Cell

Serotonin and the Neuropeptide PDF Initiate and Extend Opposing Behavioral States in *C. elegans*

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http://dx.doi.org/10.1016/j.cell.2013.08.001

SUMMARY

Foraging animals have distinct exploration and exploitation behaviors that are organized into discrete behavioral states. Here, we characterize a neuromodulatory circuit that generates long-lasting roaming and dwelling states in *Caenorhabditis elegans*. We find that two opposing neuromodulators, serotonin and the neuropeptide pigment dispersing factor (PDF), each initiate and extend one behavioral state. Serotonin promotes dwelling states through the MOD-1 serotonin-gated chloride channel. The spontaneous activity of serotonergic neurons correlates with dwelling behavior, and optogenetic modulation of the critical MOD-1-expressing targets induces prolonged dwelling states. PDF promotes roaming states through a Gαs-coupled PDF receptor; optogenetic activation of cAMP production in PDF receptor-expressing cells induces prolonged roaming states. The neurons that produce and respond to each neuromodulator form a distributed circuit orthogonal to the classical wiring diagram, with several essential neurons that express each molecule. The slow temporal dynamics of this neuromodulatory circuit supplement fast motor circuits to organize long-lasting behavioral states.

INTRODUCTION

Animal behaviors are often organized into discrete, long-lasting behavioral states. For example, many foraging animals alternate between local feeding bouts and migrations between feeding sites, with each state lasting for minutes to hours (Owen-Smith et al., 2010). Local feeding and active migration differ in arousal levels, metabolic costs, risk of predation, and the potential to discover new resources, and therefore, this bistable structure represents an example of an exploration-exploitation axis. Transitions between behavioral states can be induced by external stimuli but also occur probabilistically in the absence of specific trigger stimuli, suggesting that they can be internally generated (Goulding et al., 2008; Martin et al., 1999). The molecular and circuit mechanisms that generate stable behavioral states and transitions between them are incompletely understood. As there are similarities between these easily observable locomotor states and emotional and cognitive states, it is possible that their underlying mechanisms have common elements.

The best-characterized behavioral states are those associated with sleep, waking, or arousal levels, each of which is regulated by biogenic amine and neuropeptide neuromodulators. In mammals, arousal and waking states are stimulated by the neuropeptide orexin/hypocretin, as well as serotonin, histamine, and norepinephrine, whereas the neuropeptides galanin and MCH promote sleep states (Saper et al., 2010). In *Drosophila*, the neuropeptide pigment dispersing factor (PDF), octopamine (an invertebrate amine related to norepinephrine), and dopamine promote waking states, whereas serotonin promotes sleep states (Sehgal and Mignot, 2011). In each system, the neurons producing biogenic amines and neuropeptides are heterogeneous, with diffuse projections to numerous brain regions, and the effects of the neuromodulators differ depending on the target cell and receptor being regulated. For example, $5HT_{2C}$ serotonin receptor knockout mice have decreased NREM sleep and normal REM sleep (Frank et al., 2002), whereas $5HT_7$ serotonin receptor knockouts have decreased REM sleep and normal NREM sleep (Hedlund et al., 2005). In *Drosophila*, dopamine can promote or suppress arousal in different contexts (Lebestky et al., 2009). These results support the importance of neuromodulators as behavioral regulators but indicate that arousal is defined not by a uniform neurochemical state but by a circuit state.

Neuromodulation is widespread in the nematode *C. elegans*, whose compact nervous system consists of only 302 uniquely defined neurons. In addition to classical neurotransmitters, the *C. elegans* nervous system contains more than 100 neuropeptides and the biogenic amine modulators serotonin, dopamine, tyramine, and octopamine (Chase and Koelle, 2007; Li and Kim, 2008). Neuromodulators have long been known to drive behaviors associated with different *C. elegans* feeding states. For example, serotonin and dopamine promote food-related

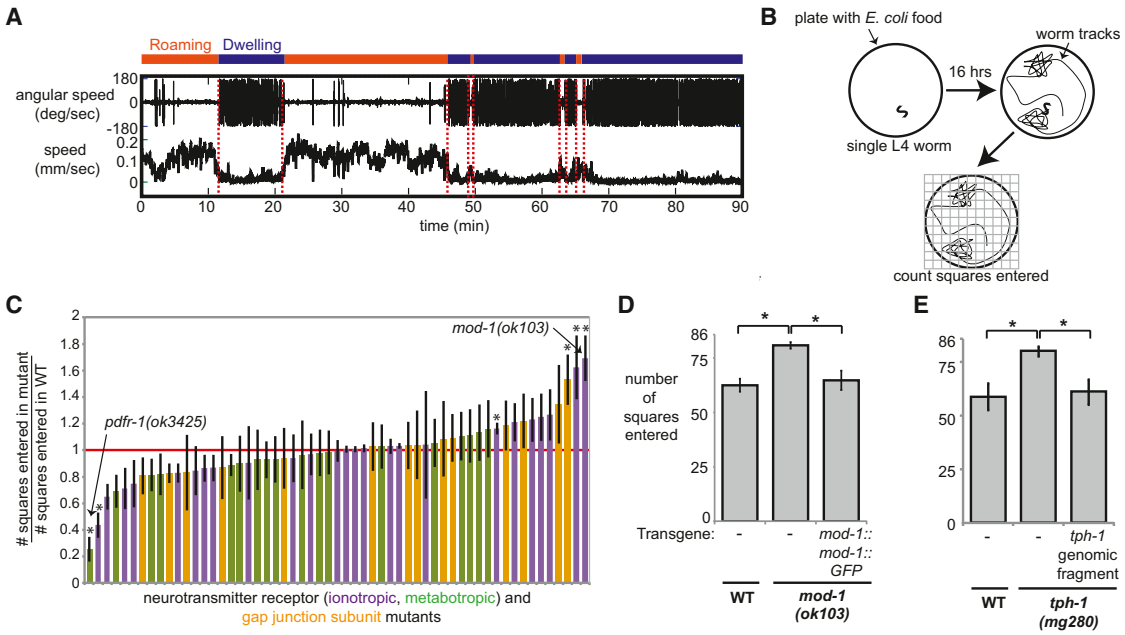

Figure 1. Serotonin Affects Exploration Behavior

(A) Locomotion of an adult wild-type animal on an *E. coli* lawn. Locomotion speed and angular speed (turning rate) differ in two distinct behavioral states, roaming and dwelling.

(B) Simplified assay for measuring exploration behavior based on movement across a 35 mm bacterial lawn. The grid has 86 squares. The exact number of squares entered can vary from day to day, so all genetic manipulations are compared to controls tested in parallel.

(C) Exploration behavior of 57 mutant strains normalized to wild-type controls. Asterisks indicate statistical significance at FDR < 0.05. For detailed results, see Table S1.

(D) Rescue of *mod-1* mutant phenotype by a *mod-1::mod-1::GFP* transgene.

(E) Rescue of *tph-1* mutant phenotype by a genomic *tph-1* transgene. For (D and E), asterisks indicate $p < 0.05$ by ANOVA and Bonferroni-Dunn post hoc test. Data are shown as means ± SEM. See also Figure S1 and Table S1.

behaviors such as eating, egg laying, and slow locomotion, whereas octopamine antagonizes these effects, mimicking the absence of food (Horvitz et al., 1982; Sawin et al., 2000). Food-related egg-laying and locomotion behaviors are also positively and negatively regulated by neuropeptides, including insulin-related and FMRFamide peptides (Li and Kim, 2008). Despite these strong effects on behavior, relatively little is known about the acute relationships between neuromodulatory signaling and neural circuit activity. Here, we examine the effects of neuromodulation on the spontaneous generation and maintenance of stable behavioral states in the presence of food.

Feeding *C. elegans* spontaneously switch between two discrete foraging states called roaming and dwelling (Ben Arous et al., 2009; Fujiwara et al., 2002). Roaming animals move quickly across a bacterial lawn and turn infrequently to explore the bacterial lawn, whereas dwelling animals move slowly and turn more frequently, remaining in a small area. Both states include common motor patterns such as forward locomotion, reversals, and turns, but these motor patterns appear in roaming- and dwelling-specific combinations that last several minutes and change through abrupt transitions (Figure 1A). The proportion of time spent roaming increases when food is limited or low in quality, suggesting that roaming behavior reflects an exploration-exploitation decision about the value of the current environment (Ben Arous et al., 2009; Shtonda and Avery, 2006). Accordingly, the relative occupancy of roaming and dwelling states is regulated partly by internal metabolic status and partly by environmental cues detected by specific sensory neurons. The cGMP-dependent protein kinase EGL-4 promotes dwelling (Fujiwara et al., 2002), a result of particular interest because its insect homolog, *foraging*, is a regulator of active and inactive foraging states (rover and sitter behaviors) in *Drosophila* and other insects (Osborne et al., 1997). The circuits that integrate these disparate cues are unknown.

