Published in final edited form as:
Nat Commun. 2013 ; 4: 2846. doi:10.1038/ncomms3846.

The neuropeptide NLP-22 regulates a sleep-like state in *Caenorhabditis elegans*

MD Nelson[1], **NF Trojanowski**[1,2], **JB George-Raizen**[1], **CJ Smith**[1], **C-C Yu**[2], **C Fang-Yen**[2,3], and **DM Raizen**[1]

[1]Department of Neurology, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA 19104, USA

[2]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104

[3]Department of Physics, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, S. Korea

Abstract

Neuropeptides play central roles in the regulation of homeostatic behaviors such as sleep and feeding. *Caenorhabditis elegans* displays sleep-like quiescence of locomotion and feeding during a larval transition stage called lethargus and feeds during active larval and adult stages. Here we show that the neuropeptide NLP-22 is a regulator of *Caenorhabditis elegans* sleep-like quiescence observed during lethargus. *nlp-22* shows cyclical mRNA expression in synchrony with lethargus; it is regulated by LIN-42, an orthologue of the core circadian protein PERIOD; and it is expressed solely in the two RIA interneurons. *nlp-22* and the RIA interneurons are required for normal lethargus quiescence, and forced expression of *nlp-22* during active stages causes anachronistic locomotion and feeding quiescence. Optogenetic stimulation of RIA interneurons has a movement-promoting effect, demonstrating functional complexity in a single neuron type. Our work defines a quiescence-regulating role for NLP-22 and expands our knowledge of the neural circuitry controlling *Caenorhabditis elegans* behavioral quiescence.

Introduction

Neuropeptides are central to mechanisms by which biological clocks temporally align animal behavior with recurring internal or external conditions. In mammals, the neuropeptide hypocretin/orexin is secreted from lateral hypothalamic neurons to promote wakefulness during the optimal time of day for foraging[1]. Other mammalian neuropeptides shown or proposed to regulate behavioral outputs of circadian clocks include prokineticin [2], epidermal growth factor (EGF)[3], neuropeptide S[4], prolactin-releasing peptide[5], urotensin II[6],

---

Corresponding author: David M. Raizen, Department of Neurology, University of Pennsylvania School of Medicine, 462 Stemmler Hall, 415 Curie Blvd, Philadelphia PA 19104, t.(215)746-4809 f.(215)746-3039, raizen@mail.med.upenn.edu.

Author Contributions

M.D.N., N.F.T., C.F-Y., and D.M.R. designed and performed experiments and wrote paper, J.B.G-R designed experiments, C.C.Y and C.J.S. performed experiments.

Competing financial Interests

The authors have no conflict of interest to declare.

and neuromedin S[7]. In the fruit fly *Drosophila melanogaster*, the neuropeptide pigment-dispersing factor (PDF) is released from central nervous system circadian neurons to control sleep/wake behavior[8–10], emphasizing a conserved role for neuropeptides in translating clock information to behavior[8].

In *Caenorhabditis elegans (C. elegans)*, sleep-like behavior is observed during lethargus periods, which occur at transitions between each of the four larval stages and between the fourth larval stage and the adult stage[11]. During lethargus, animals cease locomotion, feeding, and foraging, are less responsive to stimuli, and display a homeostatic response to forced movement[12, 13]. Signaling pathways involving cAMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinase (PKG), EGF, and pigment dispersing factor (PDF), which play roles in the regulation of mammalian and/or *Drosophila* sleep, also function in the regulation of *C. elegans* sleep-like quiescence[12, 14, 15], suggesting that *C. elegans* quiescence has fundamental similarities to sleep in other animals. Additional molecular similarity between quiescence during lethargus and mammalian sleep is demonstrated by the larval cycling of expression of the protein LIN-42, which is homologous to the core circadian protein PERIOD[16]. *lin-42* is required for proper timing of lethargus[17], much as *period* is required for proper timing of circadian sleep[18], but mechanisms by which *lin-42* may affect quiescent behavior were previously unknown.

Here we identify the gene *nlp-22* as a *lin-42*-regulated gene affecting quiescent behavior. *nlp-22* encodes a neuropeptide, whose function and expression were previously unknown. We show that *nlp-22* can induce quiescence in normally active animals and is necessary for normal lethargus quiescence. In addition, we present evidence of a surprising functional complexity of neurons expressing NLP-22. Our findings expand our knowledge of the neural circuitry controlling *C. elegans* behavioral quiescence.

Results

nlp-22 expression cycles in response to a larval clock

The activity of many mammalian neuropeptides with roles in clock regulation is controlled at the level of mRNA abundance. We therefore reasoned that a somnogenic peptide controlled by the larval clock would be up-regulated before or during lethargus. We performed a transcriptional profiling experiment (manuscript in preparation) in precisely-staged animals to identify genes with elevated expression before or during the fourth lethargus stage. mRNA abundance of the gene *nlp-22*, which encodes a protein predicted to be processed into a neuropeptide[19], was higher in the L4 stage than in lethargus. Using qRT-PCR we confirmed that *nlp-22* mRNA cycles expression with a constant phase relationship to lethargus (Fig. 1a). *nlp-22* mRNA peaked midway through the active larval stages, decreased during each lethargus stage, and was absent in adults.

Given the cyclical nature of *nlp-22* expression, we hypothesized that NLP-22 signaling is regulated by the larval clock. The *C. elegans* ortholog of the core circadian regulator PERIOD is LIN-42, which is required for synchronization of lethargus quiescence and molting[16, 17]. Like PERIOD, which shows cyclical expression with a circadian periodicity in insects and mammals[20], LIN-42 shows cyclical expression with larval periodicity in *C.*

elegans[16, 21]. LIN-42 expression has a similar phase relationship to lethargus as NLP-22. We found that LIN-42A anachronistic over-expression induced an increase in *nlp-22* mRNA (Fig. 1b), suggesting that *nlp-22* transcription is down-stream of a larval *lin-42* based clock.

nlp-22 is expressed in the pair of RIA interneurons

To determine where *nlp-22* is expressed, we generated a transcriptional reporter by placing green fluorescent protein (GFP) under the control of *nlp-22* regulatory DNA. We observed expression only in one pair of head neurons, which we identified as the RIA interneurons, based on process morphology and comparison to expression of the previously characterized gene *glr-3* (Fig. 1c and Supplementary Fig. S1a). Thus, *nlp-22* expression is cyclical and restricted to the RIA neurons.

NLP-22 is predicted to be secreted[19]. Secreted proteins in *C. elegans*, when fused to a fluorescent moiety on their C-terminus, can be visualized in cells outside of the cells in which they are expressed[22]. When we fused GFP to the C-terminus of the NLP-22 preproprotein, we observed green fluorescence in several neurons in the head as well as the ventral nerve cord (Supplementary Fig. S1b), supporting the notion that NLP-22 is a secreted peptide. When we fused GFP to the N-terminus of the NLP-22 preproprotein, expression was exclusively in the RIA neurons, presumably because the GFP protein interfered with the functionality of the signal sequence (Supplementary Fig. S1c). Thus, our data indicates that the NLP-22 peptide is processed and secreted from the RIA interneurons and may be acting by synaptic and/or neuroendocrine mechanisms.

NLP-22 induces behavioral quiescence

Based on the observation that *nlp-22* mRNA cycles expression during larval development in phase with the cycling of sleep-like behavior, we hypothesized that it regulates lethargus quiescence. To test this hypothesis, we genetically manipulated *nlp-22* expression and observed the effects on quiescence and active behavior.

We first studied the effect of temporal and spatial mis-expression of *nlp-22* during the adult stage, when the animals are typically feeding and moving. *C. elegans* moves by making dorsal and ventral body bends initiated at the anterior end of the animal[23] and feeds by rhythmic pharyngeal contractions called pumps[24]. When we over-expressed *nlp-22* using an inducible heat-shock promoter (Fig. 2a) during the normally active adult period, we observed quiescence of feeding and locomotion (Fig. 2b,c, Supplementary Movie 1, Supplementary Table S1). Two hours after heat shock, control animals made 15.9 ± 7.3 (all values presented as mean ± s.d.)(N=15) anterior body bends per minute whereas animals over-expressing *nlp-22* made 0.55 ± 1.1(N=25) body bends per minute (Fig. 2b). Wild-type animals and transgenic control animals expressing GFP under the control of a heat shock promoter pumped two hours after heat shock at rates of 264.9 ± 17.4(N=20) and 273.6 ± 17.5(N=20) pumps per minute, respectively (Fig. 2c, Supplementary Table S1). Animals carrying the integrated or extrachromosomal *Phsp16.2:nlp*-22 arrays, *qnIs142* or *qnEx95*, pumped at rates of 18.6 ± 31.1(N=37) and 25.2 ± 32.3(N=40) per minute, respectively, two hours after heat shock (Fig. 2c, Supplementary Table S1). In the absence of either the signal sequence or the dibasic residues predicted to be necessary for preproprotein cleavage[25], *nlp-22* over-expression no longer promoted behavioral quiescence (Fig. 2c), indicating that these features of the protein are important for its biological activity. When we mutated any of the four C-terminal amino acids of the predicted peptide, over-expression of the gene no longer caused quiescence (Fig. 2c), indicating that these four amino acids are important for the peptide's biological function. Nine hours after *nlp-22* over-expression, all the animals resumed feeding, indicating that the reduction in behavior was not due to an injurious effect (Fig 2d).

NLP-22-induced quiescence resembles lethargus quiescence

During lethargus, animals are less responsive to stimuli, a property shared with sleeping vertebrates[12]. In addition, worms in lethargus move backwards more often than non-lethargus worms[26]. Like wild-type worms in lethargus, adult worms over-expressing *nlp*-22 showed reduced responsiveness to optic or chemical stimulation compared to transgenic control animals that over-expressed GFP (Fig. 3a). Under mild heat shock conditions, in which *nlp-22* over-expressing adult animals were not completely quiescent, they moved backwards more often than control animals (Fig. 3b). Thus, over-expression of NLP-22 induces feeding and movement quiescence that is reminiscent of lethargus quiescence.

