The Neuropeptides FLP-2 and PDF-1 Act in Concert To Arouse *Caenorhabditis elegans* Locomotion

Didi Chen,*,†,1,2 Kelsey P. Taylor,*,†,‡,1 Qi Hall,*,† and Joshua M. Kaplan*,†,‡,3

*Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, and †Department of Neurobiology and ‡Biological and Biomedical Sciences Program, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT During larval molts, *Caenorhabditis elegans* exhibits a sleep-like state (termed lethargus) that is characterized by the absence of feeding and profound locomotion quiescence. The rhythmic pattern of locomotion quiescence and arousal linked to the molting cycle is mediated by reciprocal changes in sensory responsiveness, whereby arousal is associated with increased responsiveness. Sensory neurons arouse locomotion via release of a neuropeptide (PDF-1) and glutamate. Here we identify a second arousing neuropeptide (FLP-2). We show that FLP-2 acts via an orexin-like receptor (FRPR-18), and that FLP-2 and PDF-1 secretion are regulated by reciprocal positive feedback. These results suggest that the aroused behavioral state is stabilized by positive feedback between two neuropeptides.

KEYWORDS *Caenorhabditis elegans*; orexin; PDF; sleep; lethargus

ANIMALS undergo periods of behavioral quiescence and arousal in response to changes in their environment and metabolic state. Arousal is defined as a state of heightened responsiveness to sensory cues coupled with increased motor activity whereas quiescence is associated with diminished responsiveness and motor activity (Pfaff *et al.* 2008). Arousal is associated with fear, stress, hunger, and exposure to sexual partners (Pfaff *et al.* 2008), while quiescence is associated with sleep and satiety (Cirelli 2009). Quiescence and arousal can persist for minutes to hours. Relatively little is known about the circuit mechanisms that dictate the duration of these behavioral states, nor how transitions between these states are triggered. To address these questions, we analyzed arousal and quiescence of *C. elegans* locomotion.

During each larval molt, *Caenorhabditis elegans* undergoes a prolonged period of profound behavioral quiescence, termed lethargus behavior, during which locomotion and feeding behaviors are inactive for ~2 hr (Cassada and Russell 1975). Lethargus has properties of a sleep-like state such as reduced sensory responsiveness and homeostatic rebound of quiescence following perturbation (Trojanowski and Raizen 2016). Several genes and molecular pathways involved in lethargus behavior have been identified (Van Buskirk and Sternberg 2007; Raizen *et al.* 2008; Monsalve *et al.* 2011; Choi *et al.* 2013, 2015; Nagy *et al.* 2013, 2014; Nelson *et al.* 2013; Turek *et al.* 2013; Singh *et al.* 2014). Many sensory responses are diminished during lethargus, including those mediated by a nociceptive neuron (ASH) (Choi *et al.* 2015; Cho and Sternberg 2014), and by mechanosensory neurons (Schwarz *et al.* 2011). Diminished sensory responsiveness during lethargus is likely to be an important circuit mechanism for producing behavioral quiescence (Choi *et al.* 2013, 2015).

Mutants lacking NPR-1 Neuropeptide Y (NPY) receptors have been utilized as a model for generalized arousal. NPR-1 inhibits the activity of a central sensory circuit (defined by gap junctions to the RMG interneuron) (Macosko *et al.* 2009). In *npr-1* mutants, sensory responses mediated by the RMG circuit (*e.g.*, avoidance of pheromone, oxygen, and irritant chemicals) are exaggerated, and this heightened acuity is associated with exaggerated locomotion (both during lethargus and in adults) (Gray *et al.* 2004; Cheung *et al.* 2005; Macosko *et al.* 2009; Choi *et al.* 2013). Mutations that increase (*e.g.*, *npr-1*) and decrease (*e.g.*, *tax-4* CNG and *osm-9* TRPV) RMG circuit activity are associated with locomotion arousal and quiescence, respectively (Coates and de Bono 2002; de Bono *et al.* 2002; Choi *et al.* 2013). In *npr-1*

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[1]These authors contributed equally to this work.
[2]Present address: Department of Microbiology, UT Southwestern Medical Center, Dallas, TX 75390.
[3]Corresponding author: Department of Molecular Biology, Simches 7, 185 Cambridge St., Boston, MA 02114. E-mail: Kaplan@molbio.mgh.harvard.edu

mutants, locomotion quiescence during lethargus is nearly completely blocked (Choi *et al.* 2013; Nagy *et al.* 2014). Sensory neurons controlled by the RMG circuit arouse locomotion via secretion of a neuropeptide, pigment dispersing factor (PDF-1), and glutamate (Choi *et al.* 2013, 2015). These results raise several interesting questions. How are prolonged quiescent and aroused states established by the RMG circuit? Do the different arousing neurotransmitters (*i.e.*, glutamate and PDF-1) interact to stabilize the aroused state? Or are there additional transmitters that stabilize the aroused state?

Here we show that locomotion arousal during lethargus is promoted by the concerted action of two neuropeptides (PDF-1 and FLP-2). When animals are inactive, PDF-1 and FLP-2 secretion is diminished whereas enhanced secretion is associated with aroused locomotion. PDF-1 arouses locomotion in part via increased FLP-2 secretion, and vice versa. Thus, locomotion arousal is stabilized by reciprocal positive feedback between PDF-1 and FLP-2. A FLP-2 receptor (FRPR-18) is similar to mammalian orexin/hypocretin receptors and is required for FLP-2's arousing effects. Our results suggest that concerted signaling by two neuropeptides provides a circuit mechanism for synchronized rhythms of behavioral activity.

Materials and Methods

Strains

Strain maintenance and genetic manipulation were performed as described (Brenner 1974). Animals were cultivated at 20° on agar nematode growth media (NGM) seeded with OP50. Wild-type reference strain was N2 Bristol. Strains used in this study are as follows:

KP6048 *npr-1(ky13) X*
KP7147 *frpr-18(ok2698) V*
KP7380 *flp-2(gk1039) X*
KP7487 *frpr-18(ok2698) V; flp-2(gk1039) X*
KP7918 *frpr-18(ok2698) V; npr-1(ky13) X*
KP7420 *flp-2(gk1039) npr-1(ky13) X*
KP7488 *frpr-18(ok2698) V; npr-1(ky13) flp-2(gk1039) X*
KP7422 *nuIs513[flp-2p::flp-2::venus, vha-6p::mCherry]*
KP7435 *npr-1(ky13) X; nuIs513*
KP7398 *pdf-1(tm1996) III; npr-1(ky13) X; nuIs513*
LSC27 *pdf-1(tm1996) III*
KP6340 *pdfr-1(ok3425) III*
KP6100 *pdf-1(tm1996) III; npr-1(ky13) X*
KP6410 *pdfr-1(ok3425) III; npr-1(ky13) X*
KP7385 *pdfr-1(ok3425) III; frpr-18(ok2698) V*
KP7323 *pdfr-1(ok3425) III; flp-2(gk1039) X*
KP7384 *pdfr-1(ok3425) III; frpr-18(ok2698) V; npr-1(ky13) X*
KP7399 *pdfr-1(ok3425) III; npr-1(ky13) flp-2(gk1039) X*
KP7393 *pdf-1(tm1996) III; npr-1(ky13) flp-2(gk1039) X*
KP7384 *pdf-1(tm1996) III; frpr-18(ok2698) V; npr-1(ky13) X*
KP6693 *nuIs472 [pdf-1p::pdf-1::venus, vha-6p::mCherry]*
KP6743 *npr-1(ky13) X; nuIs472*
KP7388 *frpr-18(ok2698) III; npr-1 (ky13) X; nuIs472*
KP7386 *flp-2(gk1039) npr-1(ky13) X; nuIs472*
KP6030 *npr-1(ky13) nre-1(hd20) lin-15b(hd126) X*
KP7587-7589 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1689-1691[WRM0630bG11 fosmid;vha-6p::mCherry]*
KP7417 *nuEx1648[flp-2p::gfp,vha-6p::mCherry]*
KP7414 *nuEx1649[frpr-18p::GFP, flp-6p::NLS-mCherry, vha-6p::mCherry]*
KP7500 *nuEx1685[frpr-18p::GFP, gpa-4p::NLS-mCherry]*
KP7502 *nuEx1687[frpr-18p::GFP, flp-6p::NLS-mCherry]*
KP7562 *nuEx1688[frpr-18p::GFP, sra-6p::mCherry]*
KP7982 *nuEx1747[frpr-18p::GFP];otIs518[eat-4(fosmid::SL2::mCherry::H2B) + (pBX)pha-1(+)] III; him-5(e1490) V*
KP7983 *nuEx1747[frpr-18p::GFP];otIs544[cho-1(fosmid::SL2::mCherry::H2B) + (pBX)pha-1(+)] III; him-5(e1490) V*
KP7431-KP7434 *npr-1(ky13); nuEx1656-1659[str-3p::ced-3::GFP, daf-7p::GFP, vha-6p::mCherry]*
KP7426 *flp-2(gk1039) npr-1(ky13) X; nuEx1652[flp-2p::flp-2(gDNA), vha-6p::mCherry]*
KP7425 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1651[frpr-18p::mOxR2, vha-6p::mCherry]*
KP7593 *frpr-18(ok2698) III; npr-1(ky13) flp-2(gk1039) X; nuEx1651*
KP7890-7891 *frpr-18(ok2698) III; npr-1(ky13) X; nuEx1736-1737[gpa-4p::mOxR2, vha-6p::mCherry]*

