FMRFamide-Like Neuropeptides and Mechanosensory Touch Receptor Neurons Regulate Male Sexual Turning Behavior in *Caenorhabditis elegans*

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Caenorhabditis elegans male mating provides a powerful model to study the relationship between the nervous system, genes, and innate sexual behaviors. Male mating is the most complex behavior exhibited by the nematode *C. elegans* and involves the steps of response, backing, turning, vulva location, spicule insertion, and sperm transfer. Because neuropeptides are important neural regulators of many complex animal behaviors, we explored the function of the FMRFamide-like neuropeptide (*flp*) gene family in regulating male copulation. We found that peptidergic signaling mediated by FMRF-amide like neuropeptides (FLPs) FLP-8, FLP-10, FLP-12, and FLP-20 is required for the sensory transduction involved in male turning behavior. *flp-8*, *flp-10*, *flp-12*, and *flp-20* mutant males significantly increase repetition of substep(s) of turning behavior compared with wild-type males. Genes controlling neuropeptide processing and secretion in general, including *egl-3*, *egl-21*, *ida-1*, and *unc-31*, are also required for inhibiting repetitive turning behavior. Neuropeptidergic signaling adjusts the repetitiveness of turning independently of serotonergic modulation of the timing of turning. Surprisingly, the mechanosensitive touch receptor neurons are found to be part of the neural circuitry regulating male turning behavior, indicating the existence of functional dimorphisms in the nervous system with regard to sex-specific behaviors.

Key words: *Caenorhabditis elegans*; FMRFamide-like neuropeptide; male sexual turning; touch receptor neurons; mechanosensory behavior; sexually dimorphic function

Introduction

Sexual behavior is one of the most ancient and vital social behaviors exhibited among metazoans, yet remarkably little is known about its molecular basis. *Caenorhabditis elegans* copulation is comprised of an innate series of behavioral steps (Liu and Sternberg, 1995), raising the question of how these behaviors are genetically controlled. *C. elegans* male mating is a powerful model to study the relationship between the nervous system, genes, and behavior.

The adult male possesses 381 neurons, of which 87 are sex-specific. With the exception of four neurons in the head, the male-specific neurons are located in the copulatory structures in the tail (Sulston et al., 1980). Cell ablation experiments coupled with behavioral analysis have provided important insight into the

neuronal basis of *C. elegans* mating behavior. In wild-type males, a stereotypic behavioral sequence includes response to hermaphrodite contact (requiring male-specific ray neurons), backing along the mate's body, turning at the mate's head or tail (requiring the three most posterior pairs of rays and the male-specific serotonergic CP neurons), location of vulva (requiring the male-specific hook neurons), spicule insertion (requiring male-specific cholinergic neurons of the spicules and postcloacal sensilla), and sperm transfer into the mate's uterus (requiring the male-specific SPV spicule neurons) (Loer and Kenyon, 1993; Liu and Sternberg, 1995; Barr and Sternberg, 1999; Garcia et al., 2001). *C. elegans* male sexual behaviors are also regulated by components of the core nervous system: spicule protraction is modulated by the NSM neurosecretory head neurons, and sperm transfer requires input from the ventral nerve cord motor neurons (Gruninger et al., 2006; Schindelman et al., 2006).

Neuropeptides modulate many complex animal behaviors, including feeding, nociception, circadian rhythm, reproduction, learning, and social behaviors (Storm and Tecott, 2005; Borgland et al., 2006; Sabatier, 2006; Saper, 2006; Hill and Oliver, 2007). The *C. elegans* genome contains at least 30 FMRFamide-like neuropeptide (*flp*) genes that are predicted to encode over 70 biologically active FMRF-amide like neuropeptides (FLPs) (Li, 2005) (S. Husson and L. Schoofs, personal communication). At least 50% of *C. elegans* neurons express one or more *flp* genes (Kim and Li, 2004), indicating their potential roles in the nervous

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system. We explored *flp* gene function in regulating *C. elegans* male sexual behaviors and found that peptidergic signaling modulates the turning step. FLP-8, FLP-10, FLP-12, and FLP-20 are required to suppress the repetitive turning phenotype in *C. elegans* males. Genes controlling neuropeptide processing and secretion in general, like *egl-3*, *egl-21*, *ida-1*, and *unc-31*, are also required for inhibiting repetitive turning behavior. Moreover, peptidergic signaling regulates repetitive turning behavior independently of serotonergic and dopaminergic signaling. Interestingly, the cellular site of action for *egl-3* and *flp-20* in mediating male turning behavior is in the mechanosensitive touch receptor neurons (TRNs) found in both males and hermaphrodites, indicating that the shared nervous system has sexually dimorphic functions. Finally, we demonstrate that the MEC-4/MEC-10 channel complex is required for inhibition of repetitive turning, suggesting that a mechanosensory stimulus regulates the turning step of male mating behavior.

Materials and Methods

Strains

Routine culturing of *C. elegans* was performed as described previously (Brenner, 1974). The following alleles were used: LGI, *aex-5(sa23)*, *bli-4(e937)*, *kpc-1(gk8)*; LGII, *cat-2(e1112)*, *flp-4(yn35)*, *tdc-1(n3420)*; LGIII, *bas-1(ad446)*, *eat-4(ky5)*, *ida-1(ok409)*, *unc-47(e307)*; LGIV, *egl-21(n476)*, *flp-10(pk367)*, *him-8(e1489)*, *unc-17(e245)*, *unc-31(e169)*; LGV, *egl-3(nr2090)*, *flp-6(pk1593)*, *flp-21(pk1601)*, *him-5(e1490)*; LGX, *flp-3(pk361)*, *flp-8(pk360)*, *flp-12(n4902)*, *flp-19(pk1594)*, *flp-20(pk1596)*, *mec-4(e1611, d)*, *mec-4(e1339, rf)*, *mec-10(e1515, lf)*.