To understand how long-lasting behavioral states emerge from the interactions between neural circuits and modulatory cues, it is necessary to identify the cells that produce the modulators, the cells that detect them and mediate their effects, and the acute and long-term effects of the modulators in each behavioral state. Here, we show that long-lasting roaming and dwelling behaviors emerge from neural circuit regulation by two opposing neuromodulators, serotonin and PDF.

RESULTS

Serotonergic Signaling Promotes Dwelling Behavior

To gain insight into circuit mechanisms important for roaming and dwelling, we examined 57 mutants lacking individual neurotransmitter receptors, neuropeptide receptors, and gap junction subunits in a simple exploration assay by observing the tracks

that individual animals left on a lawn of *E. coli* over a 16 hr period (Figure 1B). This behavior will be referred to as "exploration" to distinguish it from quantitative roaming and dwelling assays. On average, wild-type animals explored about 70% of the lawn during a 16 hr interval (Figure S1A available online). Over the same interval, known mutants with elevated dwelling behavior explored less than 20% of the lawn, and the *egl-4* mutant with elevated roaming behavior explored more than 90% of the lawn (Figure S1A), indicating that the exploration assay correlated with roaming and dwelling. At a false discovery rate (FDR) of 5%, six of 57 tested mutants displayed a change in this exploration assay without any obvious uncoordinated movement (Figure 1C and Table S1). Among four mutants with elevated exploration, two were deficient in feeding (*eat-2* and *inx-20*), supporting previous observations that decreased food intake promotes roaming behavior (Ben Arous et al., 2009), and a third mutant, *lgc-47*, had a small effect. The fourth mutant, *mod-1*, had elevated exploration but is known to feed on *E. coli* as efficiently as wild-type animals (Li et al., 2012), so it was examined further.

mod-1 encodes a serotonin-gated chloride channel (Ranganathan et al., 2000), one of five known serotonin receptors in *C. elegans*. Increased exploration in *mod-1* mutants was rescued by a *mod-1* complementary DNA (cDNA) expressed under the *mod-1* promoter, confirming the involvement of the gene (Figure 1D). To gain a more general view of serotonin signaling in exploratory behavior, we examined mutations affecting serotonin synthesis, reuptake, and other serotonin receptors. The rate-limiting enzyme for serotonin synthesis is encoded by *tph-1* (Sze et al., 2000). Like *mod-1* mutants, *tph-1* mutants displayed increased exploration, which was rescued by a *tph-1* genomic transgene (Figure 1E). *mod-1 tph-1* double mutants resembled the stronger *tph-1* single mutant, suggesting that these genes act in a common pathway (Figure S1B). Conversely, mutants with enhanced serotonergic function due to a mutation in the serotonin reuptake transporter MOD-5 displayed reduced exploration (Figure S1C). None of the four other known serotonin receptors in the *C. elegans* genome (*ser-1*,*4*,*5*, and *7*) had an effect (Table S1). This result distinguishes dwelling from general slowing of locomotion because exogenous serotonin can slow *C. elegans* locomotion via either *mod-1* or *ser-4* receptors (Gürel et al., 2012). The congruent effects of *tph-1* and *mod-1*—and the opposite effect of *mod-5*—indicate that endogenous serotonergic signaling through the MOD-1 receptor suppresses exploration.

To define the precise effects of *mod-1* and *tph-1*, we used quantitative locomotion assays in which animals were filmed during 90 min of movement on large (600 cm^2) homogeneous bacterial lawns and then analyzed for the speed and turning parameters typical of roaming and dwelling (Ben Arous et al., 2009). Movement patterns scored over 10 s intervals fell into two classes: a high-speed/low-turning class (roaming) and a low-speed/high-turning class (dwelling; Figure S2A), each of which typically persisted over several minutes (Figure S2C). To segment behaviors into roaming and dwelling states in a standardized fashion, we employed a hidden Markov model (HMM), which proposes that hidden roaming and dwelling states generate the observed behavior. A two-state HMM was fit to tracking data from wild-type animals (n = 350) and validated by simulations (Figure S2B and Extended Experimental Procedures). When applied to the locomotion data from wild-type animals, the model yielded a distribution of dwelling state durations that fit a single exponential (τ = 482 s), suggesting that transitions from dwelling to roaming states follow Poisson statistics (Figure 2A). Roaming state durations fit a double-exponential distribution, indicating that there might be two populations of roaming states that differ in their durations (τ = 75 s and 520 s) (Figure 2B).

Using these quantitative methods, we found that the proportion of time spent roaming was higher in *mod-1* and *tph-1* animals than in wild-type, in agreement with the exploration assay (Figures 2C1 and 2D1). A previous study reported enhanced roaming of *mod-1* but enhanced dwelling of *tph-1* (Ben Arous et al., 2009); the discrepancy with our results probably resulted from a second linked mutation in the original *tph-1* strain (Omura, 2008). An increased proportion of time spent roaming in *mod-1* and *tph-1* mutants could be caused by different durations of dwelling, roaming, or both states. Analyzing each class of event separately showed that both were affected: dwelling durations were decreased and roaming durations were increased in *mod-1* and *tph-1* mutants (Figures 2C and 2D). Close inspection indicated that *mod-1* mutants had two classes of dwelling events: a near-normal class (31%, τ = 517 s) and a severely shortened class that was only about 1 min long (69%, τ = 63 s) (Figures S2D–S2G). Thus, serotonergic signaling through MOD-1 affects both roaming and dwelling—it extends dwelling states and truncates roaming states.

Identification of the Serotonergic Neural Circuit

To define the neural circuit by which serotonin controls exploratory behavior, we first identified the essential serotonin-producing neurons. To match serotonin levels to the normal range for each neuron, a single floxed copy of the *tph-1* gene (including promoter, exons, and introns) was inserted into a defined location on chromosome IV using the mosSCI technique (Figure 3A) (Frøkjaer-Jensen et al., 2008) and was shown to rescue the *tph-1* mutant phenotype (Figure 3B). Cell-specific transgenes expressing Cre recombinase in ADF, NSM, HSN, or ASG, as confirmed with a Cre reporter strain, were then used to eliminate serotonin production by individual neurons. Expression of Cre in either NSM or HSN (or both; Figure S3A), but not in ADF or ASG, suppressed *tph-1* rescue (Figure 3B) but had no effect in a wild-type background (Figure S3B). These results suggest that serotonin production by both NSM and HSN is required for normal adult exploration behavior.

NSM is a serotonergic/glutamatergic neuron pair in the pharynx whose axons have abundant secretory vesicles near the nerve ring, the major *C. elegans* neuropil. HSN is a hermaphrodite-specific serotonergic/cholinergic motor neuron pair in the midbody that controls egg laying; its identification as a regulator of exploration was unexpected, although its axon also enters the nerve ring. To confirm the involvement of HSN, we examined *egl-1(n986 dm)* mutants in which HSN neurons die early in development (Conradt and Horvitz, 1999). *egl-1* animals had elevated exploration, supporting the conclusion that *tph-1* in HSN suppresses exploration (Figure S3C).