All strains are available upon request.

Constructs

FLP-2 secretion construct (KP#2282): *flp-2 genomic DNA* and YFP (VENUS) containing a stop codon were each amplified by PCR and ligated into the expression vector pPD49.26 (Addgene). The construct contains 3 kb of upstream regulatory sequence 5′ to the *flp-2* start codon.

frpr-18 (KP#2276) and flp-2 (KP#2271) transcriptional reporter constructs: DNA corresponding to the *frpr-18* regulatory sequence (∼2.6 kb 5′) or *flp-2* regulatory sequence (∼3 kb 5′) was amplified by PCR and ligated into expression vectors (pPD95.75) containing GFP coding sequence.

ASI cell ablation construct (KP#2150): *ced-3* complementary DNA (cDNA) and GFP were amplified by overlapping PCR and ligated into expression vectors (pPD49.26) containing the *str-3* (∼3 kb 5′ regulatory sequence: ASI expression) promoter.

Mouse orexin receptor 2 expression constructs (KP#2290 and 3251): cDNA of mouse orexin type 2 receptor (mOxR2) (∼1.3 kb) was amplified by PCR from a mouse cDNA library and ligated into expression vectors (pPD49.26) containing the *frpr-18* (2.6 kb 5′ sequence, KP#2290) or *gpa-4* (3 kb 5′ sequence, KP#3251) promoters.

Transgenes and germline transformation

Transgenic strains were generated by microinjection of various plasmids with co-injection markers: *myo-2p*::*NLS-mCherry* (KP#1480) and *vha-6p*::*mCherry* (KP#1874).

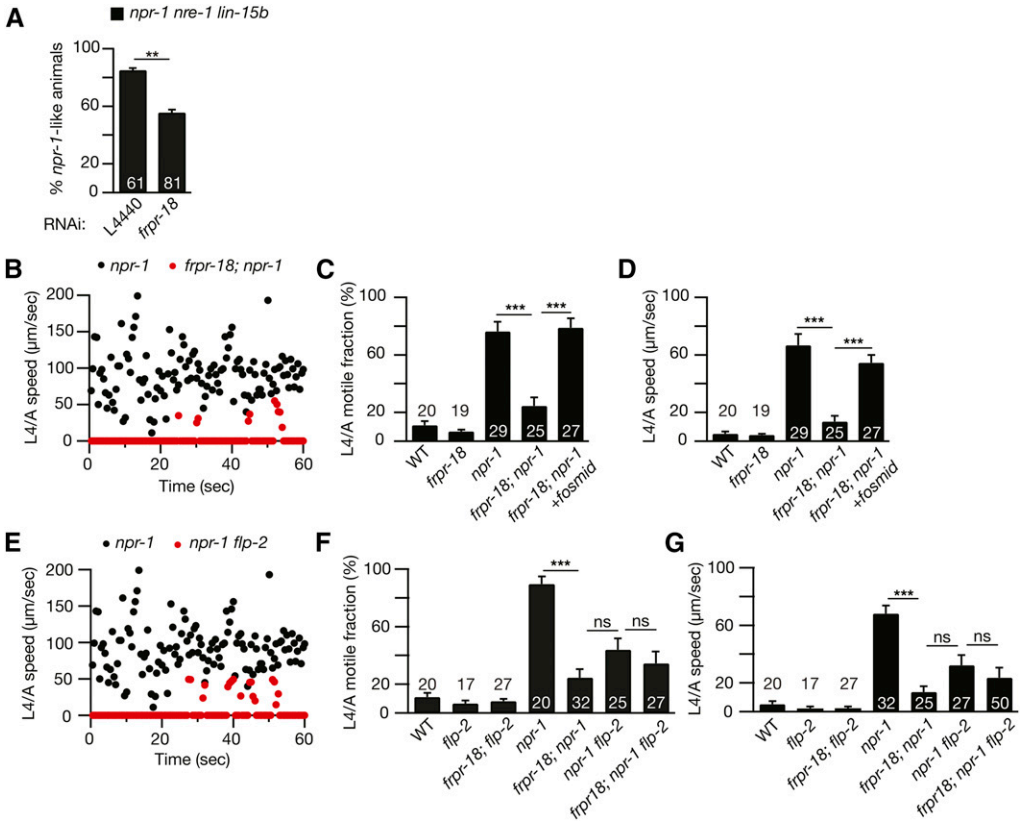

Figure 1 FRPR-18 and FLP-2 are required for aroused locomotion during molts in *npr-1* mutants. (A) The fraction of motile animals during the L4/A lethargus following treatment with RNAi is shown. RNAi was carried out using an *npr-1* mutant strain containing RNAi hyper-sensitive mutations (*npr-1 nre-1 lin-15b*) (Schmitz *et al.* 2007). Knockdown of *frpr-18* significantly suppressed the *npr-1* lethargus locomotion defect, whereas the empty vector control L4440 had no effect. The number of animals analyzed is indicated. Error bars indicate SEM. Values that differ significantly (by χ^2 test) are indicated (** $P < 0.01$). (B–G) Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (B, E), average motile fraction (C, F), and average locomotion velocity (D, G) are plotted. (B–D) The *npr-1* L4/A locomotion quiescence defect was suppressed by mutations inactivating *frpr-18* and was reinstated by a fosmid clone (WRM0630bG11) containing the *frpr-18* genomic locus in *frpr-18*; *npr-1* double mutants. (E–G) The *npr-1* L4/A locomotion quiescence defect was also suppressed by mutations inactivating the FRPR-18 ligand, FLP-2. The L4/A locomotion velocity and motile fraction of *frpr-18*; *npr-1* or *npr-1 flp-2* double mutants was not significantly different from *frpr-18*; *npr-1 flp-2* triple mutants (F–G). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*** $P < 0.001$; ns, not significant).

Injection concentration was 40–50 ng/μl for all the expression constructs and 10 ng/μl for co-injection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/μl.

Lethargus locomotion and behavior analysis

We recorded brief movies (2-Hz frame rate for 60 sec) of locomotion during the first hour of the L4/A lethargus. We chose the first hour of lethargus because this corresponds to the peak of quiescence, during which 50–80% of animals are quiescent and each quiescent bout lasts for 10–80 sec (Nagy *et al.* 2014). Thus, 60-sec movies allow us to accurately sample quiescent bouts during the peak of L4/A quiescence. These recordings were analyzed as previously described (Choi *et al.* 2013). Briefly, well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hr, locomotion of animals in lethargus (determined by absence of pharyngeal pumping) was recorded on a Zeiss Discovery Stereomicroscope using Axiovision software. Centroid velocity of each animal was analyzed at each frame using object-tracking software in Axiovision. The motile fraction of each animal was calculated by dividing the number of frames with positive velocity value by the total number of frames. The speed of each animal was calculated by averaging the velocity value at each frame. Quantitative analysis was done using a custom-written MATLAB program (Mathworks). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's *t*-test for pairwise comparison.

