nature neuroscience

FMRFamide neuropeptides and acetylcholine synergistically inhibit egg-laying by *C. elegans*

Niels Ringstad & H Robert Horvitz

Egg-laying behavior of the *Caenorhabditis elegans* hermaphrodite is regulated by G protein signaling pathways. Here we show that the egg laying–defective mutant *egl-6(n592)* carries an activating mutation in a G protein–coupled receptor that inhibits *C. elegans* egg-laying motor neurons in a G_o-dependent manner. Ligands for EGL-6 are Phe-Met-Arg-Phe-NH_2 (FMRFamide)-related peptides encoded by the genes *flp-10* and *flp-17*. *flp-10* is expressed in both neurons and non-neuronal cells. The major source of *flp-17* peptides is a pair of presumptive sensory neurons, the BAG neurons. Genetic analysis of the *egl-6* pathway revealed that the EGL-6 neuropeptide signaling pathway functions redundantly with acetylcholine to inhibit egg-laying. The retention of embryos in the uterus of the *C. elegans* hermaphrodite is therefore under the control of a presumptive sensory system and is inhibited by the convergence of signals from neuropeptides and the small-molecule neurotransmitter acetylcholine.

Network connectivity and the biophysical properties of component neurons generate motor programs that are the bases of innate behaviors. How motor programs are modulated by sensory systems and how they couple to other neural circuits to create natural behaviors remain major questions in neuroscience. The egg-laying behavior of the *C. elegans* hermaphrodite is a simple behavior that is stimulated by the detection of a bacterial food source[1,2]. The study of *C. elegans* mutants defective in egg-laying behavior offers the opportunity to identify genetic, molecular and cellular pathways that control a modulated behavior.

A simple neuromuscular circuit is required for *C. elegans* egg-laying behavior. Sixteen vulval and uterine muscles, all connected by gap junctions, contract during egg-laying. Vulval muscles receive synaptic input from two classes of motor neurons: VC neurons in the ventral nerve cord and sublateral HSN motor neurons[3]. HSN neurons provide excitatory serotonergic input to vulval muscles[1,2] and might also provide cholinergic input[4]. The VC neurons are cholinergic[4] and might also contain serotonin[5]. VC neurons have been reported to inhibit or promote egg-laying behavior under different experimental conditions[6,7].

Molecular characterization of genes required for normal egg-laying by *C. elegans* has shown that G protein signaling pathways antagonistically regulate egg-laying behavior. *C. elegans* G_q and G_o stimulate and inhibit, respectively, egg-laying behavior[8–10], as do G_q and G_o effectors[11–13]. Metabotropic serotonin receptors, which are likely to couple to a G_q signaling pathway, act on the vulval muscles[14–16]. The prohormone convertase EGL-3 and the carboxypeptidase EGL-21 promote egg laying, suggesting the existence of excitatory peptidergic signals[17,18] that might also act through G_q signaling. Neurochemical signals that activate the inhibitory G_o pathway have not been defined, although acetylcholine acting through the muscarinic receptor GAR-2 and unidentified ligands for the orphan G protein–coupled receptor (GPCR) EGL-47 are candidates[7,19].

Here we show that *egl-6*, defined by the egg-laying defective mutant *egl-6(n592)*, encodes a GPCR for FMRFamide-related peptides (FaRPs) that inhibit the HSN motor neurons in a G_o-dependent manner. One source of peptide ligands for EGL-6 is a pair of presumptive sensory neurons, the BAG neurons, suggesting that FaRP signaling integrates a sensory system with the egg-laying motor program. Loss of function of both the *egl-6* pathway and acetylcholine signaling causes a strong, synthetic hyperactive egg-laying phenotype. FaRP neuropeptides therefore act redundantly with acetylcholine to inhibit egg-laying, indicating that inhibition in a motor program can arise from the convergence of two distinct neurochemical signals.

RESULTS

egl-6(n592) causes increased activity of an orphan GPCR

The mutation *n592*, which defines the gene *egl-6*, causes a semidominant egg-laying defect that is bypassed by drugs that act directly on egg-laying muscles[2]. We hypothesized that *egl-6(n592)* activates a pathway that inhibits egg-laying, and we cloned the affected gene.

We mapped *egl-6(n592)* to a 167-kb interval on linkage group X. PCR products spanning this interval derived from wild-type genomic DNA were unable rescue the egg-laying–defective phenotype of *egl-6(n592)* mutants. We tested comparable PCR products from *egl-6(n592)* DNA for the ability to phenocopy the *n592* mutation. A PCR product predicted to contain only the gene *C46F4.1* caused a severe egg-laying–defective phenotype (**Fig. 1a**). The comparable PCR product derived from wild-type DNA also caused an egg-laying–defective phenotype (**Fig. 1a**).

Howard Hughes Medical Institute, McGovern Institute for Brain Research, Department of Biology, 68-425, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA. Correspondence should be addressed to H.R.H. (horvitz@mit.edu).

Received 14 April; accepted 17 July; published online 21 September 2008; doi:10.1038/nn.2186

a

egl-6 mutation	Egl lines
n592	15/15
Wild type	23/23
Frameshift at *Bgl*II site	0/16
Stop in exon 1a	31/31
Stop in exon 1b	15/15
Stops in exons 1a and 1b	0/7

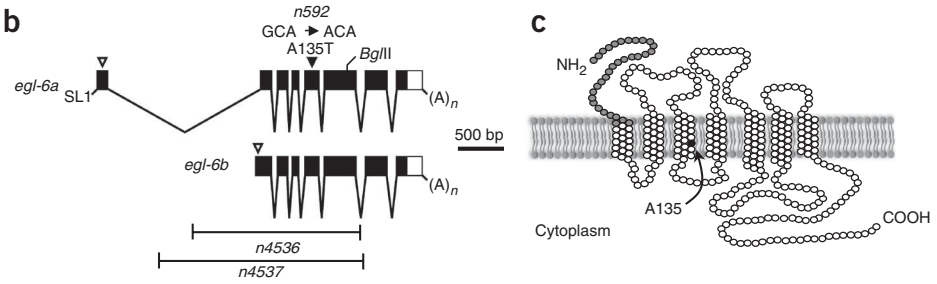


Figure 1 *egl-6* encodes two isoforms of a seven-pass transmembrane receptor. (**a**) The effects of *egl-6* mutations on egg-laying. The number of transgenic lines with egg-laying defects as a fraction of lines generated is shown for each mutation. Mutated sites are indicated in **b** as open arrowheads. (**b**) *egl-6* intron-exon structure and mutations. Coding sequences are depicted as solid boxes. Closed arrowhead indicates the position of the *n592* mutation, which is predicted to alter both isoforms by changing Ala135 to Thr (EGL-6A sequence coordinates). Sequences deleted in alleles *n4536* and *n4537*, which remove most of the *egl-6* coding sequence, are indicated. Open arrowheads indicate the positions of amber nonsense mutations introduced into an *egl-6* transgene, and a frameshift was created at the *Bgl*II site indicated. (**c**) Predicted secondary structure of EGL-6A. Both predicted *egl-6* protein products have seven transmembrane domains. The first 32 residues of EGL-6A, which are replaced by 30 different residues in EGL-6B, are shaded gray. Ala135, predicted to be mutated to threonine by the *n592* mutation, is shaded black.