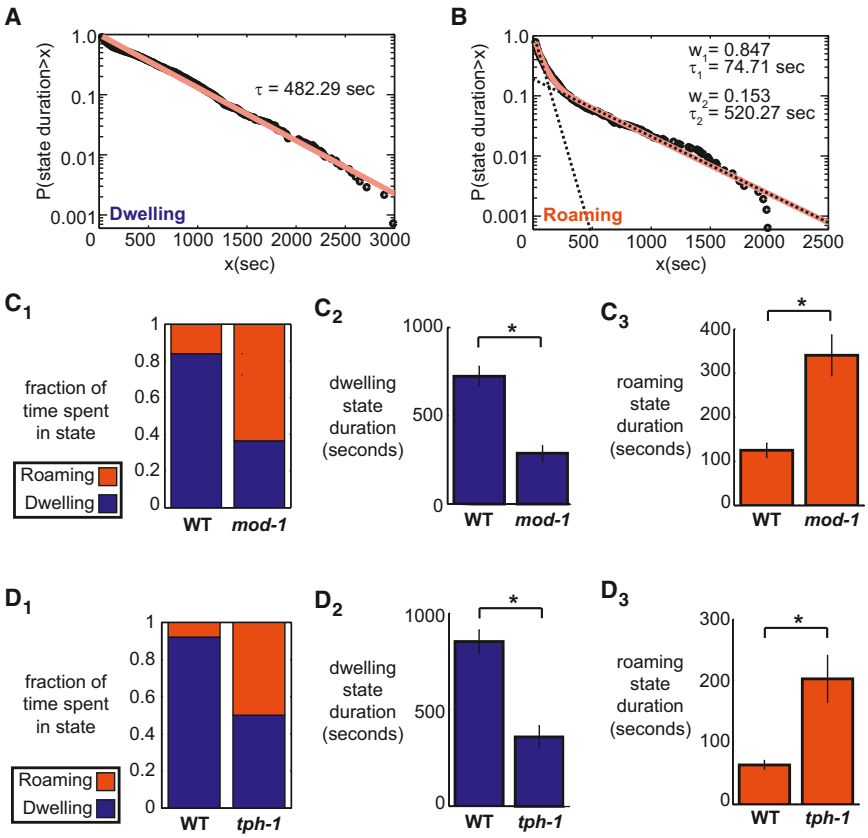

Figure 2. Behavioral State Defects in Serotonergic Signaling Mutants

(A) Complementary cumulative distribution function (ccdf) for dwelling-state durations in wild-type animals (dots) fit to a single exponential (pink line).

(B) Ccdf for roaming state durations in wild-type animals (dots) fit to a double exponential (pink line). The two exponentials are individually displayed as dashed black lines, and w_1/w_2 are the weights of each exponential, i.e., 85% of roaming states have a mean lifetime of 75 s.

(C and D) Behaviors of wild-type and mutant animals. (C_1 and D_1) Fraction of time spent in roaming and dwelling states. (C_2 and D_2) Dwelling state durations and (C_3 and D_3) roaming state durations, expressed as means of the individual animal mean ± SEM, not as event distributions as in (A and B). Asterisks indicate $p < 0.01$, t test.

See also Figure S2.

Dynamic Changes in Serotonergic Signaling Underlie Behavioral Transitions

The genetic requirement for serotonin is consistent with two general models: the serotonergic circuit (Figure 3G) could be tonically active in the presence of bacteria, providing permissive input onto a bistable circuit for roaming and dwelling, or dynamic changes in neurons that produce and detect serotonin might directly underlie bistability during constant exposure to bacteria. To distinguish between these possibilities, we monitored calcium levels as a proxy for neuronal activity in NSM and AIY as examples of serotonin-producing and -detecting neurons. Calcium was monitored in freely moving animals expressing the genetically encoded calcium indicator GCaMP5 (Akerboom et al., 2012) using a custom-designed imaging system (Figure 4A; D.R.A., J.L., and C.I.B., unpublished data). Although both dwelling and roaming behavior were observed using this imaging system, the constraints of the small viewing field truncated roaming states, which we will call "runs" rather than roams to respect this difference.

Locomotion parameters and NSM calcium signals were obtained for 20 wild-type animals over 384 min; a representative trace is shown in Figure 4B. Across the data set, NSM calcium levels were inversely correlated with locomotion speed (Figure S4A). In individual animals, NSM showed sporadic, sustained (~60 s) calcium peaks that were not associated with any apparent external event such as an encounter with the lawn edge (Figure 4B). To understand the significance of these calcium peaks, event-triggered averages were used to align all traces based on NSM peaks (Figure 4C) or behavioral transitions (Figure 4D), allowing other parameters to follow passively. NSM calcium peaks correlated with a rapid decrease in locomotion speed and termination of forward runs (Figure 4C; *NSM::GFP* controls are in Figures S4B and S4C). Conversely, NSM calcium levels reached a local minimum when runs were initiated, falling

Neurons that respond to serotonin were sought based on the expression of *mod-1*. A *mod-1* promoter fragment that rescued the *mod-1* exploration defect (Figure 1D) drove expression in AIY, RME, RID, RIF, ASI, DD1-6, and PVN neurons. We used an intersectional promoter strategy to rescue *mod-1* in subsets of these neurons (Figure 3C). The floxed *mod-1* cDNA was placed in an inverted, inactive orientation under one promoter (either *ser-2b* or *odr-2b*) and was activated by Cre expression under a second promoter (*mod-1*), which inverted the cDNA to allow functional expression in overlapping cells. This design tested sufficiency of *mod-1* expression rather than necessity as for *tph-1*, a choice made primarily for practical reasons. The enhanced exploration in *mod-1* mutants was rescued by restoring *mod-1* expression in AIY, ASI, and RID, but not by restoring expression in ASI alone or in AIY and RID (Figure 3D and data not shown). *mod-1* expression in RIF was also sufficient for rescue (Figure 3E).

To confirm the importance of *mod-1*-expressing cells in exploration, we killed neurons individually using a laser microbeam. Ablation of RID or PVN had no effect, but ablation of AIY, ASI, or RIF reduced exploration (Figure 3F). Thus AIY, ASI, and RIF neurons promote exploration, and as MOD-1 is an inhibitory serotonin receptor, these three neurons might be hyperactive in *mod-1* mutants. Together, these results suggest that serotonin produced by NSM and HSN inhibits AIY, ASI, and RIF through the MOD-1 serotonin-gated chloride channel to suppress exploration (Figure 3G).

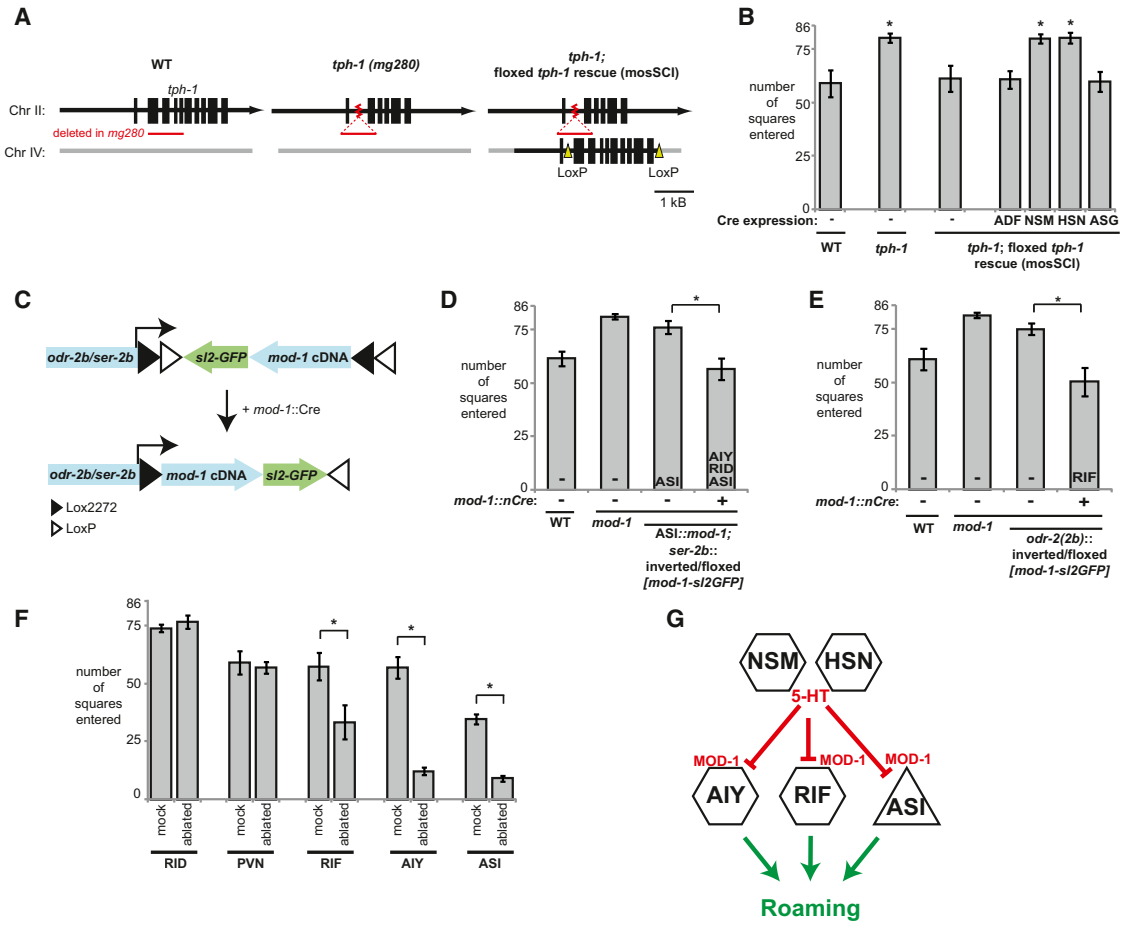

Figure 3. A Distributed Serotonergic Circuit Controls Exploration Behavior

(A and B) Cell-specific deletion of *tph-1* using a Cre/Lox strategy. (A) Schematic depicting genotypes. (B) Exploration behavior. Asterisks indicate $p < 0.01$ (versus wild-type) by ANOVA with Dunnett test.

