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Analysis of NPR-1 Reveals a Circuit Mechanism for Behavioral Quiescence in *C. elegans*

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SUMMARY

Animals undergo periods of behavioral quiescence and arousal in response to environmental, circadian, or developmental cues. During larval molts, *C. elegans* undergoes a period of profound behavioral quiescence termed lethargus. Locomotion quiescence during lethargus was abolished in mutants lacking a neuropeptide receptor (NPR-1) and was reduced in mutants lacking NPR-1 ligands (FLP-18 and FLP-21). Wild-type strains are polymorphic for the *npr-1* gene, and their lethargus behavior varies correspondingly. Locomotion quiescence and arousal were mediated by decreased and increased secretion of an arousal neuropeptide (PDF-1) from central neurons. PDF receptors (PDFR-1) expressed in peripheral mechanosensory neurons enhanced touch-evoked calcium transients. Thus, a central circuit stimulates arousal from lethargus by enhancing the sensitivity of peripheral mechanosensory neurons in the body. These results define a circuit mechanism controlling a developmentally programmed form of quiescence.

INTRODUCTION

Animals coordinately adjust their behaviors in response to changes in their environment and metabolic state. Coregulated behaviors (often termed behavioral states) can persist for minutes to hours. Increased activity (or arousal) is associated with fear, stress, hunger, and exposure to sexual partners (Pfaff et al., 2008). Conversely, decreased activity (or quiescence) is associated with sleep and satiety (Cirelli, 2009).

Many aspects of behavior and metabolism exhibit rhythmic patterns with a periodicity of approximately 24 hr, patterns generically referred to as circadian rhythms (Allada and Chung, 2010). Daily behavioral and metabolic rhythms are accompanied by a corresponding set of circadian changes in gene expression. Circadian rhythms are dictated by a cell-autonomous clock that consists of a transcriptional feedback network that exhibits intrinsically oscillating activity. The period of this circadian clock is entrained by daily changes in light and temperature, although daily rhythms persist even in constant conditions. Thus, circadian clocks provide a mechanism that allows animals to couple their behavior to anticipated changes in their environment.

Rhythmic changes in behavior and metabolism are also often coupled to developmental clocks. In the nematode *C. elegans,* molting exhibits a rhythmic pattern with a periodicity of 8–10 hr. This molting cycle is dictated by cell-intrinsic developmental clock genes (termed heterochronic genes) (Moss, 2007). The periodicity of the molting cycle is dictated by rhythmic changes in the expression of a heterochronic gene (*lin-42*), which is homologous to the fly circadian gene PERIOD (Jeon et al., 1999; Monsalve et al., 2011). Thus, circadian and heterochronic clocks are mediated by similar biochemical mechanisms.

Although a great deal is known about the biochemical and genetic mechanisms controlling circadian and heterochronic timing, relatively little is known about how these clocks are coupled to changes in behavior, i.e., to their outputs. To address this question, we analyzed the rhythmic behaviors associated with the *C. elegans* molting cycle.

During each larval molt, *C. elegans* undergoes a prolonged period of profound behavioral quiescence, whereby locomotion and feeding behaviors are inactive for ~2 hr. This molt-associated quiescence is termed lethargus behavior, and it has been observed in many wild-type nematode species (Cassada and Russell, 1975). Lethargus has properties of a sleep-like state, such as reduced sensory responsiveness and homeostatic rebound of quiescence after perturbation (Raizen et al., 2008). Several genes and molecular pathways involved in lethargus behavior have been identified (Monsalve et al., 2011; Raizen et al., 2008; Singh et al., 2011; Van Buskirk and Sternberg, 2007); however, a circuit mechanism controlling lethargus-associated quiescence has not been defined.

Here we identify a central sensory circuit that dictates entry into and exit from locomotion quiescence during lethargus. Quiescence is associated with decreased activity in this central circuit, whereas arousal is associated with increased circuit activity. This central circuit regulates motility through the action of a neuropeptide (pigment-dispersing factor-1 [PDF-1]), which enhances the sensitivity of peripheral mechanosensory receptors in the body. These results provide a circuit mechanism that controls arousal and quiescence of locomotion in *C. elegans*.

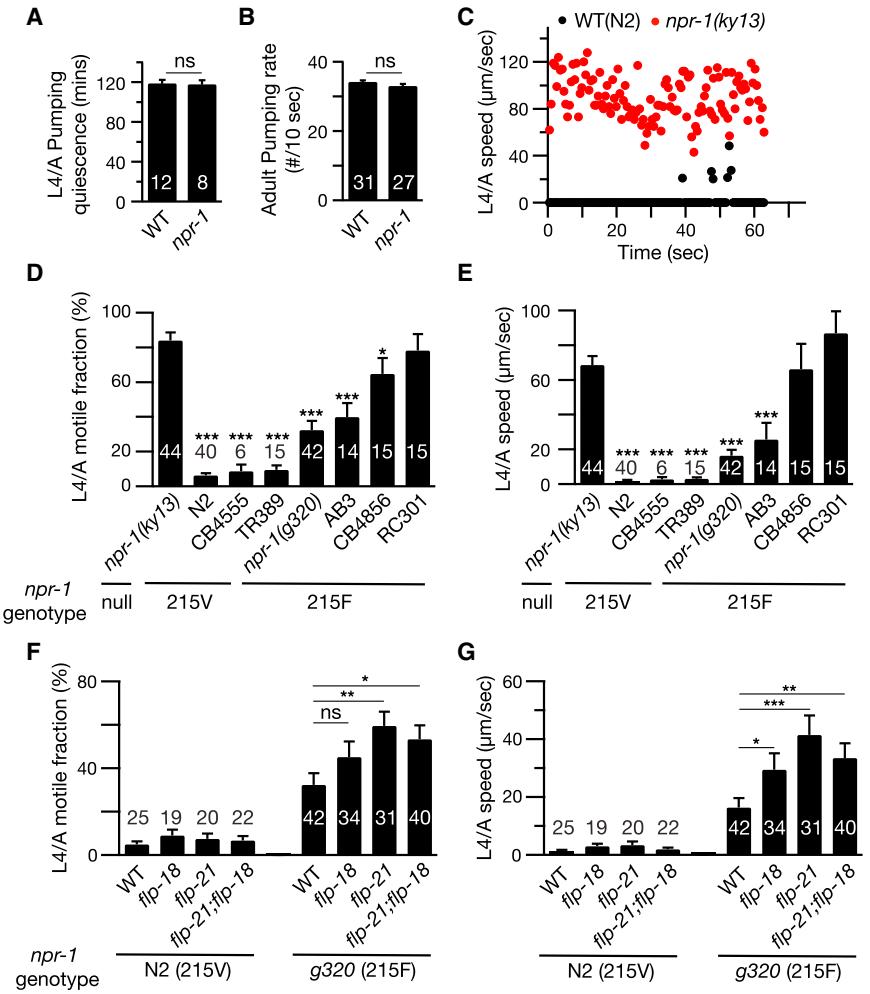

Figure 1. NPR-1 and Its Ligands FLP-18 and FLP-21 Regulate Locomotion Quiescence during Lethargus

Pharyngeal pumping (A and B) and locomotion (C–G) were analyzed in the indicated genotypes.

(A) The duration of feeding quiescence during the L4/A lethargus (defined by the absence of pharyngeal pumping) was unaltered in *npr-1* mutants.

(B) Adult pharyngeal pumping rate was also unaltered in *npr-1* mutants.

