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***C. elegans* stress-induced sleep emerges from the collective action of multiple neuropeptides**

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Summary

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.

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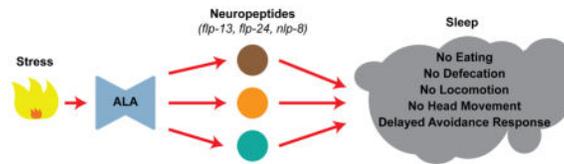
The authors declare no competing interests.

Author Contributions

E.S.C., E.M.S., R.D.N., and P.W.S. conceived the project. E.S.C. dissected ALA neurons and sequenced their amplified cDNA. E.M.S. performed the ALA transcriptome analysis. R.D.N., E.S.C. and H.W. performed *C. elegans* behavior experiments. E.S.C., H.W., and R.D.N. generated reagents. R.D.N., E.S.C., H.W., E.M.S., and P.W.S. wrote the paper.

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Sleep is a complex behavioral state that requires the coordinated regulation of multiple behaviors. Nath et al. investigate the downstream mechanism by which a neurosecretory cell coordinately promotes sleep in *C. elegans*. They discovered that sleep emerges from the collective action of three neuropeptide-encoding genes.



Keywords

Sleep regulation; RFamides; Neuropeptide modulation; single-neuron RNA-seq; genetic redundancy

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 [3–6]. One such sleep regulator is Epidermal Growth Factor Receptor (EGFR), whose activation promotes sleep in both *C. elegans* and *D. melanogaster* [7,8], and inhibits locomotion in mammals [9–11].

C. elegans sleep has been observed during developmental molting (lethargus), satiety, and Epidermal Growth Factor (EGF)/EGFR signaling [7,12–18]. Here we investigated the *C. elegans* EGF-induced sleep pathway, thought to represent a distinct molecular pathway from developmentally linked sleep (Figure 1A; [18]). The EGF-induced sleep state occurs in two contexts: by overexpressing the EGF ortholog (LIN-3C; [7]), or by EGF-signaling after stress (such as temperature elevation) in wild-type animals [14]. The EGF receptor ortholog (LET-23) is necessary for EGF-induced sleep and expressed in the ALA neuron [7]. Ablation of ALA demonstrated that it is necessary for EGF-induced sleep [7]. 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 [7,14,19]. These transcription factors control expression of genes in ALA shown to be required for EGF-induced sleep including EGFR [7,14,19]. 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 [17].

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 [20–24]. 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 [25], inhibits stress-induced sleep [7], suggesting that neuropeptide release is necessary for this state. Second, genetic axotomy of ALA does not inhibit stress-induced sleep [7], 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 [19], whereas another, *flp-13*, was previously shown to be partially required for stress-induced sleep [13]. 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 [26] 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 S1). 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 1B; Table S1). 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 1B; Table S2). *flp-24* and *flp-13* were previously found in the ALA neuron of *Ascaris suum* by single neuron mass spectrometry [27]. The *C. elegans* genome contains 122 neuropeptide genes whose mature products contain over 250 distinct neuropeptides [28]. 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 (Table S2). 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 (Table S2). 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

[13,19,29]. We verified expression of *flp-24* and *nlp-8* using GFP reporter constructs (Figure S1E–H). Previous analysis showed that each of these genes encodes a prepropeptide containing one or more mature neuropeptides ([30,31]; Figure S2; Figure S3).