Adult locomotion and behavior analysis

Locomotion of adult animals was analyzed with the same setup as L4/A locomotion analysis described above, except that well-fed adult animals were monitored 5–10 min after the transfer to full-lawn OP50 bacterial plates. Foraging behavior was analyzed as described (de Bono and Bargmann 1998). Briefly, ~150 well-fed adult animals were placed on NGM plates seeded with 200 μl OP50 *Escherichia coli* 2 days before the assay. After 3 hr, images were taken for each genotype. Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparisons and two-tailed Student's *t*-test for pairwise comparison.

RNAi screen

An RNA interference (RNAi) feeding screen was performed to identify neuropeptide receptor genes whose inactivation increases the L4/A quiescence of *npr-1* mutants, as described (Kamath *et al.* 2003). The screen utilized a strain containing mutations that increase the efficacy of RNAi: KP6030 *npr-1(ky13) nre-1 lin-15b* (Schmitz *et al.* 2007). One hundred

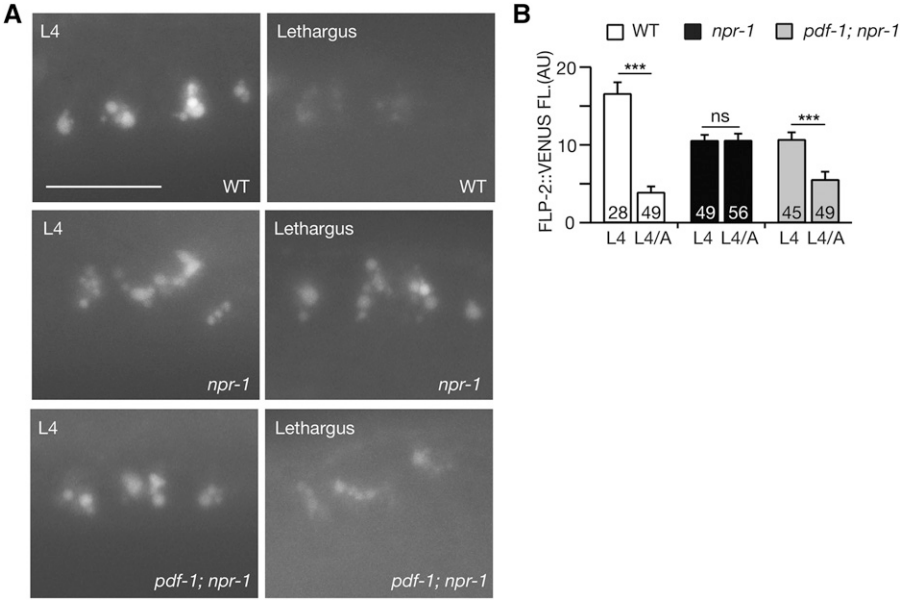

Figure 2 NPR-1 inhibits FLP-2 secretion during the L4/A molt. FLP-2 secretion was analyzed in the indicated genotypes. VENUS-tagged FLP-2 was expressed with the *flp-2* promoter. (A) Representative images and (B) summary data are shown for coelomocyte fluorescence in L4 and L4/A animals. FLP-2::VENUS coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild-type animals, but not in *npr-1* mutants. *pdf-1*; *npr-1* double mutants exhibited decreased FLP-2 secretion during lethargus. Values that differ significantly are indicated (*** $P < 0.001$; ns, not significant). Bar, 10 μm.

and fourteen neuropeptide receptor genes were selected for the screen (Frooninckx *et al.* 2012). After 5 days of RNAi treatment (two generation) at 20°, well-fed late L4 animals were transferred to full-lawn OP50 bacterial plates. After 1 hr, animals in lethargus (determined by absence of pharyngeal pumping) were scored for their motility. Statistical significance was determined using the χ^2 test.

Cell ablations

Neurons were ablated in *npr-1(ky13)* mutant worms by transgenes coexpressing CED-3 and a fluorescent protein (GFP or mCherry) under the *str-3* (ASI ablation) promoter. ASI ablation was confirmed by fluorescence microscopy.

Fluorescence microscopy and image analysis

Quantitative imaging of coelomocyte fluorescence was performed as previously described (Choi *et al.* 2013) using a Zeiss Axioskop equipped with an Olympus PlanAPO 100× (NA = 1.4) objective and a CoolSNAP HQ CCD camera (Photometrics). Worms were immobilized with 30 mg/ml 2,3-Butanedione monoxime (Sigma, St. Louis, MO). The anterior coelomocytes were imaged in L4/A lethargus (determined by absence of pharyngeal pumping), and 1 day-old adult animals. Image stacks were captured and maximum intensity projections were obtained using Metamorph 7.1 software (Universal Imaging). YFP fluorescence was normalized to the absolute mean fluorescence of 0.5 mm FluoSphere beads (Molecular Probes, Eugene, OR). Statistical significance was determined using the Kolmogorov–Smirnov test.

Identification of FRPR-18-expressing neurons

Cells expressing the *frpr-18*p::GFP reporter were identified based on their position and axon morphology. Identification of many neurons was made by analysis of *frpr-18*p::GFP coexpression with mCherry constructs expressed in other neurons. These include mCherry constructs containing the *gpa-4* (for ASI neurons), *flp-6* (for I6 and ASE neurons), and *sra-6* (for PVQ and ASH neurons) promoters. Several identifications were made by comparing expression of *frpr-18*p::GFP with *eat-4*p::RFP (Serrano-Saiz *et al.* 2013) and *cho-1*p::RFP, which have known expression patterns (O. Hobert, personal communication). Images were taken using a 60× objective (NA 1.45) on an Olympus FV-1000 confocal microscope. Maximum intensity projections of Z-series stacks were made using Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

A neuropeptide receptor (FRPR-18) is required for aroused locomotion during molts in npr-1 mutants

Locomotion quiescence during the fourth stage larva-to-adult (L4/A) molt is dramatically reduced in *npr-1* mutants (Choi *et al.* 2013; Nagy *et al.* 2014). Mutations inactivating PDF-1 or its receptor (PDFR-1) reinstate a wild-type pattern of molting-associated quiescence in *npr-1* mutants (Choi *et al.* 2013). Double mutants lacking both PDF-1 and NPR-1 exhibit a normal pattern of quiescence during arousal following molts (Choi *et al.* 2013), suggesting that changes in PDF-1 signaling are not absolutely required for the rhythmic pattern of quiescence and arousal that is coupled to the molting cycle.

To determine if additional neuropeptides are required for locomotion arousal, we used RNAi to inactivate all predicted neuropeptide receptors in *npr-1* mutants and assayed L4/A locomotion velocity and motile fraction (detailed in *Materials and Methods*). One of the genes identified in this screen was