(C–G) Locomotion behavior of single worms during the L4/A lethargus was recorded for 30–75 s and velocity was measured (2 Hz sampling). Instantaneous locomotion velocity (C), average motile fraction (D and F), and average locomotion velocity (E and G) are plotted. *npr-1* null mutants had higher locomotion during the L4/A lethargus than did WT (N2) (C–E). Wild-type strains were polymorphic for L4/A locomotion, with 215V strains being more quiescent than 215F strains (D and E). Mutations that inactivated NPR-1 ligands FLP-18 and FLP-21 decreased L4/A locomotion quiescence in animals expressing NPR-1(215F) receptors, but not in those expressing NPR-1(215V) receptors, i.e., *npr-1(g320)* mutants and N2, respectively (F and G). The number of animals analyzed is indicated for each genotype. Error bars indicate the SEM. Values that differ significantly from *npr-1(ky13)* (D and E) or *npr-1(g320)* L4/A (F and G) are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

See also Figure S1 and Movies S1 and S2.

RESULTS

Locomotion Quiescence during Lethargus Is Blocked in *npr-1* Mutants

Mutants lacking the neuropeptide receptor NPR-1 have heightened responsiveness to oxygen and pheromones, which results in altered foraging behavior and accelerated locomotion (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009). Thus, NPR-1 is proposed to set the threshold for arousal of specific behaviors. Prompted by these results, we tested the idea that NPR-1 also regulates arousal from behavioral quiescence during lethargus. To analyze animals during the L4-to-adult (L4/A) lethargus, we isolated a synchronous population of L4 animals and analyzed their behaviors during the subsequent molt. As in wild-type animals, the pharyngeal pumping of *npr-1* mutants was completely arrested during the L4/A lethargus (Figure 1A). The duration of pharyngeal pumping quiescence was unaltered in *npr-1* mutants, indicating that the duration of lethargus had not been altered (Figure 1A). Pharyngeal pumping rate was also unaltered in *npr-1* adults (Figure 1B). To assess changes in locomotion during the L4/A lethargus, we analyzed the fraction of time animals undergo active motility (motile fraction) and locomotion velocity. Unlike wild-type animals, *npr-1* mutants exhibited fast and nearly continuous locomotion during the L4/A lethargus (Figures 1C–1E; Movies S1 and S2 available online). The effects of *npr-1* on locomotion persisted throughout the L4/A lethargus (as defined by pumping quiescence) (Figures S1A and S1B). Inactivation of *npr-1* had a significantly larger effect on locomotion during the L4/A lethargus (motile fraction, 17-fold increase; velocity, 50-fold increase) than in adults (motile fraction, 1.2-fold increase; velocity, 2-fold increase) (Figures S1C and S1D). These results suggest that NPR-1 is required for locomotion quiescence during lethargus, but not for feeding quiescence.

Wild-Type Strains Are Polymorphic for Lethargus Locomotion Behavior

The *npr-1* gene is polymorphic among wild-type populations, with two frequent alleles observed (215V and 215F) (McGrath et al., 2009; Weber et al., 2010). These wild-type alleles encode receptors that differ in their affinity for NPR-1 ligands (FLP-18 and FLP-21), with 215V exhibiting higher affinity (and lower half-maximal effective concentration values) than 215F receptors (Kubiak et al., 2003; Rogers et al., 2003). To determine whether wild-type strains are also polymorphic for lethargus behavior, we analyzed locomotion during the L4/A lethargus (Figures 1D and 1E). All 215V-containing strains exhibited similar

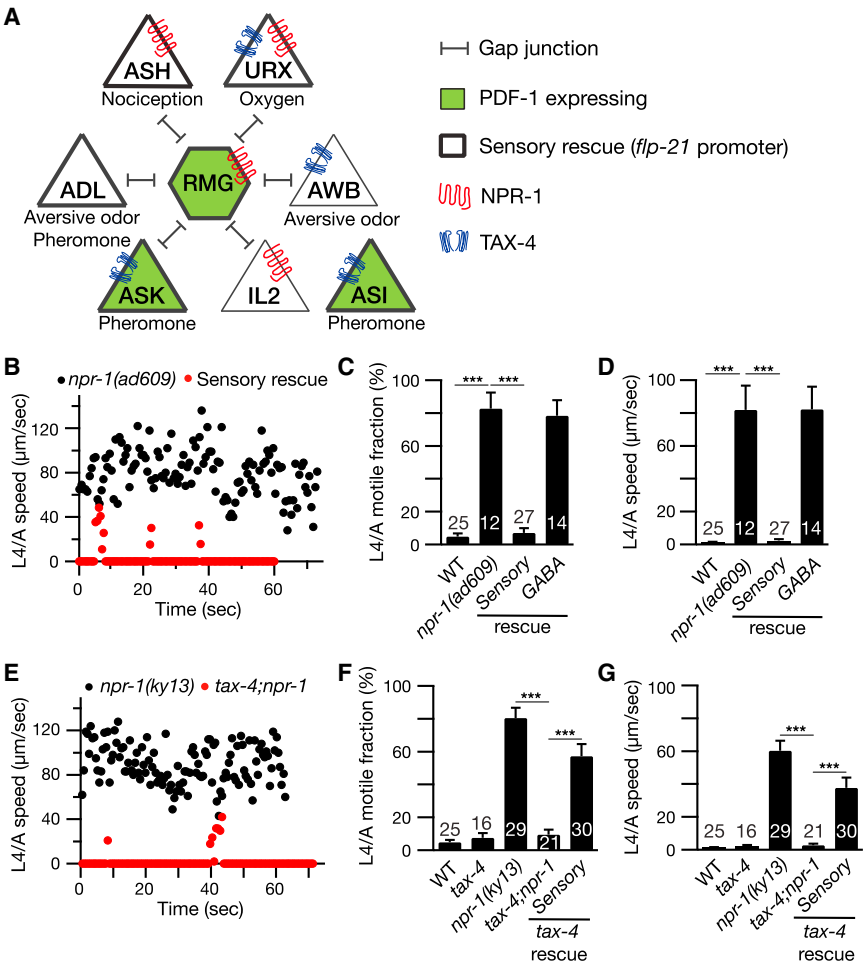

Figure 2. The npr-1 Lethargus Defect Is Mediated by Increased Sensory Activity

(A) A diagram illustrating the RMG circuit is shown. Sensory neurons (triangles) mediating the indicated aversive responses form direct gap junctions with the RMG interneuron (hexagon). Cells expressing NPR-1, TAX-4/CNG channels, PDF-1, and the *flp-21* promoter (sensory rescue) are indicated (Barrios et al., 2012; Coates and de Bono, 2002; Janssen et al., 2009; Komatsu et al., 1996; Macosko et al., 2009; Rogers et al., 2003). ASI neurons are not directly connected to RMG but are also a potential source of PDF-1. This diagram is modified from a previous work (Macosko et al., 2009).

(B–G) Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (B and E), average motile fraction (C and F), and average locomotion velocity (D and G) are plotted. The *npr-1* L4/A locomotion quiescence defect was rescued by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue, *flp-21* promoter), but not by those expressed in GABAergic neurons (GABA rescue, *unc-30* promoter), using the indicated promoters (B–D). The *npr-1* L4/A locomotion quiescence defect was suppressed in double mutants lacking TAX-4/CNG channels and was reinstated by transgenes expressing TAX-4 in the RMG circuit (Sensory rescue, *flp-21* promoter) (E–G). The number of animals analyzed is indicated for each genotype. Error bars indicate the SEM. Values that differ significantly are indicated (***$p < 0.001$).

See also Figure S2.

levels of quiescence and were significantly more quiescent than 215F strains. The quiescence observed in 215F strains was more variable, with one strain (RC301) exhibiting L4/A locomotion similar to that of *npr-1* null mutants and other strains (AB3 and CB4856) exhibiting intermediate levels of quiescence. Thus, the extent of behavioral quiescence during lethargus is polymorphic among wild-type strains. A strain carrying a 215F allele (*g320*) in the Bristol genetic background had significantly stronger quiescence than was observed in unrelated 215F wild-type strains (e.g., CB4856 and RC301). These results suggest that variation in genes other than *npr-1* also contribute to differences in the lethargus behaviors of wild-type strains.

