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Report

Neuromodulatory State and Sex Specify Alternative Behaviors through Antagonistic Synaptic Pathways in *C. elegans*

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SUMMARY

Pheromone responses are highly context dependent. For example, the *C. elegans* pheromone ascaroside C9 (ascr#3) is repulsive to wild-type hermaphrodites, attractive to wild-type males, and usually neutral to "social" hermaphrodites with reduced activity of the *npr-1* neuropeptide receptor gene. We show here that these distinct behavioral responses arise from overlapping push-pull circuits driven by two classes of pheromone-sensing neurons. The ADL sensory neurons detect C9 and, in wild-type hermaphrodites, drive C9 repulsion through their chemical synapses. In *npr-1* mutant hermaphrodites, C9 repulsion is reduced by the recruitment of a gap junction circuit that antagonizes ADL chemical synapses. In males, ADL sensory responses are diminished; in addition, a second pheromone-sensing neuron, ASK, antagonizes C9 repulsion. The additive effects of these antagonistic circuit elements generate attractive, repulsive, or neutral pheromone responses. Neuronal modulation by circuit state and sex, and flexibility in synaptic output pathways, may permit small circuits to maximize their adaptive behavioral outputs.

INTRODUCTION

Connectomics, the description of neuronal circuits based on anatomically defined synapses, is an ongoing venture in neuroscience (White et al., 1986; Lichtman and Denk, 2011). A question that is unanswered by such studies is the extent to which these synapses are functionally, as opposed to anatomically, stable in their properties. In many animals, pheromone detection results in behaviors that are highly sensitive to context (Wyatt, 2003). Here, we examine circuits for pheromone-dependent behaviors and show that a small set of common sensory inputs can give rise to multiple behavioral outputs through flexible circuit interactions.

The nematode worm *Caenorhabditis elegans* releases a pheromone mixture composed of derivatives of the dideoxysugar ascarylose (ascarosides), each of which has characteristic effects on development and behavior (Edison, 2009; Srinivasan et al., 2012). Two pheromones that have been characterized in multiple assays are C3 (ascr#5; asc ωC3) and C9 (ascr#3; asc ΔC9) ascarosides. C3 and C9 potently regulate larval entry into and exit from the alternate dauer developmental stage (Butcher et al., 2007, 2008; Kim et al., 2009) and also elicit a variety of behavioral effects in adults. Adult wild-type males accumulate in low concentrations of C9, suggesting a role in sex attraction (Srinivasan et al., 2008). Hermaphrodites with low-activity alleles of the *npr-1* neuropeptide receptor gene (henceforth "*npr-1*") are weakly attracted to ascaroside mixtures of C3 and C9 but not to either single compound alone (Macosko et al., 2009). Hermaphrodites from the standard laboratory strain N2 (henceforth "wild-type") strongly avoid C9 alone or together with C3 (Srinivasan et al., 2008; Macosko et al., 2009). The differential pheromone response in hermaphrodites correlates with aggregation behaviors: social *npr-1* animals usually aggregate into groups on food, consistent with attraction to pheromones, whereas solitary wild-type animals rarely aggregate (de Bono and Bargmann, 1998). The *npr-1* genotype appears to be a surrogate for a stress-related behavioral state, as aggregation and other *npr-1*-associated behaviors are stimulated regardless of genotype by stressful conditions (de Bono et al., 2002; Rogers et al., 2006). Thus, pheromone responses in *C. elegans* depend on sex and neuromodulatory state.

The bilateral pair of ASK sensory neurons acts with different partners in different pheromone responses. In dauer formation, ascaroside pheromones are sensed by ASK and ASI sensory

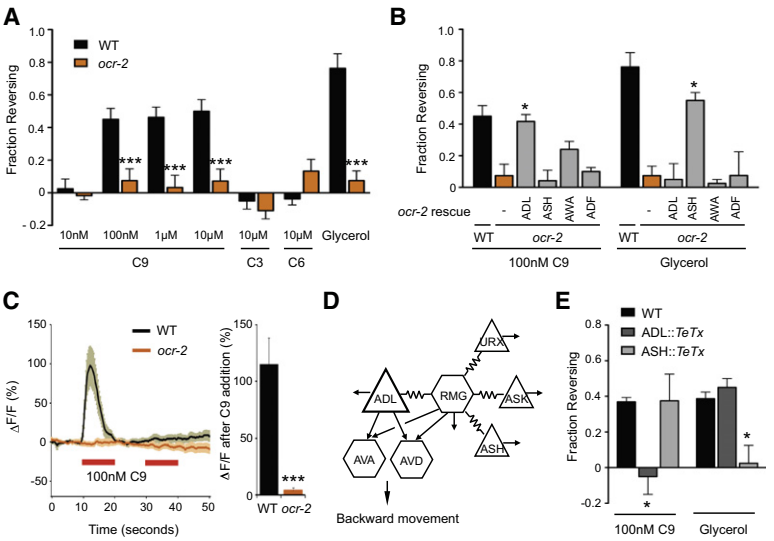

Figure 1. ADL Sensory Neurons Mediate C9 Avoidance

(A) Wild-type hermaphrodites avoid C9 in the drop test assay, but *ocr-2(ak47)* mutants do not. Assays were performed in the absence of food. *** indicates responses different from wild-type at $p < 0.001$. n = 20–100 animals each. See Figure S1A for assays on food and Figure S1B for behaviors of *osm-9* mutants. (B) *ocr-2* acts in ADL to mediate C9 avoidance. Assays were performed in the absence of food. * indicates responses different from *ocr-2* at $p < 0.05$. n = 20–100 animals each. *ocr-2* expression in AWA showed a trend toward rescue, but *odr-7* mutants with developmental defects in the AWA neurons (Sengupta et al., 1994) responded normally to C9 (Figure S1C). (C) C9-induced pheromone responses in ADL. (Left) Intracellular Ca^{2+} dynamics in GCaMP3-expressing ADL neurons in wild-type and *ocr-2(ak47)* hermaphrodites upon addition of pulses of 100 nM C9 (red horizontal bars). $n \geq 10$ neurons each. Shading around lines represents SEM. (Right) Average peak percentage changes in fluorescence upon addition of the first pulse of C9. * indicates amplitude different from wild-type at $p < 0.001$. Also see Figure S1D. (D) ADL has chemical synapses onto AVA and AVD command interneurons and is electrically coupled with the RMG hub-and-spoke circuit (adapted from White et al., 1986). Triangles represent sensory neurons and hexagons represent interneurons. Additional synapses (not shown) are indicated by arrows. (E) C9 avoidance requires ADL chemical synapses. Avoidance assays in the presence of food. * indicates responses different from wild-type at $p < 0.05$. n = 20–80 animals each. Error bars in all panels represent standard error of the mean (SEM).

