent if there was no dorsal-ventral displacement of the worm's head from the posterior of the second pharyngeal bulb to the anterior tip during 10 seconds of observation. (**B**) & (**E**) Fraction of *C. elegans* locomotion and head movement quiescent pre- and one hour post-heat shock. (**C**) Overexpression of either *flp-24*, *flp-13*, or *nlp-8*, but not *flp-7* inhibited locomotion. (**F**) Overexpression of either *flp-24* or *flp-13*, but neither *flp-7* nor *nlp-8* inhibited head movement. (**G**) *C. elegans* were presented with 30% 1-octanol and avoidance behavior was scored by video recordings before (PRE) and one hour after heat shock (POST)

induced neuropeptide overexpression. If there was no response 60 seconds after stimulus delivery, the individuals were classified as non-responsive (**Table 3.4**). (**H**) Overexpression of either *flp-24*, *flp-13*, or *nlp-8* strongly increased the response latency to aversive stimuli, while *flp-7* overexpression did not. (**A-B**) & (**D-E**) Data represents the fraction of animals quiescent from three or more independent assays, where n=total number of *C. elegans*. Data shown as mean±SEM; *p<0.05; **p<0.01; ***p<0.001; for (**A**) & (**D**) two-way ANOVA comparing transgenic strains to *ceh-14* mutants with four post-hoc contrast using Bonferroni correction for multiple comparisons. (**B**) & (**E**) Fisher's exact test. (**G**) Data shown as mean±SEM; n=11 *C. elegans* for each strain (see also **Table 3.4**); ***p<0.001; unpaired t-test with Bonferroni correction for multiple comparisons.

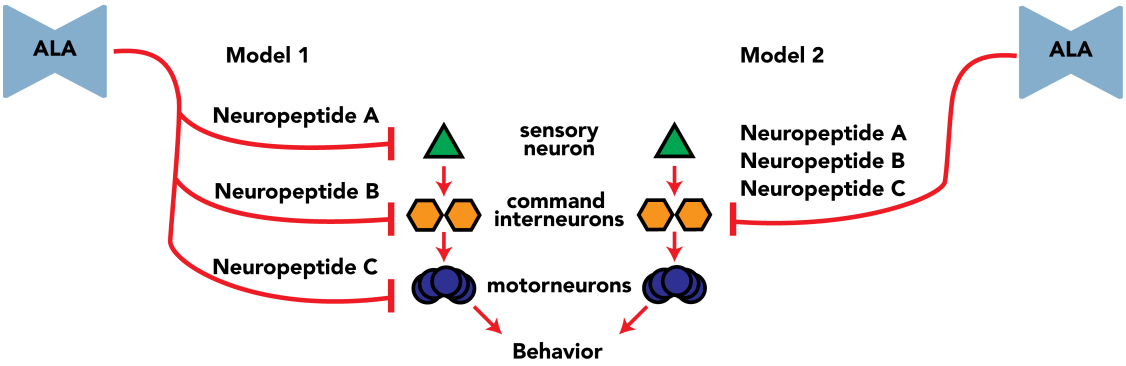

Figure 3.7: Redundancy models for the collective action of multiple neuropeptides which regulate *C. elegans* stress-induced sleep. The neurosecretory ALA is required for stress-induced sleep. ALA transcribes multiple genes encoding neuropeptides, and we have shown that three neuropeptides enriched in ALA collectively regulate *C. elegans* stress-induced sleep. Given the non-overlapping expression pattern of these neuropeptides in other neurons, it is also possible that these neuropeptides act from neurons which have a minor role in regulating stress-induced sleep. In Model 1 each neuropeptide acts on a distinct neuron within a set of neurons that regulates behavior. In Model 2 each neuropeptide acts on the same neuron within a set of neurons that regulates behavior. The principles of Model 1 and 2 also apply at the receptor and behavioral level. For instance, each neuropeptide may act at a distinct receptor, or all the neuropeptides may act on the same receptor. In addition, strong inhibition of one behavior may inhibit all other behaviors and result in sleep, or there may be shut down of multiple behaviors simultaneously. We predict that these neuropeptides regulate *C. elegans* stress-induced sleep by some combination of Model 1 and 2 at the cellular, receptor, and behavioral level.

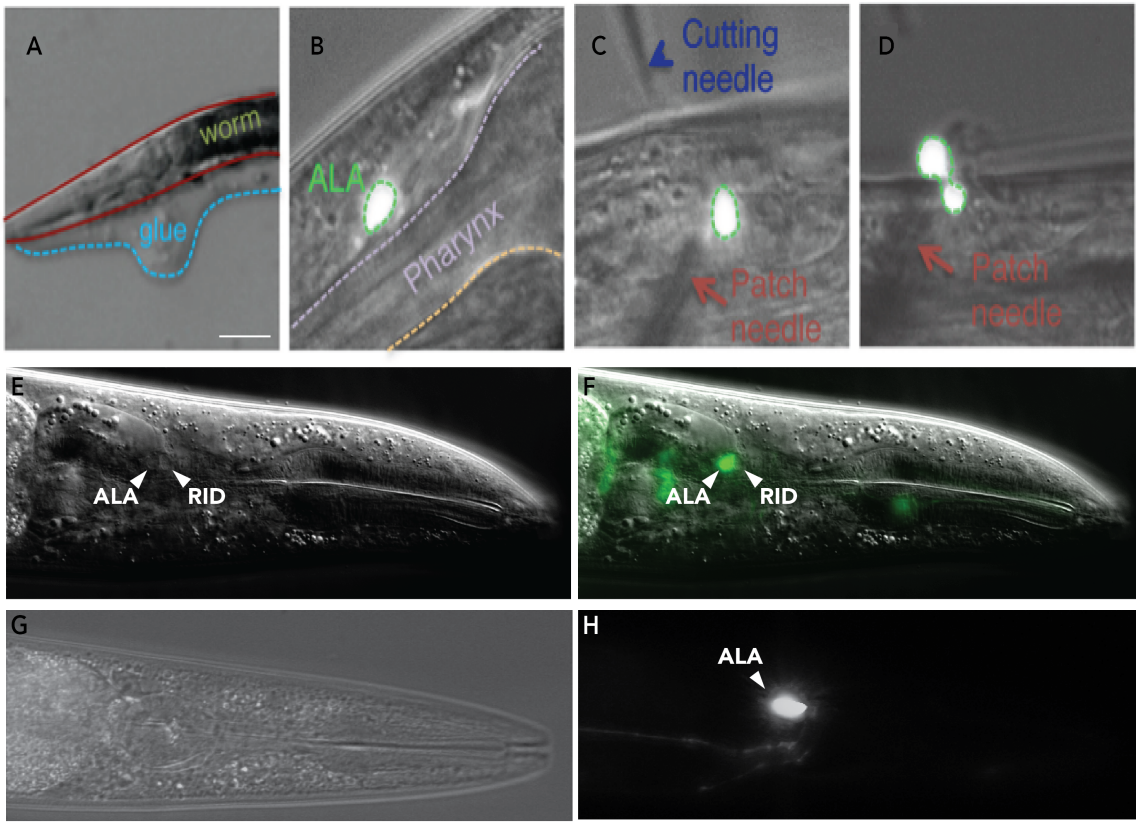

Figure 3.8: *flp-24* and *nlp-8* are expressed in ALA. (**A-D**) Microdissection of the ALA neuron from mid-L4 larva. (**A**) A mid-L4 larvae (labeled "worm") was attached to a freshly made agar pad with dental glue along the ventral bodyline [92]. (**B**) The ALA neuron was identified as dorsal to the pharyngeal isthmus and labeled with the *ceh-14* promoter driving GFP expression in the ALA neuron (green circle), the only dorsal head neuron expressing $P_{ceh\text{-}14}$*::gfp*. (**C**) A fine glass cutting needle (blue arrowhead) was used to cut open the dorsal worm body close to the vulva to release body pressure (not shown), and a small puncture was made in the dorsal head just big enough to release the ALA neuron. (**D**) A glass patch needle (red arrow) was used to collect the released ALA neuron. (**E**) DIC image of the ALA and RID neurons (white arrows). As previously reported, ALA is posterior to RID [89]. (**F**) Overlay of *nlp-8 gfp* reporter expression on DIC image. *nlp-8* is expressed in ALA, as well as other neurons in the head [97]. (**G**) Bright-field image of the ALA neuron. (**H**) *flp-24 gfp* reporter expression in ALA (white arrow) was indicated by GFP in young adult *C. elegans*. The ALA neuron is located

dorsal to the pharynx between the anterior and posterior pharyngeal bulbs. Anterior is right. Dorsal is up. Scale bar represents 20 μm.

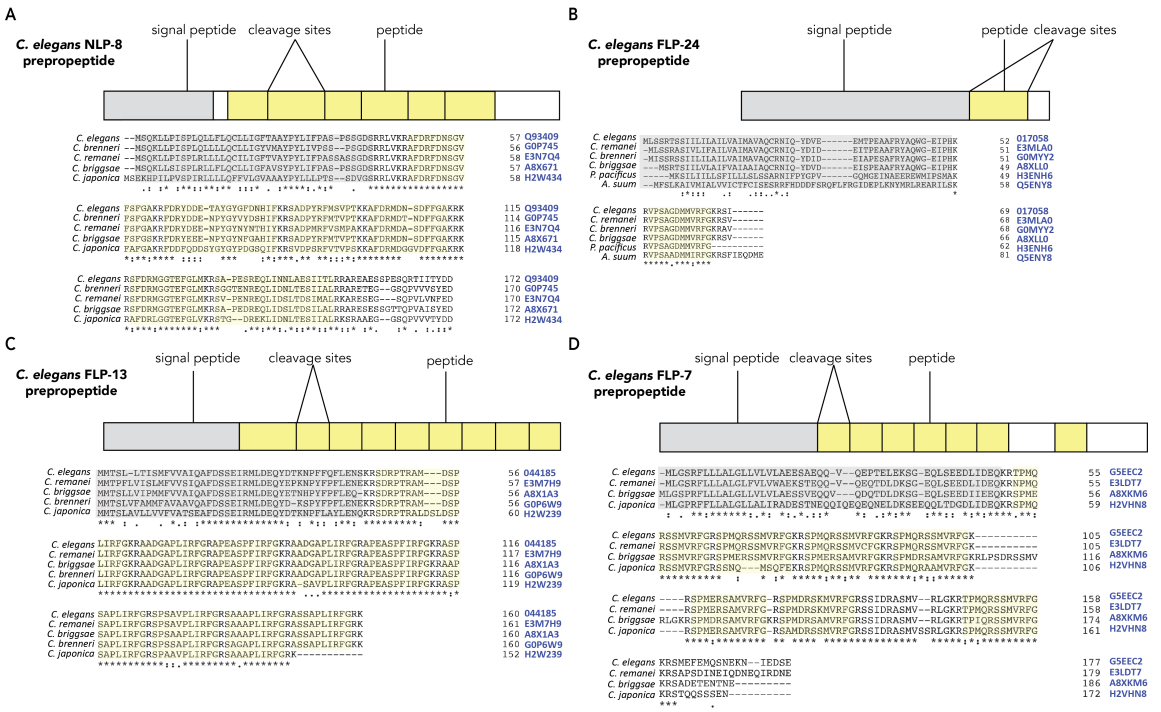

Figure 3.9: Propeptides of NLP-8, FLP-24, FLP-13, and FLP-7. (**A**) *nlp-8* encodes a propeptide that generates six candidate mature neuropeptides (P1-P6). Previous publications indicated P1, P4, and P5 as candidate peptides [97]. We propose that it is also possible for P2, P3, and P6 to serve as neuropeptides. Shown are amino acid sequences of NLP-8 in nematodes: *Caenorhabditis elegans* (Q93409), *Caenorhabditis brenneri* (G0P745), *Caenorhabditis remanei* (E3N7Q4), *Caenorhabditis briggsae* (A8X671), and *Caenorhabditis japonica* (H2W434). (**B**) *flp-24* encodes a propeptide that generates one mature neuropeptide. Shown are amino acid sequences of FLP-24 in nematodes: *Caenorhabditis elegans* (017058), *Caenorhabditis remanei* (E3MLA0), *Caenorhabditis brenneri* (G0MYY2), *Caenorhabditis briggsae* (A8XLL0), *Pristionchus pacificus* (H3ENH6), and *Ascaris suum* (Q5ENY8). (**C**) *flp-13* encodes a propeptide that generates nine mature neuropeptides (P1-P9): P2 and P4, P3 and P5 are repeated copies. Shown are amino acid sequences of FLP-13 in nematodes: *Caenorhabditis elegans* (O44185), *Caenorhabditis remanei* (E3M7H9), *Caenorhabditis briggsae* (A8X1A3), *Caenorhabditis brenneri* (G0P6W9), and *Caenorhabditis japonica* (H2W239). (**D**) *flp-7* encodes a propeptide that generates seven mature neuropeptides (P1-P7): P1 and P7 are repeated copies, and P2, P3, and P4 are also repeated copies, in confirmation of previous

work [96]. Shown are amino acid sequences of FLP-7 in nematodes: *Caenorhabditis elegans* (G5EEC2), *Caenorhabditis remanei* (E3LDT7), *Caenorhabditis briggsae* (A8XKM6), and *Caenorhabditis japonica* (H2VHN8). Neuropeptide annotation and sequence alignment were conducted via the *www.uniprot.org* alignment web server. Signal peptide: grey box; cleavage site: horizontal black line; neuropeptide: yellow boxes. "*" fully conserved residue, ":"strongly similar properties, and "." weakly similar properties.