We determined the structure of *C46F4.1* transcripts by RT-PCR and 3′ and 5′ rapid amplification of cDNA ends (RACE) experiments. *C46F4.1* generates two transcripts with alternative translational starts (**Fig. 1b**), each predicted (see below) to encode an orphan GPCR (**Fig. 1c**). Disrupting both open reading frames, either by introducing stop codons after both predicted translational start sites or by introducing a frameshift in a shared exon, abrogated the ability of *C46F4.1* transgenes to phenocopy *n592* (**Fig. 1a**); disrupting either open reading frame alone did not have this effect. We identified a missense mutation, predicted to change an alanine in the third transmembrane domain to threonine, in the *C46F4.1* coding sequence in *n592* mutants (**Fig. 1b**,**c**). Given our mapping results, the ability of *C46F4.1* transgenes to phenocopy *egl-6(n592)* and the identification of a mutation in the coding sequence of *C46F4.1*, we concluded that *egl-6* is *C46F4.1*.

We used PCR to screen a library of mutagenized worms for mutants carrying deletions in *egl-6* and found additional alleles of *egl-6*, *n4536* and *n4537*. Both deletions remove the translational start site of the *egl-6b* transcript and most of the coding sequence of both *egl-6* transcripts (**Fig. 1b**) and are likely to be null alleles of *egl-6*.

To determine how the *n592* mutation affects *egl-6*, we tested the effects of *egl-6* gene dosage on egg-laying. We quantified *C. elegans* egg-laying behavior using the developmental stage of newly laid eggs, which reflects the time embryos spent *in utero*[2] (**Fig. 2a**). Statistical comparisons between strains analyzed in this manner are presented in **Supplementary Table 1** online. Wild-type worms retained most eggs until the embryos developed to a multicellular but premorphogenic stage (**Fig. 2b**). *egl-6(n592)* mutants laid embryos that had developed to the twofold stage or later, reflecting slower rates of egg-laying and longer retention of embryos. *egl-6* deletion mutants (*egl-6(Δ)*) had wild-type egg-laying behavior, indicating that the *n592* mutation does not reduce *egl-6* gene function. By contrast, transgenic worms with extra copies of wild-type *egl-6* laid later-stage eggs than did the wild-type worms. These observations indicate that *n592* increases *egl-6* activity and that *egl-6* inhibits egg-laying. Because activation of GPCRs is accompanied by allosteric changes in the relative positions of transmembrane domains[20], *n592* might increase receptor function by stabilizing an active conformer of the receptor.

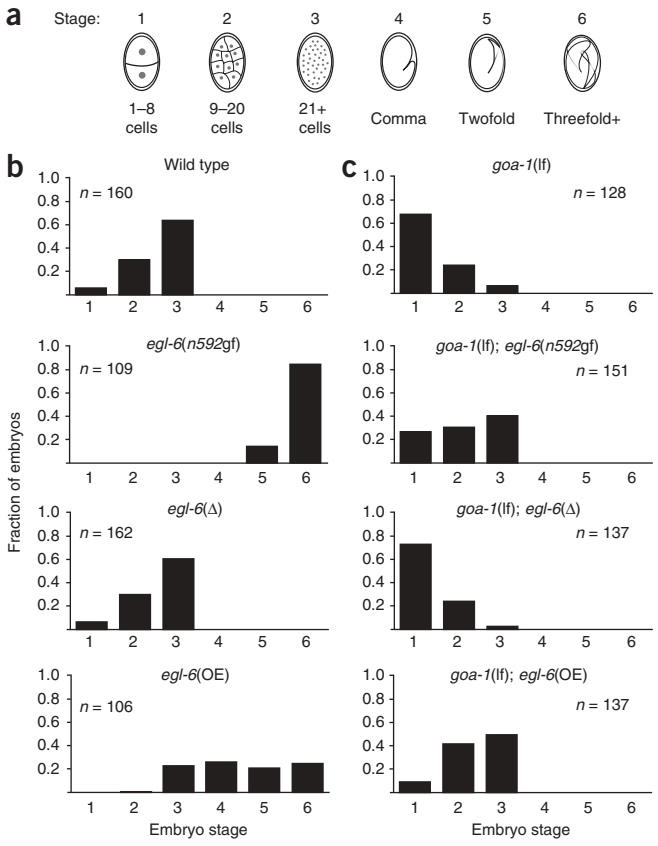
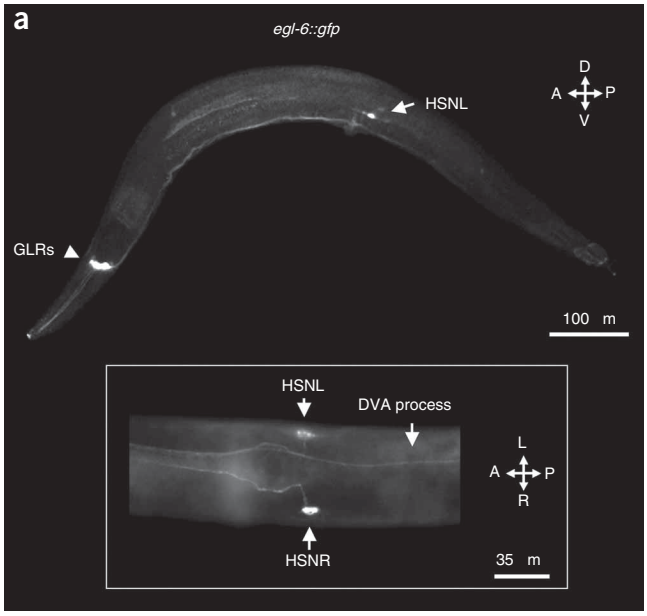


Figure 2 *egl-6*(gf) inhibition of egg-laying requires G_o signaling. (**a**) Stages of embryonic development used to assay the retention time of freshly laid eggs. (**b**) The *n592* mutation increases *egl-6* function. Distributions of the developmental stages of eggs laid by worms carrying the *n592* mutation and by worms with normal, decreased or increased *egl-6* gene dosage are shown. *egl-6(n592)* mutants and worms overexpressing (OE) *egl-6* laid later-stage embryos than did wild-type worms, indicating increased retention time *in utero*. The *egl-6* deletion allele used was *n4536*. The transgene used for *egl-6* overexpression was *nIs181*. (**c**) G_o signaling is required for EGL-6 gain-of-function mutation to inhibit egg-laying. Shown are distributions of the developmental stages of eggs laid by worms carrying a *goa-1* loss-of-function mutation together with mutations or transgenes that increase or decrease *egl-6* function. *goa-1; egl-6*(gf) double mutants were strongly suppressed for the egg-laying defect caused by *egl-6*(gf) but were still significantly different from *goa-1* single mutants ($P < 10^{-6}$) . The *egl-6* deletion allele used was *n4536*. The *goa-1* allele used was *n1134*. The transgene used for *egl-6* overexpression was *nIs181*.



b

Transgene	Egl lines
tph-1L::egl-6a (NSMs + HSNs)	5/8
tph-1L::egl-6b (NSMs + HSNs)	12/12
tph-1S::egl-6a (NSMs)	0/15
tph-1S::egl-6b (NSMs)	0/16

Figure 3 *egl-6* expression in the HSN motor neurons inhibits egg-laying. (**a**) *egl-6* is expressed in the HSN motor neurons and the GLR cells. Fainter expression in the DVA interneuron is not visible in this exposure. Occasional expression was observed in the neurons SDQL and SDQR. D, dorsal; V, ventral; A, anterior; P, posterior; L, left; R, right. (**b**) Expression of *egl-6* isoforms in the HSN motor neurons inhibits egg-laying. *egl-6a* and *egl-6b* cDNAs were under the control of *tph-1* promoter variants that drive transgene expression either in both the HSN motor neurons and the NSM pharyngeal neurons (*tph-1L*) or in the NSM pharyngeal neurons only (*tph-1S*). The number of transgenic lines with egg-laying defects as a fraction of lines generated is shown for each transgene.