neurons (Hu, 2007; Kim et al., 2009). In adult males, attraction to hermaphrodite pheromones requires ASK and the male-specific CEM sensory neurons (Srinivasan et al., 2008). In *npr-1* hermaphrodites, the ASK neurons sense pheromones and promote aggregation by cooperating with URX, ASH, and ADL sensory neurons, all of which are connected by gap junctions to the RMG inter/motorneurons in a hub-and-spoke circuit (White et al., 1986; de Bono et al., 2002; Macosko et al., 2009). The integrated input from spoke sensory neurons drives synaptic outputs from RMG and ASK to promote aggregation (Macosko et al., 2009). In wild-type animals, high NPR-1 activity in RMG inhibits this circuit (Macosko et al., 2009).

Wild-type hermaphrodites are repelled by ascarosides (Srinivasan et al., 2008; Macosko et al., 2009) but the underlying circuit mechanisms are unknown. Here we ask how repulsion from pheromones is mediated and how repulsion is transformed into neutral or attractive pheromone responses in males and in *npr-1* mutants. We find that the ADL sensory neurons promote repulsion from C9 in a sex- and *npr-1* state-dependent manner and that alternative pheromone-dependent behaviors rely differentially upon antagonistic activities of ADL chemical synapses, the RMG gap junction circuit, and ASK. Our results describe a mechanism by which overlapping, flexible circuits allow animals to integrate pheromone signals with sex and neuromodulatory state to generate a biologically appropriate behavioral response.

RESULTS

ADL Neurons Detect the Repulsive Pheromone Ascaroside C9

To identify neurons responsible for pheromone avoidance behavior, we first examined the acute responses of wild-type hermaphrodites to individual ascarosides using the drop-test assay (Hilliard et al., 2002). In this assay, a chemical diluted in buffer is presented to an animal that is moving forward, and reversal responses are compared to those to buffer alone (see Experimental Procedures). Using this behavioral response, we found that wild-type hermaphrodites specifically avoided nanomolar concentrations of ascaroside C9, but not ascarosides C3 or C6 (Figure 1A). These responses were enhanced in the presence of food, resulting in a ~10-fold increase in sensitivity (Figure S1A available online).

The neurons required for C9 avoidance were identified by examining sensory transduction mutants. *C. elegans* detects many chemical repellents with ciliated sensory neurons that signal through OSM-9 and OCR-2 TRPV channels (Bargmann, 2006). We found that both *osm-9* and *ocr-2* mutants exhibited strong defects in C9 avoidance (Figure 1A and Figure S1B). These two genes are coexpressed in four classes of head sensory neurons (Colbert et al., 1997; Tobin et al., 2002), which were individually tested for transgenic rescue of the *ocr-2* behavioral defect. The C9 avoidance defects were rescued upon expression of *ocr-2* in ADL, but not in other neurons (Figure 1B; also see Figure S1C). In control experiments, *ocr-2* expression in ADL did not rescue avoidance of high-osmotic-strength glycerol, a sensory response characteristic of ASH neurons (Bargmann, 2006) (Figure 1B). These results indicate that OCR-2 acts in the ADL neurons to mediate C9 avoidance.

To ask whether ADL responds to C9, we expressed the genetically encoded calcium (Ca^{2+}) sensor GCaMP3 (Tian et al., 2009) in ADL neurons and monitored intracellular Ca^{2+} dynamics in response to C9. A pulse of 100 nM C9 induced a rapid, transient increase in ADL intracellular Ca^{2+} levels (Figure 1C). ADL Ca^{2+} transients adapted quickly, returning to baseline within 10 s of C9 addition, and recovering ~120 s later (Figure 1C and data not shown). The response to C9 was abolished in *ocr-2* mutants