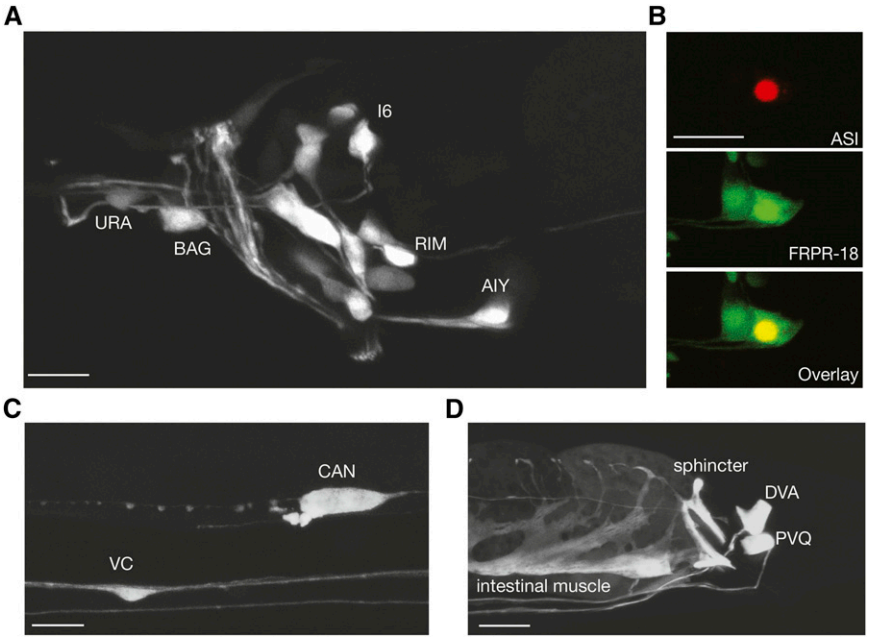

Figure 3 *frpr-18* is expressed in a subset of neurons in the head, midbody, and tail. Adult animals containing an *frpr-18* reporter construct are shown. (A) In the head, the *frpr-18* promoter expressed GFP in URA, BAG, I6, RIM, AIY neurons in the head of the worm. (B) Expression of *frpr-18* in ASI neurons was confirmed by coexpression with an ASI marker (*gpa-4*). (C, D) The *frpr-18* reporter was also expressed in the VC and CAN neurons (C), in PVQ and DVA neurons (D), and in the intestinal and sphincter muscles (D). Bar, 10 μm.

frpr-18. Inactivating FRPR-18 by either RNAi (Figure 1A) or a null mutation (*ok2698*) significantly decreased L4/A locomotion velocity and motile fraction of *npr-1* mutants, indicating that molt-associated quiescence was restored (Figure 1, B–D). The *frpr-18* null mutation had little effect on the locomotion of *npr-1* adults (Supplemental Material, Figure S1A); however, *frpr-18* single mutant adults exhibited decreased locomotion velocity (Figure S1B). A fosmid clone spanning the *frpr-18* genomic locus reinstated the L4/A locomotion quiescence defects in *frpr-18*; *npr-1* double mutants (Figure 1, C and D). Foraging behavior is also altered in *npr-1* mutants, as indicated by an increased tendency for mutant worms to form clumps at the boundaries of the bacterial lawn (de Bono and Bargmann 1998). This *npr-1* foraging defect was not suppressed in *frpr-18*; *npr-1* double mutants (Figure S2), indicating that FRPR-18 was not required for other *npr-1* phenotypes. Collectively, these results suggest that FRPR-18 signaling promotes aroused locomotion during lethargus.

An FRPR-18 ligand (FLP-2) also promotes aroused L4/A locomotion in npr-1 mutants

Prior studies found that two neuropeptides encoded by the *flp-2* gene (FLP-2A and B) activate FRPR-18 receptors expressed in transfected cells (Mertens *et al.* 2005; Larsen *et al.* 2013). Prompted by these results, we analyzed the effect of a *flp-2* mutation (*gk1039*) on lethargus locomotion. L4/A locomotion velocity and motile fraction were significantly reduced in *npr-1 flp-2* double mutants compared to *npr-1* single mutants (Figure 1, E–G). The *flp-2* mutation had little effect on *npr-1* adult locomotion (Figure S1A), although *flp-2* single mutants exhibited decreased adult locomotion velocity (Figure S1B). The *flp-2* mutation also had no effect on the *npr-1* foraging defect (Figure S2), indicating that FLP-2 was not required for other *npr-1* phenotypes.

If FLP-2 neuropeptides function as FRPR-18 ligands *in vivo* (as predicted by the cell culture data), *flp-2* and *frpr-18* mutations should not have additive effects in double mutants. Consistent with this idea, the L4/A locomotion velocity and motile fraction of *frpr-18*; *npr-1* double mutants were not significantly different from those observed in *frpr-18*; *npr-1 flp-2* triple mutants (Figure 1, F and G). Similarly, adult locomotion exhibited by *frpr-18*; *flp-2* double mutants did not differ significantly from that observed in either single mutant (Figure S1B). These results indicate that *flp-2* and *frpr-18* function together to arouse locomotion, consistent with FLP-2A/B function as FRPR-18 ligands (Mertens *et al.* 2005; Larsen *et al.* 2013).

NPR-1 inhibits FLP-2 secretion during L4/A locomotion quiescence

We previously showed that NPR-1 inhibits secretion of PDF-1 during lethargus, and that this effect is required to maintain the molt-associated locomotion quiescence (Choi *et al.* 2013). Prompted by these results, we tested the idea that NPR-1 also inhibits FLP-2 secretion during lethargus. We analyzed secretion of YFP-tagged FLP-2, expressed by the *flp-2* promoter. Secretion of FLP-2 was assessed by measuring YFP fluorescence in the endolysosomal compartment of coelomocytes, which are specialized scavenger cells that internalize proteins secreted into the body cavity (Fares and Greenwald 2001). In wild-type animals, FLP-2::YFP secretion was dramatically decreased during the L4/A molt compared to L4 stage animals (Figure 2). This molt-associated decrease in FLP-2::YFP secretion was eliminated in *npr-1* mutants, suggesting that NPR-1 inhibits FLP-2 secretion during molts and that decreased FLP-2 secretion is required for molt-associated locomotion quiescence.

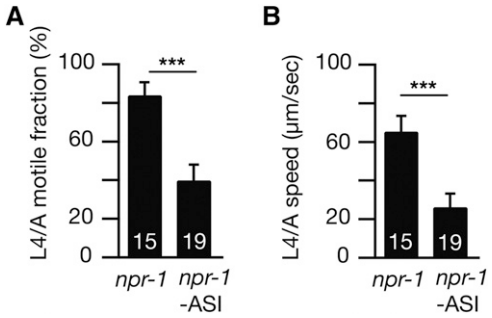

Figure 4 ASI neurons promote arousal. (A, B) The *npr-1* L4/A locomotion quiescence defect was suppressed by ASI ablation. A transgene expressing CED-3 in ASI (using the *str-3* promoter) was used to genetically ablate the ASI neurons. Summary data for (A) L4/A motile fraction and (B) locomotion velocity are shown. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*** $P < 0.001$; ns, not significant).

ASI neurons promote arousal

FLP-2 is expressed in several neurons in head ganglia, including AIA, ASI, MC, M4, I5, and RID (Kim and Li 2004). To further characterize the cellular mechanism for FLP-2's arousing effects, we identified cells that express *frpr-18*. An *frpr-18* promoter construct expressed GFP in many neurons (including AIY, ASI, BAG, URA, CAN, I6, PVQ, DVA, RIM, and VC) and in the anal sphincter and intestinal muscles (Figure 3).

ASI neurons coexpress FLP-2 (Kim and Li 2004) (Figure S3), FRPR-18 (Figure 3B), and PDF-1 (Janssen *et al.* 2009; Barrios *et al.* 2012), suggesting that these neurons play an important role in arousal. To test this idea, we genetically ablated ASI neurons in *npr-1* mutants. ASI cell death was induced by a transgene that expresses a proapoptotic caspase (CED-3) and GFP in ASI neurons (using the *str-3* promoter). ASI cell death was confirmed by the absence of GFP fluorescence in transgenic animals. The L4/A motile fraction and locomotion rate of *npr-1* worms were significantly reduced in animals lacking ASI neurons (Figure 4), confirming the role of ASI neurons in promoting arousal.

FLP-2 and PDF-1 jointly promote arousal by reciprocal positive feedback

Thus far, our results suggest that NPR-1 inhibits secretion of two arousing neuropeptides, FLP-2 (Figure 2) and PDF-1 (Choi *et al.* 2013), thereby promoting quiescence. FLP-2 and PDF-1 could act independently to arouse locomotion or they could comprise components of a single arousal pathway. If they function independently, *flp-2* and *pdf-1* mutations should have additive effects on locomotion in double mutants. Contrary to this idea, inactivating FLP-2 or its receptor (FRPR-18) did not further enhance the reduced L4/A locomotion of *pdf-1*; *npr-1* double mutants (Figure 5, A and B), nor did these mutations enhance the reduced adult locomotion defect of *pdfr-1* single mutants (Figure S1C). These results support the idea that *frpr-18*, *flp-2*, and *pdf-1* likely act in a single genetic pathway to mediate arousal.