G_o signaling inhibits *C. elegans* egg-laying behavior[8,9]. Because *egl-6* encodes a presumptive GPCR that inhibits egg-laying, we tested whether the egg-laying–defective phenotype caused by a gain-of-function (gf) mutation in *egl-6* requires G_o signaling. Loss-of-function (lf) mutations in the gene encoding *C. elegans* $G_o\alpha$, *goa-1*, increase rates of egg-laying[8,9]. We observed a corresponding change in the stage of eggs newly laid by *goa-1*(*n1134*) mutants, with a majority having eight cells or fewer (**Fig. 2c**). *goa-1*(lf) also strongly suppressed the egg-laying defects caused by either the *n592* mutation or overexpression of wild-type *egl-6*. *egl-6* deletion did not modify the egg-laying behavior of *goa-1* mutants. We observed similar results with a different *goa-1*(lf) allele, *n3055* (data not shown). Our data show that *egl-6* function strongly depends on *goa-1*, suggesting that EGL-6 couples to a G_o signaling pathway. However, some EGL-6 signaling might be independent of *goa-1*, as both the *egl-6*(*n592*gf) gain-of-function mutation and extra copies of wild-type *egl-6* slightly delayed the egg-laying behavior of *goa-1* mutants (**Fig. 2c**).

EGL-6 inhibits the HSN motor neurons

To determine where *egl-6* functions, we constructed a green fluorescent protein (GFP) reporter transgene, *egl-6::gfp*. This transgene caused an egg-laying defect that strongly depended on *goa-1* function (data not shown), suggesting that it encodes a functional receptor. We detected *egl-6::gfp* expression using an antibody to GFP and observed strong and consistent expression in HSN motor neurons (**Fig. 3a**) and in GLR cells, glia-like cells in the head. We consistently observed weaker staining of the DVA tail interneuron and occasionally observed staining of the lateral interneurons SDQL and SDQR.

HSN motor neurons innervate vulval muscles and are required for normal egg-laying[2,3]. To test whether *egl-6* expression in HSN neurons suffices to inhibit egg-laying, we used transgenes with regulatory elements from the tryptophan hydroxylase gene *tph-1* (ref. 21) to express *egl-6*. One promoter, *tph-1L*, drives gene expression in HSN neurons and pharyngeal serotonergic NSM neurons, and a truncated variant, *tph-1S*, drives gene expression primarily in NSM neurons[19]. Expression of either *egl-6* isoform under the control of the *tph-1L* promoter caused egg-laying defects, whereas the same cDNAs under the control of the NSM-specific promoter *tph-1S* did not affect egg-laying (**Fig. 3b**). Both isoforms of EGL-6 can, therefore, inhibit egg-laying. These data, together with the detection of the *egl-6* reporter in HSN motor neurons, suggest that EGL-6 receptors function in and mediate inhibition of the HSN neurons.

EGL-6 is a receptor for FLP-10 and FLP-17 FaRPs

We searched protein sequence databases using BLAST[22] and found that EGL-6 has sequence similarity to insect receptors for FaRPs[23,24]. We postulated that EGL-6 is a neuropeptide receptor and sought ligands for EGL-6 in two ways: by screening neuropeptide-encoding transgenes for the ability to inhibit egg-laying in an *egl-6*-dependent manner, and by screening a library of synthetic peptides for peptides that activate EGL-6 *in vitro*.

Our first approach to identify EGL-6 ligands was based on the hypothesis that overexpression of a ligand-encoding gene would inhibit egg-laying as does *egl-6* overexpression. We generated transgenic worms carrying extra copies of neuropeptide genes, including *flp-1* through *flp-23*, which are predicted to encode FMRFamide-like peptides, and ten *nlp* genes, which are predicted to encode neuropeptide-like peptides[25,26]. *flp-10* and *flp-17* transgenes inhibited egg-laying by wild-type worms but not by *egl-6*(Δ) mutants (see below), suggesting that *flp-10* and *flp-17* encode ligands for EGL-6. The introduction of stop codons or frameshift mutations into the *flp-10* and *flp-17* coding sequences abrogated their ability to inhibit egg-laying behavior (data not shown). To quantify the egg-laying defects caused by these transgenes and the dependence of these defects on the EGL-6 receptor, we generated strains carrying stably integrated versions of *flp-10* and *flp-17* transgenes. Stably integrated *flp-10* or *flp-17* transgenes inhibited egg-laying in wild-type worms, and these defects required the *egl-6* gene (**Fig. 4a**,**b**).

We also tested peptides for EGL-6 agonist activity *in vitro*. The coupling of GPCRs to G protein–activated inwardly rectifying K^+ (GIRK) channels in heterologous expression systems has been used to match ligands to orphan GPCRs (for example, in ref. 27). We coexpressed EGL-6 and GIRKs in *Xenopus laevis* oocytes and tested pools of synthetic peptides corresponding to predicted and observed *C. elegans* neuropeptides[25,26] for the ability to activate GIRK conductance using a two-electrode voltage clamp. We tested 55 peptides in five pools (**Supplementary Table 2** online). Two pools, B and D, evoked inward currents (**Fig. 4c**) that were observed only in oocytes coexpressing EGL-6 and GIRKs (data not shown). These pools therefore contained EGL-6 agonists. Pool B contained the predicted *flp-10* peptide product QPKARSGYIRFamide, and pool D contained both predicted *flp-17* peptide products, KSAFVRFamide and