The NPR-1 Ligands FLP-21 and FLP-18 Regulate Lethargus Behavior

Two NPR-1 ligands have been identified, the neuropeptides FLP-18 and FLP-21 (Kubiak et al., 2003; Rogers et al., 2003). Both neuropeptides bind and activate NPR-1 receptors expressed in transfected cells; however, NPR-1 exhibits significantly stronger affinity for FLP-21. We found that mutations inactivating FLP-18 and FLP-21, and double mutants inactivating both ligands, had no effect on the L4/A locomotion behavior of worms expressing high-affinity NPR-1(215V) receptors (Figures 1F and 1G). By contrast, when either FLP-18 or FLP-21 was inactivated in a Bristol strain expressing low-affinity NPR-1(215F) receptors, i.e., *npr-1(g320)* mutants, locomotion quiescence was significantly decreased (Figures 1F and 1G). These results suggest that FLP-18 and FLP-21 function as endogenous NPR-1 ligands to regulate lethargus behavior in strains expressing NPR-1(215F) receptors.

The *npr-1* Lethargus Defect Is Mediated by Increased Sensory Activity

NPR-1's effects on foraging are mediated by its expression in a sensory circuit in the head that is defined by gap junctions to the RMG interneuron (Figure 2A) (Macosko et al., 2009). Hereafter, we refer to this circuit as the RMG circuit. In addition to the RMG circuit, NPR-1 is also expressed in GABAergic motor neurons in the ventral nerve cord (Coates and de Bono, 2002). We did two experiments to determine where NPR-1 functions to regulate motility during lethargus. First, an *npr-1* transgene expressed in the RMG circuit (using the *flp-21* promoter) (Figure 2A) completely rescued the lethargus locomotion defect of *npr-1* mutants, whereas a transgene expressed in GABAergic motor neurons (using the *unc-30* promoter) had no rescuing activity (Figures 2B–2D). Second, the lethargus locomotion defect of

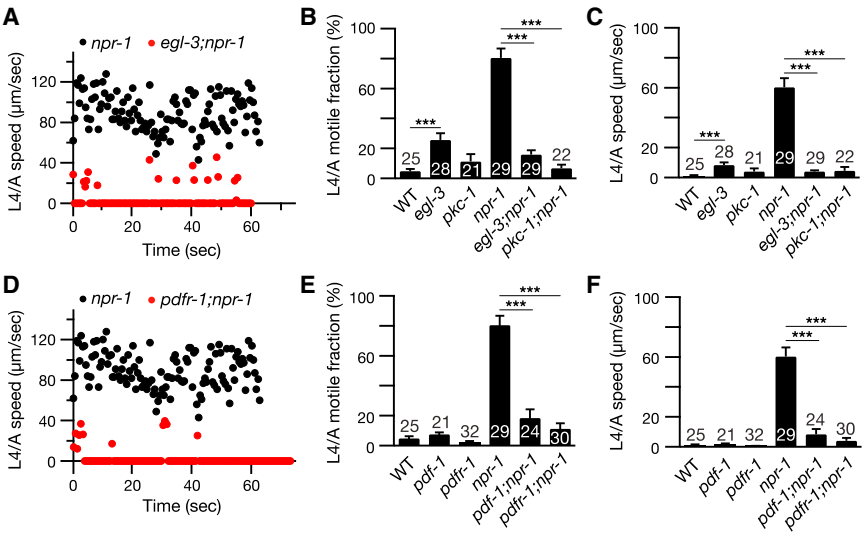

Figure 3. PDF-1 and PDFR-1 Mediate Arousal from Locomotion Quiescence during Lethargus

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A and D), average motile fraction (B and E), and average locomotion velocity (C and F) are plotted. The *npr-1* L4/A locomotion quiescence defect was suppressed by mutations that block neuropeptide processing (*egl-3* PC2 mutants [A–C]) and dense core vesicle exocytosis (*pkc-1* PKCε mutants [A–C]), and by mutations inactivating PDF-1 and PDFR-1 (D–F). The number of animals analyzed is indicated for each genotype. Error bars indicate the SEM. Values that differ significantly are indicated (***$p < 0.001$).
See also Figure S3.

npr-1 mutants was abolished by mutations inactivating ion channels required for sensory transduction, such as TAX-4/CNG and OSM-9/TRPV channels (Figures 2E–2G, S2A, and S2B). A transgene expressing TAX-4 in the RMG circuit reinstated the L4/A quiescence defect in *tax-4; npr-1* double mutants (Figures 2F and 2G). These results suggest that the *npr-1* defect in locomotion quiescence during lethargus was caused by heightened sensory activity in the RMG circuit.

Pigment-Dispersing Factor Is Required for the *npr-1* Lethargus Defect

Neuropeptides play a pivotal role in sleep and wakefulness in other systems. For example, hypocretin/orexin regulates sleep, arousal, feeding, and metabolism in vertebrates (Sutcliffe and de Lecea, 2002). Thus, we tested whether neuropeptides are required for the *npr-1* lethargus defect. Consistent with this idea, the *npr-1* lethargus quiescence defect was eliminated by mutations inactivating *egl-3* PC2 and *pkc-1* PKCε (Figures 3A–3C), which are required for proneuropeptide processing and dense-core-vesicle (DCV) exocytosis, respectively (Husson et al., 2006; Kass et al., 2001; Sieburth et al., 2007). These results suggest that the *npr-1* lethargus defect was mediated by an endogenous neuropeptide.

In *Drosophila*, the neuropeptide PDF regulates circadian rhythms and promotes wakefulness (Parisky et al., 2008; Renn et al., 1999). Prompted by PDF's role in *Drosophila*, we tested the idea that PDF mediates the lethargus quiescence defect in *npr-1* mutants. *C. elegans* PDF peptides (PDF-1 and PDF-2) and their receptor (PDFR-1) were previously identified (Janssen et al., 2008, 2009). PDF-1 is expressed in several classes of sensory neurons and interneurons, including ASK chemosensory neurons and RMG interneurons in the RMG circuit (Barrios et al., 2012; Janssen et al., 2009) (Figure 2A). The locomotion rate and motile fraction of *pdf-1; npr-1* and *pdfr-1; npr-1* double mutants during the L4/A lethargus were significantly lower than in *npr-1* single mutants (Figures 3D–3F). Inactivating PDF-1 and PDFR-1 had a much less dramatic effect on adult locomotion in *pdf-1; npr-1* and *pdfr-1; npr-1* double mutants (Figure S3A). Thus, increased signaling by PDF-1 and PDFR-1 in *npr-1* mutants was required for the increased motility during lethargus. The *npr-1* foraging defect was unaltered in *pdf-1; npr-1* and *pdfr-1; npr-1* double mutants (Figure S3B), indicating that PDF was not required for other *npr-1* phenotypes. Inactivating PDF-2 had little effect on the locomotion of *npr-1* mutants during lethargus (Figures S3C and S3D), indicating that PDF-1 is the major form of PDF involved in lethargus behavior. Collectively, these results suggest that PDF-1 functions as an arousal peptide in *npr-1* mutants, preventing locomotion quiescence during lethargus. PDF-1's effects on arousal were specific, because knockdown of 14 other neuropeptides expressed in the RMG circuit had no effect on the *npr-1* lethargus defect (Figure S3E).