(C–E) Intersectional cell-specific rescue of *mod-1* using an inverted Cre-Lox strategy. (C) Schematic depicting transgenes. (D) Rescue of *mod-1* by expression in AIY, RID, and ASI. (E) Rescue of *mod-1* by expression in RIF. For (D and E), asterisks indicate $p < 0.05$ by ANOVA with Bonferroni-Dunn post hoc test.

(F) Laser ablations of individual *mod-1*-expressing neurons. RID, PVN, and RIF were ablated in *mod-1* mutants; AIY and ASI were ablated in wild-type animals. Asterisks indicate $p < 0.05$, t test.

(G) Serotonin promotes dwelling by inhibiting MOD-1-expressing neurons that promote roaming.

All data are shown as means ± SEM. See also Figure S3.

for at least 1 min before a run (Figure 4D). These results indicate that NSM calcium peaks correlate with acute decreases in speed and suppression of forward runs.

In *mod-1* mutants, NSM neurons had normal calcium peaks, but these peaks were less strongly associated with locomotion speed and forward run probability (Figure 4E). Forward runs were initiated without a preceding reduction in NSM calcium levels, although calcium levels were still reduced during runs (Figure 4F). These results indicate that *mod-1* helps couple NSM calcium levels to behavior.

Calcium levels in the MOD-1-expressing AIY neuron were reciprocally correlated with behaviors compared to NSM. AIY calcium levels were consistently highest during forward runs (Figures S4D and S4F), with gradual increases preceding forward run initiation (Figure 4G; *AIY::GFP* controls in Figures S4D and S4E). Together, these results indicate that a serotonin-producing neuron (NSM) and a serotonin-detecting neuron (AIY) both show dynamic changes in calcium levels correlated with behavioral states and transitions over a minutes-long timescale.

Optogenetic Manipulations of the Serotonergic Circuit

Serotonin mutants have long roaming and short dwelling states (Figure 2). To ask whether serotonin signaling directly drives roaming animals into the dwelling state, we performed optogenetic experiments to manipulate the serotonergic circuit in roaming animals. To depolarize serotonergic neurons, we used channelrhodopsin-2*(ChR2-C128S), an increased sensitivity variant that is activated by blue light at levels that do not induce endogenous behavioral responses in *C. elegans* (Berndt et al., 2009; Schultheis et al., 2011). 1 min of ChR2-mediated depolarization of the serotonergic neurons (Figures 5A and S5A) caused

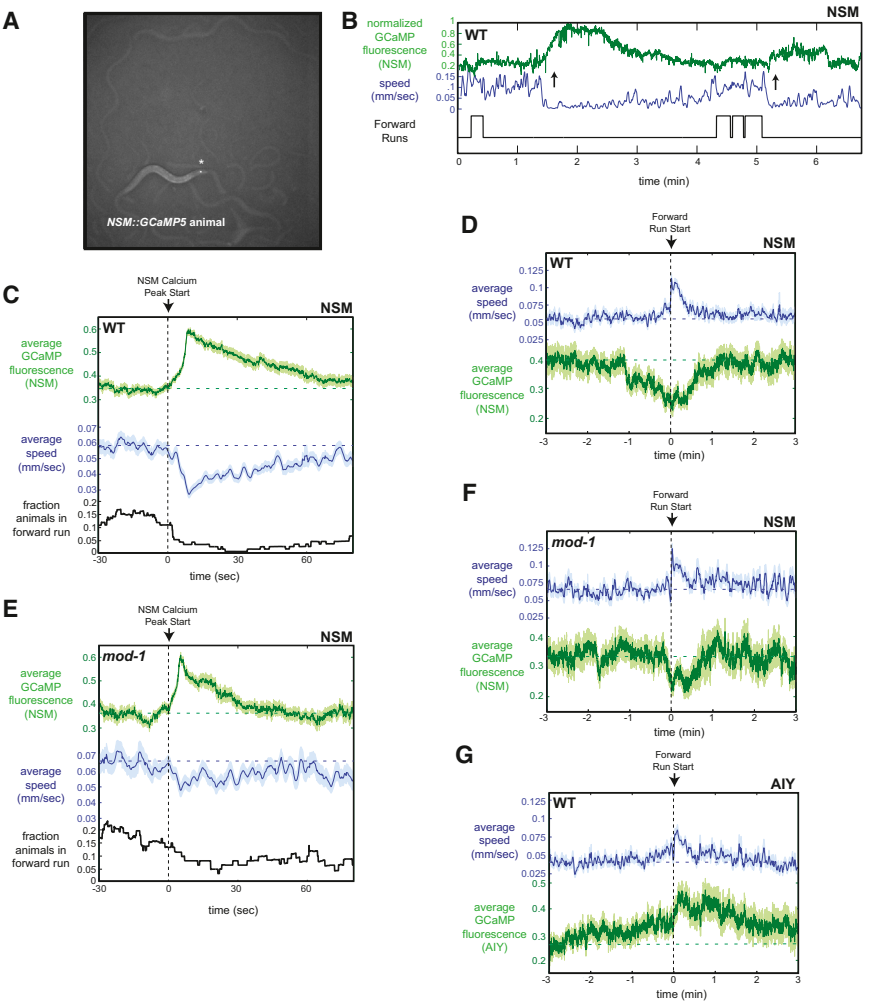

Figure 4. Changes in NSM and AIY Calcium Levels Correlate with Behavioral Transitions

(A) Representative image from a video recording of a freely moving *NSM::GCaMP5* transgenic animal on a bacterial lawn. Asterisk indicates the position of the NSM neuron; gut autofluorescence is also visible.

(B) NSM calcium imaging in a freely moving animal as in (A). Arrows mark calcium peaks (see Extended Experimental Procedures for identification criteria). Roaming states are abbreviated in the calcium-imaging device due to the small viewing field and bacterial lawn; nevertheless, we observe clusters of forward runs that are related to roaming states.

(C–F) Averaged NSM calcium levels, speeds, and forward runs in wild-type and *mod-1* animals. (C and E) Event-triggered average aligning NSM calcium peaks with locomotion speed and forward runs in wild-type animals (C, n = 112; $p < 0.001$ for calcium levels and speed, before versus during peak, paired t test) and *mod-1* mutants (E, n = 61; $p < 0.001$ for speed during calcium peak in wild-type versus *mod-1*, t test on values normalized to pre-event baseline; $p < 0.001$ for fraction of animals in forward run, wild-type versus *mod-1*, chi-square test). (D and F) Event-triggered average aligning forward runs with NSM calcium signal in wild-type animals (D, n = 51; $p < 0.01$ for NSM calcium during pre-event baseline versus forward run or −60 to −20 s, paired t test) and *mod-1* mutants (F, n = 32; $p < 0.01$ for NSM calcium during pre-event baseline versus forward run, paired t test, no significant difference at −60 to −20 s).

(G) Event-triggered average aligning forward runs with AIY calcium signal in wild-type animals (n = 27; $p < 0.01$ for AIY calcium during pre-event baseline versus forward run, paired t test). For (C–G), horizontal dashed lines indicate pre-event baseline calcium signals and speed.

All data are shown as means ± SEM. See also Figure S4.