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 [12,14,17,32]. 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 2; Figure 3; [7,14]). 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 2; Figure 3; Figure S3; Figure S4; Table S3–S5). 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 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 2A), confirming the results of Nelson et al. [13]. 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 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 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 2B; [13]). *ceh-14* and *ceh-17* mutants, previously shown to be strongly resistant to heat shock [7, 14], 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 2B). The difference in our results could be due to differences in heat shock protocol or scoring (Supplemental Information). 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 1; Figure S1; Table S2) 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 (Supplemental Information) 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 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 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 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 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 2E). The resistance of *nlp-8; flp-13* double mutants and *nlp-8; flp-24; flp-13* triple mutants were similar to *ceh-14* mutants in locomotion quiescence (*ceh-14*: 42±9% locomotion quiescent; $p = 0.1$; Figure 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 [12,32,33]. 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; Table S4; $n = 10$). 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 3B; Table S5). The defecation motor program comprises posterior body wall contraction, anterior body wall contraction, and expulsion [34]. 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 S5), 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 ($n = 10$; $p > 0.5$; Figure 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 3B). Resistance to the suppression of defecation in the triple mutant was indistinguishable from *ceh-14* and *ceh-17* animals ($p > 0.4$; Figure 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 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 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 [14]. 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 4A; [7,14,19]).

flp-13 overexpression inhibits pharyngeal pumping

Following the heat shock protocol illustrated in Figure 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*: $73 \pm 1\%$ pumping quiescent compared to *ceh-14*: $0 \pm 0\%$ pumping quiescent, at one hour post-heat shock; $p < 0.001$; Figure 4B,C). We conclude that overexpression of *flp-13*, but neither *flp-24*, *flp-7*, nor *nlp-8*, is sufficient to inhibit pumping (Figure 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 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 5A; Figure S5A). 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 5A; Figure S5A). 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 S5B), 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* ($p < 0.04$; Figure S5B).

Since arrested defecation might be a consequence of halted feeding [34,35], 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 5B). Consistent with Figure 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 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*: $31 \pm 5\%$ locomotion quiescent one hour post-heat shock compared to $4 \pm 4\%$ locomotion quiescent pre-heat shock; $p < 0.001$; Figure 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*: $41 \pm 6\%$ locomotion quiescent one hour post-heat shock compared to $0 \pm 0\%$ locomotion quiescent pre-heat shock; $p < 0.001$; and compared to *ceh-14*: $31 \pm 5\%$ locomotion quiescent one hour post-heat shock; $p = 0.3$; Figure 6A,B). By contrast, worms overexpressing *flp-24* showed severe inhibition of locomotion (HS::*flp-24*; *ceh-14*: $68 \pm 9\%$ locomotion quiescent one hour post-heat shock compared to $0 \pm 0\%$ locomotion quiescent pre-heat shock; $p < 0.001$; and compared to *ceh-14*: $31 \pm 5\%$ one hour post-heat shock; $p < 0.01$; Figure 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 (Video S1). As with pumping, *ceh-14* mutants showed no background quiescence for head movement after heat shock (Figure 6D). Overexpression of either *nlp-8* or *flp-7* failed to suppress head movement (Figure 6D,E). However, overexpression of either *flp-24* or *flp-13* inhibited head movement (HS::*flp-24*; *ceh-14*: $47 \pm 10\%$ head movement quiescent; HS::*flp-13*; *ceh-14*: $40 \pm 12\%$ head movement quiescent compared to *ceh-14*: $0 \pm 0\%$ head movement quiescent; $p < 0.01$; Figure 5D; Video S2). 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 6; Video S3). 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 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 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 S6). Thus, overexpression of *flp-24*, *flp-13*, or *nlp-8*, but not of *flp-7*, inhibited the avoidance response (Figure 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 [7]. 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 7). For example, the avoidance response results from sensory neurons, command interneurons, and motor neurons working in series (Figure 7; [32]). 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 [32], 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 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 [13,19,29,31,36–38]. 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 [38], and thus might play a role in inhibiting defecation during mating [39]. 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 [40], and at least 20 of which may have important functions in sleep-wake

regulation [24]. 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.

Materials and Methods

Strains were grown on nematode growth medium (NGM) agar Petri plates seeded with *E. coli* strain OP50 and maintained at 20°C under standard conditions [41]. Our wild-type strain was N2 (Bristol). The following can be found in the Supplemental Experimental Procedures: characterization of behavioral assays, single ALA neuron dissection and transcriptome profiling, data availability, information about strains.