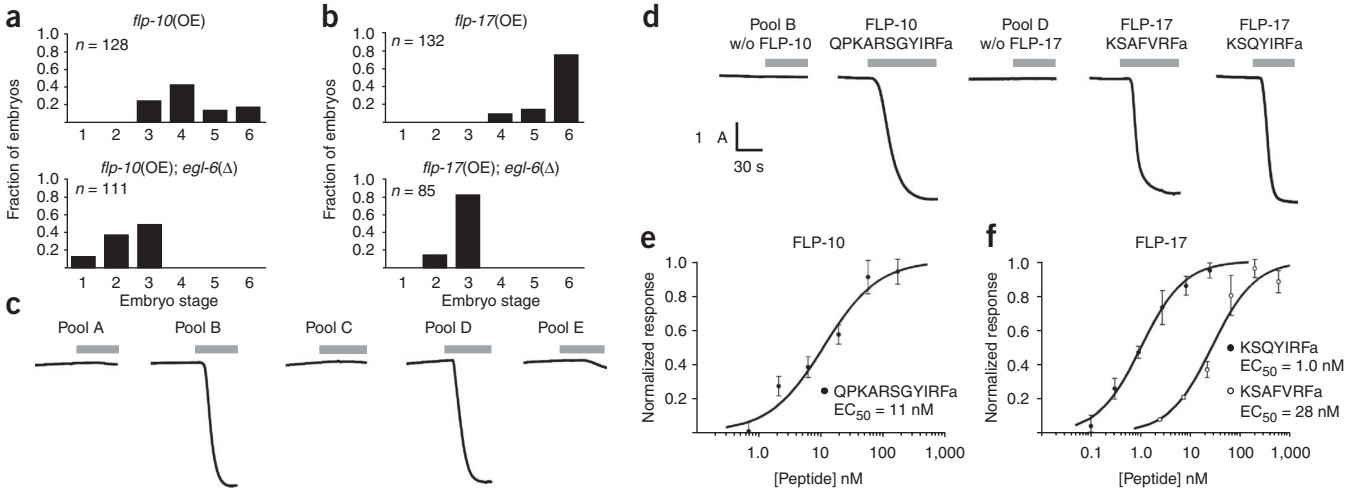


Figure 4 *flp-10* and *flp-17* encode ligands for the EGL-6 receptor. (**a**,**b**) Overexpression of *flp-10* or *flp-17* inhibits egg-laying behavior of wild-type worms but not of *egl-6*(Δ) mutants. Shown are distributions of developmental stages of eggs laid by transgenic worms carrying extra copies of *flp-10* (**a**) or *flp-17* (**b**) in the presence or absence of an *egl-6* deletion allele. *flp-10* and *flp-17* overexpression increased egg retention times compared to wild type, and deletion of *egl-6* suppressed the egg-laying defects caused by *flp-10* and *flp-17* overexpression. The transgene used for *flp-10* overexpression was *nIs209*. The transgene used for *flp-17* overexpression was *nIs211*. The *egl-6* deletion allele used was *n4536*. (**c**,**d**) Identification of EGL-6 ligands by screening pools of synthetic peptides. Pools of synthetic peptides (see **Supplementary Table 1**) were applied to *X. laevis* oocytes coexpressing EGL-6 and GIRK channels. The final concentration of each peptide in the pools was 1 μM. Whole-cell currents were monitored using a two-electrode voltage clamp. Gray bars indicate the periods during which peptide pools were applied. (**e**,**f**) Peptides encoded by *flp-10* (**e**) and *flp-17* (**f**) activate EGL-6 at nanomolar concentrations. Dilution series of the indicated peptides were applied to *X. laevis* oocytes coexpressing EGL-6 and GIRK channels. The response was calculated as the ratio of the peptide-evoked inward K^+ current and the peptide-independent inward K^+ current. Data were fitted to the Hill equation and normalized to the calculated maximum current. The means of at least three experiments ± s.e.m. are plotted. EC_{50}, half-maximal effective concentration.

KSQYIRFamide. Omitting these peptides from the active pools eliminated agonist activity; furthermore, each of these peptides could function as agonists when applied individually (**Fig. 4d**). *flp-10* and *flp-17* peptides were effective at nanomolar concentrations in this assay (**Fig. 4e**,**f**).

We concluded that *flp-10* and *flp-17* encode ligands for the EGL-6 receptor, because (i) the egg-laying defects caused by *flp-10* and *flp-17* overexpression required the *egl-6* receptor gene *in vivo*, and (ii) *flp-10* and *flp-17* peptides activated the EGL-6 receptor *in vitro*.

Activated mutant EGL-6 receptor is ligand dependent

We isolated deletion alleles of *flp-10* and *flp-17* (**Fig. 5a**). The *n4543* deletion removes the translational start site of *flp-10* and most of the coding sequence, including sequences encoding the active peptide. The *n4894* mutation removes the translational start site of *flp-17* and is likely to eliminate the production of both FaRPs encoded by *flp-17*.

Deletion of *flp-10* and *flp-17* did not grossly affect egg-laying behavior, either individually or in combination (**Fig. 5b**). Deletion of *flp-10*, however, suppressed the egg-laying defect caused by the *n592*gf mutation of *egl-6*, and this suppression was enhanced by deletion of *flp-17* (**Fig. 5c**). These data show that the activity of both the wild-type receptor and the mutant receptor encoded by *egl-6(n592*gf*)* is regulated by *flp-10* and *flp-17* peptides *in vivo*.

flp-10 and *flp-17* can function in different cells

To identify the sources of *flp-10* and *flp-17* peptides, we generated *flp-10::gfp* and *flp-17::gfp* reporters and detected their expression using antibody to GFP (**Fig. 6**). We observed consistent *flp-10::gfp* expression in ASIL, ASIR, DVB, PVCL, PVCR and PVR neurons and in vulval tissue, uterine cells, spermathecae and the head mesodermal cell (**Fig. 6a**). We also observed faint and inconsistent staining of a small number of unidentified neurons. The pattern of *flp-10::gfp* expression we observed was similar but not identical to a *flp-10* expression pattern previously observed using a transcriptional reporter[28]. Specifically, we did not observe *flp-10* expression in the BAG neurons, and the previous study did not note *flp-10* expression in the uterus, spermathecae or head mesodermal cell[28]. We observed expression of *flp-17::gfp* expression primarily in a pair of anterior sensory neurons, BAGL and BAGR (**Fig. 6b**). The *flp-17::gfp* expression we observed was similar to that previously reported[28]. We also observed faint but consistent expression of *flp-17::gfp* in three pairs of unidentified head neurons.

To test whether expression of *flp-10* and *flp-17* in specific neurons is required for these genes to inhibit egg-laying, we laser-ablated neurons in transgenic worms overexpressing *flp-10* or *flp-17*. Ablation of BAG neurons or of six neurons expressing *flp-10::gfp* together had no marked effect on egg-laying by wild-type worms (**Fig. 6c**). We then ablated six neurons expressing *flp-10::gfp* in worms overexpressing *flp-10* to test whether these neurons were required for the egg-laying defect conferred by the *flp-10* transgene. Ablation of ASIL, ASIR, DVB, PVCL, PVCR and PVR together did not strongly modify the egg-laying phenotype of worms overexpressing *flp-10* (**Fig. 6d**), although the small change we observed in the distribution of egg stage might be significant ($P = 0.0193$). The six neurons we identified as expressing *flp-10::gfp* expressing were not, therefore, strictly required for *flp-10* function. By contrast, laser ablation of the BAG neurons strongly suppressed the egg-laying defect conferred by *flp-17* overexpression (**Fig. 6e**), suggesting that the BAG neurons are a principal site of *flp-17* expression. Ablation of BAG cells also partially suppressed the egg-laying defects conferred by *flp-10* overexpression (**Fig. 6d**).