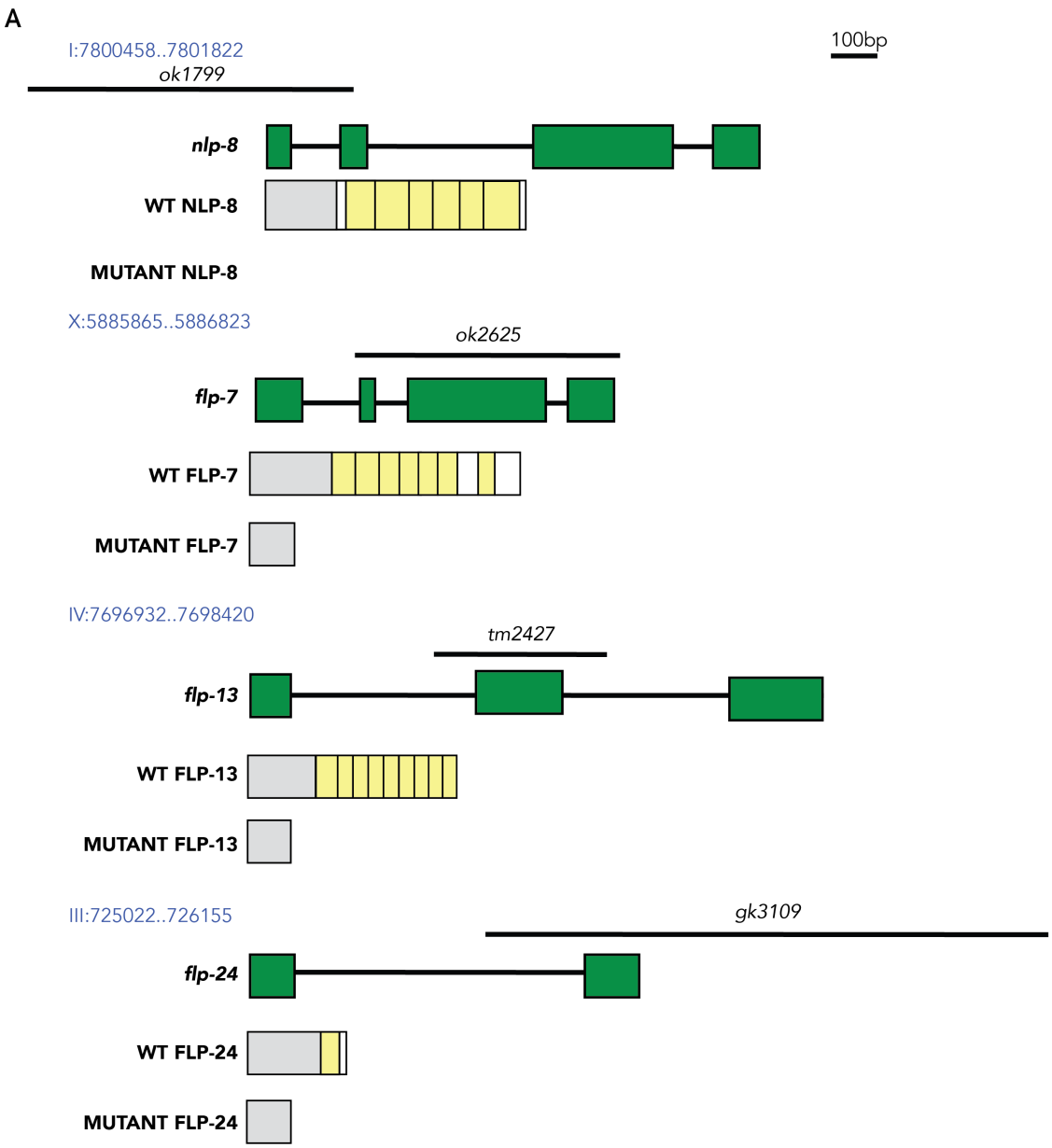

Figure 3.10: Gene model with deletions in mutant alleles. Shown are gene models of *nlp-8, flp-7, flp-13*, and *flp-24* indicating the positions of the relevant deletion mutations, along with the structures of the wild-type proteins and the predicted mutant proteins, with domains annotated as in **Figure 3.9**. Horizontal black bars labeled with allele numbers indicate genomic deletions and green blocks represent exons of coding genes. Grey boxes indicate signal peptide and yellow boxes indicate mature neuropeptides. Orientation of genes and protein structures are 5' to 3', and N-terminal to C-terminal, respectively. Genomic positions are provided for each gene in blue.

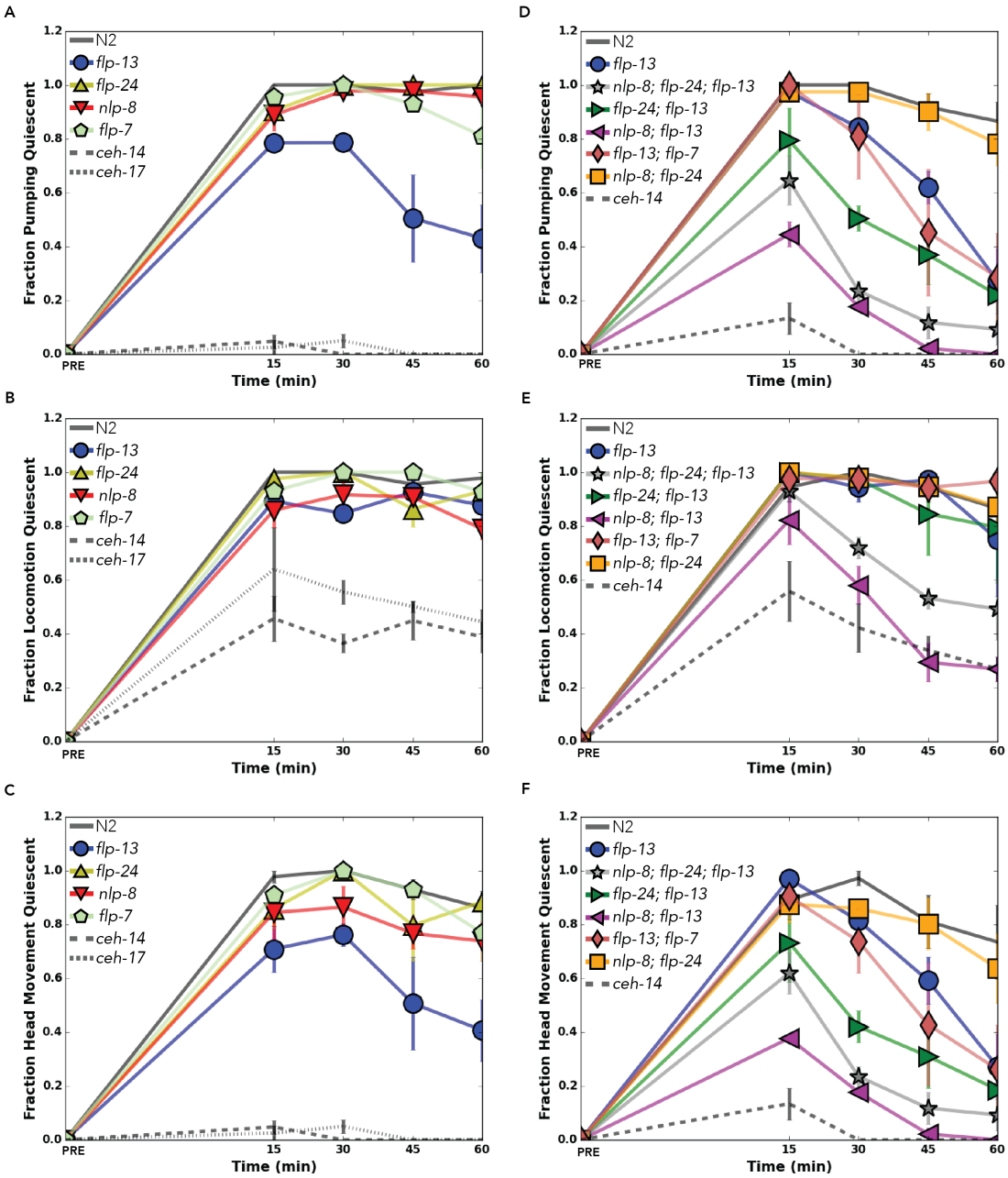

Figure 3.11: Variability in waking times post-heat shock of single, double, and triple neuropeptide mutants. (**A-F**) The fraction of single, double, and triple mutants pumping, locomotion, and head movement quiescent before (PRE) and up to one hour after heat shock. Behavior was scored at 15 minute intervals after heat shock. (**A**) & (**D**) *C. elegans* were scored as quiescent for pumping if there was no pumping during 10 seconds of observation. (**B**) & (**E**) *C. elegans* were scored as quiescent for locomotion if

there was no centroid movement during 10 seconds of observation. (**C**) & (**F**) *C. elegans* were scored as quiescent for head movement if there was no head movement in the dorsal-ventral directions during 10 seconds of observation. Data represents the fraction of animals quiescent from three independent assays, where n≥33 *C. elegans* for each strain. Data shown as mean ± SEM.

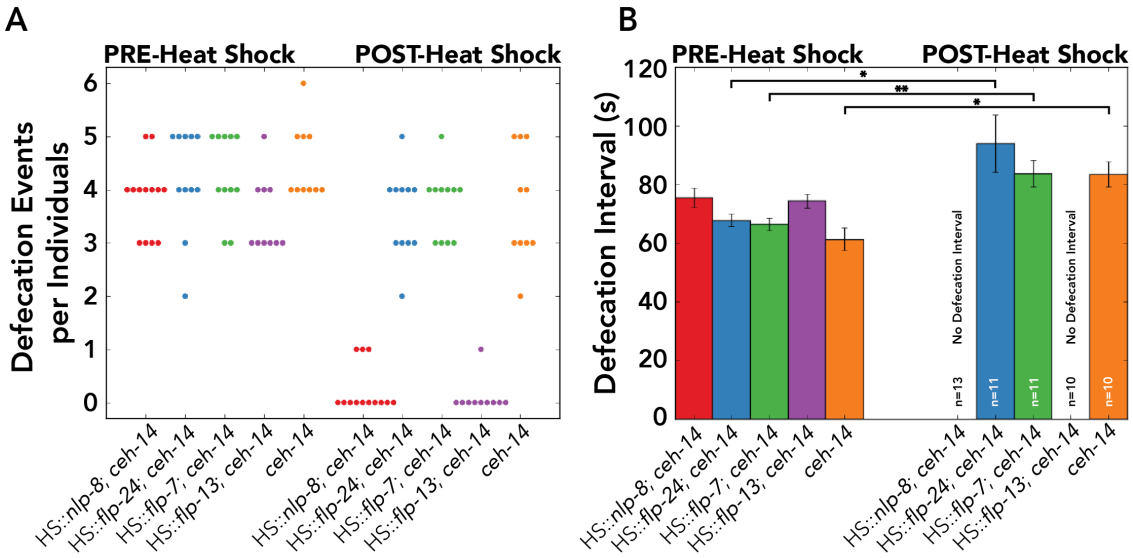

Figure 3.12: FLP-13 and NLP-8 reduce the total number of defecation events. (**A**) Total number of defecation events for individuals five minutes before (PRE) and one hour after (POST) heat shock. Each dot represents the total number of defecation events for an individual during five minutes of observation. Overexpression of either *flp-13* or *nlp-8* inhibited defecation. (**B**) The time between defecation events represents the defecation interval. *ceh-14* animals exhibited a longer defecation interval post-heat shock compared to pre-heat shock (*ceh-14*: 61.3±3.9 seconds pre-heat shock compared to 83.5±4.3 seconds post-heat shock). No significant difference was observed post-heat shock between *ceh-14* and animals overexpressing *flp-24* or *flp-7* (HS*::flp-24, ceh-14*: 94.0±9.7 seconds post-heat shock; HS*::flp-7, ceh-14*: 83.7±4.5 seconds post-heat shock; compared to *ceh-14*: 83.5±4.3 seconds post-heat shock; $p \geq 0.4$). (**B**) Data shown as mean±SEM; n=total number of *C. elegans*; *p<0.05; **p<0.01; paired t-test.