Supplementary Material

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Acknowledgments

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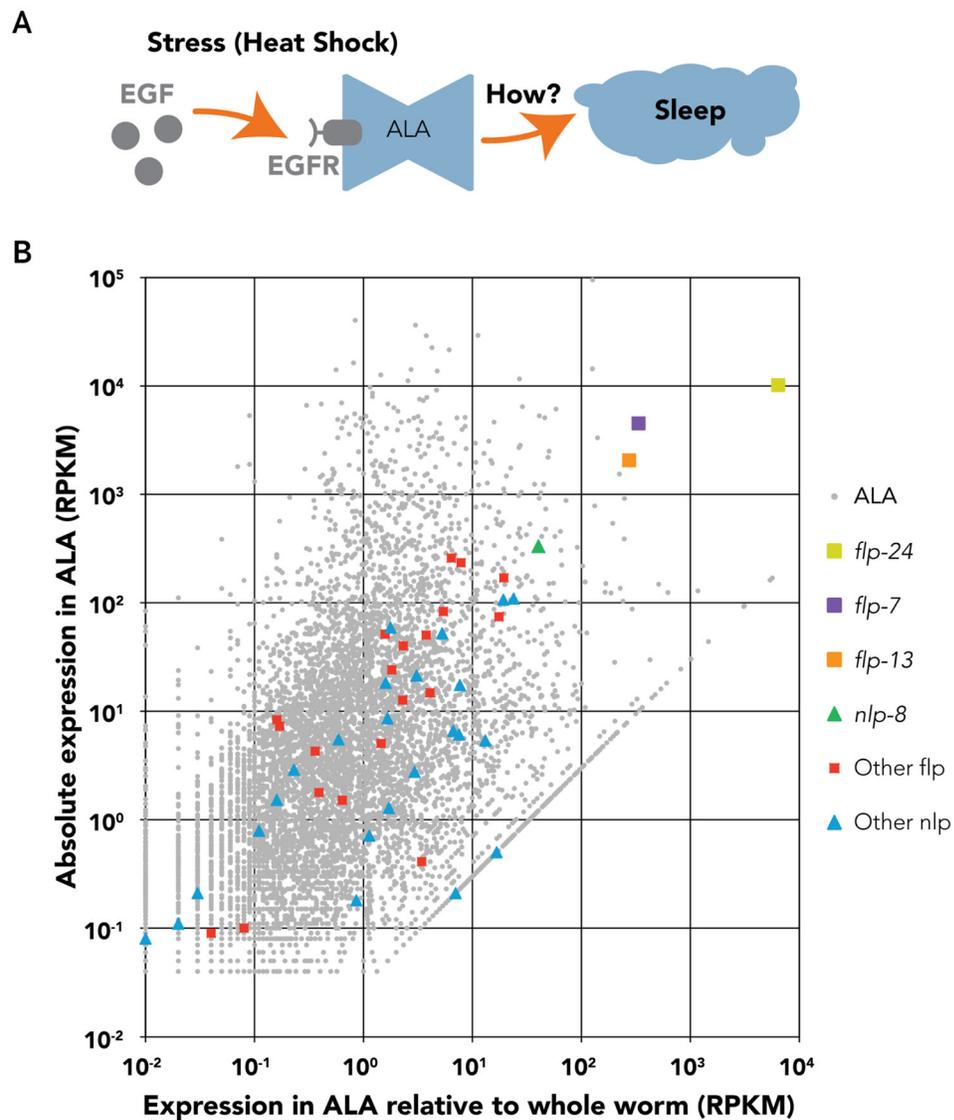
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Highlights

- RNA-seq of key sleep neuron ALA reveals enrichment of neuropeptide-encoding genes
- A triple-null mutant of three ALA-enriched neuropeptide genes abolishes sleep
- Three neuropeptides were each sufficient to induce specific sleep behaviors
- The collective action of three neuropeptide genes (*nlp-8*, *flp-24*, *flp-13*) induces sleep