flp mutant isolation

flp deletion alleles were isolated by PCR screening of populations of EMS-mutagenized strains (Jansen et al., 1997). *flp-6(pk1593)* is a 1717 bp deletion that spans from –85 bp upstream of the ATG start codon to 32 bp downstream of the TAA stop codon (flanking sequences, tgcttcgtcttaaggattctatctcata and ggctcacacatttaactattttaaataca). The deletion removes the entire coding sequences of the *flp-6* gene. *flp-8(pk360)* is a 1414 bp deletion that removes the entire coding region and some 5' and 3' neighboring sequences of the *flp-8* gene (flanking sequences, aatcaatttcagataattctcagaacaagc and ataatttggcgtcatagttttgatagaa). *flp-10(pk367)* is a 1883 bp deletion that spans 1.3 kb upstream of the start codon, exon 1, and 5' part of intron 1 of the *flp-10* gene (flanking sequences, tattttctt-gcttattctcgctccatc and agtaaaactattcttaataatccattcca). *flp-12(n4902)* is a 1732 bp deletion that removes most of the *flp-12* gene except exon 1 and adjacent 5' part of intron 1 (flanking sequences, aaatgtaacactagacat-agctctccatgt and caatttgaatttctgaataagagcccgaa). *flp-20(pk1596)* is a 1343 bp deletion that removes the entire coding region and some 5' and 3' neighboring sequences of the *flp-20* gene (flanking sequences, acgt-cacggaattcttttaaatgaatgga and ttgacgtattataaaagtaatcgttttgga). None of the above deletions disrupt the promoter region or coding sequence of neighboring genes. *flp-19(pk1594)* is a 1946 bp deletion that removes the 5'-UTR, the entire coding sequence, and 3'-UTR of *flp-19* gene (flanking sequences, cattagcattatattccatagctgccacc and atgcggtgagattgtctgcata-gacgga). It is important to note that the deletion also removes the 3'-UTR, the last exon, the last intron, and the final 17 bp of the penultimate exon of *abl-1* gene. Each *flp* mutant strain was outcrossed at least 10 times.

Male mating behavior

Generating males. To increase the frequency of males, the *him-8* mutation was crossed into *flp-6*, *flp-8*, *flp-12*, *flp-19*, *flp-20*, *egl-3*, *bli-4*, *aex-5*, *kpc-1*, and *ida-1* mutant strains, and the *him-5* mutation was crossed into *flp-10*, *flp-10*; *flp-12 flp-20 flp-8*, *mec-4(e1611)*, *bas-1*, and *mec-4(e1611)*; *bas-1* mutant strains. For other mutant backgrounds, males were generated through the heat shock method (Hodgkin, 1983).

Mating behavior assay. Males were isolated at L4 stage, cultured at 15°C overnight, and the resulting young virgin adults were used for mating assays. Adult, mix-staged (~1–2 d of age) *unc-31* hermaphrodites were used as mates. Mating plates were prepared by inoculating 5 cm nema-

tode growth medium plates with 10 μ l of OP50 (outer diameter, 1.0) and culturing at room temperature (22–23°C) for 1 d. For mating behavior observations, 16–18 hermaphrodites were transferred to the mating plate and allowed to evenly distribute on the bacterial lawn. One male was added to the mating plate for each observation. The observation period was 3 min or until spicule insertion occurred. Behavioral observations were done at room temperature with a Leica (Heerbrugg, Switzerland) MZ12.5 stereomicroscope under 125 $\times$ magnification. To score the repetitiveness of male turning behavior, we calculated T_{avg} (population average of maximum trials per turn exhibited by individual males of a particular genotype). Intergenotypic comparisons of T_{avg} were made using Kruskal–Wallis test (nonrepeated and nonparametric ANOVA test).

Transformation rescue of *egl-3(nr2090)* mutants

Plasmids were constructed by standard molecular cloning techniques, and sequences were verified when necessary. Plasmid KP509 contains the *glr-1* promoter and 4.0 kb of *egl-3* genomic coding region. Plasmid KP454 contains 4.2 kb endogenous *egl-3* promoter, 4.0-kb *egl-3* genomic coding fragment, 2.3 kb 3'-UTR, and an in-frame green fluorescent protein (GFP) fusion to the C terminus (Kass et al., 2001). Plasmid TL1115 and plasmid TL9284 both contain the same *egl-3* genomic coding fragment excised from KP509, but under the control of the *flp-8* and *mec-7* promoter, respectively (plasmid sequence information available in supplemental material, available at www.jneurosci.org). Transgenic strains were generated by microinjecting different plasmid constructs at 30–50 ng/ μ l together with 60 ng/ μ l cotransformation marker coelomocyte::GFP (Miyabayashi et al., 1999) into *egl-3*; *him-8* hermaphrodites. Male turning behavior was examined as described above.

Transformation rescue of *flp-10(pk367)* and *flp-20(pk1596)* mutants

Wild-type genomic *flp-10* and *flp-20* clones (promoter, coding region, 3'-UTR) were amplified with PCR using high-fidelity *TaKaRa LA Taq* polymerase. Primers FLP90 (5'-TAC TCG GCT AAT GAC TAG TGT T-3') and FLP490 (5'-GAG AAC TCA AAG TCT TGA GC-3') were used for *flp-10* locus amplification. The purified *flp-10(+)* PCR product was injected at 33 ng/ μ l into *flp-10*; *him-5* hermaphrodites. Primers FLP285 (5'-GGA AAC ATT GGT CGG GAG ATG-3') and FLP485 (5'-TAG AAT AGG GGT CAA ATA GGA GG-3') were used for *flp-20* locus amplification. The purified *flp-20(+)* PCR product was injected at 17 ng/ μ l into *flp-20*; *him-8* hermaphrodites. *flp-8(+)* or *flp-12(+)* genomic fragments were amplified in a similar manner, and the PCR products were injected at various concentrations (50 ng/ μ l, 5 ng/ μ l, 3 ng/ μ l, and 1 ng/ μ l) into *flp-8*; *him-8* or *flp-12*; *him-8* hermaphrodites, respectively. However, none of the 400+ F1 *flp-8(+)* or 100+ F1 *flp-12(+)* transgenic lines segregated the extrachromosomal array in the F2 generation, possibly because of toxic effects of the transgenes. Cotransformation marker coelomocyte::GFP (Miyabayashi et al., 1999) was injected at 50–100 ng/ μ l to achieve a total DNA concentration of ~100 ng/ μ l. Male turning behavior was examined as described above.

TRN-specific rescue of *flp-20(pk1596)* mutants

Plasmid TL2137 was constructed by inserting *flp-20(+)* genomic coding sequence into Fire Lab *Pmec-7* expression vector pPD52.102 using *KpnI* and *EcoRV* restriction sites. Plasmid TL2137 was microinjected into *flp-20*; *him-8* hermaphrodite constructs at 35 ng/ μ l together with cotransformation marker coelomocyte::GFP (Miyabayashi et al., 1999) at 50 ng/ μ l. Male turning behavior was examined as described above.