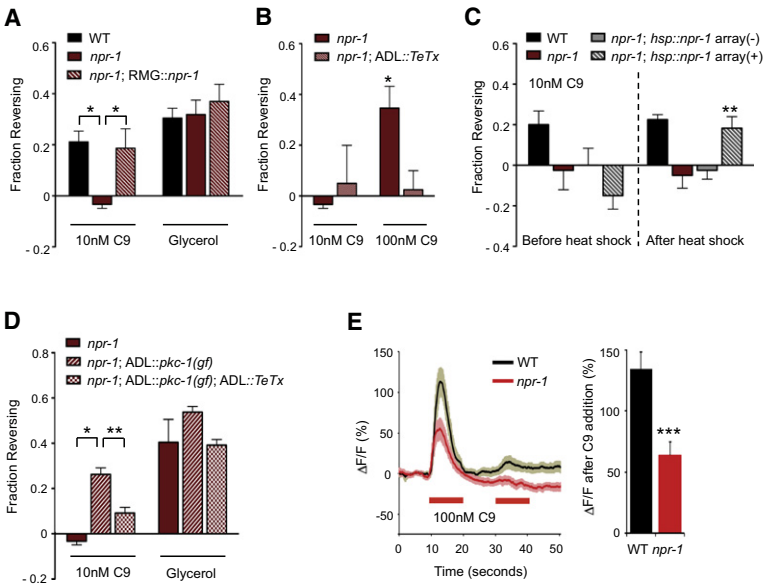

Figure 2. Reducing NPR-1 Activity in RMG Antagonizes ADL Chemical Synapses

(A) *npr-1* animals show decreased C9 avoidance. Avoidance assays in the presence of food. * indicates responses different from values indicated by brackets at $p < 0.05$. n = 40–100 animals each. (B) ADL chemical synapses can drive C9 avoidance in *npr-1*. Avoidance assays in the presence of food. * indicates responses different from wild-type at $p < 0.05$. n = 40–100 animals each. (C) Heat shock-driven expression of *npr-1* in adults rescues C9 avoidance in *npr-1* animals. ** indicates responses different from those of the same animals before heat shock at $p < 0.01$. Array(-) indicates animals from the *hsp16.2::npr-1*-expressing transgenic strain that have lost the extrachromosomal array, sibling controls for array(+) transgenic animals. Behavioral assays were performed in the presence of food before and immediately after heat shock at 33°C for 30 min. n = 40–60 animals each. (D) Strengthening ADL chemical synapses enhances C9 avoidance in *npr-1*. Avoidance assays in the presence of food. * and ** indicate responses different from values indicated by brackets at $p < 0.05$ and $p < 0.01$, respectively. n = 40–100 animals each. Also see Figure S2A. (E) (Left) Changes in GCaMP fluorescence in response to C9 in ADL neurons of wild-type and *npr-1(ad609)* hermaphrodites. Wild-type and all experimental conditions (see Figure 4C and Experimental Procedures) were examined together on multiple days. n = 12 neurons each. Shading around lines represents SEM. (Right) Average peak percentage changes in fluorescence upon addition of C9. *** indicates responses different from wild-type at $p < 0.001$. Also see Figures S2B and S2C. Error bars in all panels represent SEM.

that disrupt the sensory TRPV channel (Figure 1C). The ascaroside-evoked Ca^{2+} transients matched the behavioral results showing ADL-specific, chemically selective responses: ASH neurons did not respond to C9 or other ascarosides with Ca^{2+} transients, and no changes in Ca^{2+} dynamics were observed in the ADL neurons upon addition of C3 and C6 ascarosides (Figure S1D).

The anatomical wiring diagram of *C. elegans* hermaphrodites indicates that the ADL neurons are connected by chemical synapses to the AVA and AVD backward command interneurons, as well as other neurons (Figure 1D) (Chalfie et al., 1985; White et al., 1986). In addition, ADL neurons are spoke neurons connected by gap junctions to the RMG hub-and-spoke circuit that promotes aggregation (Figure 1D) (Macosko et al., 2009). In the simplest model, ADL-mediated avoidance behavior could be driven by synaptic output of the ADL neurons and activation of the backward command interneurons. To examine this possibility, we inhibited ADL chemical synapses by cell-specific expression of the tetanus toxin light chain (TeTx) that cleaves the synaptic vesicle protein synaptobrevin (Schiavo et al., 1992). Blocking synaptic transmission in ADL significantly suppressed C9 avoidance responses (Figure 1E), but not osmotic avoidance behavior mediated by the ASH neurons. Conversely, expression of similar transgenes in ASH blocked high-osmolarity glycerol avoidance but did not affect C9 avoidance (Figure 1E). Thus, the ADL neurons drive C9 avoidance through their chemical synapses.

Reducing *npr-1* Activity in the RMG Hub-and-Spoke Circuit Suppresses C9 Avoidance

npr-1 animals show reduced avoidance of repulsive pheromones in accumulation assays (Macosko et al., 2009), consistent with their increased aggregation behaviors. As the aggregation behaviors are most prominent on food (de Bono and Bargmann, 1998; de Bono et al., 2002), we included food when comparing *npr-1* and wild-type responses to C9. *npr-1* mutants did not avoid 10 nM C9 in the drop test, although they avoided higher concentrations (Figures 2A and 2B). High-osmolarity glycerol avoidance was unaffected by *npr-1* (Figure 2A). Silencing ADL synaptic output by TeTx expression in *npr-1* mutants did not further affect their behavioral responses to 10 nM C9, but reduced their avoidance of 100 nM C9 (Figure 2B). These results suggest that ADL chemical synapses can drive C9 avoidance in *npr-1* animals, but with reduced sensitivity compared to wild-type.

Previous studies have indicated that the ADL neurons promote aggregation in *npr-1* mutants, in apparent contradiction to their role in C9 avoidance in wild-type (de Bono et al., 2002). A possible explanation of this paradox is provided by the proposed circuit for aggregation, which involves gap junctions between ADL and RMG neurons rather than ADL chemical synapses (Figure 1D) (White et al., 1986; Macosko et al., 2009). Aggregation through the RMG circuit is inhibited by *npr-1* expression in RMG (Macosko et al., 2009). We hypothesized that this gap junction circuit might antagonize or inactivate C9 avoidance mediated by ADL chemical synapses. Indeed, the C9 avoidance defects in *npr-1* animals were fully rescued by a transgene expressing an *npr-1* cDNA in RMG (Figure 2A), indicating that NPR-1 acts in RMG to enhance C9 avoidance behaviors initiated by ADL.

To determine whether NPR-1 acts during development to affect connectivity, or in adults to regulate circuit function, we asked whether expression of NPR-1 during the adult stage could rescue C9 avoidance in *npr-1* animals. An *npr-1* cDNA