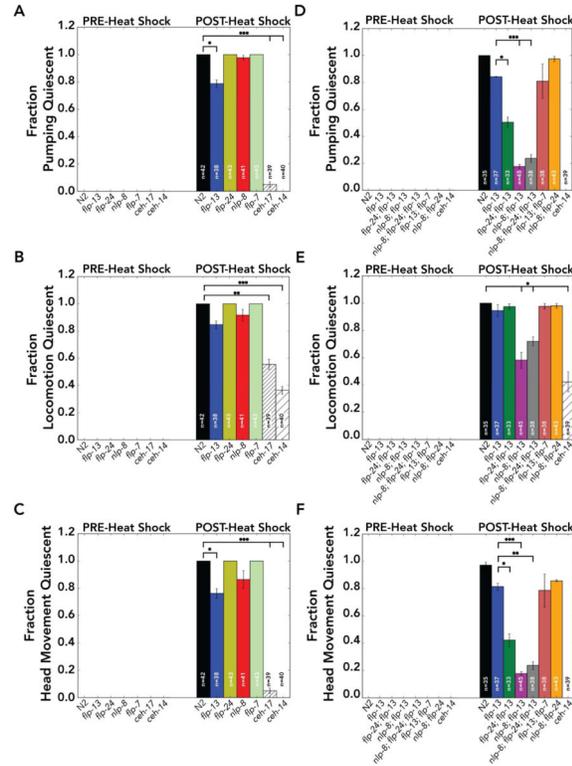


Figure 2. Double and triple mutants of ALA-enriched neuropeptides suppress pumping, head movement, and locomotion quiescence during stress-induced sleep
 (A–C) The fraction of single-null mutants pumping, locomotion, and head movement quiescent before (PRE) and 30 minutes after (POST) heat shock (a 35°C 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 S3, Figure S4, and Table S3.