Results

We used two strategies to study the role of neuropeptides in *C. elegans* male mating behaviors. First, we analyzed the behavior of mutants defective in *flp* genes (*flp-3*, *flp-4*, *flp-6*, *flp-8*, *flp-10*, *flp-12*, *flp-19*, *flp-20*, and *flp-21*), neuropeptide processing (*egl-3* and *egl-21*), or neuropeptide secretion (*ida-1* and *unc-31*). Second, we determined the cellular site of action of these neuropeptidergic components, which provided insight into the cellular and molecular basis of turning behavior.

The turning repetitiveness in wild-type populations

C. elegans male mating behavior includes the sequential steps of response to hermaphrodite contact, backing, turning, location of vulva, spicule insertion, and sperm transfer (Liu and Sternberg, 1995). Turning behavior can be dissected into three substeps: approaching the mate's body end while backing (a), initiating the turn with a small ventral tail arch (b), and completing the turn with a sharp ventral tail flexure and continued backing along the other side of the hermaphrodite (c) (Fig. 1A) (supplemental Movie 1, available at www.jneurosci.org as supplemental material).

The majority of wild-type males followed the turning behavioral sequence of "a-b-c" for each turn during mating (85% for *him-5* and 88% for *him-8*) (Fig. 1B). A low percentage of wild-type males repeated a turn once or twice (13% for *him-5* and 10% for *him-8*) (Fig. 1B). In this case, the male backed to the mate's body end and initiated a small ventral tail arch, but instead of proceeding to substep (c), he switched to moving forward along the mate's body. He traveled for one-eighth to one-fourth of the hermaphrodite body length before resuming backing and turning, exhibiting the behavioral sequence of "a-b-forwarding-a-b-c." In rare instances, a wild-type male repeated a turn three times before turn execution and continued backing (2% for both *him-5* and *him-8*) (Fig. 1B). We observed similar phenomena in heat-shock-generated N2 males (data not shown). Herein, we refer to two or more turning attempts as a repetitive turn. In summary, repetitive turns exist in wild-type populations but in a low percentage of animals and with a low degree of repetitiveness.

To quantify the turning repetitiveness for a given population, we developed a parameter, T_{avg} , which is the population average of maximum trials per turn. T_{avg} is 1.23 ± 0.06 for *him-5* and 1.18 ± 0.04 for *him-8* (no statistical difference between these two controls) (Fig. 2A). Because T_{avg} takes into account both the number of males exhibiting repetitive turnings and the maximum degree of repetitiveness exhibited by each male, T_{avg} can be used for intergenotypic comparisons of the repetitiveness of male sexual turning behaviors.

FLP signaling pathways regulate male turning behavior

To determine whether *C. elegans* FMRF-amide like neuropeptides regulate male mating behavior, we analyzed the mating behavior of available *flp* mutant males (Table 1). *flp-8(pk360)*, *flp-10(pk367)*, *flp-12(n4902)*, and *flp-20(pk1596)* single mutants exhibited a male repetitive turning phenotype, which is defined as T_{avg} significantly higher than the wild-type control (Fig. 2A). All other single *flp* mutant males successfully executed the mating behavioral steps of response, backing, turning, vulva location, and spicule insertion (Table 1).

The T_{avg} score is designed to evaluate the turning repetitiveness of a given population. However, one cannot easily deduce from T_{avg} how often males fail to initiate a turn at the first attempt or whether the repetitive turning phenotype is progressive (i.e., more severe with subsequent attempts). We therefore examined the percentage of males that exhibit repetitive turning behavior during their first turn. $10 \pm 3\%$ *him-5* males and $5 \pm 2\%$ *him-8* males failed to complete their first turn in one trial (Fig. 2B). There was no significant statistical difference between the two wild-type populations ($p > 0.05$), consistent with results using T_{avg} score.

Consistently, for *flp-8*, *flp-10*, *flp-12*, and *flp-20* mutants, the percentages of males exhibiting repetitive turning behavior in their first turn were all significantly higher than corresponding wild-type controls (Fig. 2B).

Of the turning-defective mutants, *flp-8* and *flp-12* are ex-

Figure 1. The repetitiveness of male sexual turning behavior in wild-type populations. **A**, Turning behavior can be dissected into three substeps: approaching the mate's body end while backing (**a**), initiating the turn with a small ventral tail arch (**b**), and completing the turn with a sharp ventral tail flexure and continued backing along the other side of the hermaphrodite (**c**). Arrows indicate the typical behavioral sequence of a nonrepetitive turning (a-b-c). **B**, Plots of percentage of males against maximum number of trials per turn. The majority of wild-type males completed each turn in the first attempt before moving on to subsequent steps of mating (85% for *him-5* and 88% for *him-8*). A low percentage of wild-type males required two or three trials before completing a turn (13% for *him-5* and 10% for *him-8*). In rare instances, a wild-type male repeated three times for one turn (2% for both *him-5* and *him-8*). The plot curves show that repetitive turns exist in the wild-type populations but at a low percentage of animals and with a low degree of repetitiveness. The number of animals observed per genotype is indicated in parentheses. Values shown are mean $\pm$ SEM.

pressed in a subset of male-specific neurons as well as neurons in the mechanosensory touch circuit (Table 1). To our surprise, *flp-10* and *flp-20* are not expressed in any male-specific neurons (Table 1). Rather, *flp-10* is expressed in the PVR interneuron (White et al., 1986) and *flp-20* in PVR and the touch receptor neurons AVM, ALM, PVM, and PLM (Chalfie et al., 1985), suggesting that the touch circuitry of the core nervous system contributes to turning behavior.