Given their function in a single genetic pathway, we next asked if FLP-2 promotes PDF-1 secretion. To test this idea, we analyzed PDF-1::YFP secretion in *flp-2* mutants. In wild-type animals, PDF-1::YFP secretion is inhibited during the L4/A molt and this effect was eliminated in *npr-1* mutants, as shown in our prior study (Choi *et al.* 2013). Inhibition of PDF-1::YFP secretion during lethargus was restored in both *npr-1 flp-2* and in *frpr-18*; *npr-1* double mutants (Figure 5, C and D). These results suggest that FRPR-18 and FLP-2 stimulate PDF-1 secretion, which could contribute to FLP-2's arousing effects. Analogous experiments suggest that PDF-1 promotes FLP-2 secretion (Figure 2). These results suggest that FLP-2 and PDF-1 secretion are regulated by reciprocal positive feedback. Positive feedback between FLP-2 and PDF-1 could provide a mechanism for stabilizing (or prolonging) aroused locomotion in *npr-1* mutants.

FLP-2 and FRPR-18 are functionally analogous to vertebrate orexin and orexin receptors

The neuropeptide orexin promotes wakefulness and feeding in vertebrates (Chemelli *et al.* 1999; Lin *et al.* 1999). Orexin orthologs have not been described in invertebrates, suggesting that their arousal is mediated by a distinct mechanism. Interestingly, FRPR-18 has significant similarity to mammalian orexin type 2 receptors (mOxR2) (BLAST score: $E = 1e{-}08$), implying that FRPR-18 and mOxR2 could perform analogous functions. To test this idea, we asked if a transgene expressing the mouse mOxR2 could rescue the *frpr-18* mutant defect. Consistent with this idea, expressing an mOxR2 transgene with the *frpr-18* promoter (Figure 6, A and B) or exclusively in ASI neurons (with the *gpa-4* promoter) (Figure 6, C and D) reinstated the L4/A locomotion quiescence defect in *frpr-18*; *npr-1* double mutants. Thus, mOxR2 expression compensates for the absence of FRPR-18 receptors, supporting the idea that FRPR-18 and orexin receptors perform analogous functions in arousal.

To test the idea that FLP-2 is functionally analogous to vertebrate orexins, we analyzed the effect of *flp-2* mutations on the rescuing activity of the mOxR2 transgene. We found that inactivating FLP-2 blocked the ability of mOxR2 to promote aroused L4/A locomotion in *frpr-18*; *npr-1* double mutants (Figure 6, A and B). This result suggests that endogenously expressed FLP-2 neuropeptides are required for mOxR2's arousing effects. Taken together, these results suggest that FLP-2 and FRPR-18 are functionally analogous to mammalian orexin and mOxR2 receptors.

Discussion

Our results lead to three principal conclusions. First, we identify FLP-2 as a new arousing neuropeptide in *C. elegans*. Second, we show that FLP-2 and its receptor (FRPR-18) are functional analogs of vertebrate orexin and OxR2 receptors. Third, we show that secretion of two arousing neuropeptides (FLP-2 and PDF-1) is regulated by reciprocal positive feedback. Below we discuss the significance of these findings.

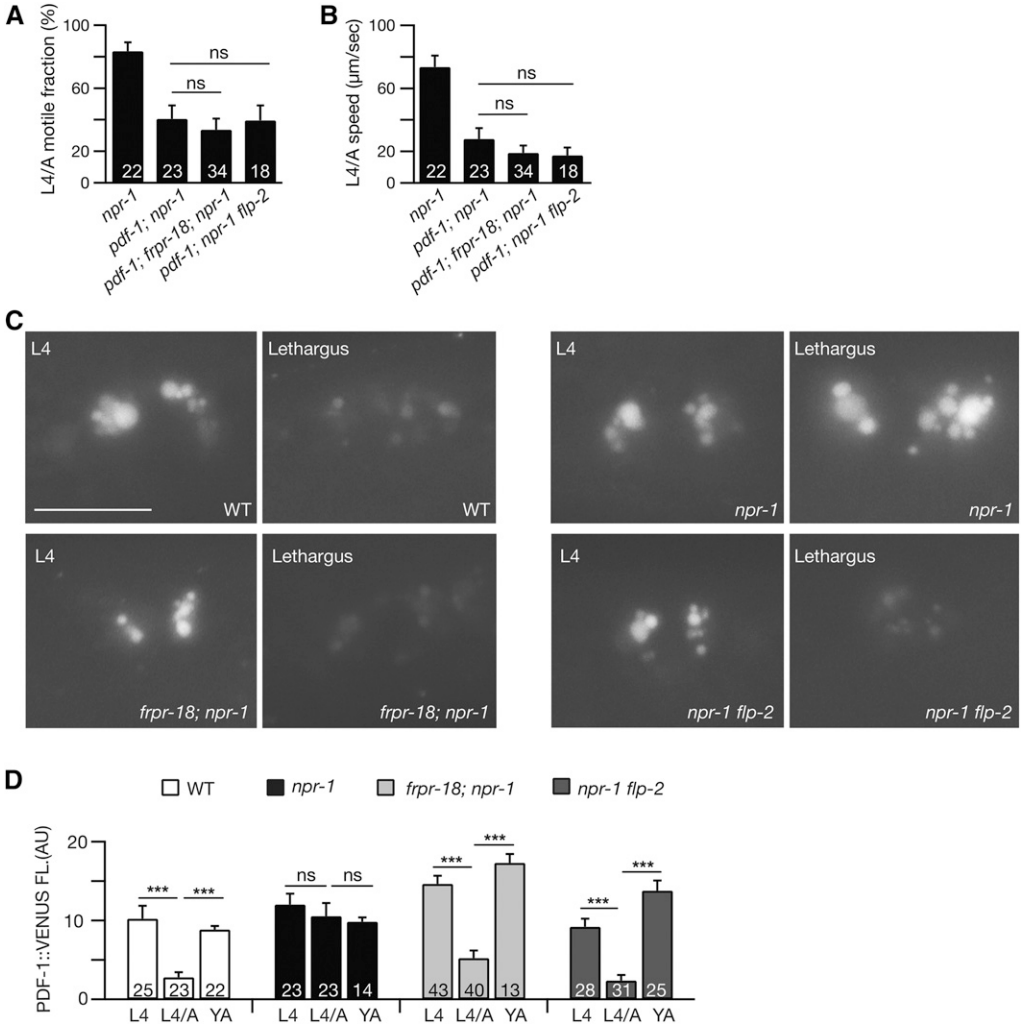

Figure 5 FLP-2 and PDF-1 jointly promote arousal by reciprocal positive feedback. (A, B) Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Inactivation of *frpr-18* or *flp-2* did not further decrease the locomotion of *pdf-1*; *npr-1* mutants. Average motile fraction (A) and average locomotion velocity (B) are plotted. (C, D) PDF-1 secretion was analyzed in the indicated genotypes. VENUS-tagged PDF-1 was expressed with the *pdf-1* promoter. (C) Representative images and (D) summary data are shown for coelomocyte fluorescence in L4, L4/A, and young adults (8 hr post L4) of the indicated genotypes. PDF-1::VENUS coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild-type animals, but not in *npr-1* mutants, as previously reported (Choi *et al.* 2013). *frpr-18*; *npr-1* and *npr-1 flp-2* double mutants exhibited decreased PDF-1 secretion during lethargus. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*** $P < 0.001$; ns, not significant). Bar, 10 μm.

Several results support the idea that FLP-2 promotes locomotion arousal during lethargus. Mutations inactivating FLP-2 or FRPR-18 restore molt-associated quiescence to *npr-1* mutants. FLP-2 secretion is inhibited during molts (when locomotion is quiescent) and this inhibition is blocked in *npr-1* mutants (which lack molt-associated locomotion quiescence). Thus, locomotion quiescence and arousal are linked to decreased and increased FLP-2 signaling, respectively.