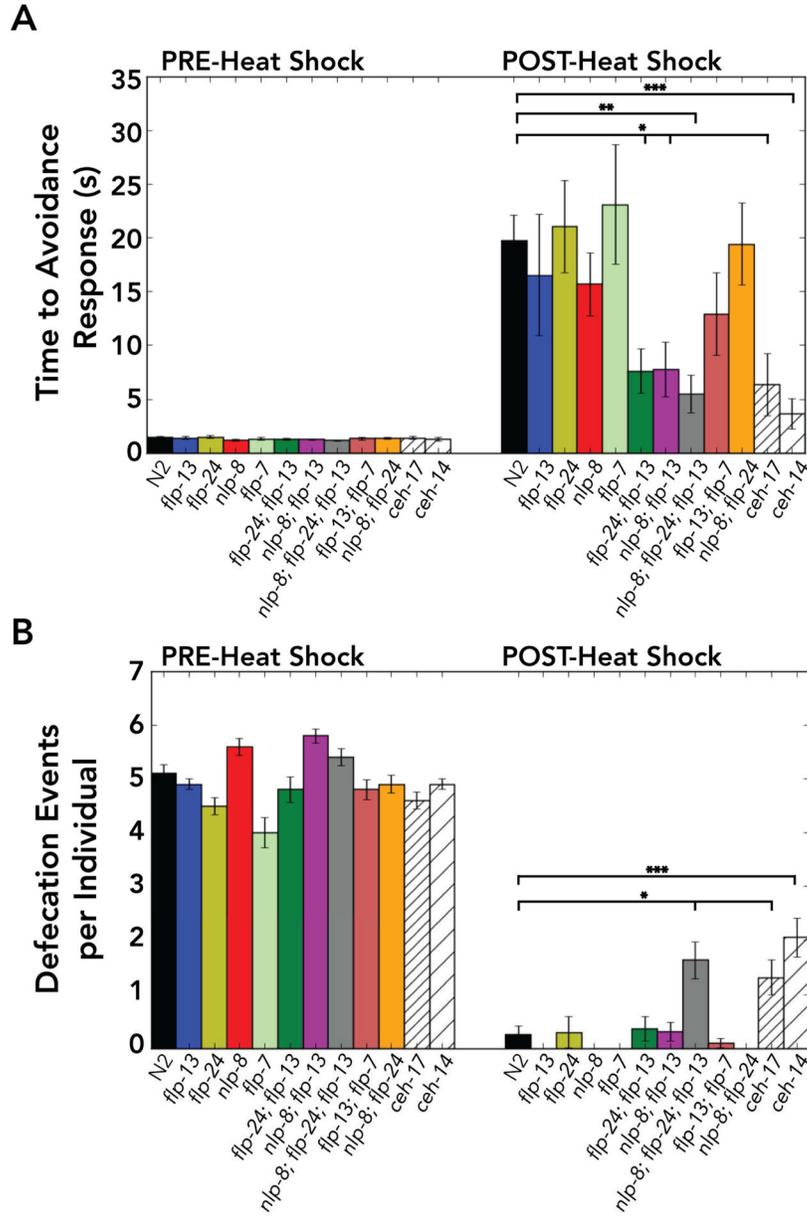


Figure 3. Double and triple mutants of ALA-enriched neuropeptides suppress the increased response latency to aversive stimuli, while only the triple mutant suppresses defecation quiescence during stress-induced sleep

(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 S4). 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 (Supplemental Information). We found that our negative controls *ceh-14* and *ceh-17* had background suppression of defecation at 30

minutes (Table S5). 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 \pm SEM; n 10 *C. elegans* for each strain (see also Table S4–S5); * $p<0.05$; ** $p<0.01$; *** $p<0.001$; unpaired t-test with Bonferonni correction for multiple comparison. Statistical comparisons are indicated for comparisons to N2.

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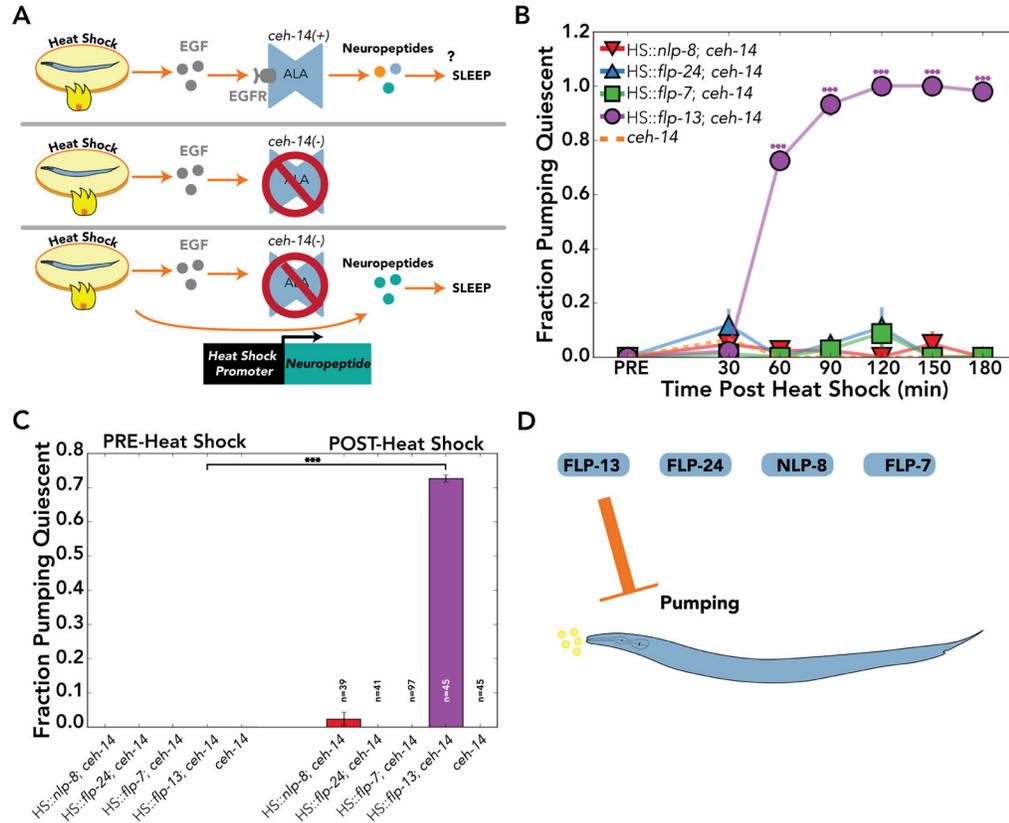