That the *flp-10* transgene functioned in the absence of the six neurons that expressed *flp-10::gfp* suggested that non-neuronal—possibly vulval or gonadal—expression of *flp-10* inhibits egg laying. It is also possible that our *flp-10::gfp* reporter was expressed in only a

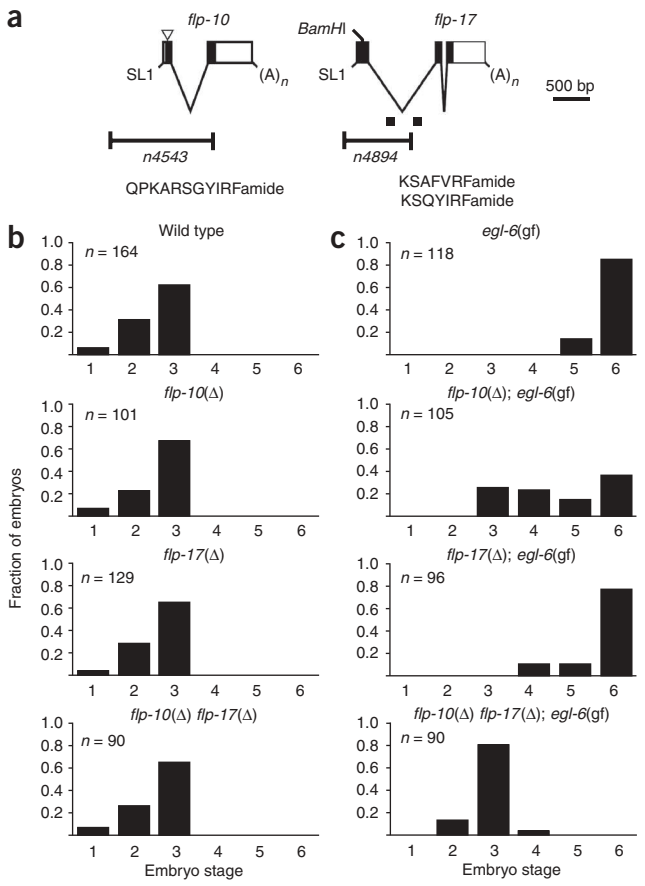


Figure 5 The EGL-6 receptor is regulated by *flp-10* and *flp-17 in vivo*. (**a**) *flp-10* and *flp-17* intron-exon structure. Coding sequences are depicted as solid boxes. The *flp-10* and *flp-17* loci are both on linkage group IV but in opposing orientations. Sequences deleted in *flp-10(n4543)* and *flp-17(n4894)* are indicated. The open arrowhead indicates the position of an amber nonsense mutation that abrogated the ability of the *flp-10* transgene to inhibit egg-laying. A frameshift that abrogated the ability of the *flp-17* transgene to inhibit egg-laying was introduced at the indicated *BamH*I site. Closed squares indicate the presence of 21U RNA-encoding genes within the *flp-17* locus. The predicted peptide products of *flp-10* and *flp-17* are shown beneath the corresponding gene models. (**b**) *flp-10* and *flp-17* mutants lay normally staged embryos. The distributions of developmental stages of embryos laid by wild-type worms, *flp-10* mutants, *flp-17* mutants and *flp-10 flp-17* double mutants are shown. (**c**) *flp-10* and *flp-17* mutations partially suppress the egg-laying defect caused by the *egl-6* gain-of-function mutation. Shown are the distributions of developmental stages of embryos laid by *egl-6*(gf) mutants and *egl-6*(gf) mutants with mutations that delete the ligand-encoding genes *flp-10* and *flp-17*. The *egl-6* gain-of-function allele used was *n592*. The *flp-10* and *flp-17* deletion alleles used were *n4543* and *n4894*, respectively.

subset of *flp-10*-expressing neurons, in which case we would not have ablated all *flp-10*-expressing neurons. To test whether non-neuronal cells are competent to express and process *flp-10* peptide, we expressed *flp-10* specifically in vulval cells and spermathecae, non-neuronal tissues that expressed the *flp-10::gfp* reporter. We expressed *flp-10* in vulval cells, in spermathecae and pan-neuronally using heterologous promoters fused to the *flp-10* cDNA (see **Supplementary Methods** online). Egg-laying defects were observed in three of four transgenic lines carrying the vulval expression construct, five of five lines carrying the spermathecal expression construct and three of six lines carrying the pan-neuronal expression construct. Non-neuronal cells can therefore express functional FLP-10 peptide. Together with our laser ablation studies, these data suggest that non-neuronal cells are one source of endogenous FLP-10 peptide.

The BAG neurons inhibit *C. elegans* egg-laying behavior

egl-6 overexpression caused an egg-laying defect that was partially suppressed by *flp-17* deletion (**Fig. 7a,b**). The egg-laying defect of worms overexpressing *egl-6* therefore partly depends on *flp-17*. To test whether ablation of BAG cells would phenocopy *flp-17* deletion and

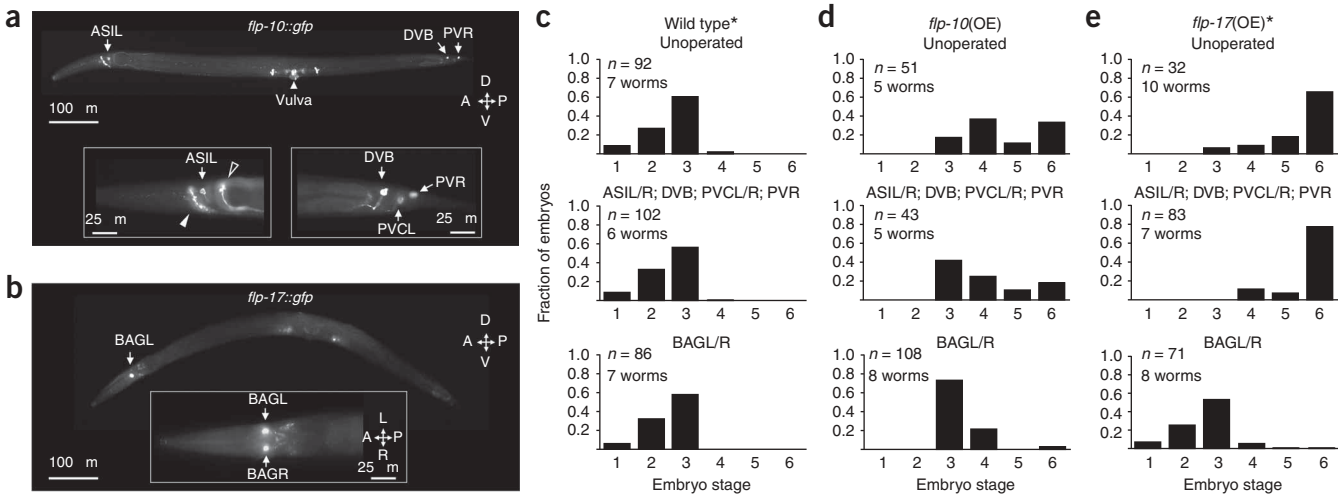


Figure 6 *flp-10* and *flp-17* are expressed in different cells. (**a**,**b**) Expression of *flp-10::gfp* and *flp-17::gfp* was detected by immunocytochemistry. (**a**) *flp-10::gfp* expression was observed in the ASI sensory neurons, the DVB motor neuron, the PVC interneurons and the PVR neuron. *flp-10::gfp* expression was also observed in the head mesoderm cell (open arrowhead), vulval cells, uterus and spermathecae. The ASI dendrites contained high levels of *flp-10::gfp* (closed arrowhead). Both micrographs are of the same worm. (**b**) *flp-17::gfp* expression was detected primarily in a pair of anterior sensory neurons, BAGL and BAGR. Both micrographs are of the same worm. (**c**–**e**) BAG neurons are required for *flp-17* function. Neurons expressing *flp-10* and *flp-17* reporter transgenes were ablated with a laser microbeam in wild-type animals (**c**) and in animals overexpressing *flp-10* (**d**) or *flp-17* (**e**). The distributions of developmental stages of eggs laid by operated animals are shown. The transgene used for *flp-10* overexpression was *nIs209[flp-10(+)]*. The transgene used for *flp-17* overexpression was *nIs211*. Asterisks denote the presence of the *gcy-33::gfp* transgene, *nIs242*, used in some strains to facilitate identification of BAG neurons.