FRPR-18 has significant sequence homology to vertebrate OxR2 receptors, suggesting that the *flp-2*-encoded peptides (FLP-2A and B) are functionally analogous to vertebrate orexins. To further test this idea, we showed that mOXR2 expression rescues the *frpr-18* mutant defect in locomotion arousal and that this rescuing activity requires expression of endogenous FLP-2 peptides. These results support the idea that FRPR-18 and FLP-2A/B are functional analogs of vertebrate mOXR2 and orexins and that FLP-2 peptides may function as mOxR2 ligands. These results are surprising because FLP-2A and B are RFamide peptides (defined by their C-terminal RF motifs) while Orexin A and B have conserved C-terminal sequences lacking the RF motif. Like mOxR2, FRPR-18 receptors are coupled to G_q (Mertens *et al.* 2005; Larsen *et al.* 2013); consequently, FLP-2 peptides are likely to activate neurons that express FRPR-18.

We also show that ASI neurons play an important role in promoting locomotion arousal. Three arousal-inducing genes (*pdf-1*, *flp-2*, and *frpr-18*) are expressed in ASI. A transgene expressing mOxR2 in ASI reinstated the lethargus quiescence defect in *frpr-18*; *npr-1* double mutants, suggesting that FRPR-18 acts in ASI to promote arousal. Genetic ablation of ASI significantly decreased locomotion arousal during molts in *npr-1* mutants. FRPR-18 is expressed in several other neurons that control locomotion including AIY, DVA, and RIM (Alkema *et al.* 2005; Gray *et al.* 2005; Li *et al.* 2006; Piggott *et al.* 2011; Flavell *et al.* 2013). This expression pattern suggests that FLP-2's arousing effects could be mediated by multiple classes of neurons.

Mutations disrupting orexin or orexin receptors are associated with narcolepsy in humans, dogs, and mice (Chemelli *et al.* 1999; Lin *et al.* 1999). In orexin and mOxR2 knockout mice, the total amount of sleep is unaltered; however, the duration of awake bouts is dramatically reduced (Willie *et al.* 2003; Mochizuki *et al.* 2004). This fragmentation of wakefulness in narcoleptic mice has been interpreted to mean that orexin

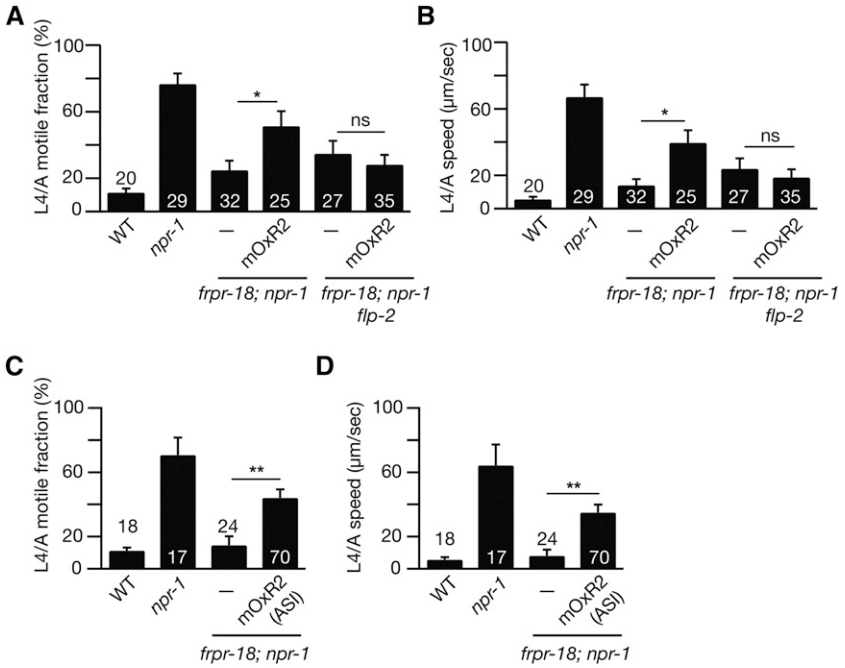

Figure 6 FLP-2 and FRPR-18 are functionally analogous to vertebrate orexin and orexin receptors. Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. The *npr-1* L4/A quiescence defect was reinstated in *frpr-18*; *npr-1* double mutants by transgenes expressing mouse OxR2 with the *frpr-18* promoter (A, B) or the ASI-specific promoter *gpa-4* (C, D). The transgene-expressing mouse OxR2 under the *frpr-18* promoter did not reinstate the *npr-1* locomotion quiescence defect in *frpr-18*; *npr-1 flp-2* triple mutants (A, B). Average motile fraction (A, C) and average locomotion velocity (B, D) are plotted. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (* $P < 0.05$, ** $P < 0.01$; ns, not significant).

stabilizes wakefulness. Our results support two mechanisms for stabilizing aroused states. First, FLP-2 and FRPR-18 are coexpressed in ASI neurons, suggesting that ASI is regulated by autocrine positive feedback. Second, we find that FLP-2 and PDF-1 secretion are regulated by reciprocal positive feedback, *i.e.*, FLP-2 promotes PDF-1 secretion and vice versa. We propose that these circuit motifs stabilize the aroused state. Interestingly, both motifs are conserved in mammalian arousal circuits. Mouse orexin directly activates orexin-expressing neurons via activation of mOxR2 receptors (Yamanaka *et al.* 2010). Orexin also activates neurons that express several other arousing neurotransmitters (*e.g.*, cholinergic, histaminergic, noradrenergic, and serotonergic neurons) (Brown *et al.* 2012). Thus, autocrine positive feedback and reciprocal positive feedback are conserved motifs found in arousal circuits.

Prior studies suggest that switching between sleep and awake states in mammals is mediated by reciprocal inhibition between sleep- and awake-promoting neurons, creating a flip-flop switch (Saper *et al.* 2010). For example, mouse NPY inhibits orexin-positive neurons in the hypothalamus (Fu *et al.* 2004). Mouse orexin also inhibits NPY-positive neurons in the rat thalamus, through OxR1 and OxR2 receptors (Palus *et al.* 2015). It will be interesting to determine if behavioral state switching in *C. elegans* is also mediated by reciprocal inhibition between neurons expressing arousing (FLP-2 and PDF-1) and quiescence promoting (FLP-21) neuropeptides. Consistent with this idea, we find that NPR-1 inhibits secretion of two arousing neuropeptides (FLP-2 and PDF-1).

In conclusion, we identified a new arousing neuropeptide (FLP-2) that regulates *C. elegans* molting-associated quiescence through an orexin-like receptor (FRPR-18). FLP-2 acts in concert with PDF-1 through reciprocal positive feedback to promote arousal. The many mechanistic parallels between mammalian orexin and FLP-2/FRPR-18 suggest that *C. elegans* may be a powerful genetic invertebrate system to study the role of orexin in behavioral arousal.

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Literature Cited

Alkema, M. J., M. Hunter-Ensor, N. Ringstad, and H. R. Horvitz, 2005 Tyramine functions independently of octopamine in the Caenorhabditis elegans nervous system. Neuron 46: 247–260.

Barrios, A., R. Ghosh, C. Fang, S. W. Emmons, and M. M. Barr, 2012 PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in C. elegans. Nat. Neurosci. 15: 1675–1682.

Brenner, S., 1974 The genetics of Caenorhabditis elegans. Genetics 77: 71–94.

Brown, R. E., R. Basheer, J. T. McKenna, R. E. Strecker, and R. W. McCarley, 2012 Control of sleep and wakefulness. Physiol. Rev. 92: 1087–1187.

Cassada, R. C., and R. L. Russell, 1975 The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev. Biol. 46: 326–342.

Chemelli, R. M., J. T. Willie, C. M. Sinton, J. K. Elmquist, T. Scammell *et al.*, 1999 Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98: 437–451.

Cheung, B. H., M. Cohen, C. Rogers, O. Albayram, and M. de Bono, 2005 Experience-dependent modulation of C. elegans behavior by ambient oxygen. Curr. Biol. 15: 905–917.