NLP-22 is required for proper quiescence during lethargus

Our over-expression studies demonstrate sufficiency of *nlp-22* for the induction of behavioral quiescence. However, over-expression experiments are likely to result in supra-physiological concentrations of the peptide as well as spatially ectopic expression. Therefore, we proceeded to study a loss of function *nlp-22* mutation in order to ask the question: Is *nlp-22* necessary for quiescence? The *nlp-22* allele *gk509904* has a point mutation that introduces a stop codon prior to the NLP-22 peptide (Fig. 2a); thus, it is likely to fully eliminate *nlp-22* function. We measured quiescence in wild-type and *nlp-22(gk509904)* animals from the active L3 stage through development into adulthood. Total quiescence during L4 lethargus of *gk509904* mutants was reduced in comparison to total quiescence of wild-type animals (Fig. 4a,c and Supplementary Table S2). The quiescence defect of *gk509904* mutants was rescued by expression of a genomic fragment spanning the *nlp-22* locus (Fig. 4a,c and Supplementary Table S2). To test for the functional relevance of the observed RIA gene reporter expression, we expressed *nlp-22* double stranded RNA (dsRNA) to knockdown *nlp-22* mRNA specifically in RIA, and, as a control, in the hypodermis. Expression in the RIA neurons but not in the hypodermis reduced lethargus quiescence (Fig. 4b,c and Supplementary Table S2). In addition to reduced quiescence, *nlp-22(gk509904)* mutants showed enhanced responsiveness to optic sensory stimulation during L4 lethargus quiescence bouts (Fig. 4d), demonstrating reduced sleep-like behavior. Thus, *nlp-22* is required for appropriate *C. elegans* sleep-like quiescence during lethargus.

NLP-22 signals through a PKA-dependent mechanism

To determine mechanisms by which *nlp-22* induces sleep-like behavior, we crossed animals carrying *nlp-22* over-expression arrays into a subset of the genetic backgrounds that have been previously shown to affect *C. elegans* quiescence (*kin-2*[27], *egl-4*[12] and *ceh-17*[14]) and into genetic backgrounds known to affect neuropeptide processing or release (*egl-3*[28], *egl-21*[29] and *unc-31*[30]) (Supplementary Table S1). In all cases but one, the quiescence-inducing effects of *nlp-22* over-expression were no different in the mutant background from these effects in the wild-type background. The one observed exception was in *kin-2(ce179)* mutants, in which the normal inhibition of the catalytic subunits of the cAMP-dependent protein kinase PKA by the regulatory subunits is impaired due to a mutation in the regulatory subunit, resulting in excess activity of the catalytic subunit[31]. Excessive PKA activity causes increased wakefulness in *C. elegans* and other species[32]. *kin-2* mutants are hyperactive[33], are hyper-responsive to stimuli during lethargus, and show reduced lethargus quiescence[27]. In the *kin-2* mutant background P*hsp16.2:nlp-22* transgenic animals continued to feed and move from 2.5 to 4.0 hours after heat shock (Fig. 5a,b). Thus, a reduction in PKA activity is required for NLP-22-induced quiescence. Though *kin-2* mutants continued to pump upon *nlp-22* over-expression, pumping rates were reduced relative to *kin-2* mutant controls (Supplementary Table S1), indicating that either residual *kin-2* activity in the mutant analyzed is sufficient to reduce PKA activity in response to *nlp-22* over-expression, or that there are also PKA-independent pathways regulating feeding and locomotion quiescence in response to *nlp-22*.

The RIAs have quiescence- and movement-promoting properties

Because the RIA neurons express the somnogenic peptide NLP-22, we hypothesized that they are quiescence-promoting neurons. To test this hypothesis, we used the strain VM1345, which expresses the cell death gene Interleukin-lβ-Converting Enzyme (ICE)[34–36] in the RIA neurons. Because of the incomplete penetrance of the RIA ablation phenotype in VM1345, in a subset of the animals only one of the two RIAs died. We compared L4 lethargus quiescence in animals lacking one or two RIAs to a control transgenic strain with both RIA neurons intact. There was a significant reduction in lethargus quiescence in animals lacking both RIA neurons (Fig. 6a), consistent with the hypothesis that RIA neurons promote quiescence. Animals lacking only one of the RIA neurons also showed a small but significant reduction in L4 lethargus quiescence compared to control transgenic animals (Fig 6a).

This neuron ablation experiment supports the notion that the RIA neurons promote sleep-like behavior. However, removing the neuron by genetic ablation is a chronic manipulation that removes all functions of the neurons, including both those related to rapid signaling via membrane voltage changes as well as chronic signaling via mechanisms independent of membrane voltage. To test whether acute depolarization of the RIA neurons also induces quiescence, we made transgenic animals expressing the blue light-activated cation channel channelrhosopsin-2 (ChR2)[37] in the RIA neurons. We optogenetically depolarized the RIA neurons during the active L4 stage as well as during L4 lethargus. Animals carrying the ChR2 transgene in the RIA neurons were cultivated in the absence (N=4) or presence (N=7) of the ChR2 co-factor all-*trans* retinal (ATR) and followed for eight hours during the active L4 stage. The animals were exposed to blue light for 15 seconds every 30 minutes and monitored for locomotion and feeding quiescence. Stimulating the RIA neurons during the active L4 stage caused no induction of behavioral quiescence. In contrast, optogenetic depolarization of the RIA neurons during L4 lethargus had a locomotion-stimulating effect.

Compared to control animals lacking the ChR2 transgene and to control animals carrying the transgene but lacking ATR, stimulation of the RIA neurons increased movement during lethargus (Fig. 6b). Thus, acute depolarization of the RIA neurons during lethargus promotes wake-like behavior.

We conclude that the RIA neurons have both quiescence-promoting effects, revealed by chronic ablation, and wake-promoting effects, revealed by acute depolarization. Thus, the RIA neurons are complex regulators of lethargus quiescence with both movement- and quiescence-promoting roles.

NLP-22 is structurally similar to mammalian Neuromedin S

The NLP-22 predicted peptide has a glycine at its C-terminus. In prior analyses, *C. elegans* neuropeptides with a C-terminal glycine have been shown to be deglycinated and then amidated during peptide maturation[38]. Hence, NLP-22 has been termed an FRPamide peptide, corresponding to a Phenylalanine(F)-Arginine(R)-Proline(P) motif[19] (Fig. 2a). Using the alignment program Muscle (version 3.7)[39] on the Phylemon 2.0 platform[40], we found that the predicted NLP-22 neuropeptide in *C. elegans* is nearly identical to orthologous proteins of four other nematodes (Fig. 8a), indicating strong functional selection on the neuropeptide sequence. Comparing the NLP-22 neuropeptide with human neuropeptides, we noted similarities with neuromedin S (NMS). Both the NLP-22 and NMS peptides are located near the C-terminus of the preproprotein (Fig. 8b) and are composed of the dipeptide Glycine(G)-Arginine(R) located in the middle of the peptide and the tripeptide FRP located at or close to the C-terminus (Fig. 8b).

To further compare NLP-22 to NMS, we used the peptide structure algorithm Pep-Fold[41] which predicts the three-dimensional structures of small peptides. Pep-Fold predicts that NLP-22 forms an anti-parallel beta-sheet where the conserved GR dipeptide is within the loop and the C-terminal FRP is near the end of the second sheet (Fig. 8c). Interestingly, human NMS is predicted to form the same general structure (Fig. 8c). These structural similarities suggest that NLP-22 and NMS may be orthologous peptides.

Discussion

Wake-promoting neuropeptides such as hypocretin/orexin in vertebrates and PDF in *Drosophila* have well-defined roles as modulators of sleep/wake as an output of the circadian clock[9]. However, few physiologically-relevant sleep-promoting peptides have been identified[3, 5, 42] and their relationship to the circadian clock is poorly understood. We have identified NLP-22 as a *C. elegans* somnogenic peptide. NLP-22 activity is partially regulated at the level of mRNA abundance in response to a LIN-42/PER-based larval clock, suggesting that similar somnogens may function as the output to the PER-based circadian clock in other species.

How might LIN-42 be regulating *nlp-22* expression? *Drosophila* PER acts as a cell-autonomous negative regulator of transcription of genes that are positively regulated by the transcription factors CLK and CYC[20]. In response to ectopic expression of LIN-42, we observed *nlp-22* mRNA induction only after 6–8 hours, which is delayed relative to a typical heat shock response[43]. If LIN-42 were functioning in *C. elegans* similarly to PER function in *Drosophila*, then LIN-42 could repress transcription of a gene that negatively regulates *nlp-22* expression in the same cell. Alternatively, the interaction could be non-cell-autonomous. In support of a possible non-cell-autonomous interaction, restoration of LIN-42 in the hypodermis of *lin-42* mutants partially rescues the asynchronous nature of lethargus quiescence[17] yet *nlp-22* is expressed outside the hypodermis, in the RIA interneurons. Even if LIN-42 were regulating *nlp-22* expression directly, then it must also regulate other genes, since *nlp-22* mutants do not show other phenotypes observed for *lin-42* mutants such as heterochronic defects, slow growth, and molting defects[17, 44].

What is the role of the RIA interneurons during lethargus quiescence? The RIAs are a highly connected pair of interneurons that are required for thermotaxis[45] and aversive/associative learning[35, 46–48]. Our data suggests that the RIAs also control sleep-like behavior. Surprisingly, our experiments revealed both quiescence- and movement-promoting functions for the RIA neurons. Our results suggest that the RIAs release two factors with different behavioral effects: the neuropeptide NLP-22, which decreases PKA activity in an unidentified cell type (Fig. 7) to promote quiescent behavior; and an unknown transmitter, which promotes movement. Since acute depolarization of RIA promotes movement and not quiescence, the mechanism of NLP-22 release may be different from that of the movement-promoting transmitter. NLP-22 release may be regulated by second messengers independently from the membrane voltage. For example, a local calcium increase without membrane potential change could promote NLP-22 secretion without affecting release of wake-promoting factor. Consistent with this possibility, the RIA neurons show sub-cellular compartmentalization of intracellular calcium levels[49]. In addition, NLP-22 availability is regulated at least partially at the level of mRNA abundance. Regardless of the explanation for these results, they highlight both the limitation of relying on ablation alone to infer neuron function and the benefit of using acute optogenetic activation to understand neuronal function.