70% of roaming animals to transition into dwelling states (versus 19% of controls). Activation of NSM alone had a similar but weaker effect (Figure S5B). The induced dwelling states continued after ChR2-C128S inactivation by green light, with a long duration similar to endogenous dwelling states (Figure S5C). *tph-1* and *mod-1* mutants were less responsive to ChR2 (Figures S5D and S5E), supporting a role for serotonin in dwelling. In wild-type animals, hyperpolarizing serotonergic neurons with the green-light-activated proton pump archaerhodopsin-3 (ARCH) (Chow et al., 2010) caused 24% of dwelling animals to initiate roaming states (versus 3% of controls); these roaming states ended when the green light was extinguished (Figure 5B). Thus, acute activation of serotonergic neurons in roaming animals increases the probability of roaming-to-dwelling transitions, and acute inhibition of serotonergic signaling in dwelling animals transiently increases roaming behavior.

To mimic serotonin's effect on the *mod-1*-expressing neurons, we hyperpolarized them with ARCH. The majority of roaming animals rapidly entered a dwelling state that outlasted the light stimulus (Figure 5C). Conversely, ChR2-mediated activation of the *mod-1*-expressing neurons (Figures 5D and S5F) or a subset of these neurons (RIF and AIY; Figure S5G) caused animals that were dwelling to enter roaming states that were similar in duration to endogenously generated roaming states (Figure S5H). The reciprocal effects of ARCH and ChR2 on roaming and dwelling transitions demonstrate a key role for the *mod-1*-expressing neurons in these behavioral states.

mod-1::ARCH also prolonged dwelling states. 2 min of light exposure caused existing dwelling states to extend 8 min longer than control dwelling states (Figure 5E), indicating that inhibition of *mod-1*-expressing neurons maintains dwelling states as well as inducing them. Serotonin contributes to these long-lasting states, as dwelling states induced by *mod-1::ARCH* activation in *mod-1* or *tph-1* mutants were shorter than those induced in a wild-type background (Figures S5C, S5I, and S5J). Together, these experiments indicate that serotonergic signaling can initiate and maintain dwelling states.

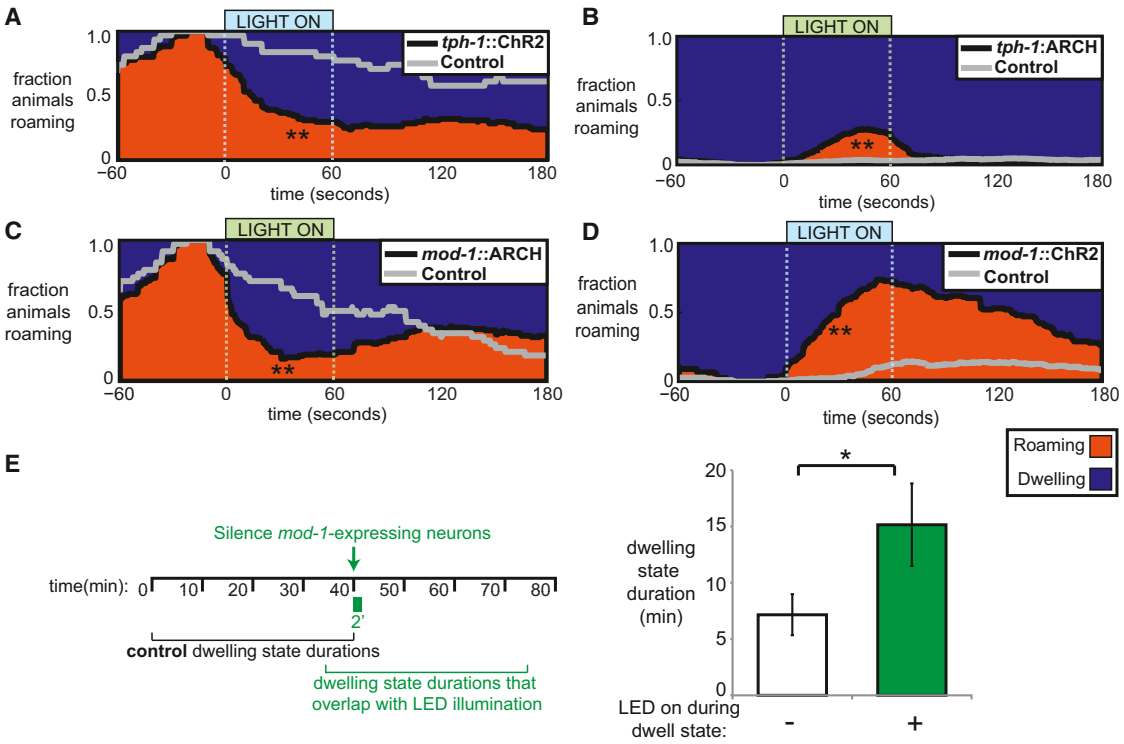

Figure 5. Optogenetic Manipulations of a Serotonergic Neural Circuit

(A) ChR2-mediated activation of serotonergic neurons in animals that were roaming (red) prior to LED illumination.

(B) ARCH-mediated silencing of serotonergic neurons in animals that were dwelling (blue) prior to LED illumination.

(C) ARCH-mediated silencing of *mod-1*-expressing neurons in animals that were roaming prior to LED illumination.

(D) ChR2-mediated activation of *mod-1*-expressing neurons in animals that were dwelling prior to LED illumination. For (A–D), asterisks indicate $p < 0.001$ compared to control strain, chi-square test, and controls (gray lines) were identically treated wild-type animals. Blue light delivery was followed by 60 s of green light delivery to inactivate ChR2(C128S).

(E) Long-lasting effects of *mod-1::ARCH* activation. Left: experimental design. Right: durations of control dwelling states and dwelling states that overlapped with ARCH activation. Data are shown as medians ± 95% confidence intervals. Asterisk indicates $p < 0.05$, Wilcoxon rank-sum test.

See also Figure S5.

PDF Signaling Promotes Roaming Behavior

Among the candidates tested in the initial exploration screen, effects reciprocal to those of the serotonin pathway were observed in mutants with disrupted PDF neuropeptide signaling (Figure 1C). *C. elegans* has two genes encoding PDF neuropeptides and one gene encoding a PDF receptor; defects in PDF signaling cause uncharacterized locomotion defects in hermaphrodites and eliminate mate search behaviors in males (Barrios et al., 2012; Janssen et al., 2008). We found that *pdf-1* mutants, *pdf-1; pdf-2* double mutants, and mutants for the one known *C. elegans* PDF receptor, *pdfr-1*, had greatly reduced exploration behavior (Figure 6A) but had near-normal sinusoidal locomotion and responses to touch (Figure S6A and Movies S1 and S2). Quantitative analysis of *pdfr-1* and *pdf-1; pdf-2* mutants demonstrated that both strains had prolonged dwelling states, shortened roaming states, and reduced speed during roaming (Figures 6B, 6C, and S6B). The truncated roaming states in *pdfr-1* mutants were reasonably well fit by a single exponential that resembled the shorter of the two wild-type roaming states (τ = 101 s, Figures S6C and S6D).

The neural circuit for PDF neuropeptide signaling was analyzed by neuron-specific *pdf-1* depletion using a floxed *pdf-1* gene and Cre recombination (Figure 6D). A *pdf-1* cDNA under its own promoter rescued roaming and was expressed in AVB, SIAD, SIAV, PVP, and AIM neurons (Figures 6E, S6E, and S6F). Cre expression in AVB, PVP, and SIAV neurons eliminated rescue (Figure 6E), and expression in subsets of these neurons had intermediate effects, identifying AVB, PVP, and SIAV as potential PDF-1 sources for roaming behavior.