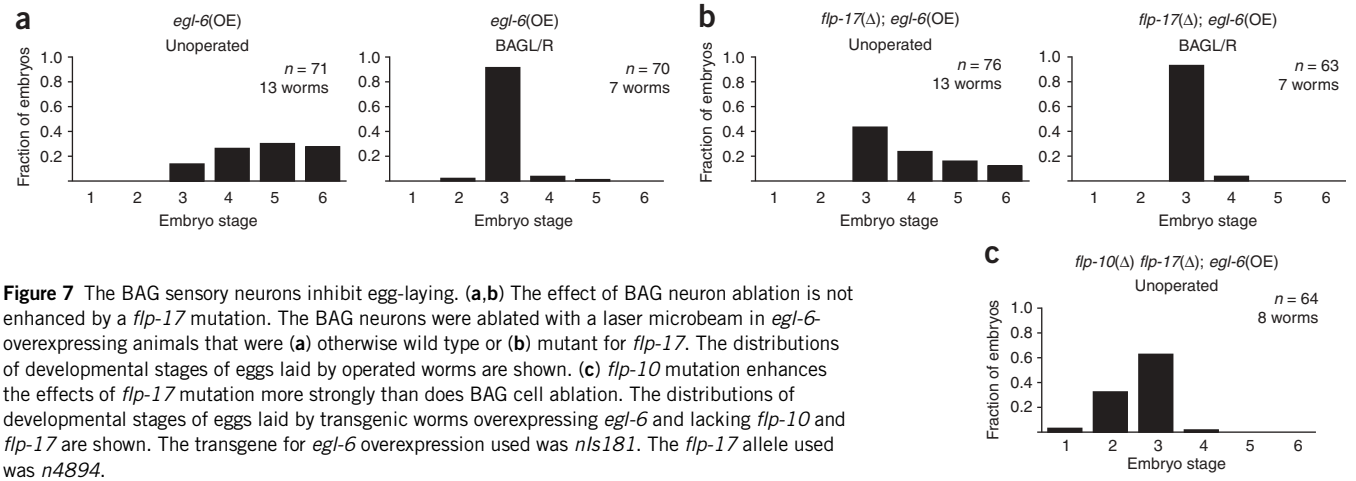


Figure 7 The BAG sensory neurons inhibit egg-laying. (**a**,**b**) The effect of BAG neuron ablation is not enhanced by a *flp-17* mutation. The BAG neurons were ablated with a laser microbeam in *egl-6*-overexpressing animals that were (**a**) otherwise wild type or (**b**) mutant for *flp-17*. The distributions of developmental stages of eggs laid by operated worms are shown. (**c**) *flp-10* mutation enhances the effects of *flp-17* mutation more strongly than does BAG cell ablation. The distributions of developmental stages of eggs laid by transgenic worms overexpressing *egl-6* and lacking *flp-10* and *flp-17* are shown. The transgene for *egl-6* overexpression used was *nIs181*. The *flp-17* allele used was *n4894*.

whether *flp-17* has a BAG cell–independent function, we ablated the BAG cells in worms overexpressing *egl-6*, with or without a *flp-17* deletion allele (**Fig. 7a**). BAG cell ablation suppressed the egg-laying defect of worms overexpressing *egl-6*, but this suppression was not enhanced by *flp-17* deletion—that is, deleting *flp-17* had no effect in the absence of BAG cells (**Fig. 7b**). The suppression by *flp-17* deletion of the egg-laying defect caused by *egl-6* overexpression was enhanced by *flp-10* deletion (**Fig. 7c**).

These data, together with the expression of *flp-17::gfp* in BAG cells and the requirement of BAG cells for the function of the *flp-17* transgene, indicate that BAG cells are the principal source of endogenous FLP-17 peptides. That the effect of BAG cell ablation on egg-laying behavior was stronger than that of deleting *flp-17* indicates that BAG cells provide one or more inhibitory signals in addition to FLP-17 peptides. Although some inhibition provided by BAG cells might require *flp-10*, we observed that *flp-10* deletion has a larger effect than does BAG cell ablation (**Fig. 7**), suggesting that *flp-10* can function in cells in addition to BAG cells. Our observation that BAG cell ablation only partially suppressed the egg-laying defect caused by *flp-10* overexpression (**Fig. 6d**) is consistent with this hypothesis.

EGL-6 and acetylcholine redundantly modulate egg-laying

Although activation of the *egl-6* pathway by mutation or overexpression inhibited egg-laying, mutants lacking *egl-6* or the ligand-encoding genes *flp-10* and *flp-17* had grossly normal egg-laying behavior (**Figs. 2b** and **4c**). In addition to using the developmental stage of newly laid eggs to assay egg-laying behavior, we analyzed the timing of egg-laying events by *egl-6(Δ)* mutants (**Supplementary Fig. 1** online) and the modulation of egg-laying behavior of *egl-6(Δ)* mutants by the presence or absence of a bacterial food source (data not shown). These experiments did not reveal notable differences between *egl-6(Δ)* mutants and wild-type worms.

We tested whether redundancy between the *egl-6* signaling pathway and another signaling pathway might explain our observation that mutants defective in the *egl-6* pathway have grossly normal egg-laying behavior. We constructed strains with multiple mutations in *egl-6* and genes required for the synthesis or storage of specific neurotransmitters (GABA, dopamine, glutamate, tyramine, octopamine, serotonin or acetylcholine) and examined these strains for synthetic egg-laying defects. Genes tested for interactions with the *egl-6* pathway were *unc-25*, which is required for GABA synthesis[29]; *cat-2*, which is required for dopamine synthesis[30]; *eat-4*, which encodes a vesicular glutamate transporter[31]; *tdc-1*, which is required for tyramine and octopamine synthesis[32]; *tph-1*, which is required for serotonin synthesis[21]; and *unc-17* and *cha-1*, which encode a vesicular acetylcholine transporter and a choline acetyltransferase required for acetylcholine synthesis, respectively[33,34].