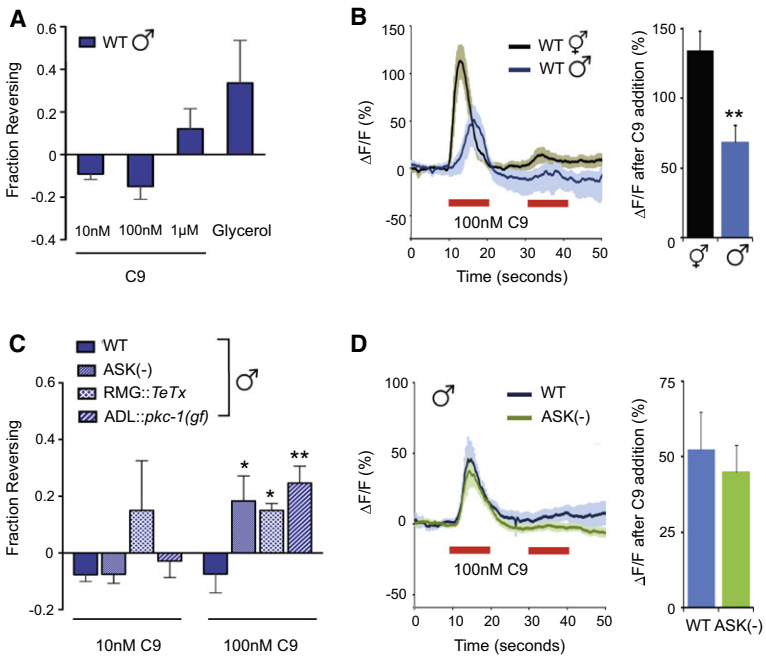

Figure 3. ASK Antagonism of ADL Decreases C9 Avoidance in Males

(A) Wild-type males do not avoid C9. Avoidance assays were performed in the presence of food. n = 20–70 animals each. (B) (Left) Changes in intracellular fluorescence in GCaMP3-expressing ADL neurons of wild-type hermaphrodites and males upon addition of 100 nM C9. n ≥ 12 neurons each. Shading around the lines represents SEM. (Right) Average peak percentage change in fluorescence upon C9 addition. ** indicates responses different from wild-type at $p < 0.01$. Also see Figures S3A and S3B. (C) ASK and RMG antagonize ADL in wild-type males. Avoidance of C9 in the presence of food. * and ** indicate responses different from wild-type at $p < 0.05$ and $p < 0.01$, respectively. n = 20–120 animals each. Also see Figure S3C. (D) C9 responses in wild-type male ADL neurons are unaffected by ASK ablation. Ca^{2+} transients upon C9 addition (red horizontal bar) in ADL in wild-type and ASK-ablated animals. n ≥ 10 neurons each. Error bars in all panels represent SEM.

expressed under a heat shock promoter (Coates and de Bono, 2002) restored C9 avoidance fully after 30 min of heat shock (Figure 2C), suggesting that NPR-1 acts acutely to regulate neuronal responses and circuit output.

The model that *npr-1* in RMG antagonizes ADL chemical synapses predicts that increased ADL synaptic function might restore avoidance. To test this prediction, we used an ADL-specific promoter to drive *pkc-1(gf)*, a constitutively active protein kinase C isoform that enhances neuronal synaptic output (Okochi et al., 2005; Sieburth et al., 2007; Tsunozaki et al., 2008; Macosko et al., 2009). Expression of *pkc-1(gf)* in ADL enhanced C9 avoidance in *npr-1* animals (Figure 2D), and blocking ADL chemical synapses with TeTx eliminated C9 avoidance in the *pkc-1(gf)* strain (Figure 2D). Expression of *pkc-1(gf)* in ADL neurons of wild-type animals had little effect on C9 avoidance (Figure S2A). These results suggest that strengthening ADL chemical synapses can override the effect of the *npr-1* mutation.

ADL Ca^{2+} transients were slightly but significantly reduced in amplitude in *npr-1* as compared to wild-type animals (Figures 2E and S2B). Two results suggest that this small change in amplitude is due to indirect effects of RMG on ADL. First, ADL Ca^{2+} responses were rescued by expressing *npr-1* under a promoter that is expressed in RMG (as well as a few other neurons) but not in ADL (Figure S2B). Second, the effect of *npr-1* on ADL Ca^{2+} responses was reversed in animals mutant for *unc-9*, which encodes a gap junction subunit that is broadly expressed in muscles and neurons (Liu et al., 2006; Starich et al., 2009) (Figure S2C). This observation suggests that gap junctions are required for NPR-1 to affect ADL, as predicted by the hub-and-spoke model. However, *unc-9* has stronger effects on ADL Ca^{2+} responses than *npr-1* (Figure S2C) and acts at multiple sites, so it may have either direct or indirect effects on ADL. In summary, *npr-1* has a strong effect on C9 avoidance behavior that is mediated by RMG and an indirect effect on ADL Ca^{2+} responses. Our results suggest that *npr-1* functions primarily by changing activity of the RMG gap junction circuit relative to ADL chemical synapses, and not solely by changing ADL sensory properties.

Reduced ADL C9 Responses and ADL-ASK Antagonism Abolish C9 Avoidance in Wild-Type Males

Unlike wild-type hermaphrodites, wild-type *C. elegans* males accumulate in low concentrations of C9, a behavior that requires the ASK neurons and the male-specific CEM sensory neurons (Srinivasan et al., 2008). In agreement with this result, we found that wild-type males did not avoid either 10 nM or 100 nM C9 in the drop test, although they exhibited robust avoidance of high-osmolarity glycerol (Figure 3A).

This sexually dimorphic behavioral response to C9 was accompanied by sexually dimorphic Ca^{2+} responses in ADL neurons. C9-induced Ca^{2+} transients in male ADL neurons were delayed by several seconds and reduced in amplitude compared to responses in hermaphrodites (Figures 3B, Figure S3A). Because slow changes in neuronal responses are often associated with neuropeptide signaling, we investigated ADL C9 responses in *egl-3* and *egl-21* mutants, which lack processed neuropeptides (Kass et al., 2001; Jacob and Kaplan, 2003). Both males and hermaphrodites showed sex-appropriate ADL Ca^{2+} transients in neuropeptide mutant backgrounds (Figure S3A), suggesting that classical neuropeptide signaling is not essential for this sexual dimorphism. Thus, altered male behaviors are associated with decreased and delayed pheromone signaling by the ADL neurons, which might or might not be intrinsic to ADL.