Figure 2. Disruption of *flp* signaling and neuropeptide release increases repetitive turning. **A**, T_{avg} values for *flp-8*, *flp-10*, *flp-12*, and *flp-20* mutants, neuropeptide processing enzyme mutants *egl-3*(*nr2090*) and *egl-21*(*n476*), neuropeptide secretion mutants *ida-1*(*ok409*) and *unc-31*(*e169*), TRN genetically ablated mutants *mec-4*(*e1611*, *d*), and glutamatergic signaling mutants *eat-4*(*ky5*). All mutants are significantly different compared with wild type ($p < 0.001$). T_{avg} of *flp* quadruple mutants is significantly higher than that of *flp* single mutants ($p < 0.01$). **B**, Percentage of males exhibiting repetitive turning behavior in their first turn. All mutants are significantly different compared with wild type ($p < 0.01$ for *eat-4* mutants, and $p < 0.001$ for other mutants). The number of animals observed per genotype is indicated in parentheses. Values shown are mean $\pm$ SEM.

Table 1. *flp* gene expression pattern^a and mutant male mating behavior

Genes	Male-specific neurons	Touch receptor neurons	Select other neurons ^b	Male mating ^c
<i>flp-8</i>	CP9	PVM		Repetitive turning (T_{avg} , 1.76 ± 0.11)
<i>flp-10</i>			PVR	Repetitive turning (T_{avg} , 2.27 ± 0.22)
<i>flp-12</i>	Rays 1, 4, 5, 7 ^d ; CP9		PVR, SDQ	Repetitive turning (T_{avg} , 2.58 ± 0.18)
<i>flp-20</i>		AVM, ALM, PVM, PLM	PVR	Repetitive turning (T_{avg} , 1.93 ± 0.12)
<i>flp-3</i>	Spicule SP(V/D); CP9			WT (T_{avg} , 1.27 ± 0.27)
<i>flp-4</i>		AVM		WT (T_{avg} , 1.06 ± 0.06)
<i>flp-6</i>	Rays 2B, 5B, 6B, 7B ^d			WT (T_{avg} , 1.16 ± 0.08)
<i>flp-19</i>	Rays 5B, 7B, 9B ^d ; CEM			WT (T_{avg} , 1.15 ± 0.07)
<i>flp-21</i>	Spicule SP; CP6–9			WT (T_{avg} , 1.26 ± 0.17)

WT, Wild type.

^aKim and Li, 2004; Lints et al., 2004. We did not observe sexual dimorphism with regard to *flp* gene expression in shared neurons (e.g., *flp-8* is expressed in PVM in both hermaphrodites and males).

^bPVR and SDQ are interneurons connected to the touch circuit via gap junctions or chemical synapses (White et al., 1986).

^cThe mating behavioral steps of response, backing, turning, vulva location, and spicule insertion were scored. Turning repetitiveness (T_{avg}) is shown as mean $\pm$ SEM.

^d*flp-6*, *flp-12*, and *flp-19* reporter strains have mosaic expression in ray sensory neurons.

To establish a causal relationship between the *flp* mutation and the male turning phenotype, we performed transformation rescue experiments on *flp* mutants. The wild-type copy of *flp-10* or *flp-20* gene completely rescued the male repetitive turning phenotype of the corresponding mutant (Fig. 3), indicating that the abnormal male turning phenotype of *flp-10* and *flp-20* mutants is caused by a single gene mutation. We were unable to obtain stable transgenic lines expressing wild-type *flp-8*(+) or *flp-12*(+), perhaps because of toxic effects of the transgenes.

To examine genetic interactions, we constructed a *flp-10*; *flp-12* *flp-20* *flp-8* quadruple mutant and analyzed the turning behavior. The male repetitive turning phenotype of the quadruple

mutants was significantly more severe, with T_{avg} increasing from the 1.8–2.6 range in single mutants to 3.5 (Fig. 2A), indicating that these genes act in a partially redundant manner. Combined, these results demonstrate that multiple FLP peptidergic signaling pathways mediated by FLP-8, FLP-10, FLP-12, and FLP-20 function to regulate *C. elegans* male turning behavior.

The neuropeptide processing enzymes EGL-3 and EGL-21 and neuropeptide secretion regulators IDA-1 and UNC-31 are required for male turning behavior

Newly translated neuropeptide precursors are targeted to the endoplasmic reticulum–Golgi pathway for posttranslational modification, and the final mature products are stored in dense-core vesicles (DCVs) for secretion. Several genes involved in these processes have been identified and characterized in *C. elegans*, including the *kex-2*/subtilisin like proprotein convertase (PC) gene *egl-3*, the carboxypeptidase gene *egl-21*, the DCV cargo release regulatory gene *ida-1*, and the calcium-dependent activator protein for secretion gene *unc-31* (Livingstone, 1991; Kass et al., 2001; Zahn et al., 2001; Jacob and Kaplan, 2003). Each neuropeptide processing and secretion component controls multiple behavioral programs. Although immunostaining and mass spectrometry studies have shown that *egl-3* and *egl-21* mutants have significantly reduced levels of mature FMRamide-related peptides (Jacob and Kaplan, 2003; Husson et al., 2006), there have been no functional studies linking EGL-3, EGL-21, IDA-1, or UNC-31 to any of the *C. elegans* neuropeptides. Our newly identified male repetitive turning phenotype of the *flp* mutants provided an opportunity to genetically address the question.

egl-3, *egl-21*, *ida-1*, and *unc-31* single mutants all displayed the repetitive turning phenotype exhibited by *flp* mutants. The T_{avg} scores of all four mutants are significantly higher than wild-type control (Fig. 2A), and the percentages of males exhibiting repetitive turning behavior in

their first turn were also significantly higher than wild type (Fig. 2B). *egl-3*(*nr2090*) mutants have the most severe male repetitive turning phenotype (supplemental Movie 2, available at www.jneurosci.org as supplemental material). The T_{avg} for *egl-3*(*nr2090*) is 5.30 ± 0.31 , and $78 \pm 3\%$ *egl-3* males failed to complete their first turn in one trial (Fig. 2A,B). To confirm that the *egl-3* repetitive turning phenotype is attributable to deletion of the *egl-3* gene, we performed transformation rescues. Expression of wild-type *egl-3*(+) under its endogenous promoter rescued the male repetitive turning phenotype, and T_{avg} was decreased to 1.82 ± 0.15 ($p > 0.05$, compared with wild type) (Fig. 4A). Mutations in the other *C. elegans* PC genes *bli-4*, *aex-5*, and

Figure 3. Transformation rescue of the *flp-10* and *flp-20* mutant repetitive turning phenotype. **A**, Distribution curves of “percentage of males” versus “maximum trials per turn” for *him-5* wild-type control (WT), *flp-10* mutant, and *flp-10* rescue populations. The *flp-10* mutant population has a plot curve significantly different from the WT population, whereas the *flp-10* rescued population shows a plot curve similar to WT. T_{avg} of *flp-10* mutants is 2.27 ± 0.22 ($p < 0.001$ compared with wild type), whereas T_{avg} is lowered to 1.31 ± 0.10 in *flp-10* rescued males ($p > 0.05$ compared with wild type). **B**, Distribution curves of “percentage of males” versus “maximum trials per turn” for *him-8* wild-type control, *flp-20* mutant, and *flp-20* rescue populations. The *flp-20* mutant population has a plot curve significantly different from the WT population, whereas the *flp-20* rescued population shows a plot curve similar to WT. T_{avg} of *flp-20* mutants is 1.93 ± 0.12 ($p < 0.001$ compared with wild type), whereas T_{avg} is lowered to 1.23 ± 0.09 in *flp-20* rescued males ($p > 0.05$ compared with wild type).