We have shown that NLP-22 and mammalian NMS are structurally similar (Fig. 8). In addition, NLP-22 and NMS show intriguing functional similarities. NMS shows a diurnal pattern of mRNA expression and is predominately expressed in a small set of central nervous system neurons in the suprachiasmatic nucleus[7]. Like NMS, NLP-22 cycles its expression, only as the output of a larval-based clock, and like NMS, NLP-22 is selectively expressed, in a single neuron pair, the RIAs (Fig. 1c). While the suprachiasmatic nucleus is well known as the master circadian clock pacemaker[50], a role for the RIAs in regulating the analogous larval clock in *C. elegans* has not been reported. The RIAs are required for *C. elegans* memory behaviors[35, 48], behaviors that in mammals are sensitive to the sleep-wake history of the animal[51].

Central nervous system administration of NMS into mammals and birds inhibits feeding behavior[52–55]. NLP-22 over-expression causes an almost complete block of feeding in *C. elegans* (Fig. 2). Central nervous system administration of NMS in rats causes an inhibition of locomotion and a phase shift of the circadian behavioral rhythm[7]. NLP-22 over-expression causes behavioral quiescence during the normally active adult stage (Fig. 2). Our work predicts effects of NMS on sleep behavior, but to our knowledge this has not yet been tested.

While there are functional and structural similarities between NLP-22 and NMS, there are also clear differences. Mammalian NMS has a highly conserved C-terminal RN motif that is not present in *C. elegans* NLP-22. When we over-expressed human NMS(17–33), which contains the 17 C-terminal amino acids of NMS including the amino acids conserved with NLP-22 and which is predicted to form a similar three-dimensional structure as NLP-22 and full length NMS (Supplementary Fig. S2a), we did not observe behavioral quiescence in *C. elegans* (Supplementary Fig. S2b). Furthermore, we could not identify genes coding for proteins predicted to be processed into NLP-22 or NMS-like peptides in the genomes of insects or fish. Finally, loss of function of each of four G-protein coupled receptors that are weakly similar to a mammalian NMS receptor[56] did not suppress the NLP-22-induced quiescence (Supplementary Table S1). Neuropeptides and neuropeptide receptors often show co-evolution, leading to difficulty in identifying homologs across phyla[57]. Thus, future additional data, including the identification of the NLP-22 post-synaptic signaling pathway, will be required to determine whether NLP-22 and NMS are true homologs.

In summary, our work defines the first role for a somnogenic neuropeptide in *C. elegans* and identifies a pair of neurons, the RIAs, in the regulation of *C. elegans* sleep-like behavior. While *C. elegans* sleep-like behavioral research is still in its nascent stage, the simplicity of this model animal's nervous system combined with recent progress in the identification of cells involved in sleep-like behavioral regulation[13–15, 58, 59] holds promise for rapid future progress in delineating the genes and circuits regulating sleep and wake.

MATERIALS AND METHODS

Cultivation methods and Strains

Animals were cultivated on NGM agar and, unless noted otherwise, were fed the OP50 *E. coli* derivative strain DA837[60]. All experiments were performed on hermaphrodites. The following strains were used in this study: **N2** (Bristol), **EG4322** *ttTi5605 II; unc-119(ed3)III*, **TJ375** *gpIs1[Phsp-16.2::gfp]*, **NQ156** *lin-15*(*n765ts*)X; *qnEx48*[P*ins-4:gfp:PEST; lin-15*(+)]**,** **NQ216** *unc-119(ed3)III;qnEx95*[P*hsp-16.2::nlp-22; Pmyo-2:mCherry; unc-119(+)*], **IB16** *ceh-17*(*np1*)I, **NQ230** *ceh-17*(*np1*)I; *qnEx95*, **NQ235** *nmur*-4(ok1381)I; *qnEx95*, **NQ250** *qnIs101[Pnlp-22:nlp-22:gfp; Pmyo-2:mCherry; unc-119*(+)], **NQ251** *qnIs142[Phsp-16.2:nlp-22; Phsp-16.2:gfp; Pmyo-2*:mCherry, *unc-119*(+)], **DA509** *unc-31(e928)IV*, **NQ256** *unc-31*(*e928*)*IV;qnEx95*, **NQ266** *unc-119*(*ed3*)III;*qnEx128*[P*nlp-22*:gfp:*nlp-22; Pmyo-2:mCherry; unc-119*(+)], **NQ269** *unc-119*(*ed3*)III;*qnEx131*[P*hsp-16.2:nlp-22*(No Signal Sequence); P*myo-2*:mCherry; *unc-119(+)*], **NQ270** *unc-119*(*ed3*)*III;qnEx132[Phsp-16.2:nlp-22*(No Signal Sequence); P*myo-2*:mCherry; *unc-119*(+)], **NQ274** *nmur*-1(ok1387)X; *qnEx95*, **NQ275** *nmur-3*(*ok2295*)X; *qnEx95*, **NQ282** *unc-119*(*ed3*)III;*qnEx139[Phsp-16.2:nlp-22*(No Signal Sequence); *Pmyo-2:mCherry; unc-119*(+)], **NQ285** *nmur-2*(*ok3502*)II; *qnEx95*, **NQ305** *sid-1*(*pk3321*)V; *qnIs137*[P*dpy-7:nlp-22(RNAi); Pmyo-2:mCherry; unc-119(+)*], **NQ314** *unc-119*(*ed3*)III; *qnEx160[Phsp-16.2:nlp-22*(KR→AA); *Pmyo-2:mCherry; unc-119*(+)],

KP1873 *egl-3(nu349)V*, **NQ319** *egl-3*(*nu349*)V; *qnEx95*, **KP2018** *egl-21(n476)IV***, NQ320** *egl-21*(*n476*)IV;*qnEx95*, **MT1074** *egl-4*(*n479*)IV**, NQ321** *egl-4*(*n479*)V;*qnEx95*, **NQ333** *unc-119*(*ed3*)III; *qnEx162*[P*nlp-22*:Intron:*gfp*; P*myo-2*:mCherry; *unc-119*(+)], **NQ367** *unc-119*(*ed3*)III; *qnEx178*[P*hsp-16.2:nlp-22*(KR→AA); *Pmyo-2:mCherry; unc-119*(+)], **NQ374** *unc-119*(*ed3*)III; *qnEx185*[P*hsp-16.2:nlp-22*(FRPG→MRPG); P*myo-2*:mCherry; *unc-119*(+)], **NQ375** *unc-119*(*ed3*)III;*qnEx186*[P*hsp-16.2:nlp-22*(FRPG→MRPG); P*myo-2*:mCherry; *unc-119*(+)], **NQ376** *sid-1*(*pk3321*)V; *qnIs157*[*glr-3:nlp-22(RNAi); myo-2:mCherry; unc-119(+)*], **NQ378** *unc-119*(*ed3*)III;*qnEx187*[P*hsp-16.2:nlp-22*(FRPG→FEPG); P*myo-2*:mCherry; *unc-119*(+)], **NQ379** *unc-119*(*ed3*)III;*qnEx187*[P*hsp-16.2:nlp-22*(FRPG→FEPG); P*myo-2*:mCherry; *unc-119*(+)], **NQ383** *unc-119*(*ed3*)III; *qnEx192*[P*hsp-16.2:nlp-22*(FRPG→FRPE); P*myo-2*:mCherry; *unc-119*(+)], **NQ392** *unc-119*(*ed3*)III;*qnEx188*[P*hsp-16.2:nlp-22*(FRPG→FREG); P*myo-2*:mCherry; *unc-119*(+)], **NQ393** *unc-119*(*ed3*)III;*qnEx189*[P*hsp-16.2:nlp-22*(FRPG→FREG); P*myo-2*:mCherry; *unc-119*(+)], **NQ448** *unc-119*(*ed3*)III; *qnEx224*[P*glr-3*:ChR2::YFP (Regular) + *unc-122*:gfp + *unc-119*(+)] **ARF240** *aaaIs1*[*Phsp-16::lin-42a::unc-54utr;* P*myo-2:dsRed*], **NQ596** *nlp-22*(*gk509904*)X, **NQ603** *nlp-22*(*gk509904*)X; *qnEx311*[*nlp-22*(+); *Pmyo-2:Cherry*], **KG532** *kin-2(cd179)*X, **NQ606** *kin-2*(*cd179*)X; *qnIs142*; **VC40199** *nlp-22*(*gk509904*)X; **NQ667** *kin-2*(*ce179*)X; *qnEx95*, **NQ668** *egl-4*(*n479*)IV; *qnIs142*, **NQ670** *qnEx95*, **VM1345** *lin-15*(*n765ts*)X; *akEx211*[P*glr-3:gfp*; P*glr-3*:ICE; *lin-15*(+)], **RB1288** *nmur-1*(*ok1387*)X, **RB2526** *nmur-2*(*ok3502*)II, **VC1974** *nmur-3*(*ok2295*)X, **RB1284** *nmur-4*(*ok1381*)I

Molecular biology and transgenesis

All DNA constructs were made using overlap-extension PCR[61]. Briefly, to fuse two or more DNA fragments, we amplified (Herculase Enhanced DNA Polymerase, Agilent Technologies) the individual DNA fragments with oligonucleotides that included 5′-extensions complementary to the fusion target. Individually-amplified DNA fragments were then mixed without primers in the presence of DNA polymerase in order to anneal and extend the fused product. The fused product was purified (QIAquick PCR Purification Kit, Qiagen) and amplified using nested primers. The size of the final PCR product was verified by gel electrophoresis[61]. Oligonucleotides used for making these constructs are listed in Supplementary Table S3. For *nlp-22* over-expression, the promoter of *hsp-16.2* was amplified from the vector pPD49.83 (obtained from Addgene) and fused to the genomic sequence of *nlp-22*(+1 to +1451) (where nucleotide number refers to the nucleotide position relative to the start site of translation). Both the extrachromosomal array, *qnEx95*, and the integrated array, *qnIs142*, were analyzed in each behavioral assay. The mutated *nlp-22* over-expression constructs (No signal sequence (SS), KR→AA, FRPG→MRPG, FRPG→FEPG, FRPG→FREG, FRPG→FRPE) were created by introducing the desired mutations by overlap-extension PCR using oligonucleotide 5′-extensions that encoded the amino acid changes[61]. Each mutated fused PCR product was cloned into a pCR™2.1-TOPO® TA Vector (Invitrogen™) and then sequenced to verify that the intended mutation but no other mutation was introduced. At least two and often three independent lines were analyzed for each behavioral analysis. To make a fusion protein in which GFP is at the c-terminus of the