Cho, J. Y., and P. W. Sternberg, 2014 Multilevel modulation of a sensory motor circuit during C. elegans sleep and arousal. Cell 156: 249–260.

Choi, S., M. Chatzigeorgiou, K. P. Taylor, W. R. Schafer, and J. M. Kaplan, 2013 Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in C. elegans. Neuron 78: 869–880.

Choi, S., K. P. Taylor, M. Chatzigeorgiou, Z. Hu, W. R. Schafer *et al.*, 2015 Sensory neurons arouse C. elegans locomotion via both glutamate and neuropeptide release. PLoS Genet. 11: e1005359.

Cirelli, C., 2009 The genetic and molecular regulation of sleep: from fruit flies to humans. Nat. Rev. Neurosci. 10: 549–560.

Coates, J. C., and M. de Bono, 2002 Antagonistic pathways in neurons exposed to body fluid regulate social feeding in Caenorhabditis elegans. Nature 419: 925–929.

de Bono, M., and C. I. Bargmann, 1998 Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. Cell 94: 679–689.

de Bono, M., D. M. Tobin, M. W. Davis, L. Avery, and C. I. Bargmann, 2002 Social feeding in Caenorhabditis elegans is induced by neurons that detect aversive stimuli. Nature 419: 899–903.

Fares, H., and I. Greenwald, 2001 Genetic analysis of endocytosis in Caenorhabditis elegans: coelomocyte uptake defective mutants. Genetics 159: 133–145.

Flavell, S. W., N. Pokala, E. Z. Macosko, D. R. Albrecht, J. Larsch *et al.*, 2013 Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in C. elegans. Cell 154: 1023–1035.

Frooninckx, L., L. Van Rompay, L. Temmerman, E. Van Sinay, I. Beets *et al.*, 2012 Neuropeptide GPCRs in C. elegans. Front Endocrinol (Lausanne) 3: 167.

Fu, L. Y., C. Acuna-Goycolea, and A. N. van den Pol, 2004 Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: tonic depression of the hypothalamic arousal system. J Neurosci 24: 8741–8751.

Gray, J. M., D. S. Karow, H. Lu, A. J. Chang, J. S. Chang *et al.*, 2004 Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. Nature 430: 317–322.

Gray, J. M., J. J. Hill, and C. I. Bargmann, 2005 A circuit for navigation in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 102: 3184–3191.

Janssen, T., S. J. Husson, E. Meelkop, L. Temmerman, M. Lindemans *et al.*, 2009 Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in Caenorhabditis elegans. J. Neurochem. 111: 228–241.

Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin *et al.*, 2003 Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421: 231–237.

Kim, K., and C. Li, 2004 Expression and regulation of an FMRFamide-related neuropeptide gene family in Caenorhabditis elegans. J. Comp. Neurol. 475: 540–550.

Larsen, M. J., E. R. Lancheros, T. Williams, D. E. Lowery, T. G. Geary *et al.*, 2013 Functional expression and characterization of the C. elegans G-protein-coupled FLP-2 Receptor (T19F4.1) in mammalian cells and yeast. Int. J. Parasitol. Drugs Drug Resist. 3: 1–7.

Li, W., Z. Feng, P. W. Sternberg, and X. Z. Xu, 2006 A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. Nature 440: 684–687.

Lin, L., J. Faraco, R. Li, H. Kadotani, W. Rogers *et al.*, 1999 The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98: 365–376.

Macosko, E. Z., N. Pokala, E. H. Feinberg, S. H. Chalasani, R. A. Butcher *et al.*, 2009 A hub-and-spoke circuit drives pheromone attraction and social behaviour in C. elegans. Nature 458: 1171–1175.

Mertens, I., T. Meeusen, T. Janssen, R. Nachman, and L. Schoofs, 2005 Molecular characterization of two G protein-coupled receptor splice variants as FLP2 receptors in Caenorhabditis elegans. Biochem. Biophys. Res. Commun. 330: 967–974.

Mochizuki, T., A. Crocker, S. McCormack, M. Yanagisawa, T. Sakurai *et al.*, 2004 Behavioral state instability in orexin knockout mice. J. Neurosci. 24: 6291–6300.

Monsalve, G. C., C. Van Buskirk, and A. R. Frand, 2011 LIN-42/PERIOD controls cyclical and developmental progression of C. elegans molts. Curr. Biol. 21: 2033–2045.

Nagy, S., C. Wright, N. Tramm, N. Labello, S. Burov *et al.*, 2013 A longitudinal study of Caenorhabditis elegans larvae reveals a novel locomotion switch, regulated by G(αs) signaling. eLife 2: e00782.

Nagy, S., N. Tramm, J. Sanders, S. Iwanir, I. A. Shirley *et al.*, 2014 Homeostasis in C. elegans sleep is characterized by two behaviorally and genetically distinct mechanisms. eLife 3: e04380.

Nelson, M. D., N. F. Trojanowski, J. B. George-Raizen, C. J. Smith, C. C. Yu *et al.*, 2013 The neuropeptide NLP-22 regulates a sleep-like state in Caenorhabditis elegans. Nat. Commun. 4: 2846.

Palus, K., L. Chrobok, and M. H. Lewandowski, 2015 Orexins/hypocretins modulate the activity of NPY-positive and -negative neurons in the rat intergeniculate leaflet via OX1 and OX2 receptors. Neuroscience 300: 370–380.

Pfaff, D., A. Ribeiro, J. Matthews, and L. M. Kow, 2008 Concepts and mechanisms of generalized central nervous system arousal. Ann. N. Y. Acad. Sci. 1129: 11–25.

Piggott, B. J., J. Liu, Z. Feng, S. A. Wescott, and X. Z. Xu, 2011 The neural circuits and synaptic mechanisms underlying motor initiation in C. elegans. Cell 147: 922–933.

Raizen, D. M., J. E. Zimmerman, M. H. Maycock, U. D. Ta, Y. J. You *et al.*, 2008 Lethargus is a Caenorhabditis elegans sleep-like state. Nature 451: 569–572.

Saper, C. B., P. M. Fuller, N. P. Pedersen, J. Lu, and T. E. Scammell, 2010 Sleep state switching. Neuron 68: 1023–1042.

Schmitz, C., P. Kinge, and H. Hutter, 2007 Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive *Caenorhabditis elegans* strain nre-1(hd20) lin-15b(hd126). Proc. Natl. Acad. Sci. 104: 834–839.

Schwarz, J., I. Lewandrowski, and H. Bringmann, 2011 Reduced activity of a sensory neuron during a sleep-like state in Caenorhabditis elegans. Curr. Biol. 21: R983–R984.

Serrano-Saiz, E., R. J. Poole, T. Felton, F. Zhang, E. D. De La Cruz *et al.*, 2013 Modular control of glutamatergic neuronal identity in C. elegans by distinct homeodomain proteins. Cell 155: 659–673.

Singh, K., J. Y. Ju, M. B. Walsh, M. A. DiIorio, and A. C. Hart, 2014 Deep conservation of genes required for both Drosphila melanogaster and Caenorhabditis elegans sleep includes a role for dopaminergic signaling. Sleep 37: 1439–1451.

Trojanowski, N. F., and D. M. Raizen, 2016 Call it worm sleep. Trends Neurosci. 39: 54–62.

Turek, M., I. Lewandrowski, and H. Bringmann, 2013 An AP2 transcription factor is required for a sleep-active neuron to induce sleep-like quiescence in C. elegans. Curr. Biol. 23: 2215–2223.

Van Buskirk, C., and P. W. Sternberg, 2007 Epidermal growth factor signaling induces behavioral quiescence in Caenorhabditis elegans. Nat. Neurosci. 10: 1300–1307.

Willie, J. T., R. M. Chemelli, C. M. Sinton, S. Tokita, S. C. Williams *et al.*, 2003 Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. Neuron 38: 715–730.

Yamanaka, A., S. Tabuchi, T. Tsunematsu, Y. Fukazawa, and M. Tominaga, 2010 Orexin directly excites orexin neurons through orexin 2 receptor. J. Neurosci. 30: 12642–12652.