We observed that strains carrying mutations in the *egl-6* pathway and genes required for acetylcholine signaling had a strong synthetic hyperactive egg-laying phenotype (**Fig. 8a**). *unc-17* and *cha-1*, which are required for acetylcholine synthesis and storage, are in an operon. The *unc-17(e113)* mutation reduces the function of both *unc-17* and *cha-1* (ref. 35). The egg-laying behavior of *unc-17(e113)* mutants (**Fig. 8a**) was similar to that of wild-type worms and *egl-6* deletion mutants (**Fig. 2b**). By contrast, *unc-17(e113); egl-6(Δ)* double mutants laid early-stage embryos with eight or fewer cells at a high frequency, reflecting decreased time spent *in utero*. Deletion of *flp-10*, *flp-17* or both affected the egg-laying behavior of *unc-17(e113)* mutants similarly. We saw comparable interactions between another deletion allele of *egl-6* and *unc-17(e113)* and between both deletion alleles of *egl-6* and three other mutations affecting the *unc-17 cha-1* operon: *e876*, *p1152* and *n2421* (data not shown). We also ablated BAG cells, which are a principal site of *flp-17* expression, in *unc-17(e113)* mutants and observed a higher frequency of early-stage embryos laid (**Fig. 8a**). Given that potentiation of acetylcholine signaling by mutation of the acetylcholinesterase-encoding genes *ace-1* and *ace-2* causes locomotion defects[36] and inhibits egg-laying behavior[7], we also tested whether deletion of the EGL-6 signaling pathway could modify the egg-laying defect of acetylcholinesterase mutants. The egg-laying defect of *ace-2; ace-1* double mutants was suppressed by deletion of *egl-6*, *flp-10* and *flp-17* (**Fig. 8b**). These data show that EGL-6 signaling and cells required for the production of EGL-6 ligands function redundantly with acetylcholine to inhibit egg-laying.

Interactions between mutations that affect *egl-6* signaling and mutations that alter acetylcholine signaling are consistent with the hypothesis that EGL-6 functions upstream of or in parallel to an acetylcholine signal to inhibit egg-laying. The egg-laying defect of *ace-2; ace-1* double mutants was suppressed by deletion of the *egl-6* pathway, but the egg-laying behavior of suppressed worms was still distinguishable from that of wild-type worms, suggesting that acetylcholine does not inhibit egg-laying behavior by acting strictly upstream of *egl-6* signaling. The locomotion defects of mutants with reduced acetylcholine signaling (*unc-17* and *cha-1* mutants) or excess acetylcholine signaling (*ace-2; ace-1* double mutants) were not modified by mutations in *egl-6* or in the *flp-10* and *flp-17*

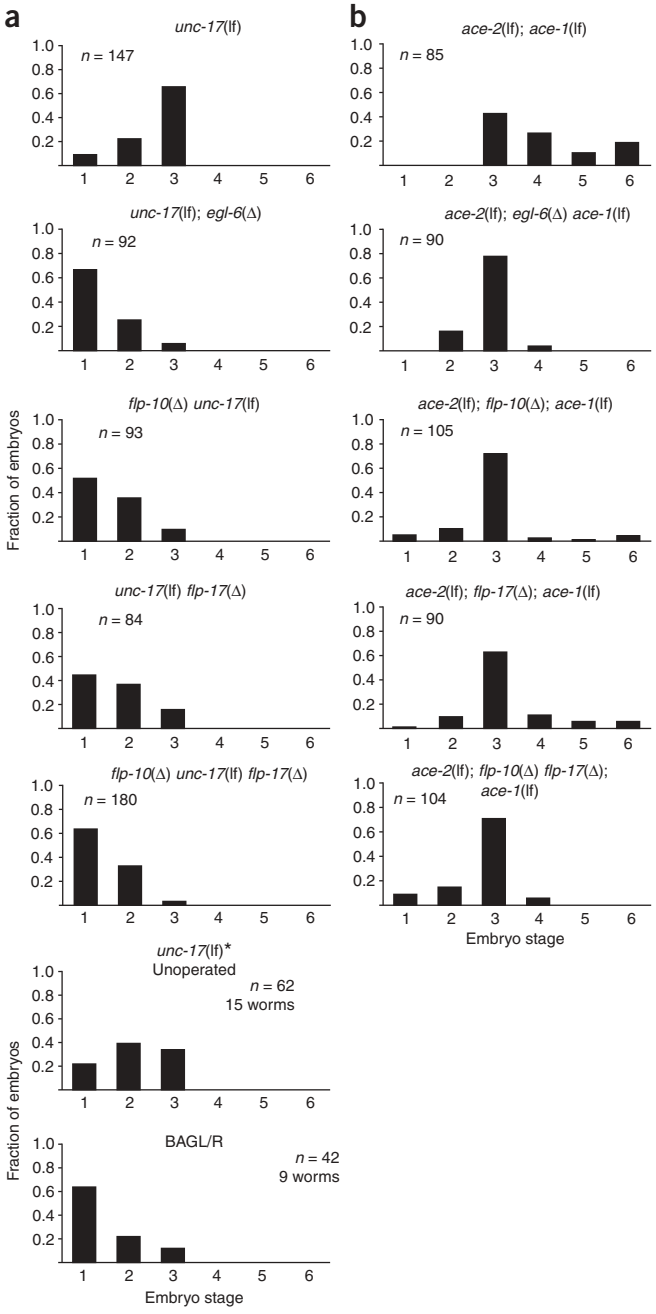


Figure 8 EGL-6 signaling inhibits egg-laying behavior redundantly with acetylcholine. (**a**) Loss of function in both EGL-6 signaling and acetylcholine signaling decreases egg retention time. The distribution of developmental stages of embryos laid by *unc-17* mutants (defective in vesicular acetylcholine transport) is shown together with the distributions of stages of eggs laid by worms mutant for *unc-17* and genes in the *egl-6* pathway or *unc-17* mutants lacking BAG neurons. BAG neurons were ablated with a laser microbeam in *unc-17* mutants. An asterisk denotes the presence of the *gcy-33::gfp* transgene *nIs242*, used in some strains to facilitate identification of BAG neurons. The *unc-17* allele used was *e113*. The *egl-6*, *flp-10* and *flp-17* alleles used were *n4536*, *n4543* and *n4894*, respectively. (**b**) Loss of function in the *egl-6* pathway suppresses the egg-laying defect of mutants with excess acetylcholine signaling. Shown are distributions of developmental stages of eggs laid by *ace-2; ace-1* double mutants (defective in acetylcholinesterase function) and by worms multiply mutant for acetylcholinesterase-encoding genes and genes in the *egl-6* pathway. The *ace-1* allele used was *p1000*. The *ace-2* allele used was *g72*. The *egl-6*, *flp-10* and *flp-17* alleles used were *n4536*, *n4543* and *n4894*, respectively.

ligand-encoding genes (data not shown), suggesting that EGL-6 signaling does not function in all cholinergic circuits.

DISCUSSION

Through a molecular genetic analysis of *C. elegans* mutants defective in egg-laying behavior, we identified a FaRP signaling pathway that inhibits motor neurons in the *C. elegans* egg-laying system in a G_o-dependent manner. This pathway consists of the EGL-6 GPCR and two classes of peptide ligands encoded by the genes *flp-10* and *flp-17*. We found that the *flp-17* peptides KSAFVRFamide and KSQYIRFamide are provided by a pair of presumptive sensory neurons, the BAG cells. The *flp-10* peptide QPKARSGYIRFamide is expressed by both neuronal and non-neuronal cell types, and our data suggest that non-neuronal cells are a source of this inhibitory peptide. We conclude that the EGL-6 receptor transduces inhibitory signals to the HSN motor neurons from multiple cell types.