The *pdfr-1* gene is predicted to have two alternative promoters (Figure S6G). Expression of *pdfr-1* from the distal promoter fully rescued the *pdfr-1* phenotype, as did a genomic fragment spanning both promoters; a genomic fragment with the proximal promoter did not rescue (Figures S6H and S6I). The distal promoter served as a starting point to identify relevant *pdfr-1*-expressing neurons using an inverted, floxed *pdfr-1* cDNA and intersectional promoters (Figure 6F). Partial rescue of roaming was observed upon *pdfr-1* expression in AIY, RIM, and RIA neurons and complete rescue with broader neuronal expression (Figures 6G and 6H). These experiments suggest that *pdfr-1*

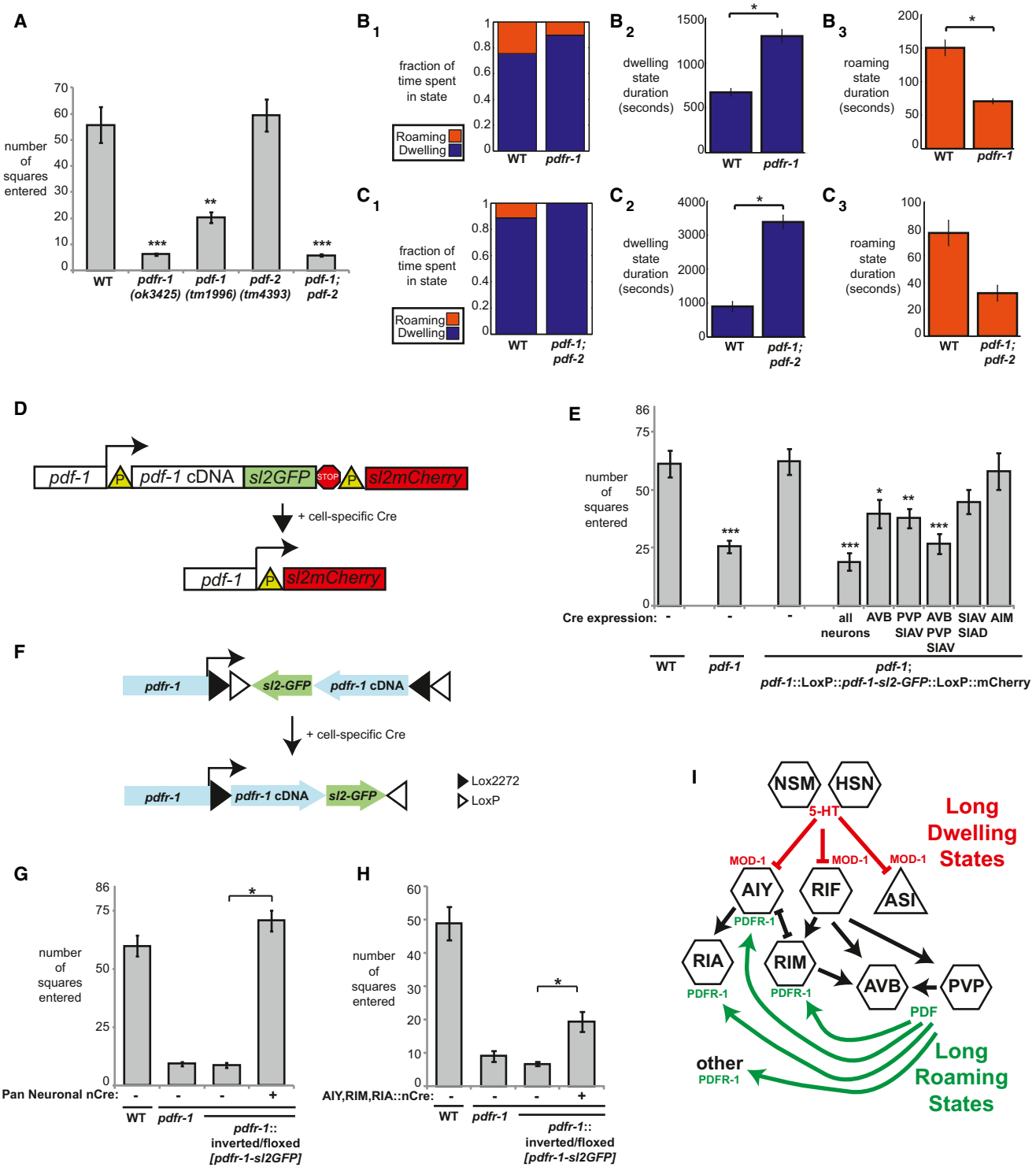

Figure 6. PDF Signaling Controls Exploration Behavior

(A) Exploration behavior of PDF signaling mutants. **p < 0.01 and ***p < 0.001 by ANOVA and Bonferroni-Dunn post hoc test.

(B and C) Fraction of time spent in roaming and dwelling states and dwelling and roaming state durations in (B) *pdfr-1* and (C) *pdf-1; pdf-2* mutants, shown as in Figures 2C and 2D. Asterisks indicate p < 0.01, t test.

(D and E) Cell-specific deletion of *pdf-1* using a Cre/Lox strategy. (D) Schematic depicting transgenes. (E) Exploration behavior. *p < 0.05, **p < 0.01, and ***p < 0.001 by ANOVA with Dunnett test.

(legend continued on next page)

expression in AIY, RIM, RIA, and other neurons promotes roaming behavior.

Acute cAMP Signaling in PDFR-1-Expressing Neurons Triggers Roaming

PDFR-1, a secretin-receptor family G-protein-coupled receptor (GPCR), stimulates Gαs to increase cyclic AMP (cAMP) levels when expressed in heterologous cells (Janssen et al., 2008), like the *Drosophila* PDF receptor. To ask whether cAMP mimics endogenous PDFR-1 signaling, we expressed a constitutively active version of the *C. elegans* adenylyl cyclase, ACY-1(P260S) (Saifee et al., 2011), in PDFR-1-expressing neurons. This transgene caused a dramatic increase in roaming behavior, prolonging roaming states and truncating dwelling states (Figure 7A). Expression restricted to the AIY, RIM, and RIA neurons also increased exploration (Figure S7A). *pdfr-1::acy-1(P260S)* rescued exploration in a *pdfr-1* mutant (Figure 7B), which is consistent with the possibility that the adenylyl cyclase acts downstream of *pdfr-1*.

As an optogenetic approach to mimic acute activation of PDFR-1, we expressed a blue-light-activated adenylyl cyclase, BlaC, under the distal *pdfr-1* promoter (Figure 7C) (Ryu et al., 2010). 1 min of blue light illumination caused 77% of dwelling *pdfr-1::BlaC* animals to switch to roaming states (versus 11% of controls) (Figure 7D), which lasted at least as long as endogenous roaming states (Figure S7B). This behavioral effect was abolished by a point mutation (D265K) that inactivates the adenylyl cyclase activity of BlaC (Figure 7D) (Ryu et al., 2010).

Parallel, Antagonistic Functions of Serotonin and PDF

PDF prolongs roaming and shortens dwelling states, whereas serotonin has reciprocal effects. To ask how these systems interact, we generated *mod-1; pdfr-1* double mutants and compared their locomotion to wild-type, *mod-1*, and *pdfr-1* mutants. *mod-1; pdfr-1* double mutants had short dwelling states that resembled *mod-1* single mutants and had short roaming states that resembled *pdfr-1* single mutants (Figure 7E). These results indicate that the prolonged roaming states observed in *mod-1* mutants require *pdfr-1*, and the prolonged dwelling states observed in *pdfr-1* mutants require *mod-1* (Figure S7C); in animals lacking both modulators, both dwelling and roaming states are short.

To further characterize the interaction between these two circuits, we examined the effects of optogenetic manipulations in *mod-1; pdfr-1* double mutants. Imitating PDF signaling with *pdfr-1::BlaC* initiated roaming states in double mutants, and imitating serotonin signaling with *mod-1::ARCH* initiated dwelling states (Figures 7F and 7G). Thus, each modulatory circuit can independently regulate the initiation of its corresponding foraging state.

DISCUSSION

Distributed Circuits that Signal through Serotonin and PDF

Our results show that serotonergic signaling through *mod-1* initiates and extends dwelling states, whereas PDF signaling through *pdfr-1* initiates and extends roaming states.

What does a complete neuromodulatory circuit look like in its organization and its relationship to fast circuits? Despite the compact size of the *C. elegans* nervous system, the serotonin and PDF that regulate roaming and dwelling each have several important sources, and their receptors each act in several target neurons. These modulatory circuits include sensory neurons, interneurons, and motor neurons, but their organization does not follow the dominant sensory-to-motor hierarchy of the classical synaptic wiring diagram (Figure 6I). The serotonin sources, NSM and HSN, are both motor neurons (although NSM may have sensory functions as well), and the PDF sources are interneurons; the targets include sensory neurons (ASI) and multiple interneurons. The serotonin and PDF circuits are mostly nonoverlapping, intersecting only at the AIY neurons. Moreover, although serotonin- and PDF-expressing neurons have chemical and electrical synapses in the *C. elegans* wiring diagram (black arrows in Figure 6I), these synapses do not overlap with the neuromodulatory connections inferred from our genetic mapping experiments (red and green arrows in Figure 6I). Thus, the neuromodulatory circuit for long-lasting behavioral states is essentially orthogonal to the synaptic connectivity diagram.