We next probed the roles of other sexually dimorphic neurons in C9 avoidance. The male-specific CEM sensory neurons are required for male accumulation at low C9 concentrations (Srinivasan et al., 2008), but were not central to C9 avoidance: sex-appropriate behaviors to C9 were observed both in males lacking CEM neurons (*ceh-30(lf)*) and in hermaphrodites

with ectopic CEM neurons (*ceh-30(gf)*) (Schwartz and Horvitz, 2007) (Figure S3B). The ASK neurons are pheromone-sensing neurons that participate in the RMG gap junction circuit (Macosko et al., 2009) (Figure 1D), and these neurons are functionally dimorphic between males and hermaphrodites (Srinivasan et al., 2008, 2012). Males whose ASK neurons were killed with a mouse caspase gene (Kim et al., 2009) exhibited significant avoidance of 100 nM C9, unlike wild-type males (Figure 3C). Ablation of ASK had little effect on wild-type hermaphrodite C9 avoidance (Figure S3C). Thus, ASK effectively antagonizes ADL-mediated C9 avoidance in wild-type males, but not in wild-type hermaphrodites. ASK ablation did not affect C9-induced Ca^{2+} transients in male ADL neurons (Figure 3D), suggesting that ASK acts at a circuit level to suppress C9 avoidance.

Reasoning by analogy to the *npr-1* circuit, we asked whether synaptic output of the RMG gap junction circuit antagonizes C9 avoidance in males. Indeed, expression of TeTx in the RMG neurons led to robust C9 avoidance behavior in wild-type males (Figure 3C). Expression of *pkc-1(gf)* in ADL also led to C9 avoidance, indicating that a strongly activated ADL neuron can drive repulsion in males (Figure 3C), as it can in *npr-1* hermaphrodites (Figure 2D). These results suggest that ADL has a latent ability to drive C9 avoidance in males, but this activity is inhibited by ASK and RMG.

Male Sexual Identity and *npr-1* Have Additive Effects on C9 Responses

Both males and *npr-1* hermaphrodites have decreased C9 avoidance (compare Figures 2A and 3A), and males also resemble *npr-1* hermaphrodites in their avoidance of high oxygen, their rapid movement on food, and their propensity to aggregate (Figures S4A and S4B). Despite this similarity, behavioral analysis of *npr-1* males suggests that *npr-1* mutations and male sex have independent effects on C9 responses. First, in *npr-1* males C9 failed to induce reversals as it did in *npr-1* hermaphrodites and wild-type males, but instead suppressed spontaneous reversals (Figure 4A). Based on the biased random walk model for *C. elegans* chemotaxis, the suppression of reversals suggests that *npr-1* males are attracted to C9 (Pierce-Shimomura et al., 1999; Luo et al., 2008). The suppression of reversals was eliminated by each of the genetic manipulations that increased C9 repulsion in wild-type males: killing ASK with the caspase transgene, reducing RMG synaptic output with TeTx, or enhancing ADL output with *pkc-1(gf)* (Figure 4B). Like other effects of *npr-1*, the effect on males was rescued by *npr-1* expression in RMG neurons (Figure 4B) and was rapidly reversed after acute expression of *npr-1* in adults (Figure S4C).

Additive effects of *npr-1* and male sex were also observed in Ca^{2+} imaging. The majority of ADL neurons in *npr-1* mutant males failed to modulate Ca^{2+} after C9 addition (Figure 4C, right panel). This reduction in ADL Ca^{2+} responses exceeded that of wild-type males or *npr-1* hermaphrodites, even considering only the small subset of *npr-1* males that did modulate ADL Ca^{2+} in response to C9 (Figure 4C, left panel).

The strong reduction in ADL Ca^{2+} transients might explain the loss of C9 avoidance in *npr-1* males but would not predict the appearance of the new behavior of C9 attraction (strictly speaking, reversal suppression). Therefore, we sought another sensory neuron that enhances C9 attraction in *npr-1* males. ASK was a plausible candidate to drive C9 attraction based on the behavioral analysis (Figure 4B), so we asked whether its pheromone sensitivity was altered by *npr-1*. Indeed, ASK neurons showed much stronger C9-evoked Ca^{2+} transients in *npr-1* males than in wild-type males (Figure 4D). A similar enhancement of ASK responses was present in *npr-1* hermaphrodites, whose C9 avoidance is also antagonized by ASK (Figures 4D and S3C).

Together, these results indicate that *npr-1* males have enhanced ASK C9 responses and decreased ADL C9 responses compared to wild-type males and that these changes drive attraction to C9 through RMG chemical synapses. Circuit changes driving sexually dimorphic and NPR-1-dependent C9 pheromone responses are summarized in Figure 4E.

Pheromone Blends Are Integrated by the RMG Circuit

The results described above suggest that antagonism between repulsive signaling from ADL chemical synapses and attractive signaling mediated by ASK and the RMG gap junction circuit determine whether C9 is repulsive, neutral, or attractive. We considered what this might mean for the pheromone-dependent behaviors of *npr-1* hermaphrodites, which are weakly attracted to mixtures of ascarosides, including C9 and C3, but not to either C3 or C9 alone (Srinivasan et al., 2008; Macosko et al., 2009). By analogy with the detection of pheromone blends in other animals (Kaissling, 1996), synergistic attraction to ascaroside blends could result from cooperation of multiple pheromone-sensing neurons. Hermaphrodite ASK neurons detect C3 at nanomolar concentrations (Kim et al., 2009), and ASK pheromone responses are stronger in *npr-1* than in wild-type hermaphrodites (Macosko et al., 2009). If antagonism between ASK and ADL applies to pheromone blends, C3 detection by ASK should suppress repulsion to C9 detected by ADL. To address this hypothesis, we used the drop-test assay to detect behavioral interactions between pheromones.

In agreement with the observation that C3 is not highly attractive or repulsive on its own (Macosko et al., 2009), C3 did not induce or suppress reversals in wild-type or *npr-1* hermaphrodites (Figure 4F). However, C3 did modify the response to C9 in *npr-1* hermaphrodites, suppressing their avoidance of 100 nM C9 almost to baseline levels (Figure 4F). No suppression was observed in wild-type hermaphrodites, indicating that the interaction depends on *npr-1* and the gap junction circuit. Genetic ablation of the ASK neurons in *npr-1* hermaphrodites abolished the interaction between C3 and C9 (Figure 4F). These results support the model that ASK suppresses ADL-mediated avoidance and additionally are consistent with a circuit that can evaluate pheromone blends, so that the combination of C3 detected by ASK and C9 detected by ADL is less repulsive than C9 alone.