	Locomotion	Head Movement	Pumping
N2	-	-	-
flp-13	-	+	+
flp-24	-	-	-
nlp-8	-	-	-
flp-7	-	-	-
flp-24; flp-13	-	++	++
nlp-8; *flp-13*	+++	+++	+++
nlp-8; *flp-24*; *flp-13*	+++	+++	+++
flp-13; *flp-7*	-	+	+
nlp-8; *flp-24*	-	-	-
ceh-17	+++	+++	+++
ceh-14	+++	+++	+++

Table 3.1: Summary of neuropeptide loss-of-function results for locomotion, head movement, and pumping. Degree of suppression of locomotion, head movement, and pumping behavior indicated: "+" weak suppression, "++" moderate suppression, and "+++" strong suppression.

Worm	**N2**	***flp-13***	***flp-24***	***nlp-8***	***flp-7***	***flp-24*; *flp-13***	***nlp-8*; *flp-13***	***nlp-8*; *flp-24*; *flp-13***	***flp-13; flp-7***	***nlp-8*; *flp-24***	***ceh-17***	***ceh-14***
1	14.7	**NR**	18.8	4.9	51.8	**NR**	7.3	16	1.5	38.5	1.2	3.4
2	12.0	12.3	45.0	2.4	10.1	14.3	14.2	1.0	17.1	15.8	2.0	1.0
3	16.0	50.5	40.9	18.6	14.4	16.2	11.4	1.4	29.0	26.0	1.0	2.2
4	16.0	1.0	15.2	28.8	**NR**	1.4	1.2	12.0	8.6	38.5	2.0	1.8
5	18.4	34.0	36.4	26.2	18.4	7.2	1.4	1.1	**NR**	1.0	8.5	1.9
6	26.3	14.9	11.6	8.6	**NR**	1.0	2.2	3.6	**NR**	13.7	2.7	16.6
7	33.1	**NR**	9.5	26.6	38.0	1.2	27.6	12.8	7.2	24.4	1.2	1.7
8	20.0	7.5	8.0	11.0	**NR**	11.1	9.1	3.8	24.9	7.3	11.5	2.5
9	31.3	2.9	6.4	21.0	9.0	1.7	2.4	1.7	**NR**	21.0	31.6	1.0
10	9.5	9.3	18.7	8.8	20.0	14.5	1.1	1.6	2.0	8.0	2.0	4.0

Table 3.2: Summary of neuropeptide loss-of-function results for avoidance. Avoidance response times of individuals to 30% 1-octanol 30 minutes after heat shock. NR = No response.

Worm	**N2**	***flp-13***	***flp-24***	***nlp-8***	***flp-7***	***flp-24*; *flp-13***	***nlp-8*; *flp-13***	***nlp-8*; *flp-24*; *flp-13***	***flp-13; flp-7***	***nlp-8*; *flp-24***	***ceh-17***	***ceh-14***
1	0	0	0	0	0	1	0	4	0	0	0	3
2	0	0	0	0	0	0	0	0	0	0	1	0
3	0	0	0	0	0	0	0	4	0	0	3	1
4	0	0	0	0	0	4	0	3	0	0	3	0
5	0	0	0	0	0	0	0	4	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	2
7	0	0	0	0	0	0	0	4	0	0	0	3
8	0	0	3	0	0	0	0	4	0	0	0	3
9	0	0	0	0	0	2	0	0	1	0	1	0
10	0	0	0	0	0	0	0	0	0	0	3	4
11	3					0	0	3			4	3
12	0					0	0	0			3	0
13	0					0	0	0			3	0
14	0					0	0	0			2	4
15	0					0	3	0			2	4
16	0					0	2	0			0	4
17	0					0	0	1			0	3
18	0					0	0	0			0	3
19	0					0	0	0			0	2
20	0						0	0				
21	3						0	4				
22	0						3	0				
23	0						0	5				
24	0						0	0				
25							0	0				
26							0	3				
27								3				
28								0				
29								4				
30								3				

Table 3.3: Summary of neuropeptide loss-of-function results for defecation. Number of defecation events for individuals during five minutes of observation 30 minutes after heat shock-induced sleep.

Worm	HS::*nlp-8; ceh-14*	HS::*flp-24; ceh-14*	HS::*flp-7*; *ceh-14*	HS:: *flp-13; ceh-14*	*ceh-14*
1	46.8	8.8	1.2	**No Response**	3.0
2	**No Response**	8.2	1.5	**No Response**	1.5
3	**No Response**	17.3	1.0	**No Response**	1.0
4	1.4	42.8	1.5	14.4	2.2
5	22.0	44.0	1.3	**No Response**	1.0
6	23.7	40.0	2.0	**No Response**	4.0
7	22.6	23.1	1.5	26.3	1.2
8	**No Response**	**No Response**	2.0	**No Response**	1.2
9	12.3	10.8	1.7	**No Response**	0.9
10	**No Response**	**No Response**	1.4	**No Response**	1.0
11	**No Response**	18.2	1.5	**No Response**	1.5

Table 3.4: Time required for an avoidance response and number of non-responders after overexpression of FLP-24, FLP-7, FLP-13, and NLP-8. Avoidance response times of individuals to 30% 1-octanol one hour after heat shock induced overexpression of neuropeptide genes.

Methods

Single ALA neuron dissection and transcriptome profiling

Individuals from strain TB513 ($P_{ceh\text{-}14}$*::gfp*) at the mid-L4 larval stage were hand-picked and glued on an agar pad for microdissection as previously described [92] using the approach of Lockery and Goodman [107] for neuronal dissection. GFP-tagged ALA neurons were individually collected with an unpolished patch-clamp tube that served as a pipette, transferred to a prelubricated microcentrifuge tube (**Figure 3.8A-D**), and snap-frozen with liquid nitrogen. Frozen tubes containing individual ALA neurons were kept at -70°C until their RNA was amplified as described by Schwarz et al. [92], using the approach of Dulac and Axel [108]. RT-PCR, RNA-seq, and computational analysis of individual neurons were done as in Schwarz et al. [92]. To obtain RNA-seq data by Illumina sequencing, aliquots of RT-PCR from individual cells were collected into two pools (four cells and five cells). All RNA-seq reads were single-end, and originally 50 nt in length. Raw reads were quality-filtered as in Schwarz et al. [92]. They were then truncated *in silico* from 50 nt to 38 nt, the read length for previously published control data from mixed-stage whole larvae [92]. This truncation allowed the ALA reads to be mapped and quantitated using exactly the same pipeline that had been used for larval data, and thus allowed more exact comparisons between ALA and larvae. After quality filtering and truncation but before mapping, RNA-seq data from the two pools of wild-type ALA comprised 1,164,892,280 nt in 30,655,060 reads and 1,520,526,262 nt in 40,013,849 reads. Of these, 25.2% could be mapped to WS190 protein-coding gene models (i.e., 17,798,207 out of 70,668,909 reads). This relatively low rate is consistent with our previous observations in single-cell RNA-seq of linker cells, in which we found that human cDNA (probably acquired as human RNA during the manual dissection of individual *C. elegans* cells), linkers, and unmappable reads composed a significant fraction of the final RT-PCR products [92]. We used existing whole wild-type larval RNA-seq data [92] as controls for housekeeping versus ALA-enriched genes. Expression values for genes were computed as in Schwarz et al. [92]. They were defined by pooling

reads from both mid-L4 ALA neuron sets into a single set of expression values, doing likewise for both whole-animal mixed-stage larval RNA-seq sets from Schwarz et al. [92], and computing ALA/larval ratios of gene activity. We detected expression of 7,698 and 4,068 genes in the two ALA pools separately, and 8,133 genes collectively.

Data Availability

RNA-seq reads for the two pools of wild-type mid-L4 ALA neurons are available in the NCBI Sequence Read Archive (SRA), under accession number SRA: SRP038903 (*http://www.ncbi.nlm.nih.gov/sra/SRP038903*). RNA-seq reads for the two pools of whole *C. elegans* mixed-stage wild-type N2 larvae were previously published by Schwarz et al. [92], and are available in the NCBI SRA under accession number SRA: SRA058596 (*http://www.ncbi.nlm.nih.gov/sra/SRA058596*).

Strains

Wild-type *C. elegans* strain was N2 (Bristol). Mutant strains obtained from the *Caenorhabditis* Genetics Center (CGC) including RB1990 *flp-7(ok2625) X*, and VC1309 *nlp-8* (*ok1799*) *I* were provided by the *C. elegans* Gene Knockout Project at OMRF (*http://www.mutantfactory.ouhsc.edu*). VC1971 *flp-24(gk3109) III* was provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, part of the *C. elegans* Gene KO Consortium (*http://www.celeganskoconsortium.omrf.org*). Strain FX02427 *flp-13 (tm2427) IV* was obtained from the National Bioresource Project (*http://www.shigen.nig.ac.jp/c.elegans/mutants/).* Extrachromosomal arrays were *rtEx227* (P_{nlp-8}::*gfp*) [97], and transgenes generated in the course of this study, described below**.**

Mutant Strains and Alleles

PS6813: *flp-13(tm2427)* made from FX02427, outcrossed 3X

PS6814: *flp-24(gk3109)* made from VC1971, outcrossed 5X

PS6911: *nlp-8*(*ok1799*) made from VC1309, outcrossed 2X

RB1990: *flp-7(ok2625)*

TB528: *ceh-14(ch3)*. All references to *ceh-14* mutants refer to this allele

PS6991: *nlp-8*(*ok1799*); *flp-24*(*gk3109*) made from PS6911, PS6814

PS6994: *flp-24*(*gk3109*); *flp-13*(*tm2427*) made from PS6814, PS6813

PS6993: *nlp-8*(*ok1799*); *flp-13*(*tm2427*) made from PS6911, PS6813

PS6992: *nlp-8*(*ok1799*); *flp-24*(*gk3109*); *flp-13*(*tm2427*) made from PS6994, PS6993

PS7084: *flp-13*(*tm2427*); *flp-7(ok2625)* made from PS6813, RB1990

Transgenic Lines

Heat-shock transgenic strains:

Conditional expression of cDNAs was achieved by generating a fusion of the coding sequence of a gene under study to the *hsp-16.41* promoter [109]. A synthetic DNA fragment consisting of the *hsp-16.41* promoter, DNA coding sequence, and each gene's endogenous 3'-UTR was generated using fusion PCR [110]. For amplification of the coding sequence (*flp*-7, *flp-13, flp-24*, and *nlp-8* open reading frames), mixed-stage populations of wild-type animals were harvested for RNA extraction and subsequently reverse-transcribed into cDNA as previously described [92]. Their corresponding 3'-UTR regions were amplified from wild-type mixed stage animal lysates. The *hsp-16.41* [109] promoter region was amplified from plasmid pPD49.83 (AddGene). The fusion PCR product was verified by sequencing. Open reading frames and 3'-UTRs match the sequences of spliced transcripts as shown in WormBase (WS252). These constructs were injected with $P_{myo\text{-}2}$*::dsRed* as a co-injection marker and bluescript (KS+, Agilent) as a

carrier for construction of extrachromosomal arrays [111], using the concentrations as indicated below.

Reporter Expression Transgenic strains:

PT4: *him-5(e1490); lin-15(n765); rtEx227[lin-15(+), P_{nlp-8}::gfp]*. {Nathoo, 2001 #106}

PS6896: *unc-119 (ed3); syEx1422[P_{flp-24}::GFP(25ng/ul), P_{ver-3}::mCherry (25ng/ul), unc-119(+) (50ng/ul)]*. [112]

TB513: *dpy-20(e2017);chIs513[P_{ceh-14}::GFP, dpy-20(+)]*. [113]

Heat-shock transgenic strains:

PS6835: *syEx1404SP$_{hsp16-41}$::flp-13(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6563: *syEx1286[P $_{hsp16-41}$::flp-24(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6571: *syEx1294[$P_{hsp16-41}$::flp-7(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6658: *syEx1323[$P_{hsp16-41}$::nlp-8(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

Heat-shock transgenic strains in *ceh-14* background

PS6845: *ceh-14(ch3)*; *syEx1404[$P_{hsp16-41}$::flp-13(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6829: *ceh-14(ch3)*; *syEx1286[$P_{hsp16-41}$::flp-24(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6856: *ceh-14(ch3); syEx1294[$P_{hsp16-41}$::flp-7(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

PS6830: *ceh-14(ch3); syEx1323[$P_{hsp16-41}$::nlp-8(10ng/ul), P_{myo-2}::dsRed(10ng/ul), KS+(90ng/ul)]*.