NPR-1 Inhibits PDF-1 Secretion during Lethargus

If PDF-1 functions as an arousal peptide, PDF-1 expression or secretion should be inhibited during lethargus, when animals are quiescent. We did several experiments to test this idea. The abundance of *pdf-1* and *pdfr-1* mRNAs (assayed by quantitative PCR) was unaltered during the L4/A lethargus, whereas expression of *mlt-10* (a gene required for molting) was significantly increased, as expected (Figure S4A) (Frand et al., 2005). To assay PDF-1 secretion, we expressed yellow-fluorescent-protein (YFP)-tagged proPDF-1 with the *pdf-1* promoter (Figures 4A and 4B). During DCV maturation, the YFP linked to proPDF-1 is cleaved by proprotein convertases and is subsequently secreted by DCV exocytosis. To assess the level of PDF-1 secretion, we analyzed PDF-1::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; Sieburth et al., 2007). The PDF-1::YFP secretion reporter produced high levels of coelomocyte fluorescence in both L4 larvae and adults, whereas dramatically lower coelomocyte fluorescence was observed during the L4/A lethargus (Figures 4A and 4B). Coelomocyte fluorescence produced by a second secretion probe (mCherry-tagged RIG-3 expressed in cholinergic neurons) (Babu et al., 2011) was unaltered during lethargus (Figure S4B), indicating that secretion

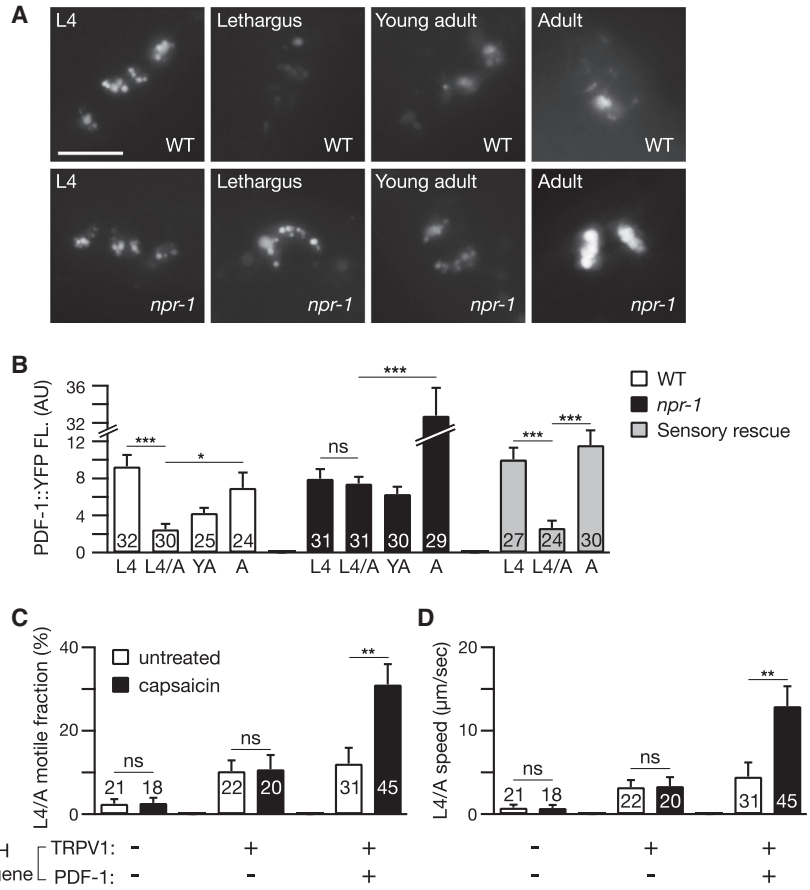

Figure 4. NPR-1 Inhibits PDF-1 Secretion during Lethargus

PDF-1 secretion (A and B) and the effect of forced secretion of PDF-1 on lethargus locomotion behavior (C and D) were analyzed in the indicated genotypes.

(A and B) YFP-tagged PDF-1 was expressed with the *pdf-1* promoter. Representative images (A) and summary data (B) are shown for coelomocyte fluorescence in L4, L4/A, young adult (0–2 eggs in uterus), and gravid adults of the indicated genotypes. PDF-1::YFP coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild-type animals, but not in *npr-1* mutants. Decreased PDF-1::YFP coelomocyte fluorescence during lethargus was reinstated by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue, *flp-21* promoter).

(C and D) Forced depolarization of PDF-1-expressing neurons decreased L4/A locomotion quiescence. PDF-1 and rat TRPV1 were ectopically expressed in ASH neurons (using the *sra-6* promoter). Locomotion behavior of transgenic worms during the L4/A lethargus was analyzed with or without capsaicin treatment (6–7 hr). Average motile fraction (C), and average locomotion velocity (D) are plotted. Capsaicin treatment decreased L4/A quiescence in transgenic animals expressing both TRPV1 and PDF-1 in ASH neurons, but not in those expressing only TRPV1. The number of animals analyzed is indicated for each genotype. Scale bar indicates 10 μm. Values that differ significantly are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

See also Figure S4.

and coelomocyte function were not globally inhibited during lethargus.

If decreased PDF-1 secretion during lethargus is a cellular mechanism for inducing quiescence, we would expect that mutants retaining or lacking locomotion quiescence would exhibit reciprocal patterns of PDF-1 secretion during lethargus. We did several experiments to test this idea. In *npr-1* mutants, which lack quiescence, the decrease in PDF-1::YFP coelomocyte fluorescence during the L4/A lethargus was eliminated (Figures 4A and 4B); the decrease was restored by a transgene expressing NPR-1 in the RMG circuit (Figure 4B). Similarly, *tax-4; npr-1* double mutants exhibited locomotion quiescence and decreased PDF-1 secretion during lethargus, and both effects were reversed by a transgene expressing TAX-4 in the RMG circuit (Figures 2F, 2G, and S4C). By contrast, RIG-3 coelomocyte fluorescence was decreased in *npr-1* mutants in both L4 and L4/A animals (Figure S4B). Consequently, the effects of NPR-1 and TAX-4 on PDF-1 coelomocyte fluorescence are unlikely to be caused either by general changes in the stability of secreted protein or by general changes in coelomocyte activity. Instead, these results suggest that heightened RMG circuit activity in *npr-1* mutants produced a corresponding increase in PDF-1 secretion from head sensory neurons, thereby increasing motility during lethargus.

The preceding results suggest that decreased and increased PDF-1 secretion during lethargus are correlated with and required for locomotion quiescence and arousal. To determine whether increased PDF-1 secretion is sufficient to arouse locomotion, we constructed transgenic animals in which PDF-1 secretion can be pharmacologically induced (Figures 4C and 4D). A prior study showed that capsaicin treatment depolarizes ASH neurons expressing rat TRPV1 channels (Tobin et al., 2002). When TRPV1 and PDF-1 were coexpressed in ASH neurons, capsaicin treatment significantly decreased locomotion quiescence during lethargus (Figures 4C and 4D). This effect was not observed when only TRPV1 was expressed in ASH. These results suggest that forced secretion of PDF-1 during lethargus was sufficient to arouse locomotion behavior.