NLP-22 protein, the genomic sequence of *nlp-22* (−450 to +701) was fused in frame to *gfp* followed by the *unc-54* 3′UTR, which was amplified from the vector pPD95.75 (Addgene). To make a fusion in which GFP is at the N-terminus of the NLP-22 protein, the promoter (−450 to +3) of *nlp-22* was fused to the coding sequence of GFP amplified from pPD95.75 only excluding the stop codon, and fused in frame to the genomic sequence of *nlp-22* (+1 to +701), which included the *nlp-22* 3′UTR. The transcriptional reporter for *nlp-22* (*Pnlp-22:Intron:gfp*) was made by fusing the promoter of *nlp-22* (−450 to +3) to the coding sequence of GFP, amplified from pPD95.75, and replacing one of the synthetic introns of *gfp* with the intron of *nlp-22*(+85 to +481). To drive expression of *nlp-22* dsRNA in the RIA neurons, the promoter of *glr-3*(−5500 to −1) was fused to the genomic sequence of *nlp-22* spanning the region +13 to +653 in the sense orientation, and, in a separate reaction, to the *nlp-22* genomic sequences spanning the same nucleotides in the anti-sense orientation. These constructs lack a start codon and 3′UTR of *nlp-22*. To drive expression of *nlp-22* dsRNA in the hypodermis, the promoter of *dpy-7* (−417 to −1) amplified from N2 genomic DNA was fused to *nlp-22* sense and anti-sense DNA. The genomic rescuing fragment of *nlp-22* spanned from −450 to +1451 relative to the *nlp-22* start codon. To over-express Neuromedin S (17-33) (Phoenix Pharmaceuticals, Inc), we replaced the coding sequence for the *nlp-22* peptide with the coding sequence for Neuromedin S (17-33) and fused the *nlp*-22-NMS(17-33) chimeric sequence to the heat shock promoter.

Transgenic animals were created by microinjection[62] using a Leica DMIRB inverted DIC microscope equipped with an Eppendorf Femtojet microinjection system. EG4322 animals were injected with 25–50ng/μl of each construct in combination with 5ng/μl pCFJ90 [P*myo-2:mCherry*], with 50–100 ng/μl of P*unc-122*:gfp, or 5ng/μl of pCFJ104, P*rab-3::mCherry*) and pCFJ151 [*unc-119*(+)] up to a total concentration of 150 ng/μl. To make the *nlp-22* RNAi strains, 25 ng/μl of *nlp-22*(sense) and *nlp-22*(anti-sense) were injected together with the transgenesis markers pCFJ90 and pCFJ151. For behavioral experiment using transgenic animals carrying extrachromosomal arrays at least two distinct lines were analyzed. In most cases, quantitative behavioral analysis was performed only on one of these transgenic lines but in all cases the other line(s) gave the same qualitative result.

The integrated transgenes *qnIs101*, *137*, *142*, and *157* were constructed by UV irradiation of the extrachromosomal transgenes *qnEx101*, *137*, *142*, and *157*[63]. A mix of approximately 100 L4 and adult transgenic animals were placed in a Spectrolinker XL-1500 UV Crosslinker (Spectronics Corporation) and exposed to 30,000 μJ/cm^2 of ultraviolet light. Irradiated animals were plated at a density of five animals per plate, and grown for multiple generations. Integrants were identified as those that produced 100% transgenic progeny. Each integrated strain was out-crossed to the wild-type strain a minimum of 3 times before analysis.

The strain VC40199 containing the *nlp-22*(*gk509904*) X allele was produced by the million mutation project[64] and obtained from the Caenorhabditis Genetics Consortium. The CGA to TGA mutation in *nlp-22* reported for this strain was verified by sequencing a genomic PCR fragment, which was PCR-amplified using the primers oNQ552 and oNQ534 (Supplementary Table S3). The strain NQ596 *nlp-22*(*gk509904*) X was generated by crossing the strain VC40199 to N2 males, and then crossing resultant males back to

VC40199. This procedure was repeated 4X. A twitcher phenotype, which was present in VC40199 animals, was not present in the out-crossed NQ596 animals.

Over-expression analysis

For all over-expression experiments, animals carrying arrays with inducible heat-shock promoters were grown to young adulthood at 20°C, transferred to the surface of NGM agar 6-cm diameter plates fully seeded with a lawn of DA837 *E. coli* bacteria, wrapped in Parafilm, and immersed in a 33°C water bath for 30 minutes. Heat-shocked animals were placed at 20°C prior to analysis. Pharyngeal pumping and body bends were counted during 20 second and 1 minute intervals, respectively. Arousal threshold was determined by measuring the latency to respond to exposure to blue light[65], or exposure to a hair dipped in a freshly prepared mixture of 30% octanol and 70% ethanol[66]. Response to octanol was defined as backwards movement of the magnitude corresponding to one third of the worm's length. Response to blue light was defined as movement (either forward or backwards) of magnitude equal to one half of the animal's body length. Arousal threshold assays of *nlp-22* mutants and *nlp-22* mutants carrying a rescuing transgene were performed by an investigator who was blinded to genotype. The blinding was setup by a colleague without the use of a computer randomization program. It was impossible to blind the investigator to the genotype of the *nlp-22*(OE) transgenic animals because these animals were extremely quiescent and retained excessive eggs.

In experiments designed to assess the effects of mild over-expression of *nlp-22* on direction of locomotion, animals were analyzed one hour after a 15 minute 33°C heat exposure. Behavior was assessed on an agar surface devoid of bacteria. During a 120-second period, we measured with a hand-held timer the total time spent moving forward as well as the total time spent moving backwards. The average of 20 experimental animals expressing *Phsp16.2:nlp-22* (*qnIs142*) was compared with the average of 20 control N2 animals.

Imaging of fluorescence

For GFP and differential interference contrast imaging, animals were mounted on 5% agar pads, immobilized with 15 mM levamisole, and observed through a 63X or 100X objective lens on a Leica DM5500B microscope. Leica LAS software was used to capture images.

Quiescence measurements

For automated measurements of lethargus quiescence we placed single larvae in molded, concave polydimethylsiloxane (PDMS) wells of diameter 3 mm and depth 2.5 mm, filled with 15 ul of NGM agar and seeded with DA837 bacteria. In each experiment, we placed one experimental and one control animal into two adjacent wells and the PDMS was then placed on a Diagnostics Instruments microscope base and illuminated for bright field microscopy, using white light supplied to the base with a fiber optic cable from a Schott DCR III light source. A camera (659x494 pixels, scA640-70fm, Basler Vision Technologies) mounted on a Zeiss Stemi 2000 stereomicroscope captured an image with 8-bit grayscale of both wells every 10 seconds. At this magnification and our camera acquisition setting, the spatial resolution was 12.5 micrometers per pixel. To quantify quiescence, we monitored animals for time a period between 12 and 48 hours, and used a machine vision frame subtraction principle to identify 10-second epochs of behavioral quiescence[12, 27, 67]. Recordings were performed in an environmental room at 19±2°C. In comparison to conditions used in prior quiescence measurements, the room temperature was lower and pixel resolution was reduced, leading to quiescence measurements that were greater than those previously reported[12]. For each experimental condition, quiescence of at least six animals was measured because we previously found this number to be sufficient to detect a difference in quiescence[12]. For most conditions, 9–11 animals were used. Data was discarded if the temperature of the room exceeded 21°C due to equipment malfunction or if one of the two worms burrowed in the agar. Quiescence data from VM1345 was discarded when, upon inspection with epifluorescence, we determined that neither RIA had died. We censored 12% of the VM1345 data for that reason.

qPCR

L2 or L3 worms were picked to fresh plates seeded with DA837 bacteria and allowed to enter lethargus. We identified worms in L2 or L3 lethargus, using a Leica MZ16 stereomicroscope, by an absence of locomotion and feeding. A subset of worms was harvested for RNA isolation in either L2 or L3 lethargus while the remaining animals were allowed to mature for additional durations prior to harvesting. The experiment was performed at 25°C to speed development. L4 lethargus animals were similarly identified by first transferring mid L4 animals to a fresh agar dish seeded with DA837, and then harvesting animals that had stopped moving and feeding.

To test *lin-42*-dependent induction of *nlp-22* mRNA, ARF240 first day adult animals, which were cultivated at 15°C, were submersed while housed on an NGM agar surface fully seeded with bacteria in a 33°C water bath for 30 minutes and then recovered at 25°C. Control N2 animals were treated identically. RNA was collected before heat-shock treatment and at 4, 6, and 8 hours after the end of the heat treatment.

Total RNA was harvested from each population using an RNAeasy mini kit (Qiagen), and cDNA was synthesized using the SuperScript® one-step RT-PCR system (Invitrogen). We performed three or more biological replicates in each experiment and for each biological replicate we used the average of two technical real-time PCR replicates. Real-time PCR was performed using Taqman® Gene Expression Mastermix on an Applied Biosystems 7500 platform at the core services within the Penn Center for AIDS Research, an NIH-funded program (P30 AI 045008). Oligonucleotides used for the real-time PCR analysis were purchased from IDT and their sequences are shown in Table S3. Relative mRNA was determined by the delta-delta method[68] by normalization to the expression of the gene *pmp-3*[69].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Amita Sehgal, Liliane Schoofs, and Lotte Frooninckx for comments. This work was supported by NIH grants T32HL07713 to M.D.N. (PI, Allan Pack), T31HL07953 to N.F.T. (PI, Allan Pack), R01NS064030 to

D.M.R., a NARSAD Young Investigator Award to D.M.R, a McCabe Foundation grant to D.M.R., a Penn University Scholars Award to C-C.Y., and by an Alfred P. Sloan Foundation Research Fellowship to C.F-Y. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The strain ARF240 was provided by A. Frand and the strain VM1345 was provided by A. Maricq.