Figure 4. Overexpression of FLP-13 inhibits pumping in *C. elegans*

(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.

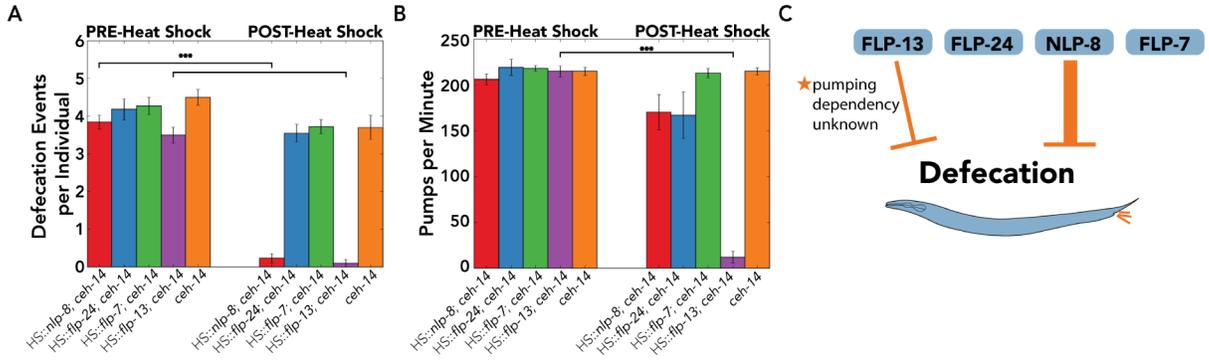


Figure 5. Overexpression of NLP-8 inhibits defecation while pumping continues
(A) Average number of defecation events for individuals five minutes before (PRE) and one hour after (POST) heat shock. Overexpressing *flp-13* or *nlp-8* inhibited defecation. **(B)** Pumping rate was scored from 10-second 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 S5A); ****p*<0.001; paired t-test.