DNA Fragment	Forward primer 5' to 3'	Reverse primer 5' to 3'
flp-24 promoter	ACGCCTAACGCATGCCTCTTAC	AAAAGGCGCGCCCGATGTGCGCG ACGACAACAT
Phsp-16.41	ACGTTGAGCTGGACGGAAAT	GCTAGCCAAGGGTCCTCCT
flp-7 ORF	AGGAGGACCCTTGGCTAGC ATGCTTGGATCCCGCTTC	TCAAGGTGTTTGCATGTACTTGTT TATTCGCTGTCCTCGATGTTC
flp-7 3' UTR	GAACATCGAGGACAGCGAATAAACAA GTACATGCAAACACCTTGA	AACAGGCGTCGGTTCTTTATTT
flp-13 ORF	AGGAGGACCCTTGGCTAGCATGATGA CGTCACTGCTCACT	TTATTTTCTGCCAAAACGAATG
flp-13 3' UTR	CATTCGTTTTGGCAGAAAATAAATTCA CTTTTTGATCTTTCTTTGTGTG	CCGGATAGAACAATTCATTTTTGT GAA
flp-24 ORF	AGGAGGACCCTTGGCTAGCATGTTGTC GTCGCGCACATCGTCCATCAT	TCAGATGCTTCTTTTTCCAAATC
flp-24 3' UTR	CGATTTGGAAAAAGAAGCATCTGATAA TATACCATCTACCGGACTTCTTAT	TTTAACACACACAAAACGGTTTAT TTCTGTT
nlp-8 ORF	AGGAGGACCCTTGGCTAGCACAAAAG CGACATGAGTCAGAA	CAACGAACAATCATCACCTATGAC GATTGA
nlp-8 3' UTR	CACCTATGACGATTGAACTTCTTGAACA ACTGG	AAATGTCAGATTTTATTCACAAAC G

Behavioral assays

Behaviors were scored at 20ºC. Sixteen hours before the start of the experiment, L4 larvae were picked so that only the behaviors of young adult animals were scored. Unless otherwise noted, between 10 and 25 animals were scored per assay. Pumping was conservatively scored as any movement of the pharyngeal grinder. Locomotion was scored as movement of the animal's centroid in the forward or reverse direction. Head movement was scored as any dorsal-ventral displacement of the animal's head from the posterior of the second pharyngeal bulb to the anterior tip. Pumping quiescence, locomotion quiescence, and head movement quiescence were scored by 10 seconds of direct observation of individual animals by an experimentalist that was blinded to genotype. Machine vision underestimates movement, and may conflate head movement

and locomotion. Our experimental design, which used a motorized stage, also eliminated handling artifacts like dish-tap or transfer of animals that would otherwise confound experiments.

Defecation was scored as follows: individuals were placed onto a tracking microscope with 5x magnification for 5 minutes of video recording. Immediately after these recordings, worm pumping rate was scored by placement on another dissecting microscope with 55x magnification, and 10 seconds of video recording were taken. Defecation and pumping rate were manually scored by examining the 5 minute and 10 second video recordings respectively. This was done pre- and post-heat shock.

Avoidance behavior was scored as follows: individuals were placed onto a fresh and thinly seeded plate: 20 µl of saturated OP50 was spread evenly around the plate 16 hours before the experiment. Video recordings were taken on a (5x) tracking microscope. Individuals were presented with 30% 1-octanol before and one-hour after heat shock [10, 98]. The response interval was manually scored by examining video recordings made pre- and post-heat shock. While scoring defecation and avoidance the experimentalist was not blinded to genotype.

Heat shock protocol

For all behaviors, unless otherwise specified: animals were placed onto a Petri plate containing 9 mL of NGM that was seeded only in the middle with 50 µl of saturated OP50 in LB, behaviors were scored before heat shock, and after heat shock at specified times. For all behaviors, only those animals on the OP50 lawn were scored. Petri plates were coded by a third party unless otherwise specified. Coded Petri plates were placed on a motorized stage to eliminate dish-tap and other behavior-modifying handling. The lid was taken off to prevent condensation, and obstruction of the view, but another glass was placed 2.3 cm above the plate so that gusts of wind did not affect behavior. Behavior was scored in the five minutes before heat shock. Parafilm was placed around the dish to create a waterproof seal.

Protocol for stress-induced sleep

Heat shock was used for our stress-induced sleep experiments. In particular, sealed plates were placed in a 35°C water bath for 30 minutes as in Hill et al. [41, 44]. We found this temperature and length of heat shock most consistent for pumping quiescence, head movement quiescence, locomotion quiescence, and timing of the increased response latency. After heat shock (POST), plates were immediately placed on the motorized stage as before heat shock (PRE). In particular, the Petri dish lids were taken off, and replaced with a shielding glass. The plates were not touched for the next 60 minutes, as a motorized stage was used to prevent dish-tap artifacts. Extensive handling of animals could lead to inconsistent results. If a motorized stage is unavailable then we suggest placing Post-heat shock plates on a large glass-slide which rests on a dissecting scope. In this case, the large glass-slide could be gently moved and the behavior of individuals could be scored (this should minimize handling).

Pumping, locomotion, and head movement were scored at 15-minute intervals for 60 minutes after heat shock. We found 30 minutes after heat shock to be the most robust and consistent time point for stress-induced sleep. Avoidance behavior was scored only at 30 minutes. To score defecation events we used a 33°C water bath for 30 minutes. This protocol gave us the most consistent results and best dynamic range between N2 and *ceh-14* or *ceh-17* (**Figure 3.3**; **Table 3.3**). We scored defecation behavior 30 minutes after heat shock.

Our stress induced-sleep protocol differs from Nelson et al. [44] in a number of ways. We handle the animals less and we concentrate on a single robust and consistent time point after heat shock (30 minutes). Further in regards to the difference in our locomotion result (**Figure 3.2**), we employ different methods of scoring locomotion than Nelson et al.: 1) we differentiate between head movement and locomotion, and 2) we concentrate on one time point after heat shock (30 minutes) rather than reporting the total time quiescent one hour after heat shock. We think that these two reasons, in addition to

different methods of handling and strength of heat shock, may account for differences in our results.

Heat shock-induced neuropeptide overexpression

Sealed plates were placed in a 33°C water bath for 30 minutes [42, 44]. After heat shock (POST), condensation was removed from the top lid, and they were placed on the lab bench agar-side up; this was done at 20°C. After 20 minutes, the plates were placed agar-side down on a motorized stage as before heat shock (PRE). In particular, the Petri dish lids were taken off, and replaced with a shielding glass. The plates were not touched for the next 160 minutes. Our overexpression experiment uses extrachromosomal arrays which are expressed in many cells, and we assume that these cells have the machinery necessary to process the neuropeptides. We also assume that these peptides reach the right target in the right amount. It is unknown if results from these experiments are hypermorphic or neomorphic. Pumping, locomotion, and head movement was scored at 30 minute intervals for 3 hours after heat shock (POST). Avoidance and defecation were scored one hour after heat shock (POST).

C h a p t e r I V

TACHYKININ PROMOTES SLEEP IN *C. ELEGANS*

Abstract

A constitutive overexpression approach was used to dissect the function of *C. elegans* neuropeptide-like protein eight (*nlp-8*) gene, which has been implicated in *C. elegans* sleep regulation. *nlp-8* is a neuropeptide gene, encoding a prepropeptide that after post-translational processing gives as many as five functional neuropeptides. Overexpression of the *nlp-8* gene induced locomotion quiescence and a modified sensory response reminiscent of *C. elegans* sleep phenotype. We discovered that a single neuropeptide encoded by *nlp-8* (*nlp-8* Peptide 3) has the strongest sleep inducing effect. The C terminus of *nlp-8* Peptide 3 is homologous to vertebrate tachykinin. Functional conservation was tested by overexpressing a Human tachykinin neuropeptide, Substance P, in *C. elegans*. These experiments demonstrated that Human Substance P induces locomotion quiescence in *C. elegans*, and also demonstrates that the –FGLM C terminus of *nlp-8* peptide 3 serves as the key domain for inducing locomotion quiescence. Given that tachykinin signaling has been shown to promotes sleep in Mice, tachykinin signaling may represent an ancient and conserved pathway for sleep regulation.

Introduction

Sleep is a genetically-encoded behavioral state [31, 67, 80, 81]. Neuropeptides are one major class of molecules that regulate sleep across the animal kingdom [21]. Neuropeptide-encoding genes have the potential to evolve over time to maintain, remove, or add new functionalities to the sleep state. Those neuropeptides with conserved function represent important sleep regulators. Here we tested if tachykinin neuropeptides are sufficient to induce sleep in *C. elegans*.

Sleep has been observed in any closely investigated animal [1-3]. There are several molecules that are conserved sleep regulators throughout the animal kingdom: clock gene

peptide, cyclic AMP, dopamine, and adenosine [1, 3, 5, 67, 81]. There are several neuropeptides that are conserved within the phylum Chordata (fish, mice, humans): orexin, epinephrine, and neuromendin U [7, 21, 114, 115]. However, there are only a handful of examples of neuropeptides conserved between invertebrate and vertebrate animals. One such vertebrate to invertebrate conserved signaling pathway for sleep regulation includes norepinephrine/octopamine [3]. We wanted to determine if there are other neuropeptides that regulate sleep across phyla. We began our study by dissecting a neuropeptide recently demonstrated to regulate *C. elegans* stress-induced sleep (SIS)[41, 43].

C. elegans SIS is regulated by a single neurosecretory cell (the ALA) which releases a cocktail of neuropeptides that shuts down various distinct behaviors [41-43, 89]. Previously, we determined that SIS is primarily regulated by three neuropeptide genes (*flp-13*, *flp-24*, *nlp-8*) [43]. The products of these three genes fall into two neuropeptide families. Neuropeptide families are classified by sequence homology of the peptide's C terminus, as this region is important for function [37, 96, 97, 106]. *flp-24* and *flp-13* are members of the –RFamide neuropeptide family [95, 96], while *nlp-8* is a member of the tachykinin neuropeptide family [97, 116, 117]. Recent work demonstrated that RFamides regulate sleep in *Drosophila* [48] and Zebrafish [49], while little is known about tachykinins role in sleep regulation [21, 118, 119]. To investigate this, we dissect the neuropeptides encoded by the *nlp-8* gene. Our results indicate that tachykinin neuropeptide signaling represents an ancient and conserved pathway for sleep regulation.

Results

Constitutive overexpression of nlp-8 *induces rapidly reversible locomotion quiescence*

The ALA neurosecretory cell releases multiple neuropeptides each of which shuts down a specific set of sleep behaviors [43]. For example, one peptide shuts down pumping and another defecation [43]. Here we focus on *nlp-8*, one of the three neuropeptides that we previously found to be necessary for stress-induced sleep and sufficient to induce a specific set of sleep behaviors when conditionally overexpressed [43]. Here we

characterize the effect of *nlp-8* constitutive overexpression (**Figure 4.1A**). When animals that constitutively overexpress *nlp-8* are transferred onto a new plate they move and explore the plate (*nlp-8* overexpressing (OE)=0±0% mean percent of the animals are locomotion quiescent ± standard error of mean (sem), n=4 trials; **Figure 4.1A,B**). After 30 minutes on the plate, the animals overexpressing *nlp-8* exhibit locomotion quiescence (*nlp-8* OE=84±2% compared to wildtype (WT)=0±0% locomotion quiescent; mean±sem; n=4 trials; $p<0.0001$; **Figure 4.1B**). Interestingly, this decrease in locomotion was rapidly reversible; locomotion resulted if the animals were poked indicating that the animals are not paralyzed. The animals enter bouts of locomotion either randomly or when disturbed. We chose to use the constitutive overexpression approach to induce the strongest effects of the neuropeptide on behavior. Henceforth, we refer to constitutive overexpression as overexpression.