Extrasynaptic function of neuromodulators is well established in *C. elegans* and other animals (Chase and Koelle, 2007), but this need not imply that serotonin and PDF act as systemic hormones. All of the relevant neurons in this neuromodulatory circuit have processes in or near the *C. elegans* nerve ring, suggesting that diffusion over ~100–200 μm would be sufficient for their communication. The graded rescue of each neuromodulator by expression in specific neurons or groups of neurons suggests that both the quantity and the source of neuromodulators contribute to their function.

The circuits that drive short roaming and dwelling states in the absence of serotonin and PDF remain to be defined, as do the neurons required for the individual motor patterns of roaming and dwelling. Importantly, each neuron in the roaming and dwelling circuit has other behavioral functions, which will provide challenges for mapping the circuits. For example, the PDF-producing AVB neuron is essential for coordinated forward locomotion,

(F–H) Intersectional cell-specific rescue of *pdfr-1* using an inverted Cre-Lox strategy. (F) Schematic depicting transgenes. (G) Rescue of *pdfr-1* by expression in all *pdfr-1*-expressing neurons. (H) Partial rescue of *pdfr-1* by expression in AIY, RIM, and RIA. For (G and H), asterisks indicate $p < 0.01$ by ANOVA and Bonferroni-Dunn post hoc test.

All data are shown as means ± SEM.

(I) Neural circuit for exploration behavior. Synapses and gap junctions in the *C. elegans* wiring diagram are in black; neuromodulatory connections defined here are in red (serotonin) and green (PDF). NSM neurons are implicated in feeding, and HSN neurons are implicated in egg laying. AIY and RIM neurons regulate reversal frequencies. ASI neurons are sensory neurons (triangles) that sense food, pheromones, and peptide cues to regulate dauer larva development. RIA interneurons regulate head curving during locomotion. AVB interneurons are forward command neurons in the motor circuit. PVP, SIAV (not diagrammed here), and RIF interneurons do not have other known functions.

See also Figure S6.

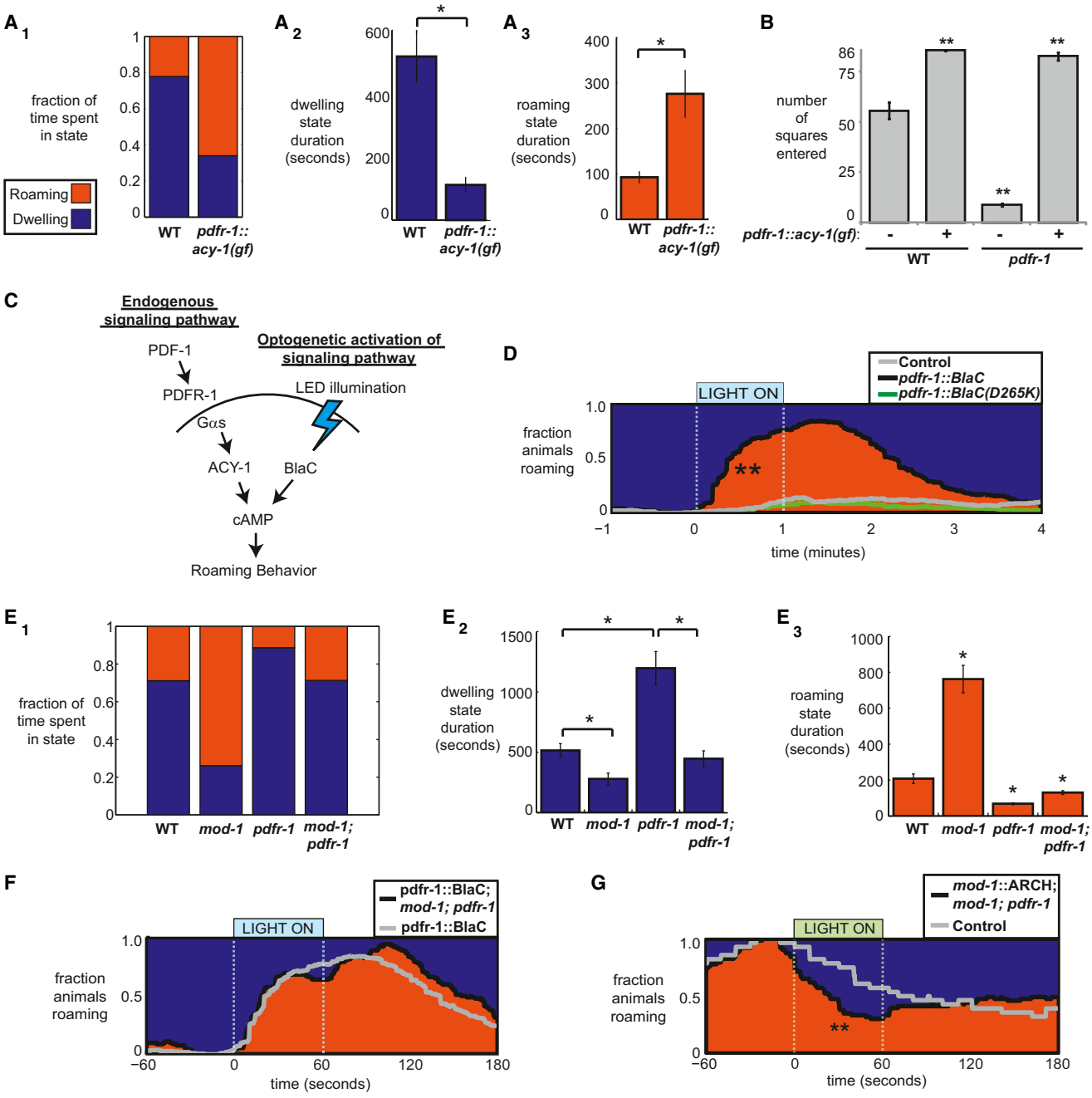

Figure 7. *pdfr-1* Acts through cAMP Signaling and Relationship of Serotonin and PDF Signaling Pathways

(A) Roaming and dwelling states in *pdfr-1::acy-1(P260Sgf)* animals, shown as in Figure 2C. (A_1) Fraction of time spent in roaming and dwelling states. (A_2) Dwelling state durations. (A_3) Roaming state durations. Asterisks indicate $p < 0.01$, t test.

(B) Exploration behavior of *pdfr-1::acy-1(P260Sgf)* animals. Asterisks indicate $p < 0.01$, ANOVA and Bonferroni-Dunn post hoc test (versus wild-type).

(C) Optogenetic strategy for mimicking acute PDFR-1 activation.

(D) BlaC activation in *pdfr-1*-expressing neurons in animals that were dwelling prior to LED illumination. Controls (gray line) were identically treated wild-type animals. Asterisks indicate $p < 0.05$, chi-square test.

(E) Roaming and dwelling in single and double mutants. (E_1) Fraction of time spent in roaming and dwelling states. (E_2) Dwelling state durations. (E_3) Roaming state durations. Asterisks indicate $p < 0.05$, ANOVA and Bonferroni-Dunn post hoc test (for E_3, each genotype compared to wild-type).

(F) *pdfr-1::BlaC* activation induces roaming behavior in *mod-1; pdfr-1* double mutants that were dwelling at the time of LED illumination.

(G) *mod-1::ARCH* activation induces dwelling behavior in *mod-1; pdfr-1* double mutants that were roaming at the time of LED illumination. Controls (gray lines) were matched mutant animals with no LED illumination. Asterisks indicate $p < 0.01$, chi-square test.

All data are shown as means ± SEM. See also Figure S7.

which it controls through chemical and electrical synapses onto motor neurons (Chalfie et al., 1985). Thus, AVB is required for coordinated forward movement at rapid timescales and releases PDF-1 peptides to regulate locomotion over longer durations. Because neurons like AVB are multifunctional, precise manipulation of neuronal signaling molecules may be needed to disentangle the activities of neurons, synapses, and modulators on behavior.