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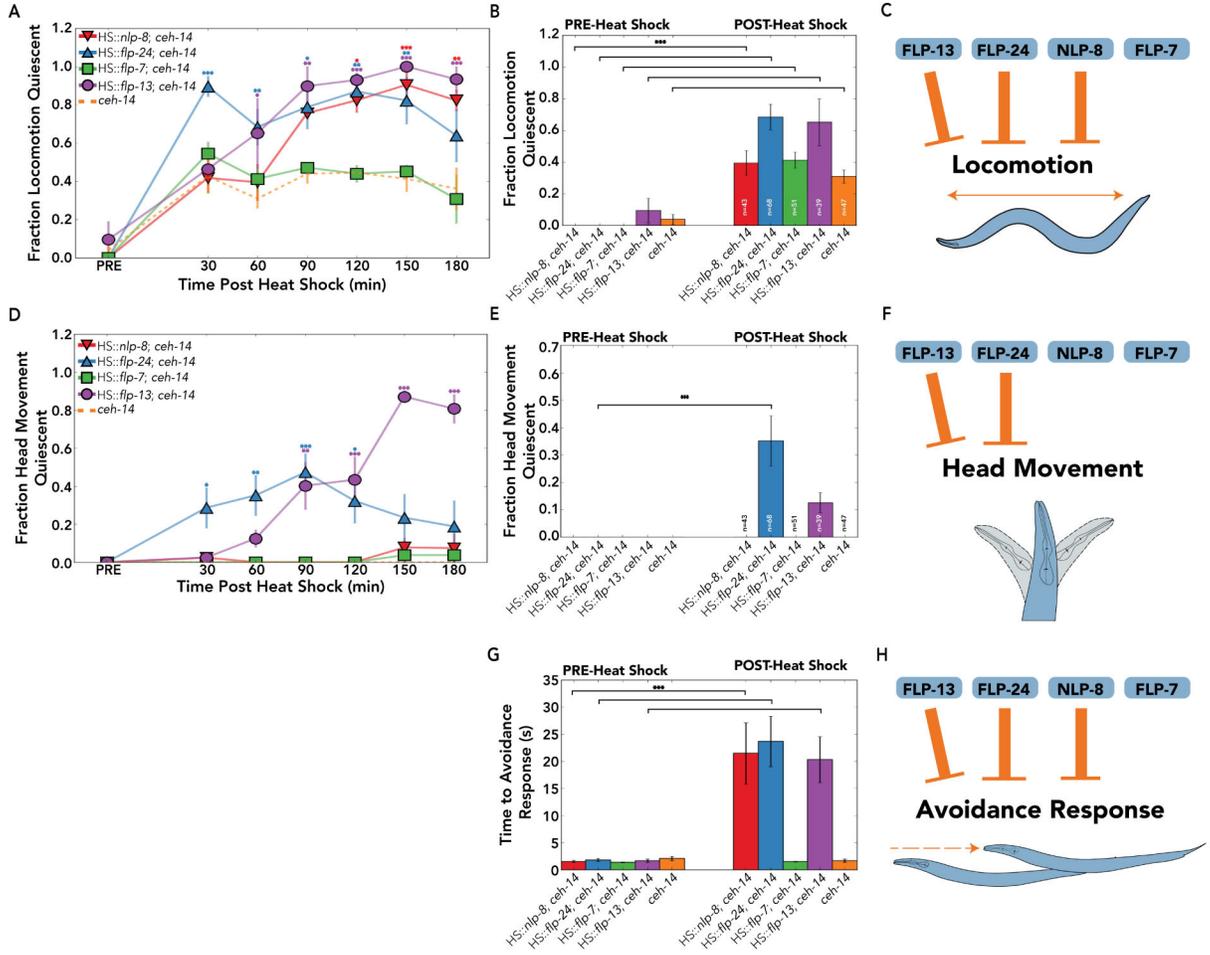


Figure 6. Overexpression of either FLP-13, FLP-24, or NLP-8 inhibit movement and the avoidance response
(A) & (D) Time-course monitoring the fraction of *C. elegans* that were locomotion and head-movement quiescent before (PRE) and up to three hours after (POST) heat shock induced neuropeptide overexpression. *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 S6). **(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 S6); *** $p < 0.001$; unpaired t-test with Bonferroni correction for multiple comparisons. See also Video S1–S3.

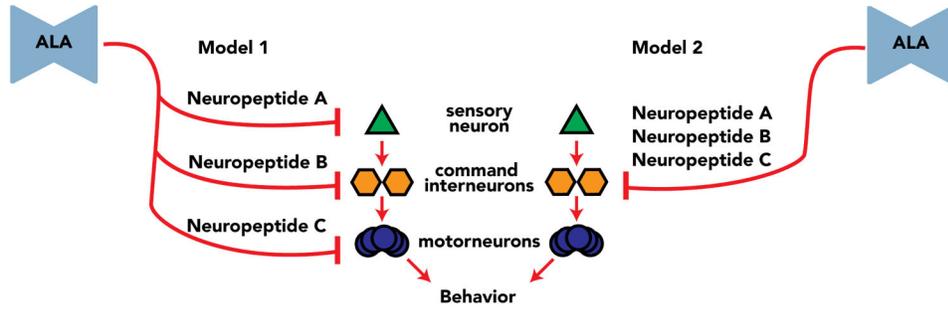


Figure 7. Redundancy models for the collective action of multiple neuropeptides which regulate *C. elegans* stress-induced sleep

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.