References

1. Lin L, et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell. 1999; 98:365–76. [PubMed: 10458611]
2. Cheng MY, et al. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature. 2002; 417:405–10. [PubMed: 12024206]
3. Kramer A, et al. Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. Science. 2001; 294:2511–5. [PubMed: 11752569]
4. Xu YL, et al. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron. 2004; 43:487–97. [PubMed: 15312648]
5. Lin SH, et al. Prolactin-releasing peptide (PrRP) promotes awakening and suppresses absence seizures. Neuroscience. 2002; 114:229–38. [PubMed: 12207968]
6. Huitron-Resendiz S, et al. Urotensin II modulates rapid eye movement sleep through activation of brainstem cholinergic neurons. J Neurosci. 2005; 25:5465–74. [PubMed: 15944374]
7. Mori K, et al. Identification of neuromedin S and its possible role in the mammalian circadian oscillator system. EMBO J. 2005; 24:325–35. [PubMed: 15635449]
8. Parisky KM, et al. PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron. 2008; 60:672–82. [PubMed: 19038223]
9. Crocker A, Sehgal A. Genetic analysis of sleep. Genes Dev. 2010; 24:1220–35. [PubMed: 20551171]
10. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell. 1999; 99:791–802. [PubMed: 10619432]
11. Singh RN, Sulston JE. Some Observations on Molting in Caenorhabditis-Elegans. Nematologica. 1978; 24:63.
12. Raizen DM, et al. Lethargus is a Caenorhabditis elegans sleep-like state. Nature. 2008; 451:569–72. [PubMed: 18185515]
13. Driver RJ, Lamb AL, Wyner AJ, Raizen DM. DAF-16/FOXO regulates homeostasis of essential sleep-like behavior during larval transitions in C. elegans. Curr Biol. 2013; 23:501–6. [PubMed: 23477722]
14. Van Buskirk C, Sternberg PW. Epidermal growth factor signaling induces behavioral quiescence in Caenorhabditis elegans. Nat Neurosci. 2007; 10:1300–7. [PubMed: 17891142]
15. Choi S, Chatzigeorgiou M, Taylor KP, Schafer WR, Kaplan JM. Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in C. elegans. Neuron. 2013; 78:869–80. [PubMed: 23764289]
16. Jeon M, Gardner HF, Miller EA, Deshler J, Rougvie AE. Similarity of the C. elegans developmental timing protein LIN-42 to circadian rhythm proteins. Science. 1999; 286:1141–6. [PubMed: 10550049]
17. Monsalve GC, Van Buskirk C, Frand AR. LIN-42/PERIOD controls cyclical and developmental progression of C. elegans molts. Curr Biol. 2011; 21:2033–45. [PubMed: 22137474]
18. Konopka RJ, Benzer S. Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A. 1971; 68:2112–6. [PubMed: 5002428]
19. Nathoo AN, Moeller RA, Westlund BA, Hart AC. Identification of neuropeptide-like protein gene families in Caenorhabditiselegans and other species. Proc Natl Acad Sci U S A. 2001; 98:14000–5. [PubMed: 11717458]
20. Dunlap JC. Molecular bases for circadian clocks. Cell. 1999; 96:271–90. [PubMed: 9988221]
21. Gissendanner CR, Crossgrove K, Kraus KA, Maina CV, Sluder AE. Expression and function of conserved nuclear receptor genes in Caenorhabditis elegans. Dev Biol. 2004; 266:399–416. [PubMed: 14738886]

22. Hu Z, Pym EC, Babu K, Vashlishan Murray AB, Kaplan JM. A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. Neuron. 2011; 71:92–102. [PubMed: 21745640]
23. Hart, AC. Wormbook, ed. Ambros, V., editor. 2006.
24. Raizen D, Song BM, Trojanowski N, You YJ. Methods for measuring pharyngeal behaviors. WormBook. 2012:1–13. [PubMed: 23255345]
25. Li C, Kim K. Neuropeptides. WormBook. 2008:1–36. [PubMed: 18819171]
26. Iwanir S, et al. The microarchitecture of C. elegans behavior during lethargus: homeostatic bout dynamics, a typical body posture, and regulation by a central neuron. Sleep. 2013; 36:385–95. [PubMed: 23449971]
27. Belfer SJ, et al. Caenorhabditis-in-drop array for monitoring C. elegans quiescent behavior. Sleep. 2013; 36:689–698G. [PubMed: 23633751]
28. Husson SJ, Clynen E, Baggerman G, Janssen T, Schoofs L. Defective processing of neuropeptide precursors in Caenorhabditis elegans lacking proprotein convertase 2 (KPC-2/EGL-3): mutant analysis by mass spectrometry. J Neurochem. 2006; 98:1999–2012. [PubMed: 16945111]
29. Husson SJ, et al. Impaired processing of FLP and NLP peptides in carboxypeptidase E (EGL-21)-deficient Caenorhabditis elegans as analyzed by mass spectrometry. J Neurochem. 2007; 102:246–60. [PubMed: 17564681]
30. Speese S, et al. UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in Caenorhabditis elegans. J Neurosci. 2007; 27:6150–62. [PubMed: 17553987]
31. Schade MA, Reynolds NK, Dollins CM, Miller KG. Mutations that rescue the paralysis of Caenorhabditis elegans ric-8 (synembryn) mutants activate the G alpha(s) pathway and define a third major branch of the synaptic signaling network. Genetics. 2005; 169:631–649. [PubMed: 15489510]
32. Zimmerman JE, Naidoo N, Raizen DM, Pack AI. Conservation of sleep: insights from non-mammalian model systems. Trends Neurosci. 2008; 31:371–6. [PubMed: 18538867]
33. Charlie NK, Schade MA, Thomure AM, Miller KG. Presynaptic UNC-31 (CAPS) is required to activate the G alpha(s) pathway of the Caenorhabditis elegans synaptic signaling network. Genetics. 2006; 172:943–61. [PubMed: 16272411]
34. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell. 1993; 75:641–52. [PubMed: 8242740]
35. Stetak A, Horndli F, Maricq AV, van den Heuvel S, Hajnal A. Neuron-specific regulation of associative learning and memory by MAGI-1 in C. elegans. PLoS One. 2009; 4:e6019. [PubMed: 19551147]
36. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 1993; 75:653–60. [PubMed: 8242741]
37. Nagel G, et al. Light activation of channelrhodopsin-2 in excitable cells of Caenorhabditis elegans triggers rapid behavioral responses. Curr Biol. 2005; 15:2279–84. [PubMed: 16360690]
38. Husson SJ, Clynen E, Baggerman G, De Loof A, Schoofs L. Discovering neuropeptides in Caenorhabditis elegans by two dimensional liquid chromatography and mass spectrometry. Biochem Biophys Res Commun. 2005; 335:76–86. [PubMed: 16061202]
39. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32:1792–7. [PubMed: 15034147]
40. Sanchez R, et al. Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing. Nucleic Acids Res. 2011; 39:W470–4. [PubMed: 21646336]
41. Maupetit J, Derreumaux P, Tuffery P. PEP-FOLD: an online resource for de novo peptide structure prediction. Nucleic Acids Res. 2009; 37:W498–503. [PubMed: 19433514]
42. Jewett KA, Krueger JM. Humoral sleep regulation; interleukin-1 and tumor necrosis factor. Vitam Horm. 2012; 89:241–57. [PubMed: 22640617]
43. Candido EP. The small heat shock proteins of the nematode Caenorhabditis elegans: structure, regulation and biology. Prog Mol Subcell Biol. 2002; 28:61–78. [PubMed: 11908066]

44. Abrahante JE, Miller EA, Rougvie AE. Identification of heterochronic mutants in Caenorhabditis elegans. Temporal misexpression of a collagen::green fluorescent protein fusion gene. Genetics. 1998; 149:1335–51. [PubMed: 9649524]
45. Mori I, Ohshima Y. Neural regulation of thermotaxis in Caenorhabditis elegans. Nature. 1995; 376:344–8. [PubMed: 7630402]
46. Chen Z, et al. Two insulin-like peptides antagonistically regulate aversive olfactory learning in C. elegans. Neuron. 2013; 77:572–85. [PubMed: 23395381]
47. Harris G, et al. Dissecting the serotonergic food signal stimulating sensory-mediated aversive behavior in C. elegans. PLoS One. 2011; 6:e21897. [PubMed: 21814562]
48. Kuhara A, Mori I. Molecular physiology of the neural circuit for calcineurin-dependent associative learning in Caenorhabditis elegans. J Neurosci. 2006; 26:9355–64. [PubMed: 16971519]
49. Hendricks M, Ha H, Maffey N, Zhang Y. Compartmentalized calcium dynamics in a C. elegans interneuron encode head movement. Nature. 2012; 487:99–103. [PubMed: 22722842]
50. Ibuka N, Kawamura H. Loss of circadian rhythm in sleep-wakefulness cycle in the rat by suprachiasmatic nucleus lesions. Brain Res. 1975; 96:76–81. [PubMed: 1175007]
51. Maquet P. The role of sleep in learning and memory. Science. 2001; 294:1048–52. [PubMed: 11691982]
52. Ida T, et al. Neuromedin S is a novel anorexigenic hormone. Endocrinology. 2005; 146:4217–4223. [PubMed: 15976061]
53. Shousha S, et al. Effect of neuromedin S on feeding regulation in the Japanese quail. Neurosci Lett. 2006; 391:87–90. [PubMed: 16159696]
54. Tachibana T, Matsuda K, Khan MS, Ueda H, Cline MA. Feeding and drinking response following central administration of neuromedin S in chicks. Comp Biochem Physiol A Mol Integr Physiol. 2010; 157:63–7. [PubMed: 20451649]
55. Atsuchi K, et al. Centrally administered neuromedin S inhibits feeding behavior and gastroduodenal motility in mice. Horm Metab Res. 2010; 42:535–8. [PubMed: 20352600]
56. Maier W, Adilov B, Regenass M, Alcedo J. A neuromedin U receptor acts with the sensory system to modulate food type-dependent effects on C. elegans lifespan. PLoS Biol. 2010; 8:e1000376. [PubMed: 20520844]
57. Moyle WR, et al. Co-evolution of ligand-receptor pairs. Nature. 1994; 368:251–5. [PubMed: 8145825]
58. Schwarz J, Lewandrowski I, Bringmann H. Reduced activity of a sensory neuron during a sleep-like state in Caenorhabditis elegans. Curr Biol. 2011; 21:R983–4. [PubMed: 22192827]
59. Gallagher T, Kim J, Oldenbroek M, Kerr R, You YJ. ASI regulates satiety quiescence in C. elegans. J Neurosci. 2013; 33:9716–24. [PubMed: 23739968]
60. Davis MW, et al. Mutations in the Caenorhabditis elegans Na,K-ATPase alpha-subunit gene, eat-6, disrupt excitable cell function. J Neurosci. 1995; 15:8408–18. [PubMed: 8613772]
61. Nelson MD, Fitch DH. Overlap extension PCR: an efficient method for transgene construction. Methods Mol Biol. 2011; 772:459–70. [PubMed: 22065455]
62. Stinchcomb DT, Shaw JE, Carr SH, Hirsh D. Extrachromosomal DNA transformation of Caenorhabditis elegans. Mol Cell Biol. 1985; 5:3484–96. [PubMed: 3837845]
63. Mello C, Fire A. DNA transformation. Methods Cell Biol. 1995; 48:451–82. [PubMed: 8531738]
64. Thompson O, et al. The Million Mutation Project: A new approach to genetics in Caenorhabditis elegans. Genome Res. 2013
65. Belfer S, et al. Caenorhabditis-in-drop array for monitoring C. elegans quiescent behavior. Sleep. 2013 In press.
66. Chao MY, Komatsu H, Fukuto HS, Dionne HM, Hart AC. Feeding status and serotonin rapidly and reversibly modulate a Caenorhabditis elegans chemosensory circuit. Proc Natl Acad Sci U S A. 2004; 101:15512–7. [PubMed: 15492222]
67. Zimmerman JE, Raizen DM, Maycock MH, Maislin G, Pack AI. A video method to study Drosophila sleep. Sleep. 2008; 31:1587–98. [PubMed: 19014079]
68. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods. 2001; 25:402–8. [PubMed: 11846609]

69. Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. BMC Mol Biol. 2008; 9:9. [PubMed: 18211699]

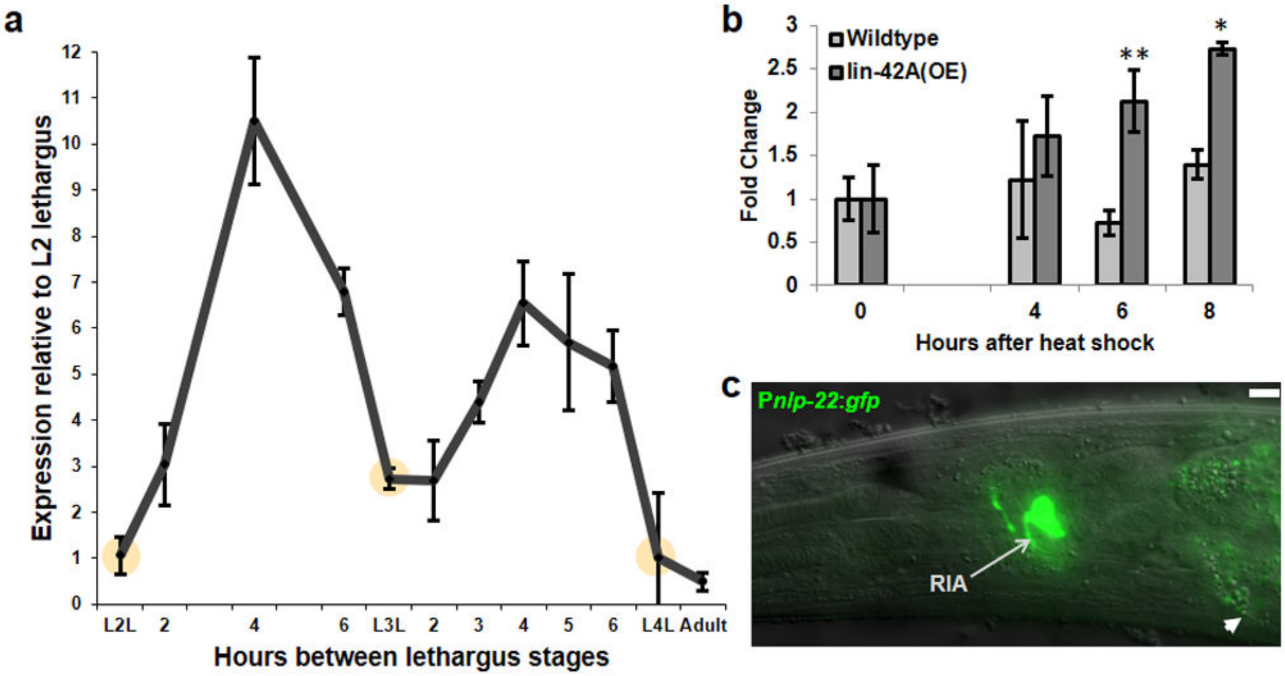

FIGURE 1.

nlp-22 is expressed in dynamic fashion in the RIA interneurons. (**a**) mRNA expression during larval development. Expression levels cycle in synchrony with each lethargus stage. Yellow circles depict each lethargus stage, as defined by cessation of feeding of the animals (Biological replicates per time point ≥3, technical replicates = 2 for each biological replicate; Error bars represent s.e.m.). (**b**) *nlp-22* expression is increased in response to *lin-42* over-expression during the adult stage. (*p<0.05, ** p<0.005, Student's two-tailed *t*-Test, Biological replicates per condition ≥5, technical replicates = 2 for each biological replicate; Error bars represent s.e.m.). (**c**) An *nlp-22* transcriptional GFP reporter is expressed in the RIA neurons (arrow). Intestinal auto-fluorescence is also observed (arrow head). Scale Bar = 5μM.

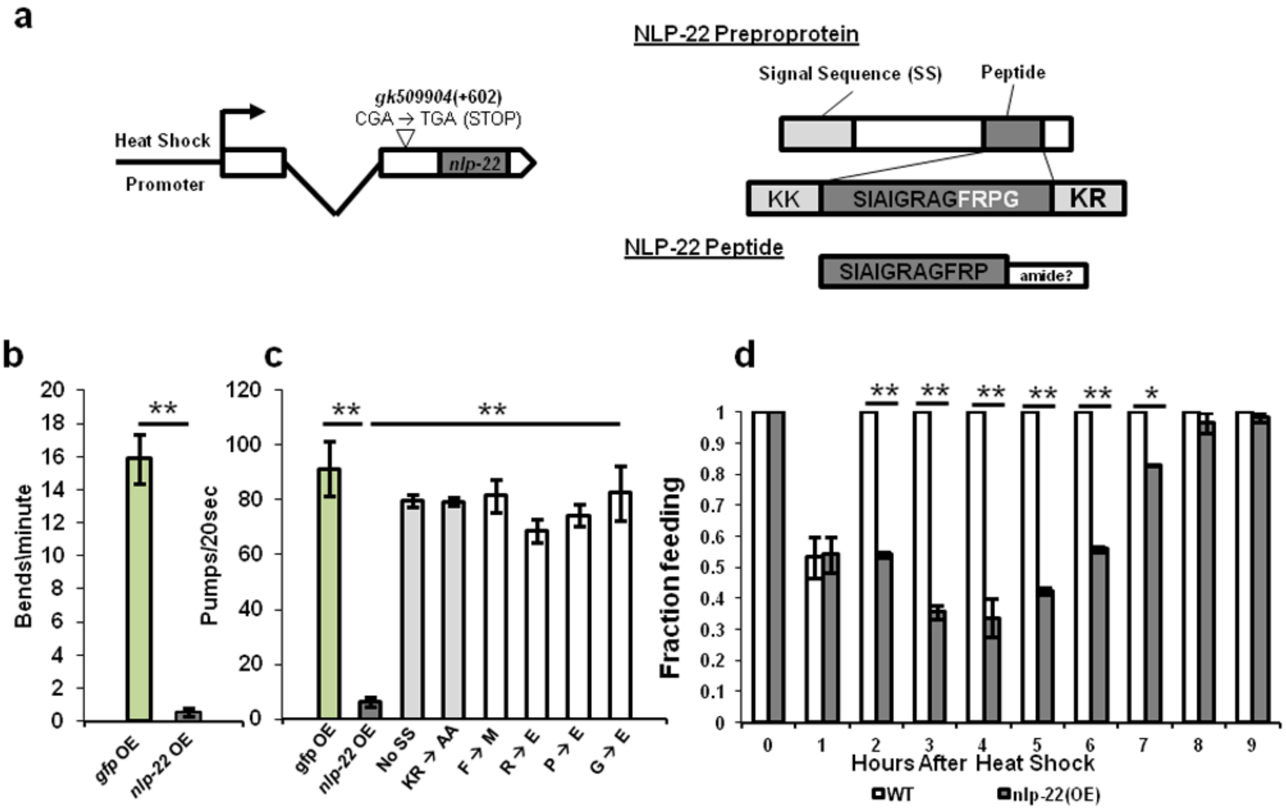

FIGURE 2.

NLP-22 induces behavioral quiescence. (**a**) Gene and protein structure of *nlp-22* including over-expression construct. 2.5 hours after *nlp-22* induction, animals show reduced movement (**b**) and feeding (**c**) relative to control animals over-expressing GFP (strain: TJ375). (**c**) Removing the signal sequence, or mutating the KR or FRPG eliminates the ability of NLP-22 to reduce pumping rate (**p<0.0001, Student's two-tailed *t*-Test, N≥20). (**d**) Animals over-expressing *nlp-22* cease pumping, but recover 9 hours after heat-shock (*p<0.001, **p<0.0001, Fisher's exact test, N≥20 animals, 2 trials; Error bars represent s.e.m.).