Figure 4. Effects of neuron-specific expression of *egl-3* and *flp-20* on the male repetitive turning phenotype of *egl-3(nr2090)* and *flp-20(pk1596)* mutants, respectively. **A**, Expression of wild-type *egl-3* under its endogenous promoter rescued the repetitive turning phenotype, and T_{avg} is decreased to 1.82 ± 0.15 ($p > 0.05$, compared with wild type). Expression of wild-type *egl-3* in all six TRNs using the *mec-7* promoter partially but significantly rescued the *egl-3* mutant repetitive turning phenotype. One representative rescued line is shown (T_{avg} , 2.17 ± 0.20 ; $p < 0.001$, compared with *egl-3*). Expression of wild-type *egl-3* exclusively in PVM using the *flp-8* promoter partially rescued the repetitive turning phenotype, although not as well as *egl-3* TRN rescue. One representative rescued line is shown (T_{avg} , 3.23 ± 0.30 ; $p < 0.001$, compared with *egl-3* mutants). Expressing *egl-3* in command interneurons using the *glr-1* promoter did not have any rescuing effect on the male repetitive turning phenotype (T_{avg} , 4.96 ± 0.51 ; $p > 0.05$ compared with the *egl-3* mutant). **B**, Expression of wild-type *flp-20* in TRNs using the *mec-7* promoter rescued the *flp-20* mutant repetitive turning phenotype. One representative rescued line is shown (T_{avg} , 1.24 ± 0.08 ; $p > 0.05$, compared with wild type). The behavioral data for *egl-3* and *flp-20* mutants are the same as shown in Figure 2A. The number of animals observed per genotype is indicated in parentheses. Values shown are mean $\pm$ SEM. WT, Wild-type control *him-8(e1489)*.

kpc-1 (Thacker and Rose, 2000) did not affect male turning behavior (data not shown), consistent with *egl-3* acting as the PC that processes FLP-8, FLP-10, FLP-12, and FLP-20.

EGL-3 acts in touch receptor neurons to regulate male turning behavior

To determine the *egl-3* site of action and gain insight into the cellular basis of turning behavior, we performed tissue-specific

rescue of the *egl-3* male repetitive turning phenotype. *egl-3* is expressed in male-specific ray neurons (data not shown) and some interneurons and sensory neurons of the shared nervous system, including the TRNs (Kass et al., 2001). Two of the *flp* genes (*flp-10* and *flp-20*) with mutants exhibiting repetitive turning are not expressed in ray neurons but rather the touch circuitry. These results led us to hypothesize that neuropeptides may function in the shared nervous system to regulate turning.

To test this hypothesis, we used different cell-specific promoters to express wild-type *egl-3*(+) in select neurons. *egl-3* is broadly expressed in the shared nervous system, and anti-*egl-3* PC2 antibody staining identified a subset of *egl-3*-expressing neurons, which includes the polymodal ASH sensory neurons that mediate nose touch response, the TRNs ALM and AVM that mediate light body touch response, another TRN PVM with no assigned function in hermaphrodite, and the command interneurons AVB, PVC, and AVD that regulate forward and reverse locomotion (Kass et al., 2001). Although there has been no report of *egl-3* expression in PLM touch receptor neurons, *egl-3* might indeed be expressed in these TRNs. Additional experimentation is needed to clarify this ambiguity. We found that expression of wild-type *egl-3* in all six TRNs using the *mec-7* promoter (Hamelin et al., 1992) partially but significantly rescued *egl-3* mutant repetitive turning phenotype. T_{avg} values were lowered from 5.30 ± 0.31 in *egl-3* mutants to 2.17 ± 0.20 and 2.19 ± 0.19 in two rescued lines, respectively ($p < 0.001$ compared with *egl-3*) (Fig. 4A). The partial rescue may reflect a requirement of *egl-3* function in other cell types, such as ray sensory neurons.

flp-8 is expressed in PVM neuron, and loss of *flp-8* induces a repetitive turning phenotype in males (Fig. 2, Table 1). These observations strongly suggest that PVM, which has no known function in hermaphrodites (Chalfie et al., 1985), acquires a mechanosensory function in males. We tested this hypothesis by restoring wild-type *egl-3* expression exclusively in PVM using the *flp-8* promoter. Two transgenic lines were partially rescued for the repetitive turning phenotype, with T_{avg} lowered to 3.23 ± 0.30 and 3.94 ± 0.50 , respectively ($p < 0.001$, compared with *egl-3* mutants) (Fig. 4A). These results indicate that the PVM neuron contributes to male turning behavior in *C. elegans*.

Consistent with a role for peptidergic transmission in the TRNs, restoring *flp-20*(+) expression in TRNs using the *mec-7* promoter completely rescued the male repetitive turning defect of *flp-20* mutants. T_{avg} values were lowered from 1.93 ± 0.12 in *flp-20* mutants to 1.24 ± 0.08 and 1.26 ± 0.10 in two rescued lines, respectively ($p > 0.05$, compared with wild type) (Fig. 4B). We conclude that *egl-3*-regulated peptidergic signaling in TRNs is required to mediate male turning. In contrast, expressing *egl-3*(+) in command interneurons with the *glr-1* promoter (Brockie et al., 2001) does not have any rescuing effect on the male repetitive turning phenotype ($p > 0.05$ compared with *egl-3*) (Fig. 4A). Because command interneurons postsynaptically couple TRNs to the locomotion circuits, we conclude that the *egl-3* repetitive turning phenotype reflects a defect in the TRNs rather than involving more downstream components.