DISCUSSION

Sex and NPR-1 neuropeptide signaling converge on a common neural circuit to regulate behavioral responses to the ascaroside C9. In each case, alternative behaviors are initiated by the ADL and ASK sensory neurons, but specific behavioral

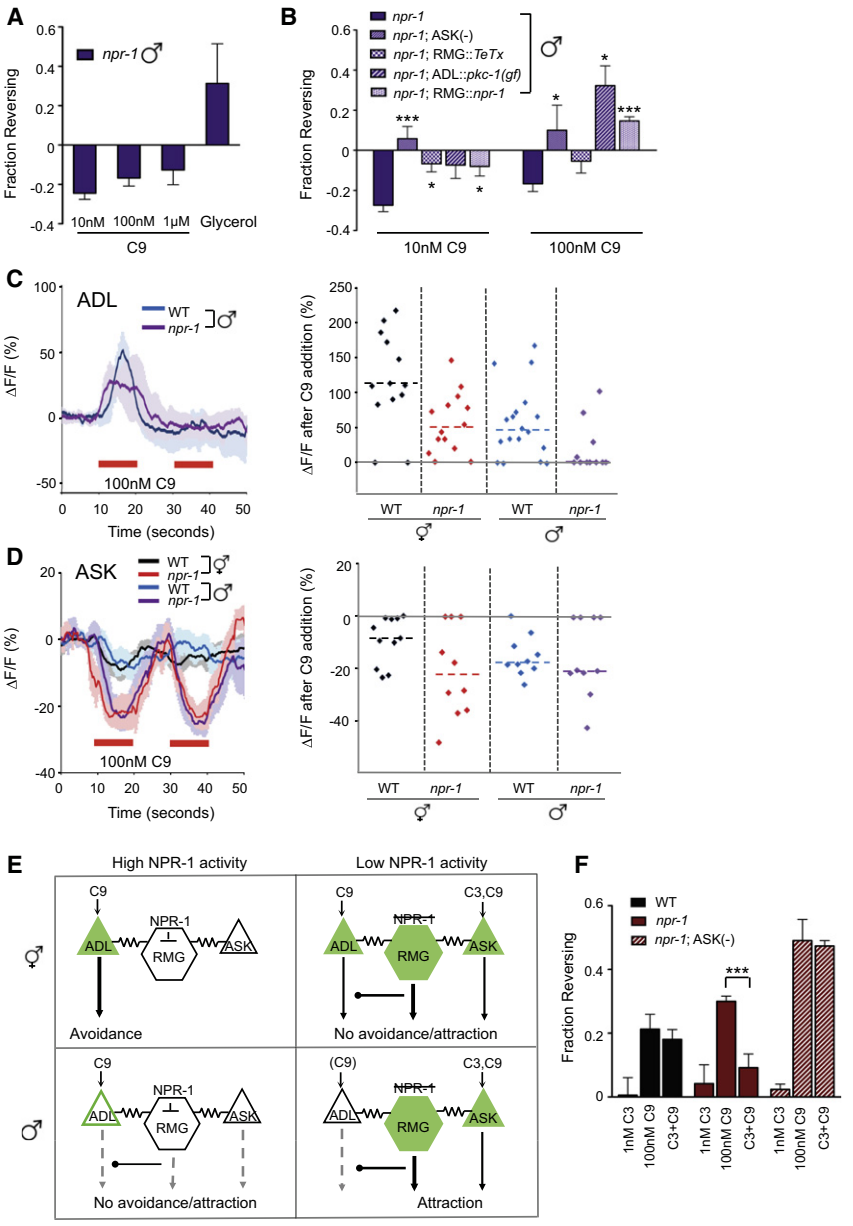

Figure 4. Masculinization and NPR-1 Exert Additive Effects on a Common Circuit

(A) Spontaneous reversals are suppressed by C9 in *npr-1* males. Avoidance assays were performed in the presence of food. n = 30–120 animals each. Responses to 10 nM and 1 μM C9 are different from those of wild-type males at $p < 0.01$ (see Figure 3A). Also see Figure S4. (B) C9-induced suppression of spontaneous reversals in *npr-1* males is eliminated by circuit manipulations that enhance C9 avoidance. * and *** indicate responses different from *npr-1* at $p < 0.05$ and $p < 0.001$, respectively. n = 40–120 animals each. (C) (Left) Intracellular Ca^{2+} dynamics in GCaMP-expressing ADL neurons of wild-type and *npr-1(ad609)* males in response to 100 nM C9. Only animals with positive responses were included in the averages at left: n = 14 (of 17) neurons for wild-type males and n = 4 (of 11) neurons for *npr-1* males. Shading around the lines represents SEM. (Right) Scatter plot showing peak percentage changes in fluorescence in individual ADL neurons of animals of the indicated genotypes in response to a pulse of 100 nM C9. Data are included from Figure 2E for comparison. Dotted lines indicate the median. ADL neurons that failed to exhibit significant changes in fluorescence: 14% in wild-type hermaphrodites (n = 14); 14% in *npr-1* hermaphrodites (n = 14); 12% in wild-type males (n = 17) and 64% in *npr-1* males (n = 11). (D) ASK responses to C9 are enhanced in *npr-1* mutants. (Left) Intracellular Ca^{2+} dynamics in GCaMP3-expressing ASK neurons (Macosko et al., 2009) of wild-type and *npr-1(ad609)* males and hermaphrodites in response to 100 nM C9 (red horizontal bars). Only animals with quantifiable changes were included in these averages; $n \geq 6$ neurons for each. Shading around lines represents SEM. (Right) Scatter plot showing peak percentage changes in fluorescence in individual ASK neurons of animals of the indicated genotypes in response to a pulse of 100 nM C9. Dotted lines indicate the median. (E) Inferred activities of ADL, ASK, and RMG neurons in animals of different sex and *npr-1* genotype. High NPR-1 activity inhibits RMG. C9 responses are high in ADL and low in ASK in wild-type hermaphrodites; ADL drives C9 avoidance via its chemical synapses. In males, ASK C9 responses remain low and ADL C9 responses are decreased due to sexual dimorphism. ASK and the RMG circuit antagonize ADL output to further reduce ADL-driven C9 avoidance. In *npr-1* animals, ASK C9 responses are increased in both males and hermaphrodites, and ADL C9 responses are significantly decreased in *npr-1* males. ASK and RMG antagonize ADL chemical synapses and decrease avoidance (hermaphrodites) or promote attraction (males). (F) Integration of pheromone blend information in *npr-1* hermaphrodites requires ASK. Assays were performed in the presence of food. *** indicates responses different from values indicated by brackets at $p < 0.001$. n = 60–80 animals each. Error bars in all panels represent SEM.