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SUPPLEMENTAL INFORMATION

Figure S1. FRPR-18 *and* FLP-2 effects on adult locomotion

(A) Inactivating FRPR-18 and FLP-2 had a significantly greater effect on the locomotion of L4/A *npr-1* animals than on *npr-1* adults. Locomotion velocity (normalized to *npr-1* mutants) is shown. Locomotion behavior of single adult worms was analyzed in the indicated genotypes and average velocity plotted (B) Adult locomotion was significantly reduced in both *flp-2* and *frpr-18* single mutants but additive effects were not observed in *frpr-18; flp-2* double mutants. (C) Mutations inactivating FLP-2 and FRPR-18 did not cause further decreases in the locomotion of *pdfr-1* adults. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, p <0.001).

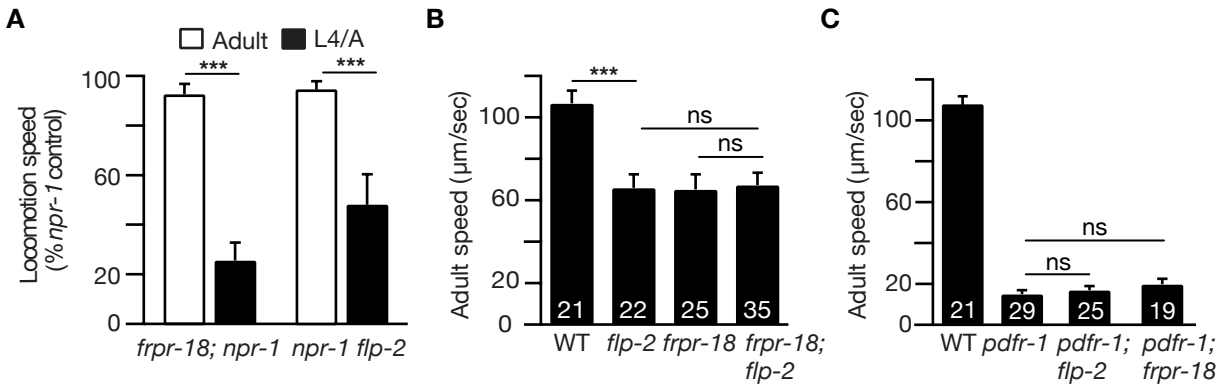
Figure S2. Inactivation of *frpr-18* or *flp-2* does not suppress *npr-1* foraging behavior

Representative images of foraging behavior on bacterial lawns are shown for the indicated genotypes. Neither *frpr-18* nor *flp-2* mutations prevented clumping of *npr-1* mutants. Scale bar indicates 1 mm.

Figure S3. *flp-2* is expressed in several head neurons including ASI

Expression of a *flp-2* promoter construct was observed in several head neurons,

including ASI. (A). Expression of *flp-2* in ASI was confirmed by co-expression with an ASI marker (*gpa-4*) (B). Scale bars indicate 10 μm.



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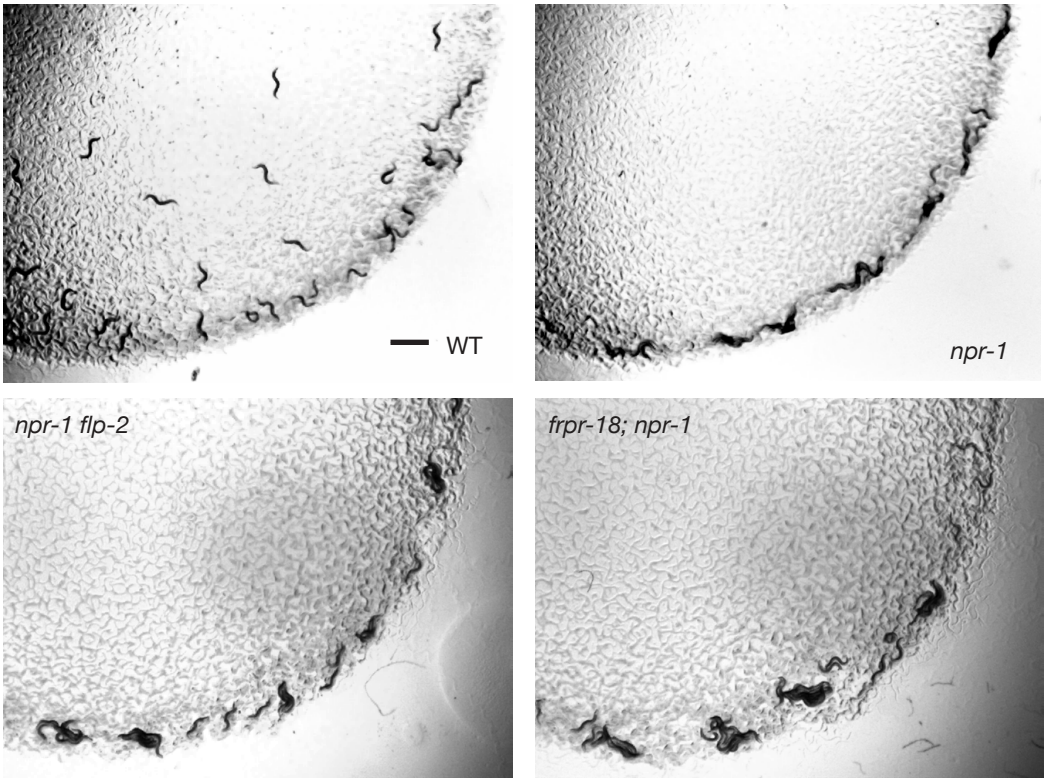

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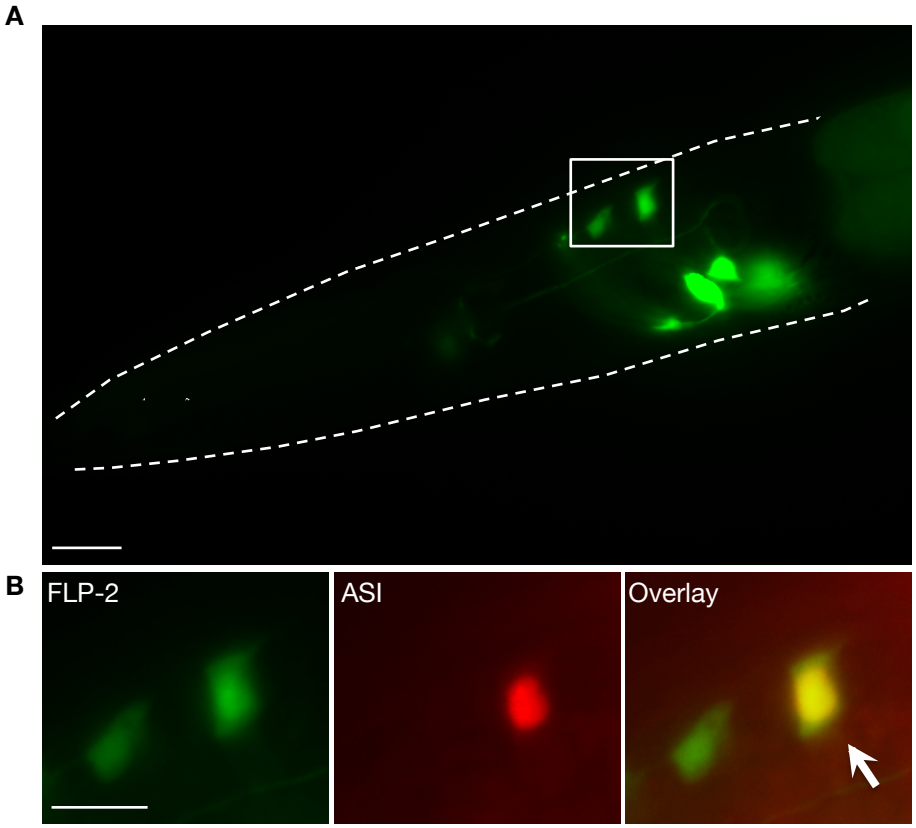

Figure S3. Chen et al.