PDF-1 Can Function in ASK Neurons to Mediate Arousal

Because RMG circuit activity controls PDF-1 secretion and locomotion arousal, a simple explanation for our data would be that PDF-1 is secreted by cells in the RMG circuit. Several results are consistent with this idea. The *pdf-1* promoter is expressed in RMG interneurons, as well as in ASK sensory neurons, which form direct gap junctions with RMG (Figure 2A) (Barrios et al., 2012; Janssen et al., 2008). Transgenes expressing PDF-1 in ASK neurons reinstated the locomotion quiescence defect in *pdf-1; npr-1* double mutants (Figures S4D and S4E). Similarly, coelomocyte fluorescence produced by PDF-1::YFP expressed in ASK neurons was decreased during lethargus in wild-type animals but not in *npr-1* mutants (Figure S4F). Thus, PDF-1

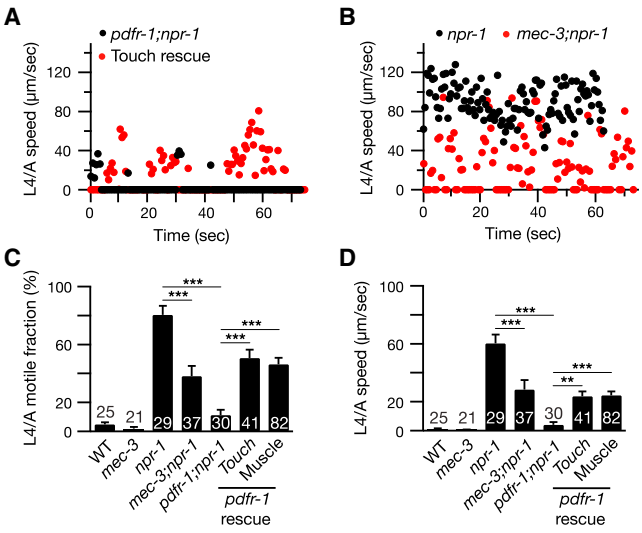

Figure 5. PDFR-1 Receptors Expressed in Touch Neurons and Body Muscles Mediate Locomotion Arousal

(A–D) Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A and B), average motile fraction (C), and average locomotion velocity (D) are plotted. The *npr-1* locomotion quiescence defect was partially reinstated in *pdfr-1;npr-1* double mutants by transgenes expressing PDFR-1 in touch neurons (*mec-3* promoter) and body muscles (*myo-3* promoter) using the indicated promoters (A, C, and D). Mutations disrupting touch neuron differentiation (*mec-3* mutants) partially suppressed the *npr-1* locomotion quiescence defect (B–D). The number of animals analyzed is indicated for each genotype. Error bars indicate the SEM. Values that differ significantly are indicated (**$p < 0.01$; ***$p < 0.001$).

expression in ASK neurons was sufficient to reconstitute NPR-1's effects on locomotion quiescence and PDF-1 secretion during lethargus. Because PDF-1 is secreted (and consequently acts in a non-cell-autonomous manner), PDF-1 secretion from other cells may also regulate lethargus behavior. Consistent with this idea, PDF-1 expression in ASI neurons also restored the L4/A quiescence defect in *pdf-1; npr-1* double mutants (Figures S4D and S4E).

PDFR-1 Acts in Mechanosensory Neurons to Mediate Arousal

How does enhanced PDF-1 secretion alter locomotion? The *pdfr-1* promoter is expressed in mechanosensory neurons that sense vibration of the body wall (the touch neurons), in body wall muscles, and in a few other classes of neurons (Janssen et al., 2008). Transgenes expressing PDFR-1 in touch neurons or in body wall muscles both partially reinstated the lethargus locomotion quiescence defect in *npr-1; pdfr-1* double mutants (Figures 5A, 5C, and 5D). These results suggest that PDFR-1 acts in both touch neurons and body wall muscles to promote arousal from locomotion quiescence during lethargus. The six touch neurons form gap junctions with the ventral cord command interneurons that control locomotion (Chalfie et al., 1985). Mutations that impair the mechanosensitivity of the touch neurons (termed Mec mutants) cause locomotion to become lethargic (Chalfie and Sulston, 1981). For these reasons, we focused our analysis on PDFR-1 function in touch neurons.

Is the *npr-1* lethargus defect mediated by increased activity of the touch neurons? We did several experiments to test this idea. First, we analyzed the lethargus behavior of *mec-3; npr-1* double mutants. The MEC-3 transcription factor is required for differentiation of touch neurons; consequently, touch responses are disrupted in *mec-3* mutants (Way and Chalfie, 1988). Mutations inactivating *mec-3* partially suppressed the lethargus locomotion defect of *npr-1* mutants (Figures 5B–5D). These results suggest that touch neuron function was required for NPR-1's effect on motility during lethargus. Partial suppression of the lethargus defect in *mec-3; npr-1* double mutants was expected, because rescue experiments suggest that PDFR-1 function is required in both touch neurons and body muscles (Figures 5A, 5C, and 5D).

Second, we measured touch-evoked calcium transients in the anterior touch neuron (ALM) of adult animals using the genetically encoded calcium indicator cameleon (Figures 6A, 6B, S5C, and S5D). Cameleon expression in touch neurons did not disrupt NPR-1 and PDFR-1 effects on L4/A locomotion quiescence (Figures S5A and S5B). Thus, calcium buffering by cameleon did not interfere with NPR-1-mediated regulation of touch cell function. PDF-1 secretion was increased in *npr-1* adults (Figures 4A and 4B); consequently, NPR-1's effects on touch sensitivity should be evident in adults. Consistent with this idea, the magnitude of touch-evoked calcium transients in ALM was significantly increased in *npr-1* mutant adults, and this defect was rescued by transgenes expressing NPR-1 in the RMG circuit (Figures 6A and 6B). The enhanced ALM touch sensitivity exhibited by *npr-1* adults was eliminated in *pdfr-1; npr-1* double mutants (Figures 6A and 6B) and was reinstated by transgenes expressing PDFR-1 in touch neurons, but not by those expressed in body wall muscles (Figures 6A and 6B). By contrast, in *pdf-1; npr-1* double mutants, heightened ALM touch responsiveness was reduced, but not eliminated (Figures S5C and S5D). The residual effect of NPR-1 on ALM touch sensitivity in *pdf-1; npr-1* double mutants was likely mediated by other PDFR-1 ligands (e.g., PDF-2). Collectively, these results suggest that increased PDF-1 secretion in *npr-1* adults was associated with enhanced touch sensitivity.

Because PDF-1 and PDFR-1 enhanced touch sensitivity in *npr-1* mutants, we would expect that *pdf-1* and *pdfr-1* single mutants would exhibit decreased touch sensitivity. Contrary to this idea, adult ALM touch responses were unchanged in either single mutant (Figures 6A, 6B, S5C, and S5D). These results do not exclude the idea that touch sensitivity was altered in these mutants. We may fail to detect differences in ALM responses for technical reasons. For example, an effect on touch sensitivity in single mutants may only be apparent at lower stimulus intensities, or upon repetitive stimulation. To further address this issue, we analyzed locomotion in the single mutants. Adult *pdf-1* and *pdfr-1* single mutants exhibited significantly slower locomotion and decreased motile fractions (Figures 6C–6E; Meelkop et al., 2012), both of which could result from diminished touch sensitivity. Consistent with this idea, the decreased locomotion rate and motile fraction of *pdfr-1* mutants was partially rescued by transgenes expressing PDFR-1 in touch neurons