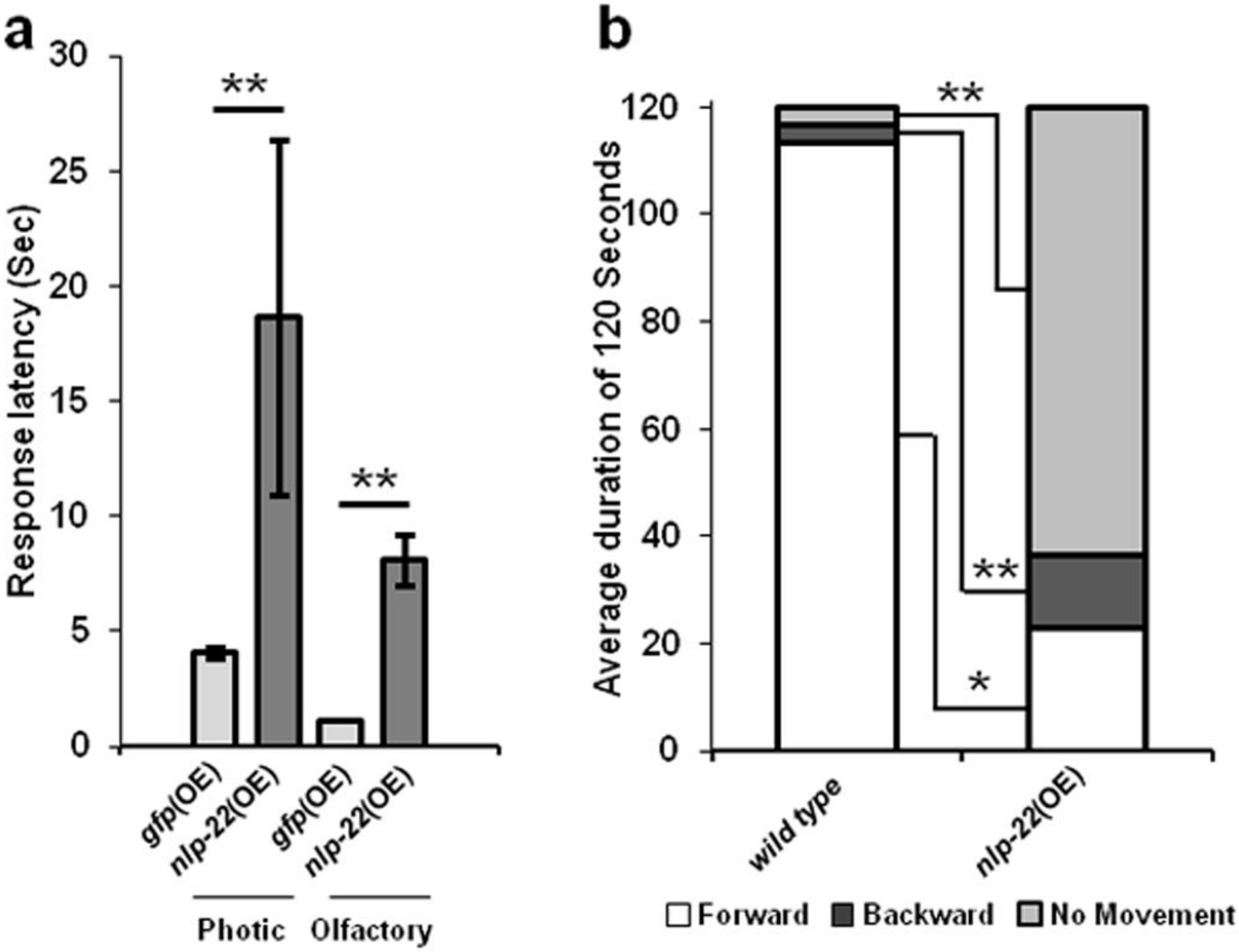

FIGURE 3.

Quiescence induced by *nlp-22* OE resembles lethargus quiescence. (**a**) Animals that over-express *nlp-22* (strain: NQ251) are less responsive to chemical (1-octanol) and optical (blue light) stimulation than control animals that over-express GFP (strain: TJ375) (**p<0.0001, Student's two-tailed *t*-Test, N≥20; Error bars represent s.e.m.). (**b**) Over-expressing *nlp-22* via a mild heat shock, such that full quiescence is not induced, results in increased backwards movement. White, dark gray, and light gray denote forward, backwards, and no movement, respectively. The average fraction of time spent moving forward or moving backward in a 120s window for *nlp-22* over-expressing animals is significantly different from WT worms (*p<0.001. **p<0.0001, Student's two-tailed *t*-Test, N=20). In addition to the bias for backwards motion, *nlp-22* over-expressing animals also moved significantly less than WT animals (**p<0.0001, Student's two-tailed *t*-Test, N=20).

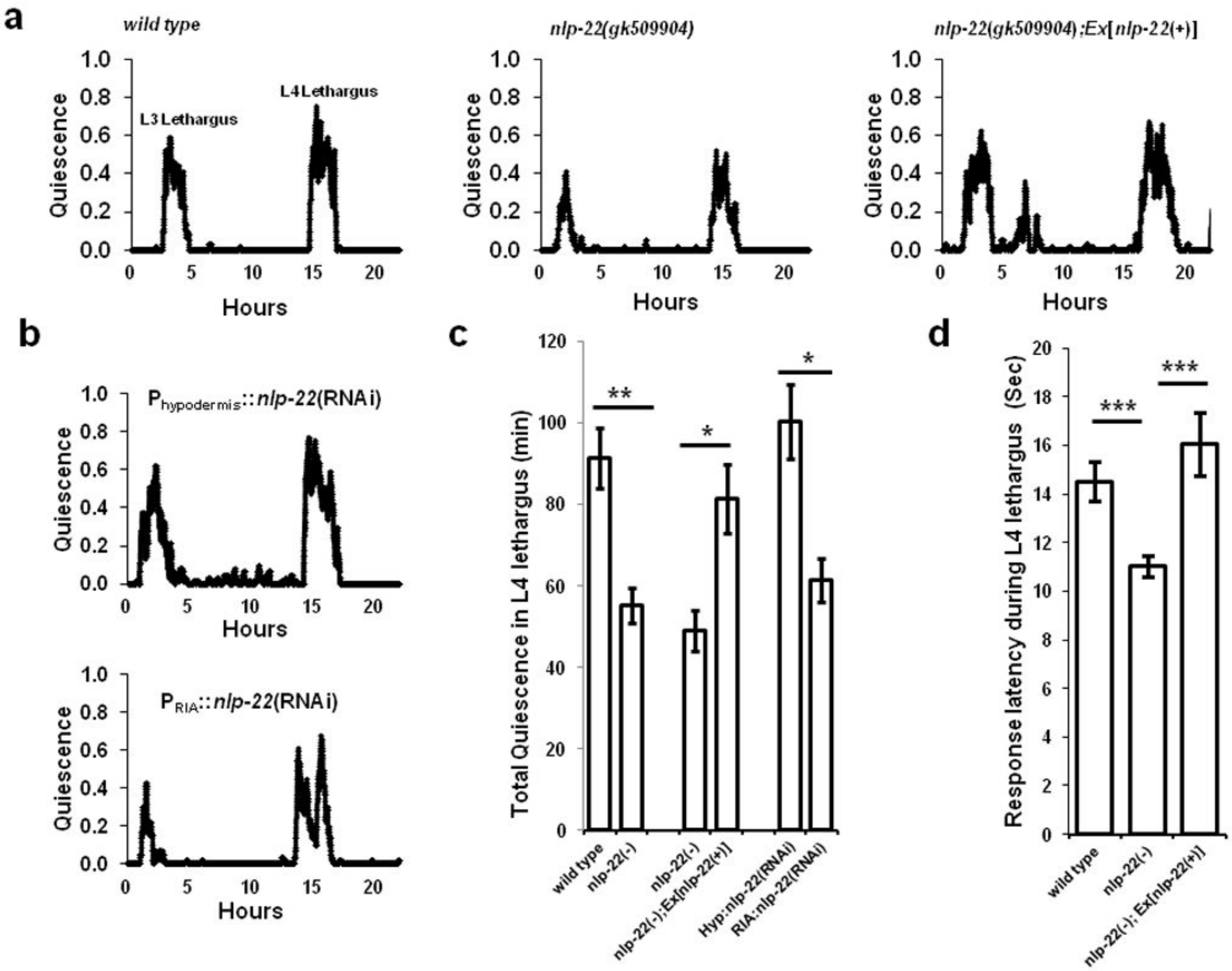

FIGURE 4.

nlp-22 is required for normal quiescence during lethargus. (**a**) Fraction of quiescence in a 10-minute moving window in a wild-type animal, an *nlp-22(gk509904)* mutant, and an *nlp-22*(*gk509904*) mutant carrying an *nlp-22* genomic rescuing transgene. The x-axis is hours from the start of recording in the late third larval stage. (**b**) Quiescence in animals expressing double stranded *nlp-22* RNA in the hypodermis and in the RIA neurons. Average quiescence (*p<0.05, **p<0.01, ***p<0.001, Student's two-tailed *t*-Test; N≥10, Error bars represent s.e.m.), (**c**) and response latencies to blue light (**d**) during fourth larval stage lethargus (***p<0.001, Student's two-tailed *t*-Test; N≥28 animals, Error bars represent s.e.m.).

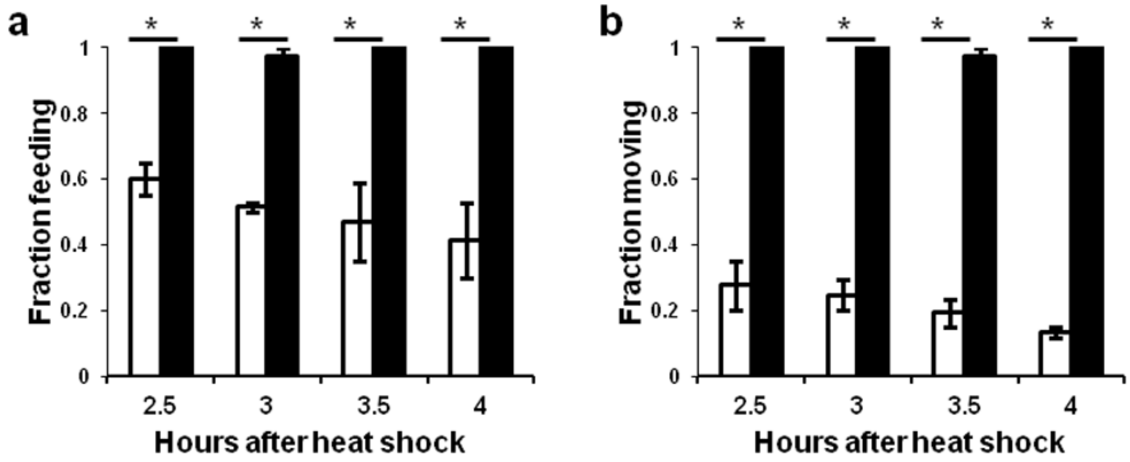

FIGURE 5.

NLP-22-induced quiescence requires the protein kinase A subunit KIN-2. Feeding (**a**) and locomotion (**b**) quiescence induced by *nlp-22* over-expression is impaired in *kin-2* mutants (*p<0.0001, Fisher's exact test, N>20 animals, 2 trials; Error bars represent s.e.m.). Unfilled bars represent animals of genotype *qnIs142*[P*hsp-16.2:nlp-22;* P*hsp-16.2:gfp;* P*myo-2:mCherry; unc-119*(+)] and filled bars represent animals of genotype *qnIs142; kin-2*(*ce179*)X.