Overexpression of nlp-8 *modulates* C. elegans *sensory response*

When an animal overexpressing *nlp-8* is placed onto a new plate, they responded to an aversive stimulus, 30% 1-octanol, within 5 seconds (**Figure 4.1C**). This is similar to the response observed in wild-type animals. However, if the animals are given 10 minutes to acclimate on the new plate and then presented with 30% 1-octanol, a delay in response to the aversive stimulus occurred (14±2 seconds compared to 4±1 seconds mean reversal time±sem; n=12 animals, $p=0.001$; **Figure 4.1C**). These data indicate that overexpression of *nlp-8* induces rapidly reversible reduced responsiveness to 30% 1-octanol. We also found that animals overexpressing *nlp-8* were delayed in responding to carbon dioxide, another aversive stimulus. Our data indicates that *nlp-8* is sufficient to induce rapidly reversible, reduced responsiveness to aversive stimuli.

We then tested the effect of *nlp-8* overexpression on chemotaxis to positive and aversive odors [120]. The odors we tested are known attractants that act through various specific sensory neuron pathway [120]. The tested stimuli that are known to attract *C. elegans* through the following sensory neurons: AWC, AWC^{ON}, AWC^{OFF}, and AWA. *nlp-8* overexpression increased *C. elegans* chemotaxis to an AWC stimulus and AWC^{ON}

stimulus, indicating that the positive valence (attractiveness) of AWC odors is increased when *nlp-8* is overexpressed (for isoamyl alcohol (AWC) WT=0.63±0.10, *nlp-8* OE=0.92±0.05, p=0.006; for 2-butanone (AWC^{ON}) WT=0.80±0.02, *nlp-8* OE=0.95±0.05, p=0.006; n=6 trials; mean chemotaxis index±sem **Figure 4.1D**). However, this trend is not generalizable to all AWC odors. For example, pentadienone, which is specific to AWC^{OFF} has a strong positive valence for both wildtype animals and those that overexpress *nlp-8*. One interpretation is that for those AWC odors that are moderately attractive, *nlp-8* overexpression increases the attractiveness of those odors, while those highly attractive odors cannot become more attractive. Another interpretation is that animals overexpressing *nlp-8* move slower and thus more efficiently locate positive odors.

If the slow locomotion explanation were true, it is not generalizable to all attractive odors. In fact, *nlp-8* overexpression decreased the attractiveness of one positive AWA odors (for diacetyl (AWA) WT=0.59±0.21, n=16, *nlp-8* OE=0.32±0.34, n=15, p=0.02; **Figure 4.1D**). These data indicate that *nlp-8* overexpression has odor specific effects, suggesting that that *nlp-8* encoded peptides act specifically on AWA or its downstream interneurons, but not on interneurons downstream of both AWA and AWC. The data for the odors presented here demonstrate that AWC odors become more attractive, while AWA odors become less attractive after *nlp-8* overexpression. In similarly designed experiments there was no difference in chemotaxis to the mildly repulsive odor 2-nonanone which is sensed by AWB. *nlp-8* overexpression broadly changes the state of *C. elegans* by promoting rapidly reversible locomotion quiescence and modifying sensory responsiveness. The *nlp-8* gene encodes at least five individual neuropeptides [43, 97], and the function of all these neuropeptides was tested.

A single neuropeptide within the nlp-8 *gene strongly induces locomotion quiescence*

Overexpression was used to determine which of the *nlp-8* neuropeptides has the strongest effect on locomotion quiescence. Previous studies indicated that the *nlp-8* neuropeptide gene has between 3 to 5 functional neuropeptides (**Figure 4.2B**; [43, 97]). First, we

constitutively overexpressed constructs where three strong candidate neuropeptides encoded by *nlp-8* Peptide 1, Peptide 2, and Peptide 3 were deleted (referred to as *nlp-8* P1, *nlp-8* P2, and *nlp-8* P3, respectively; **Figure 4.2B**). We found that animals overexpressing the *nlp-8* cDNA lacking *nlp-8* P1 through P3 did not exhibit locomotion quiescence (**Figure 4.2C,D**) demonstrating that *nlp-8* P1 through P3 are the best candidate peptides for inducing locomotion quiescence.

To test the effect of *nlp-8* peptides individually, a synthetic construct was designed which used the *nlp-8* backbone (**Figure 4.2B**). The *nlp*-8 synthetic construct (*nlp-8syn*) maintains key residues of the *nlp-8* preproprotein: the signal peptide, dibasic residues, and the preproprotein C terminal. Constitutive overexpression of the *nlp-8syn* construct had no effect on locomotion (**Figure 4.2C,D**). Next, *nlp-8* P1, P2, or P3 were individually placed into the *nlp-8syn* construct (**Figure 4.2B**) and the effect of overexpression of either *nlp-8* P1, P2, or P3 on locomotion was tested. P1 and P2 share sequence homology, suggesting they are protein paralogs (**Figure 4.2A**). When either P1 or P2 was individually overexpressed neither peptide induced locomotion quiescence (**Figure 4.2C,D**) indicating that these two peptides are not the strongest acting peptides of *nlp-8* for the locomotion sleep phenotype. All three peptides have homologous N terminal sequences, though the C terminus of *nlp-8* P3 is unique (**Figure 4.2A**). Constitutive overexpression of P3 was sufficient to induce locomotion quiescence that was similar in effect to *nlp-8* overexpression (*nlp-8* OE=83±7% compared to *nlp-8syn* P3 OE=73±3% locomotion quiescent at 30 minutes; mean±sem; n=4 trials; p=0.24). In fact, overexpression of *nlp-8* P3 exhibited reduced response to 30% 1-octanol and CO_2. These data show that *nlp-8* P3 overexpression is similar to the full *nlp-8* gene overexpression phenotypes suggesting that *nlp-8* P3 is a key regulator of the locomotion and sensory depression sleep phenotype.

Functional conservation of the C terminal -FGLM domain in C. elegans

Tachykinin neuropeptides are found amongst many animals from *Drosophila*, *C. elegans*, fish, and humans [116-119, 121, 122]. The tachykinin family of neuropeptides is divided

into two classes: invertebrate and vertebrate, with only rare instances of the vertebrate-class tachykinin found within invertebrates ([116-119, 121, 122]; **Figure 4.3A**). The invertebrate tachykinin peptides typically have the following domain F$\boldsymbol{X_1}$G$\boldsymbol{X_2}$Lamide, where $\boldsymbol{X_1}$ and $\boldsymbol{X_2}$ are variable amino acid residues [116, 117]. The vertebrate tachykinin domain typically is F***X***GLMamide, where ***X*** is a variable amino acid residue [118, 119]. The conservation of these domains has been demonstrated by cross species ligand receptor binding assays. The *C. elegans* tachykinin gene encodes both vertebrate and invertebrate tachykinin (**Figure 4.3A**), which is unique because vertebrate-class tachykinin is rarely found amongst invertebrates [116, 117]. *C. elegans* tachykinin is missing the ***X*** residues mentioned above and also lacks a Glycine residue, which is typically used for neuropeptide amidation during post-translational processing. These data suggest that *nlp-8* encodes a unique member of the tachykinin neuropeptide family.

The neuropeptide within *nlp-8* that has the strongest sleep-inducing effect is *nlp-8* P3. This peptide has C terminal homology with vertebrate tachykinin (FGLM) [118, 119]. Importantly *nlp-8* P3 and Human Substance P (vertebrate tachykinin) share the last four amino acids of their C terminus (**Figure 4.3A**). Human Substance P has diverse biological effects on the gastrointestinal tract, cardiovascular system and the immune system [118, 119, 123]. The *nlp-8syn* construct was used to test if overexpression of Human Substance P in *C. elegans* could induce locomotion quiescence. Specifically, a Human Substance P sequence, lacking a glycine residue, was inserted in the *nlp-8syn* construct (**Figure 4.3B**). The peptide induced locomotion quiescence (*nlp-8syn* Human Substance P OE=58±9% compared to wildtype (WT)=0±0% locomotion quiescent at 30 min; mean±sem; n=4 trials; $p<0.0001$; **Figure 4.3C,D)**. When we replaced the C terminal -FGLM domain with four Alanine residues, the resulting construct exhibited little to no effect on locomotion when overexpressed. These data indicate that the FGLM domain of *nlp-8* and Human Substance P are functionally conserved and critical for inducing the locomotion sleep phenotype (locomotion quiescence) in *C. elegans*.

Discussion

Sleep is an ancient animal behavior that has been observed in all closely investigated animals [1-3, 6]. *C. elegans*, *Drosophila*, Zebrafish, and mice have been deployed as model organisms for sleep research [7-10]. The biology of each of these animals provides a unique advantage to investigators, and most importantly, *C. elegans* and *Drosophila* are amenable to large-scale forward genetic screens [39]. Hypotheses generated from the study of genetic regulation of sleep in these invertebrates should be tested in vertebrates.

Sleep arises from the collective action of neuronal and genetic pathways [21, 32, 43]. The functional conservation of several genes across model organisms has been demonstrated [1, 3, 5, 81]. These genes are generally restricted to clock gene peptides, cyclic AMP, dopamine, and adenosine [1, 3, 5, 81]. While neuropeptides have been identified as key sleep regulators in animals from worm to man [3, 5, 21], functional conservation of individual neuropeptide families in sleep regulation has not been thoroughly investigated.

The family of tachykinin neuropeptides are found throughout many animal phyla [116-119, 121, 122]. They have been demonstrated to have broad and diverse roles from nociception, analgesia, opioid, stress, aggression, and inflammation [118, 123, 124]. The role of tachykinin in sleep regulation is poorly understood [21]. In humans, oral administration of tachykinin in men was found to promote sleep [125]. Similarly, there is evidence that microinjection of tachykinin into the vLPO regulates REM sleep in Mice [50]. Further, tachykinin has been recently implicated in regulating SWS in mice [126]. Experiments have shown that the effect of Substance P on sleep may be concentration dependent, and instead promotes wakefulness at low concentrations [127]. Our study attempts to test the role of tachykinin in sleep regulation using genetic experiments.

We previously demonstrated that the *nlp-8* tachykinin gene is both necessary and sufficient for sleep induction in *C elegans* [43]. The data presented here demonstrate that *nlp-8* is sufficient to induce locomotion quiescence, reduced responsiveness to aversive stimuli, and a change in the perception of attractiveness of attractive stimuli. The *nlp-8*

P3 has the strongest sleep-inducing phenotype. This peptide shares C terminal homology with vertebrate tachykinin, though the *nlp-8* P3 lacks amidation. Human Substance P, lacking amidation, was sufficient to put the worms to sleep. These experiments narrowed the functional domain of the *nlp-8* gene to the C terminal (-FGLM).

C. elegans SIS represents an ancient manifestation of sleep [1-3, 5]. The data presented here advances a recent proposition posed by Davis and Raizen [128] in which *C. elegans* stress-induced sleep could be a form of sickness sleep, implying that this sleep state is driven by strong homeostatic forces rather than circadian cycles. Additionally, Tachykinin is known to have immune-functional roles [118], and is a key inducer of migraines [129]. Tachykinin signaling via serotonergic neurons causes migraines in humans. Migraines are also associated with photophobia (aversion to light) [129]. The only treatment for intense migraine and photophobia is sleep [129].

Interestingly the two families of neuropeptides, the –RFamides and tachykinin, that regulate *C. elegans* SIS [43] have also been shown to regulate sleep in other animals [49, 50, 125, 126]. Lee et al. [49] demonstrated how –RFamides induce sleep in Zebrafish. Preliminary data indicates that tachykinin is also sufficient to induce sleep in Zebrafish (Hill and Prober, personal communications). Future studies will elucidate exactly how these peptides induce sleep. Our hypothesis is that these neuropeptides work together to construct the sleep state by shutting down specific neurons and behaviors. This construction hypothesis applies to both vertebrates and *C. elegans*.