The TRNs and MEC-4/MEC-10 DEG/ENaC channel inhibit repetitive turning behavior

To confirm the role of TRNs in mediating male turning, we examined turning behavior of males lacking these cells. The *mec-4(e1611)* gain-of-function mutation, or *mec-4(d)*, causes constitutive activation of MEC-4/MEC-10 DEG/ENaC channel and TRN degeneration (Driscoll and Chalfie, 1991; Goodman et al., 2002). *mec-4(d)* mutants exhibited the male repetitive turning

Table 2. Turning behavior of TRN mechanosensory and neurotransmitter signaling mutants

Genotype	Defective signaling pathway(s)	Male turning phenotype
<i>him-5(e1490)</i>	WT	WT (T_{avg} 1.23 $\pm$ 0.06; n = 102)
<i>mec-4(e1611, d)</i>	TRN mechanosensation	Repetitive turning (T_{avg} 2.09 $\pm$ 0.11*; n = 131)
<i>mec-4(e1339, rf)</i>	TRN mechanosensation	Repetitive turning (T_{avg} 1.65 $\pm$ 0.07**; n = 281)
<i>mec-10(e1515)</i>	TRN mechanosensation	Repetitive turning (T_{avg} 2.01 $\pm$ 0.12*; n = 181)
<i>eat-4(ky5)</i>	Glutamate	Repetitive turning (T_{avg} 2.35 $\pm$ 0.14*; n = 108)
<i>bas-1(ad446)</i>	Serotonin and dopamine	Late turn, 52% $\pm$ 7% (T_{avg} 1.28 $\pm$ 0.11; n = 46)
<i>cat-2(e1112)</i>	Dopamine	Wide turn, 43% $\pm$ 8% (T_{avg} 1.03 $\pm$ 0.03; n = 37)
<i>tdc-1(n3420)</i>	Tyramine and octopamine	WT (T_{avg} 1.13 $\pm$ 0.05; n = 40)
<i>unc-47(e307)</i>	GABA	WT (T_{avg} 1.06 $\pm$ 0.04; n = 31)

Turning repetitiveness (T_{avg}) of *mec-4(e1611, d)*, *mec-4(e1339, rf)*, *mec-10(e1515)*, and *eat-4(ky5)* mutant males are significantly higher than wild type (* p < 0.001; ** p < 0.01). T_{avg} values for *bas-1(ad446)*, *cat-2(e1112)*, *tdc-1(n3420)*, or *unc-47(e307)* mutant males are not significantly different from wild type (p > 0.05). We were unable to characterize the male turning behavior of acetylcholine signaling defective *unc-17(e245)* mutants because of their severely uncoordinated phenotype (Sandoval et al., 2006). *d*, Dominant allele; *rf*, reduction-of-function allele; WT, wild type.

phenotype with T_{avg} 2.09 $\pm$ 0.11 (p < 0.001 compared with wild type) (Table 2), indicating that TRNs provide sensory input to the neural circuit regulating male turning behavior.

To determine whether the MEC-4/MEC-10 mechanosensory complex plays a role in sexually dimorphic mechanosensation, we analyzed the male turning behavior of *mec-4(e1339)* reduction-of-function (*rf*) and *mec-10(e1515)* loss-of-function (*lf*) mutants (Chalfie and Sulston, 1981). *e1339* was selected as the *mec-4* reference allele and is not as strong as other loss-of-function mutations. Both *mec-4(rf)* and *mec-10(lf)* mutants displayed the male repetitive turning phenotype, with T_{avg} 1.65 $\pm$ 0.07 and 2.01 $\pm$ 0.12, respectively (both significantly different from wild type) (Table 2). The requirement for MEC-4/MEC-10 channels in male turning suggests the mechanosensitive nature of this sexually dimorphic behavior.

The repetitive turning defect of *mec-4(d)* males may be a direct consequence of no TRNs. Alternatively, the repetitive turning phenotype could reflect a more general problem with coordinated locomotion because of an altered balance in the activity of the AVB/PVC and AVA/AVD/AVE command interneurons. Two lines of evidence argue against the latter. First, *mec-4(d)* males respond normally to hermaphrodite contact. In contrast, *unc-47* GABAergic mutant males, which have altered command interneuron signaling and a backward locomotion defect, respond poorly to hermaphrodite contact but do not exhibit repetitive turning (Table 2). Second, *mec-4(d)* males exhibit the backing–forwarding switch specifically after the turning initiation substep but not randomly as would be predicted for command interneuron signaling defect. We conclude that the TRNs provide sensory input to the male turning neural circuit via the MEC-4/MEC-10 mechanosensory receptors.

Glutamatergic neurotransmission is required for male turning behavior

C. elegans TRNs sense mechanical stimuli and provide input to the command interneurons via glutamatergic synaptic connections (Maricq et al., 1995; Brockie et al., 2001). To determine whether glutamate influences turning behavior, we assayed the *eat-4(ky5)* mutant. *eat-4* encodes a sodium-dependent inorganic

phosphate cotransporter required for glutamatergic neurotransmission (Lee et al., 1999). *eat-4* mutants are defective in many behaviors, including chemotaxis, feeding, thermotaxis, and foraging. Interestingly, *eat-4* mutants are also defective in responses to gentle touch mediated by the TRNs (Lee et al., 1999), which is the apparent site of action for *egl-3* and several *flp* genes. We found that *eat-4(ky5)* mutants displayed the male repetitive turning phenotype (p < 0.001, compared with wild type) with a phenotypic severity similar to TRN genetically ablated *mec-4(d)* mutants (p > 0.05) (Table 2). The percentage of *eat-4* mutant males exhibiting repetitive turning behavior in their first turn was also significantly higher than wild type (p < 0.01) (Fig. 2B). We conclude that glutamatergic signaling is required for male turning behavior.