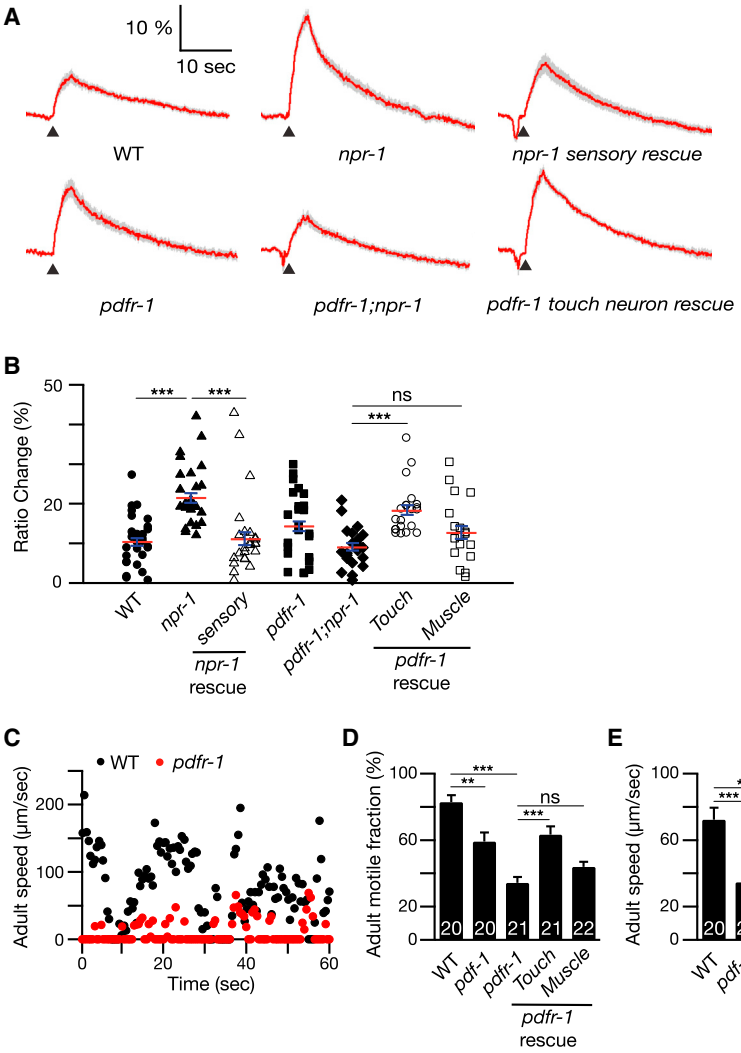

Figure 6. PDFR-1 Is Required for Enhanced ALM Touch Sensitivity in npr-1 Mutant Adults

Touch sensitivity and locomotion were analyzed in the indicated adult animals.

(A and B) Touch-evoked calcium transients in ALM were analyzed using cameleon as a calcium indicator. Responses were analyzed in adult animals. Averaged responses (A) and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates the SEM response. Touch-evoked calcium transients in adult ALM neurons were significantly larger in *npr-1* mutants. This defect was rescued by transgenes expressing NPR-1 in the RMG circuit (sensory rescue), and was suppressed by mutations inactivating PDF-1 and PDFR-1. Touch-evoked calcium transients in *pdfr-1* mutants were not significantly different from wild-type controls. Enhanced touch-evoked calcium transients in adult ALM neurons were reinstated in *pdfr-1;npr-1* double mutants by transgenes expressing PDFR-1 in touch neurons but not by those expressed in body muscles.

(C–E) Locomotion behavior of single adult worms was analyzed in the indicated genotypes. Instantaneous locomotion velocity (C), average motile fraction (D), and average locomotion velocity (E) are plotted. Both *pdf-1* and *pdfr-1* single mutants showed reduced locomotion in adult. The *pdfr-1* adult locomotion defect was partially rescued by transgenes expressing PDFR-1 in touch neurons, but not in body wall muscles. The number of animals analyzed is indicated for each genotype. Error bars indicate the SEM. Values that differ significantly are indicated (***$p < 0.001$; **$p < 0.01$; ns, not significant).

See also Figure S5.

(Figures 6D and 6E). These results support the idea that the effects of PDF-1 and PDFR-1 on touch sensitivity are not restricted to *npr-1* mutants.

To determine whether NPR-1 also regulates touch sensitivity during lethargus, we analyzed ALM calcium transients during the L4/A lethargus (Figure 7). A recent study reported that touch neuron calcium transients are significantly reduced during lethargus (Schwarz et al., 2011). Consistent with this prior study, we found that ALM touch-evoked calcium transients were significantly smaller during the L4/A lethargus; however, this effect was eliminated in *npr-1* mutants (Figure 7). The enhanced ALM touch responses exhibited by *npr-1* mutants during lethargus were eliminated in *pdfr-1; npr-1* double mutants (Figure 7). Thus, NPR-1 inhibition of PDF signaling is required for inhibition of touch sensitivity during lethargus.

DISCUSSION

We describe a circuit mechanism that controls arousal from a developmentally programmed form of behavioral quiescence in *C. elegans*. Increased RMG circuit activity in *npr-1* mutants was accompanied by increased PDF-1 secretion and heightened peripheral sensitivity to touch, thereby increasing motility during lethargus. Below we discuss the significance of these results.

Related neuropeptides mediate quiescence and arousal/motivation in worms, flies, and rodents. Peptides homologous to NPY induce locomotion quiescence in *C. elegans* (FLP-18 and FLP-21), inhibit locomotion and foraging for food in *Drosophila* (NPF) (Wu et al., 2003), and inhibit the arousing effects of hypocretin-expressing neurons in mice (NPY) (Fu et al., 2004). By contrast, peptides homologous to PDF arouse locomotion in *C. elegans* (PDF-1), arouse circadian locomotor activity, and decrease sleep duration in *Drosophila* (PDF) (Parisky et al., 2008; Renn et al., 1999), and regulate circadian behaviors and sleep in rodents (VIP) (Hu et al., 2011; Maywood et al., 2007). Thus, conserved molecular mechanisms are employed to regulate arousal and quiescence in developmentally programmed, metabolically driven, and circadian behavioral states.

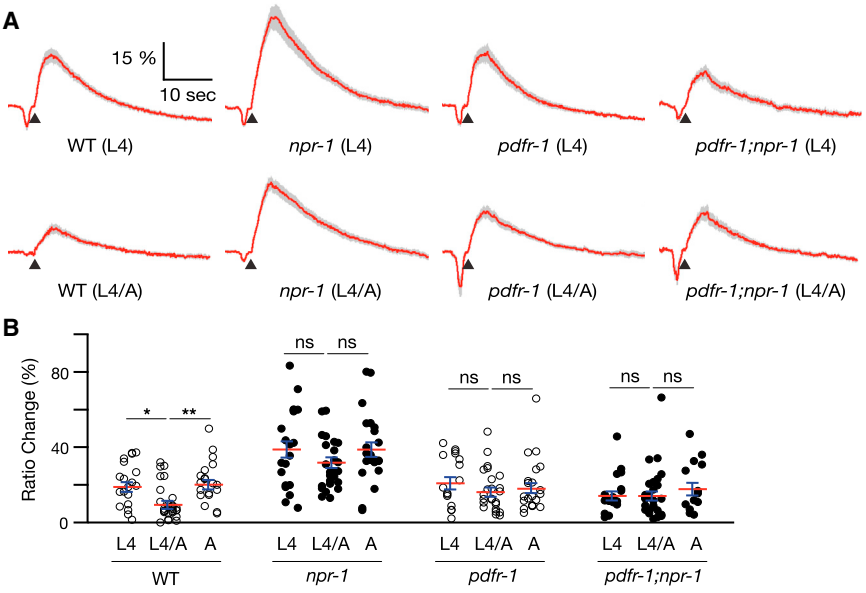

Figure 7. NPR-1 and PDFR-1 Regulate ALM Touch Sensitivity during Lethargus

(A and B) Touch-evoked calcium transients in ALM were analyzed in L4, L4/A, and adults of the indicated genotypes. Averaged responses (A) and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates the SEM response. Touch-evoked ALM calcium transients were significantly reduced during L4/A lethargus, and this effect was abolished in *npr-1* mutants. Enhanced touch-evoked calcium transients in *npr-1* mutants were suppressed by inactivating PDFR-1. Values that differ significantly are indicated (*$p < 0.05$; **$p < 0.01$; ns, not significant).

If lethargus is a sleep-like state, as previously proposed (Raizen et al., 2008; Van Buskirk and Sternberg, 2007), one would expect that disrupting quiescence during lethargus would be deleterious. Contrary to this notion, the fertility and development of *npr-1* mutants were not grossly altered, indicating that locomotion quiescence during lethargus is not essential for normal development or molting. These results do not exclude the idea that quiescence during lethargus has significant effects on health in native environments (where conditions are more variable).