Figures

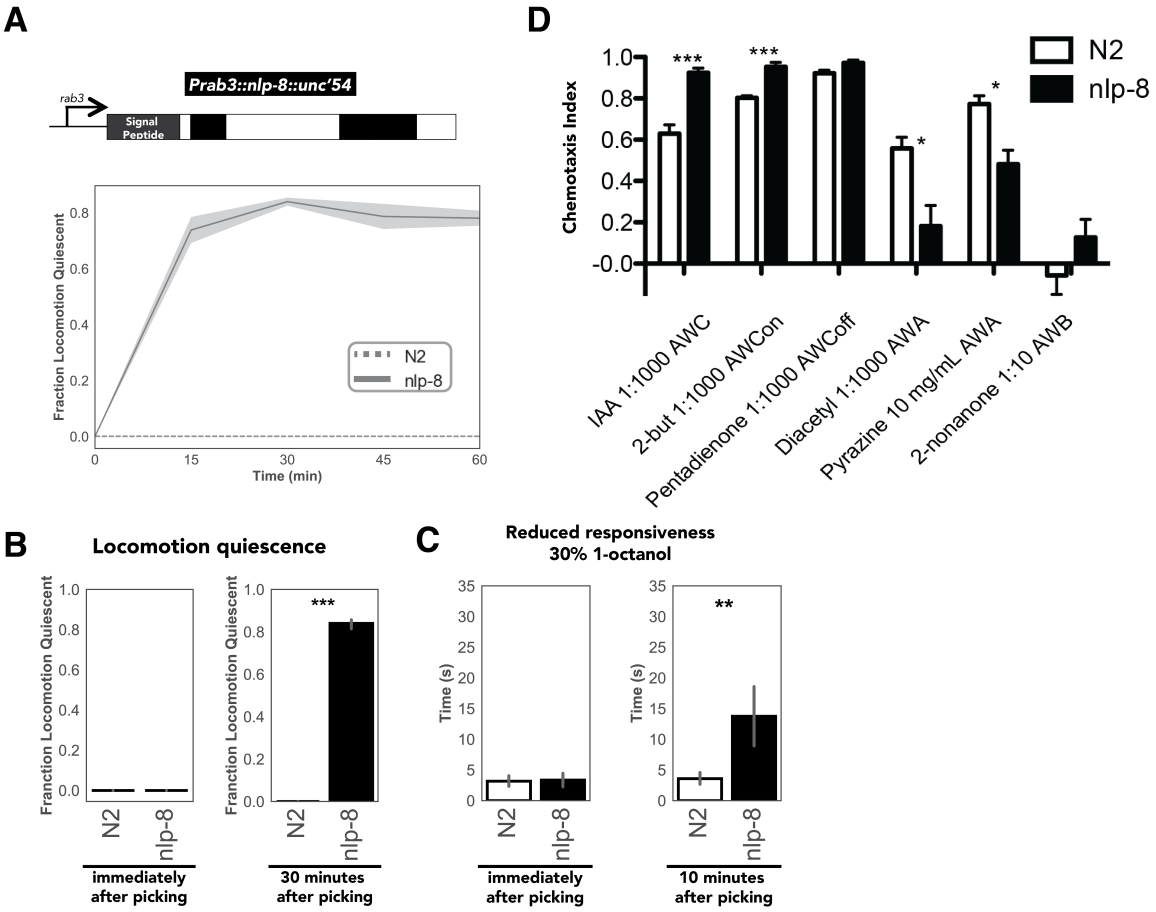

Figure 4.1: Constitutive overexpression of *nlp-8* induces locomotion quiescence and modulates *C. elegans* sensory response.

(**A**) (top) Constitutive overexpression of *nlp-8* using the pan-neuronal *rab-3* promoter, and the *unc-54* untranslated region (UTR). (bottom) Young adult animals were picked onto a new plate and locomotion was measured as the fraction of animals that are locomotion quiescent (locomotion quiescent was defined as no movement of the centroid of the animal for 10 seconds). "Fraction locomotion quiescent" was scored at 15 minute intervals for one hour. (**B**) Immediately after picking the animals, both wildtype (N2) and animals overexpressing *nlp-8* exhibited no locomotion quiescence. However, after 30 minutes, animals that overexpressed *nlp-8* exhibited strong locomotion quiescence, while wildtype animals continued to move. (**C**) The responsiveness of wildtype animals to 30% 1-octanol was tested before and after picking. Both genotypes were responsive

immediately after picking; however, 10 minutes after picking, animals overexpressing *nlp-8* exhibited a delayed response to stimulus, while wildtype animals continued to respond quickly. (**D**) Chemotaxis index to several odors for wildtype animals, as well as animals that overexpress *nlp-8*. AWC and AWA odors are attractive odors, and remain attractive for both genotypes. However, *nlp-8* overexpression may enhance the attractive experience of AWC odors, while suppressing the attractive experience of AWA odors. Meanwhile, 2-nonanone, an AWB odor, remains neutral or unattractive for both genotypes. (**A-D**) Data shown as mean±SEM, *p<0.05; **p<0.01; ***p<0.001; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2 (N2=wildtype).

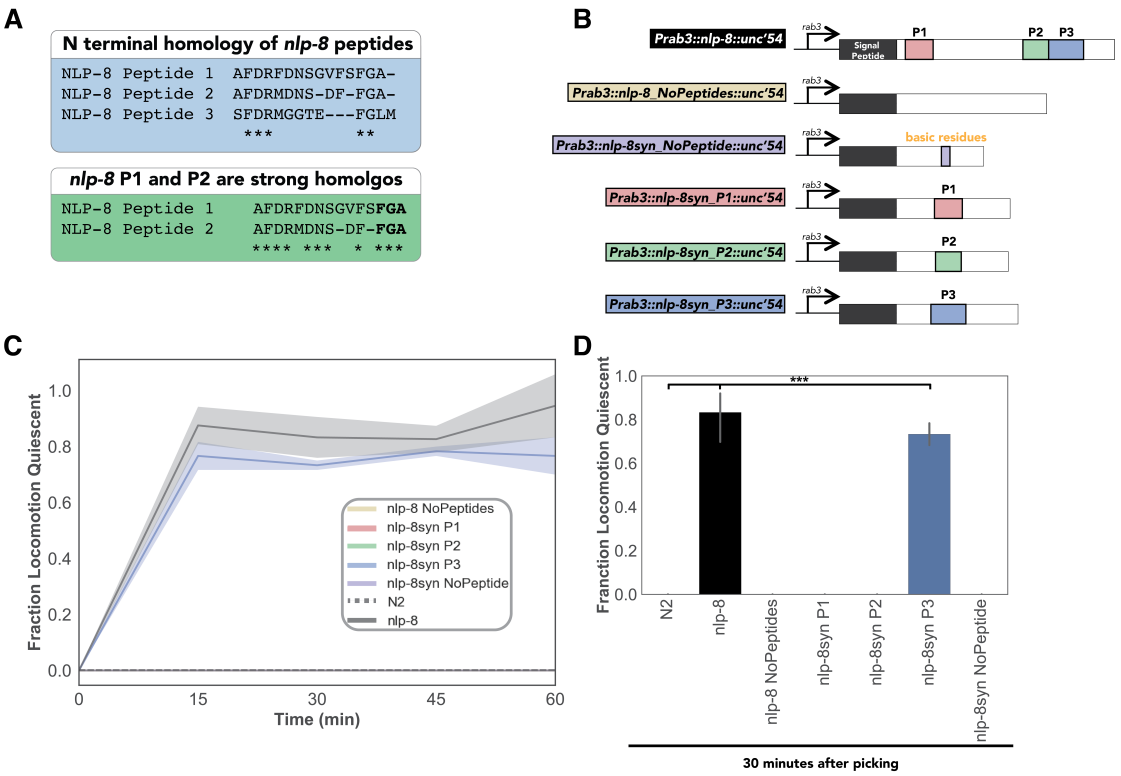

Figure 4.2: *nlp-8* P3 strongly induces locomotion quiescence.

(**A**) Alignments of neuropeptides encoded by *nlp-8* (P1, P2, P3). (top) *nlp-8* P1, P2, and P3 have homologous N termini. (bottom) *nlp-8* P1 and P2 share strong sequence homology, including the C termini [43, 97]. The C terminus of *nlp-8* P3 is unique. *=identical amino acids. (**B**) Design of constructs to genetically dissect the neuropeptides encoded by *nlp-8*. (top) the *nlp-8* gene encodes at least three neuropeptides. These neuropeptides were removed and overexpressed under the *rab-3* promoter and *unc-54* 3'UTR (nlp-8_NoPeptides). A synthetic construct was designed (nlp-8syn_NoPeptides) that maintained basic residues, the signal peptide, and the prepropeptide C terminus. Individual peptides from *nlp-8* were placed into the nlp-8syn construct (P1, P2, P3). (**C**) Young adult animals were picked onto a new plate and locomotion was measured as the fraction of animals that are locomotion quiescent (locomotion quiescent was defined as no movement of the centroid of the animal for 10 seconds). Fraction locomotion quiescent was scored at 15 minute intervals for one hour. Immediately after picking none of the genotypes tested were locomotion quiescent. The majority of animals continued to move during the one hour of observation, however animals overexpressing *nlp-8* and *nlp-*

8 P3 exhibited similar levels of locomotion quiescence. (**D**) Thirty minutes after picking, animals overexpressing *nlp-8* and *nlp-8* P3 exhibited strong locomotion quiescence, while wildtype animals and animals overexpressing *nlp-8* NoPeptides, nlp-8syn NoPeptides nlp-8syn P1 and nlp-8sy P2 continued to move. (**A-D**) Data shown as mean±SEM, ***$p<0.001$; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2 (N2=wildtype).

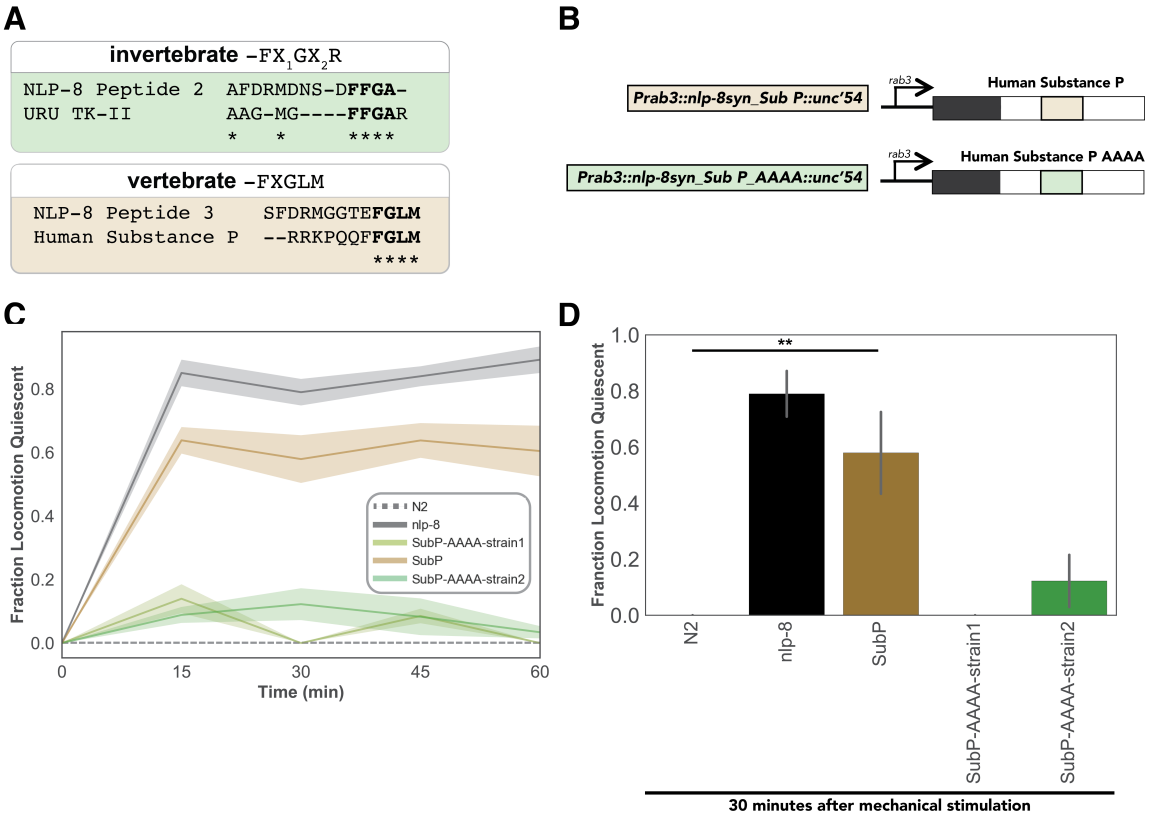

Figure 4.3: Human Substance P, lacking amidation, strongly induces locomotion quiescence.

(**A**) Alignments of neuropeptides encoded by *nlp-8* with a known invertebrate tachykinin from *Urechis unicinctus* [116, 117, 130, 131] and Human Substance P (vertebrate tachykinin homolog) [118, 119]. The C terminus of *nlp-8* P2 and tachykinin from *U. unicinctus* are homologous [116, 117, 130, 131]. The C terminus of Human Substance P and *nlp-8* P3 are homologous. *=identical amino acids. Note exclusion of amidation for Human Substance P and *U. unicinctus* tachykinin. (**B**) Design of constructs (see **Figure 4.2**). Human Substance P, lacking amidation, was placed into the *nlp-8syn* construct and overexpressed. Likewise, Human Substance P with the –FGLM C terminus replaced with four Alanine residues (SubP-AAAA) was generated to test the importance of the C teriminal –FGLM. (**C**) Young adult animals were picked onto a new plate and locomotion was measured as the fraction of animals that are locomotion quiescent (locomotion quiescent was defined as no movement of the centroid of the animal for 10 seconds). Fraction locomotion quiescent was scored at 15 minute intervals for one hour.