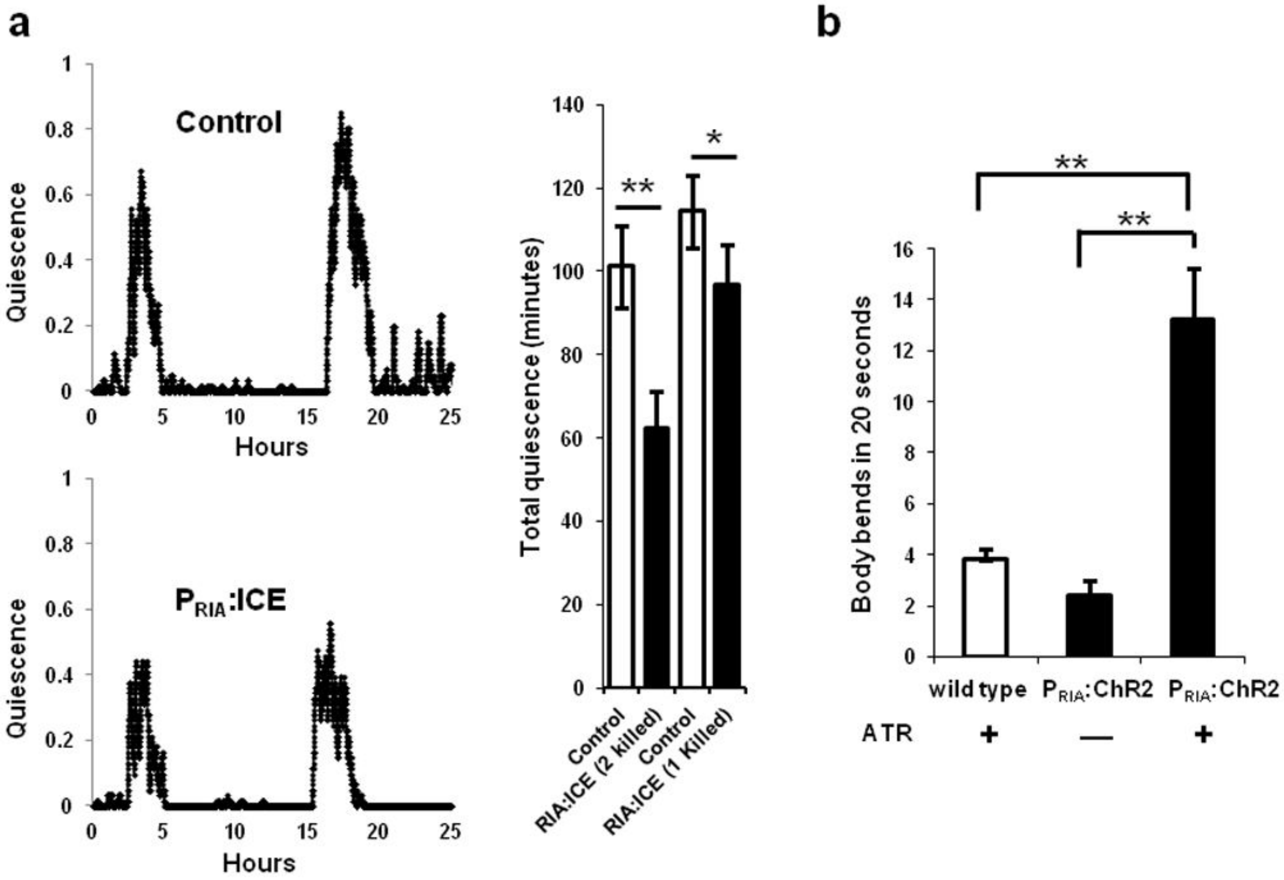

FIGURE 6.

The RIA neurons have both quiescence- and movement-promoting functions. (**a**) Genetic ablation of the RIA interneurons using the cell death protein ICE (strain:VM1345) decreases quiescence during lethargus compared to control transgenic animals (strain: NQ156). The traces depict the fraction of quiescence in a 10 minute time interval. The top trace is of a control animal of genotype *lin-15*(*n765ts*)X; *qnEx48*[P*ins-4:gfp:PEST; lin-15*(+)], and the bottom trace is of an experimental animal of genotype *lin-15*(*n765ts*)X; *akEx211*[P*glr-3:gfp*; P*glr-3*:ICE; *lin-15*(+)]. Total quiescence during L4 lethargus is lower in experimental animals lacking either one or both RIA neurons than in control animals to which they were paired (*p<0.05, **p<0.001, Student's two-tailed *t*-Test, N≥9; Error bars represent s.e.m.). (**b**) Optogenetic stimulation of the RIA neurons promotes movement during lethargus. WT animals grown on All Trans Retinal (ATR) and P_{RIA}:ChR2 worms grown either in the absence or presence of ATR, were identified as they entered L4 lethargus, transferred to a new plate and were unperturbed for 15 minutes until they became quiescent. Body bends were counted for one minute after a 15-second exposure of quiescent animals to blue light. P_{RIA}:ChR2 animals on ATR moved significantly more than control animals (*p<0.05, **p<0.001, Student's two-tailed *t*-Test, WT (N=15), No ATR vs. ATR (N≥20); Error bars represent s.e.m.).

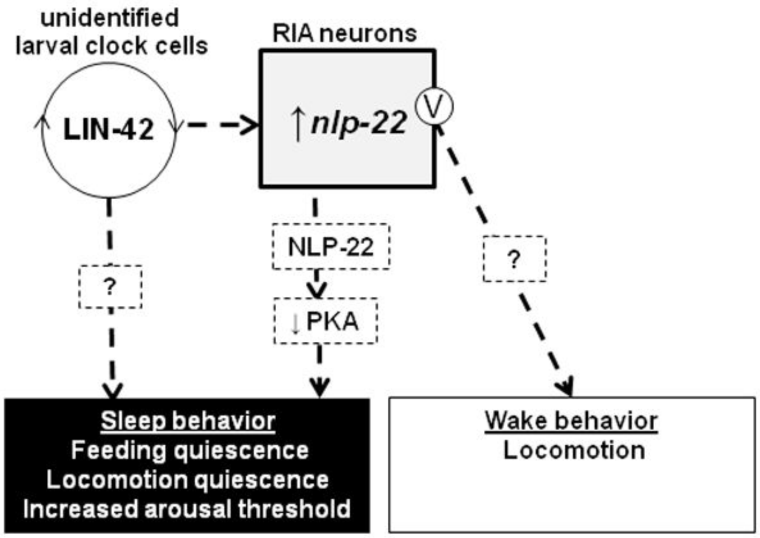

FIGURE 7.

Model for role of *nlp-22* and RIA in sleep-like quiescence regulation. LIN-42 functions in as yet unidentified larval clock cells to regulate nlp-22 expression as well as other quiescence-regulatory factors. Release of NLP-22 neuropeptide promotes sleep-like behavior via a reduction of protein kinase A (PKA) activity. RIA membrane depolarization (marked with the letter V) results in the release of an unidentified wake-promoting neurotransmitter.

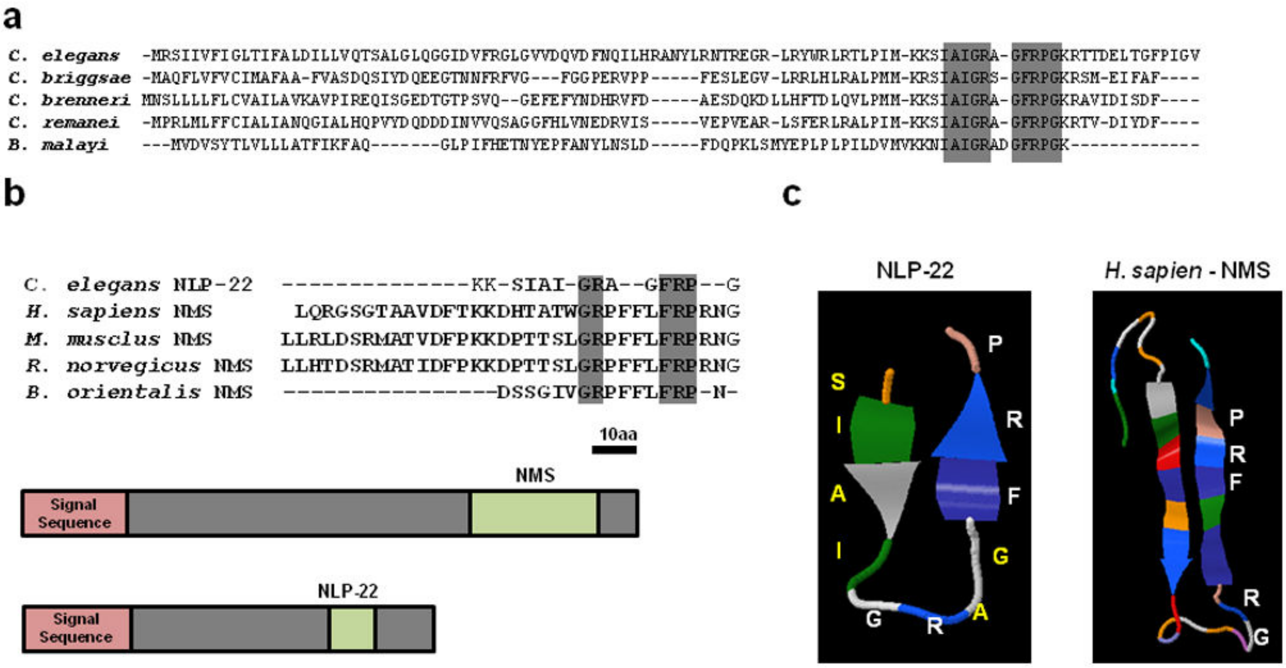

FIGURE 8.

NLP-22 is similar to Neuromedin S. (**a**) Alignment of NLP-22 preproprotein with orthologs from four nematode species. High conservation (gray box) is observed in the predicted neuropeptide sequence. (**b**) Amino acid sequence similarities (gray boxes) between NLP-22 and vertebrate Neuromedin S peptides. The preproprotein structure of human Neuromedin S and NLP-22 is also shown. Each has an N-terminal signal sequence and a single, c-terminal peptide. The preproprotein is drawn to scale (Bar = 10 Amino Acids) (**c**) Predicted structures of NLP-22 and of NMS. The conserved GR dipeptide and FRP tripeptide motifs are shown in white.