Multifunctionality is also a property of the PDF and serotonin neuromodulators, which each affect a variety of behaviors by mobilizing different sets of neurons. For example, in males, PDF-1 produced by AIM neurons signals to the PDFR-1-expressing neurons URY, PQR, and PHA to stimulate mate search (Barrios et al., 2012). Although these neurons are all present in the hermaphrodite, they appear unimportant in roaming and dwelling behavior. Similarly, serotonin regulates feeding and egg laying using receptors and neurons distinct from those defined here (Gürel et al., 2012; Li et al., 2012). In summary, neither neurons, neuromodulators, nor behaviors are subsets of one another—each represents a separate functional organization within the nervous system.

Neuromodulators Define Long-Lasting Behavioral States

Dwelling and roaming are persistent behaviors that last for several minutes. The endogenous calcium signals in serotonergic NSM neurons are long lasting, but not identical to the behavioral states—NSM calcium transients last about 1 min but predict dwelling states for several minutes thereafter. Either optogenetic excitation of NSM or optogenetic inhibition of its MOD-1-expressing target neurons with ARCH was sufficient for persistent dwelling states. As ARCH should have a direct hyperpolarizing effect on the MOD-1-expressing neurons, a persistent circuit state for dwelling may be induced by transient neuronal inhibition. The failure of *mod-1::ARCH* to induce long-lasting dwelling states in *mod-1* and *tph-1* mutants suggests that continued serotonergic signaling maintains dwelling states.

The origin of the endogenous NSM calcium signals is unknown. NSM's position in the pharynx suggests that it could detect cues associated with feeding; in addition, NSM calcium levels are indirectly regulated by attractive and repulsive odors and the biogenic amine tyramine (Li et al., 2012). Thus, NSM could detect both nutrients and sensory cues relevant to roaming and dwelling. However, nutrients and sensory cues also regulate ASI and AIY, so there are many neurons in the serotonin and PDF circuits that provide possible entry points for regulation. Understanding the relationship between *egl-4*, which functions in sensory neurons to promote dwelling (Fujiwara et al., 2002), and the circuit described here might clarify how nutrient and sensory cues are coupled to behavioral transitions.

The *C. elegans* PDF receptor is coupled to Gαs and cAMP production, so for optogenetic imitation of PDFR-1 activation, we employed the bacterial light-activated adenylyl cyclase BlaC (Ryu et al., 2010). BlaC activation in PDFR-1-expressing neurons triggered roaming states that lasted for several minutes after light cessation should terminate its catalytic activity (Ryu et al., 2010). Persistent roaming could result from sustained cAMP levels, sustained activation of the cAMP-dependent protein kinase PKA, or sustained phosphorylation of PKA targets. Taking a broader view, the slow time course of neuromodulatory G protein signaling is well suited to convert short-lasting electrical signals into longer-lasting biochemical and behavioral states.

Common Features of Behavioral Molecules and a Shared Circuit Logic in Different Animals

A role for PDF signaling in promoting roaming, an arousal state in *C. elegans*, is reminiscent of the ability of PDF to promote arousal during waking states in *Drosophila*. In flies, PDF-expressing neurons integrate the circadian cycle, light levels, and modulatory octopamine and dopamine inputs to regulate PDF release and arousal (Sehgal and Mignot, 2011). A mammalian neuropeptide, vasoactive intestinal peptide (VIP), has a similar role in stimulating arousal downstream of light inputs and neuromodulation in the suprachiasmatic nucleus (Vosko et al., 2007). PDF receptors and VIP receptors are similar in sequence, suggesting that these neuropeptide systems may have similar or even conserved roles in arousal states.

More generally, the circuit logic of roaming and dwelling resembles the logic of hypothalamic and brainstem circuits that control discrete mammalian sleep and wake behaviors (Saper et al., 2010). The transitions between wake, REM, and non-REM sleep are controlled by neuropeptides and biogenic amines produced in hypothalamic and brainstem nuclei. As is seen in roaming and dwelling, each state inhibits the others in a switch-like fashion, and loss of the neuromodulators leads to destabilized and truncated behavioral states. We suggest that these features may be signatures of a variety of discrete behavioral states.

EXPERIMENTAL PROCEDURES

Information about strains and plasmids used in this study is available in the Extended Experimental Procedures and Table S2. Nematodes were grown on agar with nematode growth medium (NGM) and OP50 bacteria. Mutant strains were backcrossed to a common strain (N2) to remove unlinked mutations prior to analysis (Table S1).

Exploration Assays

To measure exploration behavior, individual L4 animals were picked to a 35 mm agar plate uniformly seeded with *E. coli* strain OP50. After ~16 hr, plates were superimposed on a grid containing 3.5 mm squares, and the number of squares entered by the worm tracks was manually counted. Tracks could enter a maximum of 86 squares. In Figures S1B and S3A, assays were performed on 60 mm plates for 13 hr to distinguish between mutants with high levels of roaming (maximum number squares entered, 175). Transgenic and mutant strains were always compared to control animals assayed in parallel. For the candidate gene screen (Figure 1C), five animals were tested per data point, and the FDR was calculated using the Benjamini-Hochberg method. For other genotypes, 10–25 animals were tested per data point. All plates were scored by an investigator blind to the genotype of the animals.

Roaming and Dwelling Assays

High-resolution analysis of *C. elegans* locomotion was performed on 1-day-old adult animals exploring 600 cm^2 agar plates seeded with OP50. Animals were recorded for 90 min at three frames per second (fps). Worm trajectories were extracted from videos using custom Matlab scripts that calculated the speed and angular speed of each animal. Measurements were averaged over 10 s intervals, which easily distinguished roaming and dwelling intervals (Figure S2A) (Ben Arous et al., 2009), allowing us to describe the trajectory of each animal as a sequence of discrete roaming and dwelling intervals. For each experiment,

sequences from control animals were used to optimize a two-state HMM. After optimization, the most probable state path of each animal (for all genotypes) was calculated by applying the Viterbi algorithm to the sequence of discrete roaming and dwelling intervals for that animal. Additional information is available in the Extended Experimental Procedures.

Optogenetic Stimulation

L4 experimental and control animals were picked to OP50-seeded NGM plates containing 50 μm all-*trans* retinal. The next day, adult animals' locomotion was recorded using the setup described above. During video recordings, LED illumination was used to expose animals to blue (455 nm) or green (530 nm) light. For ChR2*(C128S), blue light delivery was always followed by 60 s of green light delivery to ensure full inactivation of ChR2*(C128S). We determined the behavioral states of animals using the procedure above and then aligned behavioral measurements to periods of light exposure. Additional information is available in the Extended Experimental Procedures.

Calcium Imaging

Transgenic animals expressing GCaMP5 (Akerboom et al., 2012) in NSM or AIY neurons were assayed as 1-day-old adults. Transgenic lines were generated in a *lite-1* background to prevent behavioral responses to blue light (Edwards et al., 2008). Flat NGM agar pads (~1 mm thick) on a glass microscope slide were seeded with an ~5-mm-diameter OP50 lawn. Animals were then picked to the pad, which was sealed in a small chamber to prevent evaporation. 30 min videos of the animals were recorded at 10 fps using 10 ms light exposures. In this arena, animals sometimes left the region being recorded and returned; all available data were used in subsequent analyses. Additional information is available in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, two tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.001.

ACKNOWLEDGMENTS

We thank H.R. Horvitz, S. Mitani, the National BioResource Project (NBRP), and the *Caenorhabditis* Genetics Center (CGC) for strains; L. Looger for GCaMP5; A. Gordus and A. Katsov for help with data analysis; I. Shachrai for initiating optogenetic experiments; Y. Saheki, A. Bendesky, J. Garrison, J. Greene, and other members of our laboratory for helpful discussions; and S. Kuehn and R. Axel for comments on the manuscript. This work was supported by the G. Harold and Leila Y. Mathers Foundation and the Ellison Medical Foundation (C.I.B.), a Helen Hay Whitney postdoctoral fellowship (S.W.F.), NIH grant GM07739 (E.Z.M.), and a Boehringer Ingelheim Fonds PhD Fellowship (J.L.). C.I.B. is an Investigator of the Howard Hughes Medical Institute.

Received: December 24, 2012
Revised: May 21, 2013
Accepted: July 31, 2013
Published: August 22, 2013

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