How are arousal peptides functionally coupled to circadian and developmental cycles? VIP and PDF are expressed in central clock neurons: rat VIP in the suprachiasmatic nucleus (SCN) of the hypothalamus, fly PDF in LNv neurons, and worm PDF in the RMG circuit (Helfrich-Förster, 1995; Maywood et al., 2007). Rhythmic changes in *pdf* mRNA levels were not observed in the *Drosophila* circadian and *C. elegans* molting cycles (Janssen et al., 2009; Park and Hall, 1998). Instead, PDF-1 secretion was dramatically reduced during lethargus. Inhibition of PDF-1 secretion and inhibition of locomotion during lethargus were both abolished in *npr-1* mutants. Thus, altered PDF-1 secretion provides a cellular mechanism for coupling changes in locomotor activity to the molting cycle.

How is PDF-1 secretion inhibited during lethargus? In *npr-1* mutants, pheromone and oxygen responses mediated by the RMG circuit are enhanced (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009), and we observed a corresponding enhancement of PDF-1 secretion. Similarly, inactivation and restoration of TAX-4 CNG channel expression in the RMG circuit was accompanied by parallel changes in PDF-1 secretion. Based on these results, we propose that RMG circuit activity is diminished during lethargus, thereby inhibiting PDF-1 secretion. Consistent with this idea, forced depolarization of ASH neurons expressing PDF-1 was sufficient to arouse locomotion during lethargus.

How do central clock neurons engender rhythmic behaviors? A great deal is known about how the activity and expression profile of central clock neurons are regulated. Much less is known about how clock neurons dictate circadian behaviors. In *C. elegans*, responsiveness to several sensory cues is reduced during lethargus. In particular, touch sensitivity and touch-evoked calcium transients in the touch neurons are decreased during lethargus (Raizen et al., 2008; Schwarz et al., 2011; Singh et al., 2011). Our results provide a cellular mechanism for these effects. During lethargus, NPR-1 inhibited PDF-1 secretion from the RMG circuit, thereby decreasing touch neuron sensitivity. PDF-1's effect on locomotion arousal was also mediated in part by activation of PDFR-1 receptors in body muscle. Interestingly, fly PDF and rodent VIP also have direct effects on muscle function (Talsma et al., 2012).

Although NPR-1, TAX-4, and PDF have profound effects on lethargus behavior, several results suggest that other signaling pathways must also contribute to both quiescence and arousal. For example, L4/A quiescence was restored in *pdfr-1; npr-1* double mutants (Figures 3D–3F); consequently, changes in NPR-1 and PDF signaling are not absolutely required to induce locomotion quiescence or arousal. Similarly, the locomotion of *pdfr-1* mutants during lethargus was significantly more quiescent than in adults. Thus, inactivating PDF signaling is unlikely to be the only mechanism producing L4/A quiescence. These results suggest that arousal and quiescence are behavioral states governed by multiple inputs, whose activities are integrated in the RMG circuit.

NPR-1 regulates several physiologically important traits. Inactivating NPR-1 alters sensitivity to environmental repellents (e.g., pheromones and oxygen), foraging behavior, innate immune responses, and lethargus behavior (Cheung et al., 2005; de Bono and Bargmann, 1998; Gray et al., 2004; Reddy et al., 2009; Styer et al., 2008; Figure 2A). Because NPR-1 sits at the nexus of multiple physiologically important traits, changes in NPR-1 activity and natural variation in the *npr-1* gene provide a mechanism for coupling changes in behavioral quiescence to the demands of the local environment. Specifically, changes in NPR-1 signaling could allow isolated populations to optimize

growth properties in environments with increased exposure to specific repellents or bacterial pathogens.

EXPERIMENTAL PROCEDURES

Strains

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

Wild-Type Strains

CB4555, TR389, AB3, CB4856, and RC301.

Mutant Strains and Integrants

DA609 *npr-1(ad609) X*
KP6080 *npr-1(g320) X*
KP6048 *npr-1(ky13) X*
AX1410 *flp-18(db99) X* (gift from Mario de Bono)
KP6077 *flp-21(pk1601) V*
PR678 *tax-4(p678) III*
KP3183 *osm-9(ky10) IV*
KP5966 *egl-3(nr2090) V*
KP5989 *pkc-1(nj3) V*
LSC27 *pdf-1(tm1996) III*
KP6340 *pdfr-1(ok3425) III*
KP6416 *pdf-2(tm4393) X*
CB1338 *mec-3(e1338) IV*
KP7044 *flp-21(pk1601) V; flp-18(db99) X*
KP7041 *flp-18(db99) npr-1(g320) X*
KP7042 *flp-21(pk1601) V; npr-1(g320) X*
KP7059 *flp-21(pk1601) V; flp-18(db99) npr-1(g320) X*
KP6060 *tax-4(p678) III; npr-1(ky13) X*
KP6841 *osm-9(ky10) IV; npr-1(ky13) X*
KP6054 *egl-3(nr2090) V; npr-1(ky13) X*
KP6682 *pkc-1(nj3) V; npr-1(ky13) X*
KP6100 *pdf-1(tm1996) III; npr-1(ky13) X*
KP6410 *pdfr-1(ok3425) III; npr-1(ky13) X*
KP6417 *pdf-2(tm4393) npr-1(ky13) X*
KP5364 *nre-1(hd20) lin-15b(hd126) X*
KP6050 *npr-1(ky13) nre-1(hd20) lin15b(hd126) X*
CX4978 *kyIS200[sra-6p::VR1, elt-2p::NLS-gfp]* (gift from Cori Bargmann)
KP6426 *mec-3(e1338) IV; npr-1 (ky13) X*
KP6693 *nuIS472[pdf-1p::pdf-1::YFP, vha-6p::mCherry]*
KP6744 *tax-4(p678) III; nuIS472*
KP6745 *tax-4(p678) III; npr-1(ky13) X; nuIS472*
KP6743 *npr-1(ky13) X; nuIS472*
AQ906 *bzIS17[mec-4p::YC2.12]*
KP6679 *pdfr-1(ok3425) III; bzIS17*
KP6680 *pdfr-1(ok3425) III; npr-1(ky13) X; bzIS17*
KP6681 *npr-1(ky13) X; bzIS17*
KP6699 *pdf-1(tm1996) III; npr-1(ky13) X; bzIS17*
KP6700 *pdf-1(tm1996) III; bzIS17*

Strains Containing Extrachromosomal Arrays

CX9396 *npr-1(ad609) X; kyEX1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed]* (gift from Cori Bargmann)
KP6053 *npr-1(ad609) X; nuEX1520[unc-30p::npr-1::gfp, myo-2p::NLS-mCherry]*
KP7144 *tax-4(p678) III; npr-1(ky13) X; nuEX1601[flp-21p::tax-4, vha-6p::mCherry]*
KP7141 *npr-1(ky13) X; nuIS472; nuEX1607[flp-21p::npr-1, myo-2p::NLS-mCherry]*
KP7143 *tax-4(p678) III; npr-1(ky13) X; nuIS472; nuEX1612[flp-21p::tax-4, myo-2p::NLS-mCherry]*
KP6819 *nuEX1560[unc-17p::rig-3(-GPI)::mCherry]*
KP6820 *npr-1(ky13) X; nuEX1560*
KP7053 *kyIS200; nuEX1610[sra-6p::pdf-1::venus, myo-2p::NLS-mCherry]*
KP6678 *pdf-1(tm1996) III; npr-1(ky13) X; nuEX1547[sra-9p::pdf-1::venus]*
KP6741 *pdf-1(tm1996) III; npr-1(ky13) X; nuEX1552[str-3p::pdf-1::venus, vha-6p::mCherry]*
KP6860 *nuEX1611[sra-9p::pdf-1::venus, myo-2p::NLS-mCherry]*
KP7146 *npr-1(ky13); nuEX1611*
KP6423 *pdfr-1(ok3425) III; npr-1(ky13) X; nuEX1526[mec-3p::pdfr-1b, myo-2p::NLS-mCherry]*
KP6594 *pdfr-1(ok3425) III; npr-1(ky13) X; nuEX1534[myo-3p::pdfr-1a, vha-6p::mCherry]*
KP6733 *pdfr-1(ok3425) III; npr-1(ky13) X; bzIS17; nuEX1526 [mec-3p::pdfr-1b, myo-2p::NLS-mCherry]*
KP6734 *pdfr-1(ok3425) III; npr-1(ky13) X; bzIS17; nuEX1534[myo-3p::pdfr-1a, vha-6p::mCherry]*
KP6736 *npr-1(ad609) X; bzIS17; kyEX1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed]*
KP6815 *pdfr-1(ok3425) III; nuEX1526[mec-3p::pdfr-1b, myo-2p::NLS-mCherry]*
KP6816 *pdfr-1(ok3425) III; nuEX1534[myo-3p::pdfr-1a, vha-6p::mCherry]*