We extended the survey of male turning behavior to include mutants defective in the classical neurotransmitters dopamine, serotonin, tyramine, octopamine, and GABA. Consistent with published reports, we found that 52% of the dopamine- and serotonin-deficient *bas-1(ad446)* mutant males exhibited a late-turn phenotype, and 43% of the dopamine-deficient *cat-2(e1112)* mutant males had a wide-turn phenotype (Table 2). A late turn is defined as when the male backs off the mate's body end and fails to initiate turning in a timely manner, whereas a wide turn is defined as when the male is unable to execute the sharp ventral tail coil and loses contact with mate's body after the turn (Loer and Kenyon, 1993). Tyramine- and octopamine-deficient *tdc-1(n3420)* mutants (Alkema et al., 2005) and vesicular GABA-transporting defective *unc-47(e307)* mutants (Eastman et al., 1999) were phenotypically wild type for male turning behavior (Table 2). However, none of the mutants displayed the repetitive turning phenotype (Table 2). These results indicate that dopamine, serotonin, tyramine, octopamine, and GABA do not individually contribute to turning repetitiveness.

TRNs mediate male sexual turning independently of ray neurons

Ray sensory neurons are required for the timing element of male turning. Males with the three most posterior pairs of rays ablated frequently swim off hermaphrodites instead of making turns in a timely manner (the late-turn phenotype) (Liu and Sternberg, 1995). Our finding that the TRNs inhibit the repetitiveness of male turning suggests that the mechanosensory input from TRNs is also an important regulatory factor for male turning (Fig. 5A). How do these two groups of sensory neurons function in the male turning circuitry? We propose two models (Fig. 5B). In the first “parallel” model, TRNs and ray sensory neurons independently sense hermaphrodite turning cues to coordinate the activities of their downstream neurons. In the second “linear” model, TRNs sense the change of the male's body posture resulting from tail curling (i.e., the motor output downstream of ray neurons acts as a proprioceptive signal for TRNs).

To distinguish between the two models, we examined the male turning behavior of *mec-4(d);bas-1* double mutants. *mec-4(d)* single mutant males exhibited the repetitive turning phenotype but not the late-turn phenotype (Fig. 5C). *bas-1* single mutant males presented the late-turn phenotype but not the repetitive turning phenotype (Fig. 5C). We separately calculated T_{avg} and the percentage of males exhibiting late turns for the *mec-4(d);bas-1* double mutants and found both phenotypes were intact (Fig. 5C), supporting the parallel model. We conclude that TRNs function independently of ray neurons to mediate male sexual turning behavior.

Discussion

Neuropeptide regulation of male turning behavior

Neuropeptides are important neural regulators of many complex animal behaviors such as sleep, feeding, nociception, reproduction, and social behaviors (Storm and Tecott, 2005; Borgland et al., 2006; Sabatier, 2006; Saper, 2006; Hill and Oliver, 2007). FMRamide-like neuropeptides constitute a major family of *C. elegans* neuropeptides (Li et al., 1999; Kim and Li, 2004). Although the neuronal expression patterns of the *flp* gene family have been characterized, limited evidence links neuropeptides to the regulation of genetically programmed behaviors. FLP-18 and FLP-21 have been identified as ligands differentially activating two variants of the neuropeptide Y receptor NPR-1 in the regulation of social feeding behavior (Rogers et al., 2003). Exogenously applied FLP-11A and FLP-13A inhibit pharyngeal muscle activity, whereas FLP-8, FLP-17A, and FLP-17B have excitatory effects (Papaioannou et al., 2005). Our finding that mutations in *flp-8*, *flp-10*, *flp-12*, and *flp-20* cause a male turning defect implicates FLPs in regulating the most complex form of *C. elegans* behaviors: the male copulation ritual. In support of this finding, proprotein convertase mutant *egl-3*, carboxypeptidase mutant *egl-21*, and DCV secretion regulatory mutants *ida-1* and *unc-31* exhibit the same male turning defect. The repetitive turning defect of *egl-3* mutants is much more severe than any of the *flp* single mutant or quadruple mutants, suggesting there are other unidentified neuropeptides that regulate male turning behavior. Combined, these results indicate that multiple FLP peptidergic signaling pathways regulate *C. elegans* male turning behavior.

The relationship between peptidergic and classical neurotransmitter signaling is an interesting topic. In the mammalian brain, the neuropeptide orexin A contributes to learning and addiction via modulation of glutamatergic excitability in dopaminergic neurons (Borgland et al., 2006). With respect to *C. elegans* male turning behavior and classical neurotransmitter signaling, we found that only the *eat-4* glutamate signaling mutant exhibits the repetitive turning phenotype, suggesting that FLP and glutamate signaling coregulate male turning behavior. *mec-4(e1611)* TRN-ablated males have a T_{avg} value similar to that of *flp-20* and *eat-4* mutants ($p > 0.05$), hinting that FLP-20 and glutamate act in the same pathway to regulate TRN-command interneuron synaptic transmission. Dopaminergic and serotonergic signaling are also involved in turning behavior (Loer and Kenyon, 1993; Carnell et al., 2005) but in a circuitry and genetic pathway distinct from FMRamide-like peptidergic signaling. Together, these results suggest that an intricate signaling network consisting of FLPs, dopamine, serotonin, and glutamate controls male sexual turning behavior.

C. elegans male sexual behavior and the turning step of male copulation

The innate sexual behavior of the *C. elegans* male involves a complex series of stereotyped steps that are evoked by multimodal

Figure 5. Neuronal circuitry of male turning behavior. **A**, Sensory neurons that regulate male turning behavior. An adult *C. elegans* male has six TRNs: ALML/R, AVM, PLML/R, and PVM (indicated in yellow). Each male also has nine pairs of bilaterally symmetric rays (indicated in green), each of which possesses two sensory neurons. Both groups of sensory neurons are required for male turning. This figure is modified from the study by Kaplan and Horvitz (1993). **B**, Models for male turning circuitry. In the parallel model (indicated by red arrows), TRNs and ray sensory neurons independently sense hermaphrodite turning cues to coordinate the activities of their downstream neurons. In the linear model (indicated by black arrows), TRNs sense the change of the male's body posture as a result of tail curling. Our data support the parallel model, in which two distinct sensorimotor circuits, the touch circuitry (left column) and the ray sensorimotor circuitry (right column), function in parallel to regulate male sexual turning. **C**, *mec-4(d)* males exhibit the repetitive turning phenotype (T_{avg} significantly higher than wild-type animals; $p < 0.001$) but not the late-turn phenotype. *bas-1* males exhibit the late-turn phenotype but not the repetitive turning phenotype. In the *mec-4(d);bas-1* double mutants, both turning phenotypes are intact. The black bar indicates T_{avg} , and the white bar indicates percentage of males exhibiting late turns. The number of animals observed per genotype is indicated in parentheses. Values shown are mean $\pm$ SEM. WT, Wild-type control *him-5(e1490)*.