Immediately after picking none of the genotypes tested were locomotion quiescent. Wildtype animals continued to move during the one hour of observation, however animals overexpressing *nlp-8* and Human Substance P exhibited similar levels of locomotion quiescence. Those animals overexpressing SubP-AAAA exhibited little to no locomotion quiescence. The –FGLM domain of *nlp-8* is sufficient to induce locomotion quiescence. (**D**) Thirty minutes after picking, animals overexpressing *nlp-8* and Human Substance P exhibited strong locomotion quiescence, while wildtype animals and animals overexpressing SubP-AAAA exhibited little to no locomotion quiescence. (**A-D**) Data shown as mean±SEM, $^{**}p<0.01$; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2 (N2=wildtype).

C h a p t e r V

CLOSING REMARKS

When I started my PhD in Paul Sternberg's lab my interests were genetics, neurobiology, and behavior. Throughout my studies, I have become an expert in *C. elegans*, genetics, neurobiology, neuronal imaging, evolutionary biology, behavior, and most importantly, I have become an expert in sleep. Using the jellyfish *Cassiopea*, I demonstrated that sleep is a highly conserved behavior observed across the animal kingdom (Chapter II). Next, using genetics and molecular biology, I discovered that *C. elegans* sleep is regulated by neuropeptides (Chapter III). More specifically, each neuropeptide regulates a specific suite of sleep-associated behaviors and the neuropeptides work together to collectively induce sleep. Both families of neuropeptides we identified in *C. elegans* were subsequently found to also regulate vertebrate sleep (Chapter IV). This demonstrates that *C. elegans* is a powerful model system to study the genetic basis of sleep regulation. To advance our understanding of sleep, *C. elegans* should continue to be used as a platform for the identification of novel sleep regulators.

I took a multi-phylum approach to untangle the evolutionary web of behavior, genes, and neural circuits that construct sleep. For all animals, individual behaviors serve as building blocks to construct the sleep state. These behavioral blocks are shaped by evolutionary forces such that jellyfish sleep is different than human sleep. The studies are united by our hypothesis that sleep is constructed by evolutionary forces (Chapter I). I look forward to future experiments that will test this hypothesis and further elucidate the mechanism by which sleep has evolved.

I remain interested in genetics, neurobiology, evolution, and behavior. As a post-doc I plan to develop *Nothobranchius Fuzeri* (the Turquoise African Killifish) as a new genetically tractable model system for neurobiology research.

BIBLIOGRAPHY

1. Allada, R. and J.M. Siegel, *Unearthing the phylogenetic roots of sleep.* Curr Biol, 2008. **18**(15): p. R670-R679.
2. Campbell, S.S. and I. Tobler, *Animal sleep: a review of sleep duration across phylogeny.* Neurosci Biobehav Rev, 1984. **8**(3): p. 269-300.
3. Joiner, W.J., *Unraveling the Evolutionary Determinants of Sleep.* Curr Biol, 2016. **26**(20): p. R1073-R1087.
4. Siegel, J.M., *Do all animals sleep?* Trends Neurosci, 2008. **31**(4): p. 208-13.
5. Trojanowski, N.F. and D.M. Raizen, *Call it Worm Sleep.* Trends Neurosci, 2016. **39**(2): p. 54-62.
6. Nath, R.D., et al., *The Jellyfish Cassiopea Exhibits a Sleep-like State.* Curr Biol, 2017. **27**(19): p. 2984-2990 e3.
7. Prober, D.A., et al., *Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish.* J Neurosci, 2006. **26**(51): p. 13400-10.
8. Hendricks, J.C., et al., *Rest in Drosophila is a sleep-like state.* Neuron, 2000. **25**(1): p. 129-38.
9. Shaw, P.J., et al., *Correlates of sleep and waking in Drosophila melanogaster.* Science, 2000. **287**(5459): p. 1834-7.
10. Raizen, D.M., et al., *Lethargus is a Caenorhabditis elegans sleep-like state.* Nature, 2008. **451**(7178): p. 569-72.
11. Dunn, C.W., et al., *Animal Phylogeny and Its Evolutionary Implications.* Annual Review of Ecology, Evolution, and Systematics, Vol 45, 2014. **45**: p. 371-+.
12. Erwin, D.H. and E.H. Davidson, *The last common bilaterian ancestor.* Development, 2002. **129**(13): p. 3021-32.
13. Bosch, T.C.G., et al., *Back to the Basics: Cnidarians Start to Fire.* Trends in Neurosciences, 2017. **40**(2): p. 92-105.
14. Kelava, I., F. Rentzsch, and U. Technau, *Evolution of eumetazoan nervous systems: insights from cnidarians.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2015. **370**(1684).
15. Arendt, D., M.A. Tosches, and H. Marlow, *From nerve net to nerve ring, nerve cord and brain--evolution of the nervous system.* Nat Rev Neurosci, 2016. **17**(1): p. 61-72.
16. Watanabe, H., T. Fujisawa, and T.W. Holstein, *Cnidarians and the evolutionary origin of the nervous system.* Development Growth & Differentiation, 2009. **51**(3): p. 167-183.
17. Katsuki, T. and R.J. Greenspan, *Jellyfish nervous systems.* Current Biology, 2013. **23**(14): p. R592-R594.
18. Hejnol, A. and F. Rentzsch, *Neural nets.* Current Biology, 2015. **25**(18): p. R782-R786.
19. Kristan, W.B., Jr., *Early evolution of neurons.* Curr Biol, 2016. **26**(20): p. R949-R954.
20. Bringmann, H., *Sleep-Active Neurons: Conserved Motors of Sleep.* Genetics, 2018. **208**(4): p. 1279-1289.
21. Richter, C., I.G. Woods, and A.F. Schier, *Neuropeptidergic control of sleep and wakefulness.* Annu Rev Neurosci, 2014. **37**: p. 503-31.

22. Arendt, D., *The evolution of cell types in animals: emerging principles from molecular studies.* Nat Rev Genet, 2008. **9**(11): p. 868-82.
23. Arendt, D., et al., *The origin and evolution of cell types.* Nat Rev Genet, 2016. **17**(12): p. 744-757.
24. Tessmar-Raible, K., et al., *Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution.* Cell, 2007. **129**(7): p. 1389-400.
25. Vigh, B., et al., *The system of cerebrospinal fluid-contacting neurons. Its supposed role in the nonsynaptic signal transmission of the brain.* Histol Histopathol, 2004. **19**(2): p. 607-28.
26. Smith, C.L., et al., *Novel cell types, neurosecretory cells, and body plan of the early-diverging metazoan Trichoplax adhaerens.* Curr Biol, 2014. **24**(14): p. 1565-1572.
27. Senatore, A., T.S. Reese, and C.L. Smith, *Neuropeptidergic integration of behavior in Trichoplax adhaerens, an animal without synapses.* J Exp Biol, 2017. **220**(Pt 18): p. 3381-3390.
28. Cirelli, C., *The genetic and molecular regulation of sleep: from fruit flies to humans.* Nat Rev Neurosci, 2009. **10**(8): p. 549-60.
29. Crocker, A. and A. Sehgal, *Genetic analysis of sleep.* Genes Dev, 2010. **24**(12): p. 1220-35.
30. Hendricks, J.C., A. Sehgal, and A.I. Pack, *The need for a simple animal model to understand sleep.* Prog Neurobiol, 2000. **61**(4): p. 339-51.
31. Sehgal, A. and E. Mignot, *Genetics of sleep and sleep disorders.* Cell, 2011. **146**(2): p. 194-207.
32. Lee, S.H. and Y. Dan, *Neuromodulation of brain states.* Neuron, 2012. **76**(1): p. 209-22.
33. Bargmann, C.I., *Beyond the connectome: how neuromodulators shape neural circuits.* Bioessays, 2012. **34**(6): p. 458-65.
34. Bargmann, C.I. and E. Marder, *From the connectome to brain function.* Nat Methods, 2013. **10**(6): p. 483-90.
35. van den Pol, A.N., *Neuropeptide transmission in brain circuits.* Neuron, 2012. **76**(1): p. 98-115.
36. Harris-Warrick, R.M. and E. Marder, *Modulation of neural networks for behavior.* Annu Rev Neurosci, 1991. **14**: p. 39-57.
37. Taghert, P.H. and M.N. Nitabach, *Peptide neuromodulation in invertebrate model systems.* Neuron, 2012. **76**(1): p. 82-97.
38. Marder, E., *Neuromodulation of neuronal circuits: back to the future.* Neuron, 2012. **76**(1): p. 1-11.
39. Brenner, S., *The genetics of Caenorhabditis elegans.* Genetics, 1974. **77**(1): p. 71-94.
40. White, J.G., et al., *The structure of the nervous system of the nematode Caenorhabditis elegans.* Philos Trans R Soc Lond B Biol Sci, 1986. **314**(1165): p. 1-340.
41. Hill, A.J., et al., *Cellular stress induces a protective sleep-like state in C. elegans.* Curr Biol, 2014. **24**(20): p. 2399-405.
42. Van Buskirk, C. and P.W. Sternberg, *Epidermal growth factor signaling induces behavioral quiescence in Caenorhabditis elegans.* Nat Neurosci, 2007. **10**(10): p. 1300-7.
43. Nath, R.D., et al., *C. elegans Stress-Induced Sleep Emerges from the Collective Action of Multiple Neuropeptides.* Curr Biol, 2016. **26**(18): p. 2446-2455.
44. Nelson, M.D., et al., *FMRFamide-like FLP-13 neuropeptides promote quiescence following heat stress in Caenorhabditis elegans.* Curr Biol, 2014. **24**(20): p. 2406-10.

45. Elphick, M.R., O. Mirabeau, and D. Larhammar, *Evolution of neuropeptide signalling systems.* J Exp Biol, 2018. **221**(Pt 3).
46. Jekely, G., *Global view of the evolution and diversity of metazoan neuropeptide signaling.* Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8702-7.
47. Turek, M., et al., *Sleep-active neuron specification and sleep induction require FLP-11 neuropeptides to systemically induce sleep.* Elife, 2016. **5**.
48. Lenz, O., et al., *FMRFamide signaling promotes stress-induced sleep in Drosophila.* Brain Behav Immun, 2015. **47**: p. 141-8.
49. Lee, D.A., et al., *Genetic and neuronal regulation of sleep by neuropeptide VF.* Elife, 2017. **6**.
50. Zhang, G., et al., *Substance P promotes sleep in the ventrolateral preoptic area of rats.* Brain Res, 2004. **1028**(2): p. 225-32.
51. Duboue, E.R., A.C. Keene, and R.L. Borowsky, *Evolutionary convergence on sleep loss in cavefish populations.* Curr Biol, 2011. **21**(8): p. 671-6.
52. Jaggard, J.B., et al., *Hypocretin underlies the evolution of sleep loss in the Mexican cavefish.* Elife, 2018. **7**.
53. Schlegel, P., et al., *Synaptic transmission parallels neuromodulation in a central food-intake circuit.* Elife, 2016. **5**.
54. Toonen, R.F., et al., *Dissecting docking and tethering of secretory vesicles at the target membrane.* EMBO J, 2006. **25**(16): p. 3725-37.
55. Kirszenblat, L. and B. van Swinderen, *The Yin and Yang of Sleep and Attention.* Trends Neurosci, 2015. **38**(12): p. 776-786.
56. Grimmelikhuijzen, C.J. and J.A. Westfall, *The nervous systems of cnidarians.* EXS, 1995. **72**: p. 7-24.
57. Satterlie, R.A., *Do jellyfish have central nervous systems?* Journal of Experimental Biology, 2011. **214**(8): p. 1215-1223.
58. Dupre, C. and R. Yuste, *Non-overlapping Neural Networks in Hydra vulgaris.* Curr Biol, 2017. **27**(8): p. 1085-1097.
59. Grimmelikhuijzen, C.J.P., M. Williamson, and G.N. Hansen, *Neuropeptides in cnidarians.* Canadian Journal of Zoology, 2002. **80**(10): p. 1690-1702.
60. Garm, A., et al., *Opposite Patterns of Diurnal Activity in the Box Jellyfish Tripedalia cystophora and Copula sivickisi.* Biological Bulletin, 2012. **222**(1): p. 35-45.
61. Seymour, J.E., T.J. Carrette, and P.A. Sutherland, *Do box jellyfish sleep at night?* Med J Aust, 2004. **181**(11-12): p. 707.
62. Holland, B.S., et al., *Global phylogeography of Cassiopea (Scyphozoa : Rhizostomeae): molecular evidence for cryptic species and multiple invasions of the Hawaiian Islands.* Marine Biology, 2004. **145**(6): p. 1119-1128.
63. Jantzen, C.W., C.; Rasheed, M.; El-Zibdah, M.; Richter, C., *Enhanced pore water nutrient fluxes by the upside-down jellyfish Cassiopea sp. in a Red Sea coral reef.* Mar Ecol Prog Ser, 2010. **411**: p. 117–125.
64. Santhanakrishnan, A., et al., *Flow structure and transport characteristics of feeding and exchange currents generated by upside-down Cassiopea jellyfish.* Journal of Experimental Biology, 2012. **215**(14): p. 2369-2381.
65. Zhdanova, I.V., *Sleep and its regulation in zebrafish.* Rev Neurosci, 2011. **22**(1): p. 27-36.
66. Borbely, A.A. and P. Achermann, *Sleep homeostasis and models of sleep regulation.* J Biol Rhythms, 1999. **14**(6): p. 557-68.