outcomes are determined by antagonism between ADL chemical synapses that promote repulsion and the RMG gap junction circuit that promotes attraction. These two antagonistic elements form a push-pull circuit motif, in which a single sensory input can give rise to opposite behaviors (Figure 4E). On the repulsive arm of the circuit, wild-type hermaphrodites avoid C9 through ADL chemical synapses, whose predicted targets include the backward command interneurons. Although this effect is diminished in *npr-1* mutants and males, all genotypes retain a covert ability to avoid C9. On the attractive arm, the RMG gap junction circuit suppresses C9 avoidance via RMG chemical synapses, which converge with ADL chemical synapses on the command interneurons (see Figure 1D). NPR-1 inhibits RMG through unknown molecular mechanisms; in one model, it could close the RMG gap junctions to disengage the entire hub-and-spoke circuit. The ASK neurons also sense C9 and drive attractive behavioral responses more strongly in males, in *npr-1* mutants, or in the presence of C3.

ASK and ADL form gap junctions with RMG; both behavioral results and functional imaging indicate that RMG potentiates ASK signaling and inhibits ADL signaling (Macosko et al., 2009, and this work). The attractive arm of the circuit dominates in *npr-1* males, which have minimal C9 responses in ADL, strong C9 responses in ASK, and the ability to propagate these changes through the RMG circuit.

It is likely that the alternative circuits in wild-type and *npr-1* mutants are representative of alternative neuromodulatory states that exist in all genotypes to differing degrees. The behaviors of *npr-1* animals resemble the behaviors of wild-type animals under metabolic or crowding stress, and conversely, *npr-1* animals placed in low-oxygen environments behave like wild-type animals in most respects (de Bono et al., 2002; Rogers et al., 2006). The differential modulation of ADL chemical synapses and gap junctions in overlapping circuits by *npr-1* is reminiscent of the flexible circuit states of crustacean stomach central pattern generators and vertebrate spinal cord motor circuits, which are also controlled by neuromodulatory inputs (Dickinson et al., 1990; Grillner, 2006). In males, sexual dimorphism in sensory neuron responses and circuit properties further expand this behavioral flexibility.

The RMG hub-and-spoke circuit has both similarities to and differences from the recently described RIH hub-and-spoke circuit for mechanosensation (Chatzigeorgiou and Schafer, 2011). A central hub neuron coordinates responses via gap junctions in both circuits, but RIH appears to facilitate the transfer of mechanosensory information through the circuit (Chatzigeorgiou and Schafer, 2011), whereas RMG antagonizes ADL synaptic output while facilitating ASK synaptic output, generating a consensus behavior that can be distinct from that generated by either sensory neuron. Thus, a common network motif can perform distinct computations in ways that are not evident solely from anatomical wiring diagrams.

Pheromone blends with defined concentrations of individual pheromone components elicit sex- and context-specific behaviors in many organisms (Kaissling, 1996; Slessor et al., 1988; Wyatt, 2003). The RMG circuit coordinates sensory responses via gap junctions to generate coherent responses to specific pheromones and pheromone blends. Spoke sensory neurons in the RMG circuit also respond to nonpheromone cues (Bargmann, 2006), allowing the circuit to integrate pheromones with other environmental signals. At the same time, each sensory neuron also has other outputs; for example, ASK can promote attraction to indole ascarosides via its chemical synapses in an RMG-independent manner (Srinivasan et al., 2012), and ADL alone can drive repulsion. These results reveal a multifunctional, multiplexed sensory circuit, whose compact structure integrates external context with internal states to generate a variety of adaptive behaviors.

EXPERIMENTAL PROCEDURES

Detailed protocols are listed in Supplemental Experimental Procedures.

Behavioral Assays

The drop test was performed essentially as previously described (Hilliard et al., 2002). "Fraction reversing" represents (fraction of animals reversing in 4 s to pheromone) – (fraction reversing in 4 s to buffer).

Ca^{2+} Imaging

Ca^{2+} imaging experiments were performed as previously described (Kim et al., 2009; Macosko et al., 2009) using microfluidic devices custom-designed to restrain adult hermaphrodites (Chalasani et al., 2007) or adult males (this study) (Microfluidics Facility, Brandeis Materials Research Science and Engineering Center).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.06.034.

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REFERENCES

Bargmann, C.I. (2006). Chemosensation in *C. elegans*. *WormBook*, ed. The *C. elegans* Research Community, WormBook, http://dx.doi.org/10.1895/wormbook.1.144.1, http://www.wormbook.org.

Butcher, R.A., Fujita, M., Schroeder, F.C., and Clardy, J. (2007). Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat. Chem. Biol. *3*, 420–422.

Butcher, R.A., Ragains, J.R., Kim, E., and Clardy, J. (2008). A potent dauer pheromone component in *Caenorhabditis elegans* that acts synergistically with other components. Proc. Natl. Acad. Sci. USA *105*, 14288–14292.

Chalasani, S.H., Chronis, N., Tsunozaki, M., Gray, J.M., Ramot, D., Goodman, M.B., and Bargmann, C.I. (2007). Dissecting a circuit for olfactory behaviour in *Caenorhabditis elegans*. Nature *450*, 63–70.

Chalfie, M., Sulston, J.E., White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1985). The neural circuit for touch sensitivity in *Caenorhabditis elegans*. J. Neurosci. *5*, 956–964.