Constructs

pdf-1 Expression Constructs: pdf-1p::pdf-1::YFP (KP#1861), sra-9p::pdf-1::YFP (KP#1923), str-3p::pdf-1::YFP (KP#1924), and sra-6p::pdf-1::YFP (KP#1925)

cDNAs corresponding to *pdf-1* and YFP (VENUS) containing a stop codon were each amplified by PCR and ligated into pPD49.26 (Addgene) containing the *pdf-1* (~5.4 kb 5′ regulatory sequence), *sra-9* (~3 kb 5′ regulatory sequence: ASK expression), *str-3* (~3 kb 5′ regulatory sequence: ASI expression), and *sra-6* (~3.8 kb 5′ regulatory sequence: ASH expression) promoters.

npr-1 Rescue Constructs: unc-30p::npr-1::GFP (KP#1857) and flp-21p::npr-1 (KP#1921)

npr-1 cDNA (215V) was amplified by PCR and ligated into expression vectors (pPD49.26) containing the *unc-30* promoter (~2.5 kb 5′ regulatory sequence) and GFP at the 3′ end of MCSII or the *flp-21* promoter (~4.1 kb 5′ regulatory sequence).

tax-4 Rescue Construct: flp-21p::tax-4 (KP#1922)

tax-4 cDNA was amplified by PCR and ligated into an expression vector (pPD49.26) containing the *flp-21* promoter (~4.1kb 5′ regulatory sequence).

pdfr-1 Rescue Constructs: mec-3p::pdfr-1b (KP#1863) and myo-3p::pdfr-1a (KP#1866)

pdfr-1 cDNAs were amplified by PCR and ligated into expression vectors (pPD49.26) containing the *mec-3* promoter (3.4 kb upstream of the start codon of the *mec-3* genomic region) or *myo-3* promoter (~2.4 kb 5′ regulatory sequence).

Transgenes and Germline Transformation

Transgenic strains were generated by microinjection of various plasmids with coinjection markers (*myo-2p::NLS-mCherry* (KP#1480) and *vha-6p::mcherry* (KP#1874)). Injection concentration was 40–50 ng/μl for all the expression constructs and 10 ng/μl for coinjection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/μl. Integration of transgenes was obtained by UV irradiation of strains carrying extrachromosomal arrays. All the integrants were outcrossed to wild-type strains (N2 Bristol) 10 times.

Lethargus Locomotion and Behavior Analysis

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. Locomotion was recorded at 2 Hz for 30–75 s. The 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. For long-term lethargus locomotion analysis (Figures S1A and S1B), a 1-min-long video was recorded every 20 min for each animal after the transfer to full-lawn OP50 bacterial plates, and motile

fraction was calculated for each time point. For the forced secretion of PDF-1 (Figures 4C and 4D), early L4 animals were transferred to NGM plates containing 50 μM capsaicin (with food) and treated with capsaicin for 6–7 hr. Duration of L4/A pumping quiescence was calculated by summating the time period from cessation to resumption of pharyngeal pumping. Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparison and the two-tailed Student's t test for pairwise comparison.

Adult Locomotion and Behavior Analysis

Locomotion of adult animals was analyzed with the same setup used for lethargus locomotion analysis, described above, except that well-fed adult animals were monitored within 5–10 min after the transfer to full-lawn OP50 bacterial plates. The pharyngeal pumping rate of adult animals was calculated by counting the number of pharyngeal muscle contractions for 10 s under the Leica MS5 routine stereomicroscope. Foraging behavior was analyzed as described (de Bono and Bargmann, 1998). Briefly, approximately 150 well-fed adult animals were placed on NGM plates seeded with 200 μl OP50 *E. 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 comparison and the two-tailed Student's t test for pairwise comparison.

RNAi Feeding Screen

A small-scale RNAi feeding screen was performed as described (Kamath et al., 2003). The screen was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b*) (Schmitz et al., 2007). Fifteen neuropeptide genes known to be expressed in the RMG circuit were selected for the screen (Li and Kim, 2008). After 5 days of RNAi treatment (two generations) at 20°C, 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 chi-square test.

Quantitative PCR

Total RNA was purified from synchronized animals in L4/A lethargus (determined by absence of pharyngeal pumping) and synchronized young adult animals (4–5 hr after L4/A lethargus) using standard protocol. Six biological replicates of wild-type (N2 Bristol) and *npr-1(ky13)* samples were collected on three different days. Two micrograms of total RNA was used to synthesize cDNA using RETROscript (Ambion). Real-time PCR was performed using iTaq SYBR Green Supermix with ROX (BioRad) and a 7500 Fast Real-Time PCR System (Applied Biosystems). Statistical significance was determined using the two-tailed Student's t test.

Fluorescence Microscopy and Image Analysis

Quantitative imaging of coelomocyte fluorescence was performed 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 BDM (Sigma). The anterior coelomocytes were imaged in L4, L4/A lethargus (determined by absence of pharyngeal pumping), young adult (0–2 eggs), and gravid 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). Statistical significance was determined using one-way ANOVA with Tukey test.

Calcium Imaging and Analysis

To image touch-evoked calcium transients in the ALM cell body, we used a transgenic line (*bzIs17*) that expresses the calcium-sensitive protein cameleon in touch neurons (using the *mec-4* promoter). Calcium imaging was performed on a Zeiss Axioskop 2 upright compound microscope equipped with a dual-view beam splitter and a Uniblitz shutter. Images were recorded at 10 Hz using an iXon EM camera (Andor Technology) and captured using IQ1.9 software (Andor Technology). Using Dermabond topical skin adhesive, individual worms were glued to pads composed of 2% agarose in extracellular saline (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 20 mM D-glucose, and 10 mM HEPES buffer [pH7.2]). Gentle-touch stimuli were delivered using a M-111.1DG micromanipulator. The micromanipulator was used to drive a pulled glass microcapillary with a 15-μm-diameter rounded tip against the side of the glued worm. The tip was positioned adjacent to the body wall and was driven forward to cause a 10 μm (adults, Figure 6) or 20 μm (L4/A lethargus, Figure 7) deflection of the cuticle. Optical and mechanical stimuli were synchronized by flashing a white LED on the sample a second before the stimulus was delivered. Analysis was done using a custom-written Matlab (Mathworks) program. A rectangular region of interest (ROI) was drawn surrounding the cell body and for every frame the ROI was shifted according to the new position of the center of mass. Fluorescence intensity, F, was computed as the difference between the sum of pixel intensities and the faintest 10% pixels (background) within the ROI. Statistical significance was determined using one-way ANOVA with Tukey test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two movies and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.04.002.

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