67. Zimmerman, J.E., et al., *Conservation of sleep: insights from non-mammalian model systems.* Trends Neurosci, 2008. **31**(7): p. 371-6.
68. Peres, R., et al., *Developmental and light-entrained expression of melatonin and its relationship to the circadian clock in the sea anemone Nematostella vectensis.* Evodevo, 2014. **5**: p. 26.
69. Zhdanova, I.V., et al., *Melatonin promotes sleep-like state in zebrafish.* Brain Res, 2001. **903**(1-2): p. 263-8.
70. Brzezinski, A., et al., *Effects of exogenous melatonin on sleep: a meta-analysis.* Sleep Med Rev, 2005. **9**(1): p. 41-50.
71. Tosches, M.A., et al., *Melatonin signaling controls circadian swimming behavior in marine zooplankton.* Cell, 2014. **159**(1): p. 46-57.
72. Gandhi, A.V., et al., *Melatonin Is Required for the Circadian Regulation of Sleep.* Neuron, 2015. **85**(6): p. 1193-1199.
73. Stat, M.P., X.; Cowie, R.; Gates, R.D., *Specificity in communities of Symbiodinium in corals from Johnston Atoll.* Mar. Ecol. Prog. Ser., 2010. **386**: p. 83–96.
74. Folmer, O., et al., *DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates.* Mol Mar Biol Biotechnol, 1994. **3**(5): p. 294-9.
75. van der Walt, S., S.C. Colbert, and G. Varoquaux, *The NumPy Array: A Structure for Efficient Numerical Computation.* Computing in Science & Engineering, 2011. **13**(2): p. 22-30.
76. Hunter, J.D., *Matplotlib: A 2D graphics environment.* Computing in Science & Engineering, 2007. **9**(3): p. 90-95.
77. Oliphant, T.E., *Python for scientific computing.* Computing in Science & Engineering, 2007. **9**(3): p. 10-20.
78. Abrams, M.J., et al., *Self-repairing symmetry in jellyfish through mechanically driven reorganization.* Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(26): p. E3365-E3373.
79. Treweek, J.B., et al., *Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping.* Nature Protocols, 2015. **10**(11): p. 1860-1896.
80. Singh, K., et al., *C. elegans Notch signaling regulates adult chemosensory response and larval molting quiescence.* Curr Biol, 2011. **21**(10): p. 825-34.
81. Singh, K., et al., *Deep Conservation of Genes Required for Both Drosophila melanogaster and Caenorhabditis elegans Sleep Includes a Role for Dopaminergic Signaling.* Sleep, 2014. **37**(9): p. 1439-U256.
82. Foltenyi, K., R.J. Greenspan, and J.W. Newport, *Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila.* Nat Neurosci, 2007. **10**(9): p. 1160-7.
83. Kramer, A., et al., *Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling.* Science, 2001. **294**(5551): p. 2511-5.
84. Kushikata, T., et al., *Epidermal growth factor enhances spontaneous sleep in rabbits.* Am J Physiol, 1998. **275**(2 Pt 2): p. R509-14.
85. Snodgrass-Belt, P., J.L. Gilbert, and F.C. Davis, *Central administration of transforming growth factor-alpha and neuregulin-1 suppress active behaviors and cause weight loss in hamsters.* Brain Res, 2005. **1038**(2): p. 171-82.
86. Gallagher, T., et al., *ASI regulates satiety quiescence in C. elegans.* J Neurosci, 2013. **33**(23): p. 9716-24.

87. Trojanowski, N.F., et al., *Distinct Mechanisms Underlie Quiescence during Two Caenorhabditis elegans Sleep-Like States.* J Neurosci, 2015. **35**(43): p. 14571-84.
88. You, Y.J., et al., *Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in C. elegans: a model for satiety.* Cell Metab, 2008. **7**(3): p. 249-57.
89. Van Buskirk, C. and P.W. Sternberg, *Paired and LIM class homeodomain proteins coordinate differentiation of the C. elegans ALA neuron.* Development, 2010. **137**(12): p. 2065-74.
90. Lee, D.A. and S. Blackshaw, *Feed your head: neurodevelopmental control of feeding and metabolism.* Annu Rev Physiol, 2014. **76**: p. 197-223.
91. Lin, X.G., et al., *UNC-31/CAPS docks and primes dense core vesicles in C. elegans neurons.* Biochem Biophys Res Commun, 2010. **397**(3): p. 526-31.
92. Schwarz, E.M., M. Kato, and P.W. Sternberg, *Functional transcriptomics of a migrating cell in Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 2012. **109**(40): p. 16246-51.
93. Jarecki, J.L., et al., *Mapping Neuropeptide Expression by Mass Spectrometry in Single Dissected Identified Neurons from the Dorsal Ganglion of the Nematode Ascaris suum.* Acs Chemical Neuroscience, 2010. **1**(7): p. 505-519.
94. Hobert, O., *The neuronal genome of Caenorhabditis elegans.* WormBook, 2013: p. 1-106.
95. Kim, K. and C. Li, *Expression and regulation of an FMRFamide-related neuropeptide gene family in Caenorhabditis elegans.* J Comp Neurol, 2004. **475**(4): p. 540-50.
96. Li, C., et al., *Neuropeptide gene families in the nematode Caenorhabditis elegans.* Ann N Y Acad Sci, 1999. **897**: p. 239-52.
97. Nathoo, A.N., et al., *Identification of neuropeptide-like protein gene families in Caenorhabditis elegans and other species.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(24): p. 14000-14005.
98. Cho, J.Y. and P.W. Sternberg, *Multilevel modulation of a sensory motor circuit during C. elegans sleep and arousal.* Cell, 2014. **156**(1-2): p. 249-60.
99. Schwarz, J., I. Lewandrowski, and H. Bringmann, *Reduced activity of a sensory neuron during a sleep-like state in Caenorhabditis elegans.* Current Biology, 2011. **21**(24): p. R983-R984.
100. Thomas, J.H., *Genetic-Analysis of Defecation in Caenorhabditis-Elegans.* Genetics, 1990. **124**(4): p. 855-872.
101. Liu, D.W.C. and J.H. Thomas, *Regulation of a Periodic Motor Program in C-Elegans.* Journal of Neuroscience, 1994. **14**(4): p. 1953-1962.
102. Etchberger, J.F., et al., *The molecular signature and cis-regulatory architecture of a C-elegans gustatory neuron.* Genes & Development, 2007. **21**(13): p. 1653-1674.
103. Shan, G., et al., *Convergent genetic programs regulate related motor neuron classes similarities and differences between in Caenorhabditis elegans.* Developmental Biology, 2005. **280**(2): p. 494-503.
104. Yu, H., et al., *Distinct roles of transcription factors EGL-46 and DAF-19 in specifying the functionality of a polycystin-expressing sensory neuron necessary for C-elegans male vulva location behavior.* Development, 2003. **130**(21): p. 5217-5227.
105. Liu, K.S. and P.W. Sternberg, *Sensory Regulation of Male Mating-Behavior in Caenorhabditis-Elegans.* Neuron, 1995. **14**(1): p. 79-89.
106. Burbach, J.P., *What are neuropeptides?* Methods Mol Biol, 2011. **789**: p. 1-36.
107. Lockery, S.R. and M.B. Goodman, *Tight-seal whole-cell patch clamping of Caenorhabditis elegans neurons.* Methods Enzymol, 1998. **293**: p. 201-17.

108. Dulac, C. and R. Axel, *A novel family of genes encoding putative pheromone receptors in mammals.* Cell, 1995. **83**(2): p. 195-206.
109. Stringham, E.G., et al., *Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans.* Mol Biol Cell, 1992. **3**(2): p. 221-33.
110. Hobert, O., *PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans.* Biotechniques, 2002. **32**(4): p. 728-30.
111. Mello, C. and A. Fire, *DNA transformation.* Methods Cell Biol, 1995. **48**: p. 451-82.
112. Maduro, M. and D. Pilgrim, *Identification and cloning of unc-119, a gene expressed in the Caenorhabditis elegans nervous system.* Genetics, 1995. **141**(3): p. 977-88.
113. Kagoshima, H., et al., *The LIM homeobox gene ceh-14 is required for phasmid function and neurite outgrowth.* Dev Biol, 2013. **380**(2): p. 314-23.
114. Singh, C., G. Oikonomou, and D.A. Prober, *Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish.* Elife, 2015. **4**: p. e07000.
115. Chiu, C.N., et al., *A Zebrafish Genetic Screen Identifies Neuromedin U as a Regulator of Sleep/Wake States.* Neuron, 2016. **89**(4): p. 842-56.
116. Nassel, D.R., *Tachykinin-related peptides in invertebrates: a review.* Peptides, 1999. **20**(1): p. 141-58.
117. Van Loy, T., et al., *Tachykinin-related peptides and their receptors in invertebrates: a current view.* Peptides, 2010. **31**(3): p. 520-4.
118. Severini, C., et al., *The tachykinin peptide family.* Pharmacol Rev, 2002. **54**(2): p. 285-322.
119. Maggio, J.E., *Tachykinins.* Annu Rev Neurosci, 1988. **11**: p. 13-28.
120. Bargmann, C.I., *Chemosensation in C. elegans.* WormBook, 2006: p. 1-29.
121. Vanden Broeck, J., et al., *Tachykinin-like peptides and their receptors. A review.* Ann N Y Acad Sci, 1999. **897**: p. 374-87.
122. Pennefather, J.N., et al., *Tachykinins and tachykinin receptors: a growing family.* Life Sci, 2004. **74**(12): p. 1445-63.
123. Shimizu, Y., et al., *Tachykinins and their functions in the gastrointestinal tract.* Cell Mol Life Sci, 2008. **65**(2): p. 295-311.
124. Asahina, K., et al., *Tachykinin-expressing neurons control male-specific aggressive arousal in Drosophila.* Cell, 2014. **156**(1-2): p. 221-35.
125. Lieb, K., et al., *Effects of the neuropeptide substance P on sleep, mood, and neuroendocrine measures in healthy young men.* Neuropsychopharmacology, 2002. **27**(6): p. 1041-9.
126. Zielinski, M.R., et al., *Substance P and the neurokinin-1 receptor regulate electroencephalogram non-rapid eye movement sleep slow-wave activity locally.* Neuroscience, 2015. **284**: p. 260-72.
127. Andersen, M.L., et al., *Sleep disturbance induced by substance P in mice.* Behav Brain Res, 2006. **167**(2): p. 212-8.
128. Davis, K.C. and D.M. Raizen, *A mechanism for sickness sleep: lessons from invertebrates.* J Physiol, 2017. **595**(16): p. 5415-5424.
129. Messina, A., et al., *Non-Rapid Eye Movement Sleep Parasomnias and Migraine: A Role of Orexinergic Projections.* Front Neurol, 2018. **9**: p. 95.
130. Ikeda, T., et al., *Two novel tachykinin-related neuropeptides in the echiuroid worm, Urechis unicinctus.* Biochem Biophys Res Commun, 1993. **192**(1): p. 1-6.
131. Kawada, T., et al., *A novel tachykinin-related peptide receptor. Sequence, genomic organization, and functional analysis.* Eur J Biochem, 2002. **269**(17): p. 4238-46.