Chatzigeorgiou, M., and Schafer, W.R. (2011). Lateral facilitation between primary mechanosensory neurons controls nose touch perception in *C. elegans*. Neuron *70*, 299–309.

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

Colbert, H.A., Smith, T.L., and Bargmann, C.I. (1997). OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in *Caenorhabditis elegans*. J. Neurosci. *17*, 8259–8269.

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

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

Dickinson, P.S., Mecsas, C., and Marder, E. (1990). Neuropeptide fusion of two motor-pattern generator circuits. Nature *344*, 155–158.

Edison, A.S. (2009). *Caenorhabditis elegans* pheromones regulate multiple complex behaviors. Curr. Opin. Neurobiol. *19*, 378–388.

Grillner, S. (2006). Biological pattern generation: the cellular and computational logic of networks in motion. Neuron *52*, 751–766.

Hilliard, M.A., Bargmann, C.I., and Bazzicalupo, P. (2002). *C. elegans* responds to chemical repellents by integrating sensory inputs from the head and the tail. Curr. Biol. *12*, 730–734.

Hu, P.J. (2007). Dauer. *WormBook*, ed. The *C. elegans* Research Community, WormBook, http://dx.doi.org/10.1895/wormbook.1.144.1, http://www.wormbook.org.

Jacob, T.C., and Kaplan, J.M. (2003). The EGL-21 carboxypeptidase E facilitates acetylcholine release at *Caenorhabditis elegans* neuromuscular junctions. J. Neurosci. *23*, 2122–2130.

Kaissling, K.E. (1996). Peripheral mechanisms of pheromone reception in moths. Chem. Senses *21*, 257–268.

Kass, J., Jacob, T.C., Kim, P., and Kaplan, J.M. (2001). The EGL-3 proprotein convertase regulates mechanosensory responses of *Caenorhabditis elegans*. J. Neurosci. *21*, 9265–9272.

Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P. (2009). Two chemoreceptors mediate developmental effects of dauer pheromone in *C. elegans*. Science *326*, 994–998.

Lichtman, J.W., and Denk, W. (2011). The big and the small: challenges of imaging the brain's circuits. Science *334*, 618–623.

Liu, Q., Chen, B., Gaier, E., Joshi, J., and Wang, Z.W. (2006). Low conductance gap junctions mediate specific electrical coupling in body-wall muscle cells of *Caenorhabditis elegans*. J. Biol. Chem. *281*, 7881–7889.

Luo, L., Gabel, C.V., Ha, H.I., Zhang, Y., and Samuel, A.D. (2008). Olfactory behavior of swimming *C. elegans* analyzed by measuring motile responses to temporal variations of odorants. J. Neurophysiol. *99*, 2617–2625.

Macosko, E.Z., Pokala, N., Feinberg, E.H., Chalasani, S.H., Butcher, R.A., Clardy, J., and Bargmann, C.I. (2009). A hub-and-spoke circuit drives pheromone attraction and social behaviour in *C. elegans*. Nature *458*, 1171–1175.

Okochi, Y., Kimura, K.D., Ohta, A., and Mori, I. (2005). Diverse regulation of sensory signaling by *C. elegans* nPKC-epsilon/eta TTX-4. EMBO J. *24*, 2127–2137.

Pierce-Shimomura, J.T., Morse, T.M., and Lockery, S.R. (1999). The fundamental role of pirouettes in *Caenorhabditis elegans* chemotaxis. J. Neurosci. *19*, 9557–9569.

Rogers, C., Persson, A., Cheung, B., and de Bono, M. (2006). Behavioral motifs and neural pathways coordinating O_2 responses and aggregation in *C. elegans*. Curr. Biol. *16*, 649–659.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature *359*, 832–835.

Schwartz, H.T., and Horvitz, H.R. (2007). The *C. elegans* protein CEH-30 protects male-specific neurons from apoptosis independently of the Bcl-2 homolog CED-9. Genes Dev. *21*, 3181–3194.

Sengupta, P., Colbert, H.A., and Bargmann, C.I. (1994). The *C. elegans* gene *odr-7* encodes an olfactory-specific member of the nuclear receptor superfamily. Cell *79*, 971–980.

Sieburth, D., Madison, J.M., and Kaplan, J.M. (2007). PKC-1 regulates secretion of neuropeptides. Nat. Neurosci. *10*, 49–57.

Slessor, K.N., Kaminski, L.A., King, G.G.S., Borden, J.H., and Winston, M.L. (1988). Semiochemical basis of the retinue response to queen honey bees. Nature *332*, 354–356.

Srinivasan, J., Kaplan, F., Ajredini, R., Zachariah, C., Alborn, H.T., Teal, P.E., Malik, R.U., Edison, A.S., Sternberg, P.W., and Schroeder, F.C. (2008). A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. Nature *454*, 1115–1118.

Srinivasan, J., von Reuss, S.H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M.C., O'Doherty, O.G., Edison, A.S., Sternberg, P.W., and Schroeder, F.C. (2012). A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. PLoS Biol. *10*, e1001237.

Starich, T.A., Xu, J., Skerrett, I.M., Nicholson, B.J., and Shaw, J.E. (2009). Interactions between innexins UNC-7 and UNC-9 mediate electrical synapse specificity in the *Caenorhabditis elegans* locomotory nervous system. Neural Dev. *4*, 16.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.

Tobin, D., Madsen, D., Kahn-Kirby, A., Peckol, E., Moulder, G., Barstead, R., Maricq, A., and Bargmann, C.I. (2002). Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. Neuron *35*, 307–318.

Tsunozaki, M., Chalasani, S.H., and Bargmann, C.I. (2008). A behavioral switch: cGMP and PKC signaling in olfactory neurons reverses odor preference in *C. elegans*. Neuron *59*, 959–971.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philos. Trans. R. Soc. Lond. B Biol. Sci. *314*, 1–340.

Wyatt, T.S. (2003). Pheromones and Animal Behavior:Communication by Smell and Taste (Cambridge: Cambridge University Press).