sensory information provided by his mating partner. First, a long-range pheromone acts to attract males (Chasnov et al., 2007). Next, a short-range and short-lived hermaphrodite signal triggers male searching behavior (Lipton et al., 2004). A distinct, diffusible hermaphrodite cue influences male locomotion (Simon and Sternberg, 2002). Once in close proximity to a potential mate, the male initiates the copulatory behavioral program that includes the steps of response to hermaphrodite contact, backing, turning, location of vulva, spicule insertion, and sperm transfer. Each step is genetically separable and controlled by its own neural circuitry (Loer and Kenyon, 1993; Liu and Sternberg, 1995; Barr and Sternberg, 1999; Garcia et al., 2001; Schindelman et al., 2006). Each step likely involves distinct sensory cues from the mate. For example, the response and vulva location steps may involve chemical and mechanical signals from the hermaphrodite's body (J. Wang, M.M.B., and P. W. Sternberg, unpublished observation). The sperm transfer step requires the hermaphrodite's uterus (Emmons and Sternberg, 1997). The identity of the turning cue is not known but likely mechanosensory in nature, based on our findings that the TRNs regulate turning repetitiveness.

Male turning behavior involves spatial and temporal regulation to execute a sharp ventral tail coil (Loer and Kenyon, 1993; Liu and Sternberg, 1995). Herein, careful observation of turning behavior in wild-type males ($n = 278$) reveals a third criterion (i.e., turning repetitiveness) as gauged by T_{avg} (population average of maximum trials per turn exhibited by individual male). We propose that a low degree of turning repetitiveness ($T_{avg} \sim 1.18$ – 1.23 in wild-type males) may serve to ensure accurate timing for turning. The practice of increasing redundancy to enhance accuracy is used in several systems, including the control of behavioral performance by the rat motor cortex and in diagnostic reasoning by the human brain (Bloch et al., 2003; Narayanan et al., 2005).

When a *C. elegans* male is engaged in mating behavior, his decision to turn is constantly challenged, particularly in the natural environment with a free-moving partner. A mechanism for fine-tuning and adjusting turning behavior is essential for successful copulation. In contrast, excessive repetitiveness of turning (the repetitive turning phenotype, as defined by T_{avg} value significantly higher than wild-type males) is likely to lower behavioral efficiency and hence put the male at a reproductive disadvantage.

We cannot rule out the possibility that repetitive turning phenotype is a consequence of a failure in the turning program. However, we think the latter possibility is unlikely based on three lines of evidence. First, repetitive turning behavior exists in the wild-type population. Second, *bas-1(ad446)* mutant males are defective in turning timing but do not have the repetitive turning phenotype. Third, both wild-type (Loer and Kenyon, 1993) and *egl-3(nr2090)* mutant males (T.L. and M.M.B., unpublished observation) produce sharp ventral tail coils when soaked in exogenous serotonin. Therefore, we favor the theory that repetitive turning is a normal behavior, and the repetitive turning phenotype results from an abnormal expansion of a wild-type behavior.

The neuronal regulation of male sexual turning behavior

The complexity of male turning behavior is reflected in its underlying neural circuitry. Several male-specific nervous system components have been identified previously as key components of the circuitry. At the sensory input level, the serotonergic ray neurons signal the male when to turn (Sulston and Horvitz, 1977; Loer and Kenyon, 1993; Liu and Sternberg, 1995). At the motor output level, CP motor neurons and diagonal muscles are required for the execution of ventral tail flex (Loer and Kenyon, 1993). Several turning-defective mutants further support the roles of these male-specific structures in mediating male turning. For instance, *MSI-1*, a neural RNA-binding protein involved in posttranscriptional gene regulation, is expressed in ray sensory neurons and CP neurons, and *msi-1* mutant males have the wide-turn and late-turn phenotypes (Yoda et al., 2000). *MAB-23* is a DM domain transcription factor necessary for the differentiation of sex-specific tissues, and *mab-23* males are unable to adopt the appropriate turning posture because of a diagonal muscle differentiation defect (Lints and Emmons, 2002). The IP_3 receptor gene *itr-1* is expressed in CP8/9, and diagonal muscles and *itr-1* mutant males also exhibit the wide-turn and late-turn phenotypes (Gower et al., 2005). Hence, the “ray sensorimotor circuit” is one element required for copulatory turning (Fig. 5B, right column).

Here, we demonstrate that the touch circuitry of the core nervous system (Chalfie et al., 1985; White et al., 1986) acts as a second element required for proper turning. First, we observed a repetitive turning phenotype in *flp-10* and *flp-20* mutants, yet *flp-10* and *flp-20* are not expressed in any male-specific neurons or muscles. We also showed that *egl-3* neuropeptide processing enzyme mutant males exhibit the same repetitive turning defect. Additionally, restoring *egl-3(+)* expression in all six TRNs partially but significantly rescues the *egl-3* mutant turning phenotype. In addition, *flp-20* TRN-specific expression completely rescues the *flp-20* mutant turning phenotype. Finally, we found that genetic ablation of TRNs in *mec-4(d)* animals or genetic disruption of the MEC-4/MEC-10 DEG/ENaC mechanosensory channel produces the male repetitive turning phenotype. These observations support the conclusion that mechanosensory TRNs of the shared nervous system are required to mediate the male sexual turning behavior. Also interesting is the fact that PVM-specific expression of *egl-3(+)* partially rescues the *egl-3* mutant turning

defect. These data provide the first genetic evidence that PVM neuron functions in the male mating behavioral program and exemplifies the functional dimorphism of the *C. elegans* nervous system.

Our model of male turning involves two distinct sensorimotor circuits acting in concert to control a behavioral outcome. We propose that the touch circuit coordinates backward movement with the tail turning event, which is controlled by the ray sensorimotor circuit (Fig. 5B). Cross talk between both circuits can be readily embraced into the current model. How the core and sex-specific nervous systems communicate and how gender-specific modifications of the shared nervous systems contribute to copulatory behaviors are not known. Reconstruction of the male *C. elegans* nervous system will enable dissection of the neural circuitries that generate complex sexual behaviors.

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