Because presumptive sensory neurons are one source of EGL-6 ligands, EGL-6 signaling might modulate egg-laying behavior in response to environmental cues. BAG cells have specialized cilia characteristic of sensory neurons[37,38] but have not been associated with a sensory modality. BAG cell processes have goblet-shaped termini surrounding a region of hypodermal tissue, but the processes are not exposed to the external environment[37,38] and might therefore function as receptors for factors that can permeate the cuticle or are generated internally. Synapses between the BAG cells and the HSN motor neurons have not been observed by electron microscopic reconstruction of the *C. elegans* nervous system[3]. Therefore, *flp-17*-encoded FaRPs are likely to function as paracrine or endocrine signals in the control of egg-laying behavior.

Our data suggest that non-neuronal expression of *flp-10* peptides inhibits egg-laying behavior. Most non-neuronal expression of *flp-10* that we observed was in components of the hermaphrodite's reproductive system: vulval, uterine and spermathecal cells. We further found that expression of *flp-10* in vulval and spermathecal cells using heterologous promoters suffices to inhibit egg-laying. It is not fully understood how somatic components of the hermaphrodite's reproductive system might modulate egg-laying behavior. Others have proposed that mechanical stimuli trigger release of neuromodulators from neuroendocrine cells at the junction of the vulva and uterus[39]. Oocytes passing through the spermathecae, distension of the uterus and mechanical stimulation of vulval tissue might similarly trigger FLP-10 release and allow ovulation, egg retention and the act of egg-laying to alter the activity of HSN motor neurons.

EGL-6 signaling is dispensable for the egg-laying behavior of wild-type worms under normal laboratory conditions. By contrast, the egg-laying behavior of acetylcholine signaling mutants was strongly affected by perturbations of the EGL-6 pathway (**Fig. 8**). Loss of *egl-6* function or of the function of one or both genes encoding EGL-6 ligands in *unc-17* mutants resulted in the expulsion of immature embryos from the uterus. The egg-laying behavior of acetylcholinesterase-deficient mutants, which are egg-laying defective, was also sensitive to mutation of one or both genes that encode EGL-6 ligands. These data indicate that even partial loss of function in EGL-6 signaling can alter the egg-laying behavior of mutants with abnormal acetylcholine signaling.

What is the circuit that provides inhibitory cholinergic input to the egg-laying system? The interactions between the EGL-6 pathway and acetylcholine signaling support models in which FLP-10 and FLP-17

FaRPs work either in parallel to or upstream of acetylcholine to inhibit egg laying. We have tested whether EGL-6 signaling functions redundantly with the GAR-2 metabotropic acetylcholine receptor, which is expressed on HSN motor neurons and has been proposed to negatively regulate HSN function[7,40]; we did not observe egg-laying defects in *gar-2; egl-6* double mutants. Similarly, ablation of the cholinergic VC4 and VC5 egg-laying motor neurons did not modify the egg-laying behavior of *egl-6* mutants (data not shown). It is therefore likely that the cholinergic pathway that functions redundantly with EGL-6 signaling requires cells and receptors previously not implicated in the control of egg-laying behavior. We observed that the hyperactive egg-laying phenotype of *unc-17; egl-6* double mutants is comparable to that of *goa-1* mutants (**Figs. 2** and **8**). If cholinergic inhibition of egg-laying is mediated by metabotropic acetylcholine receptors, a significant fraction of inhibitory G_o signaling in the egg-laying system might be controlled by these receptors together with EGL-6. Three metabotropic acetylcholine receptors have been found in *C. elegans*[40–42]. Other receptors that might mediate inhibitory cholinergic signaling are acetylcholine-gated chloride channels[43].

Our data and those of others show that the *C. elegans* egg-laying system is regulated by multiple neurotransmitters, including serotonin, acetylcholine and FaRPs. Interactions among these neurotransmitter systems are critical for the generation and regulation of egg-laying behavior. There is evidence that acetylcholine signaling is required for the stimulatory effects of serotonin on egg-laying, as if these two neurochemical signals constitute a logical AND gate in the control of egg-laying[6,44]. We have described a different relationship between neurotransmitter systems in the *C. elegans* egg-laying system: cholinergic and peptidergic signals synergistically inhibit egg-laying, constituting a logical OR gate. Cholinergic and peptidergic inhibition of egg-laying might be independently invoked by sensory systems or other cellular circuits to stop egg-laying by *C. elegans* in unfavorable conditions. The logical relationships among neurotransmitter systems that control *C. elegans* egg-laying behavior might have general implications for the study and manipulation of neural circuits. We suggest that even if inactivation of individual neurochemical pathways has little or no effect on the function of a target circuit, the combinatorial perturbation of pathways might cause strong and specific alterations of circuit function.

Note added in proof: While this manuscript was in review, Hallem and Sternberg[45] identified a role for the BAG neurons in carbon dioxide–avoidance behavior of C. elegans.

METHODS

Strains and transgenes. A detailed description of strains used and construction of transgenes can be found in **Supplementary Methods**.

Mapping and cloning of *egl-6(n592).* We isolated egg-laying–defective Mec non-Lon and Mec non-Lon recombinants from the strain MT16027 *lon-2(e678) egl-6(n592) mec-7(e1506)* after crossing with the polymorphic wild-type strain CB4856 and determined crossover sites as described[46]. We mapped *n592* to a 167-kb region between SNPs on the cosmids F13D11 and F22F4. PCR products spanning this interval were derived from wild-type and *n592* genomic DNA, injected into the wild-type strain N2 and scored for the ability to phenocopy the *n592* mutation. A transgene containing only the gene *C46F4.1* phenocopied *n592*. The predicted gene structure of *C46F4.1* was confirmed by RT-PCR. We used 5′ RACE (Invitrogen) to identify a transcript with an alternative start and an SL1 *trans*-spliced leader sequence. The 3′ sequences of *egl-6* transcripts were amplified using PCR from a *C. elegans* cDNA library (Stratagene) using gene-specific primers and primers directed against the cloning vector. Sequences of PCR-amplified regions of genomic DNA comprising all exons and splice junctions of *C46F4.1* from *n592* mutants and wild-type worms were determined using an ABI Prism 3100 Genetic Analyzer.

Isolation of deletion alleles. Libraries of mutagenized animals were constructed and screened by PCR for deletion alleles of *egl-6*, *flp-10* and *flp-17* essentially as described[47]. Deletion mutants were isolated from frozen stocks and backcrossed to wild-type worms at least four times. Sequences deleted in each allele are described in **Supplementary Methods**.

Immunocytochemistry. Worms were fixed, permeabilized and stained as previously described[48] using a 1:50 dilution of monoclonal antibody to GFP (Millipore) and a 1:100 dilution of Alexa488-conjugated goat antibody to mouse (Invitrogen). Samples were viewed using a Zeiss Axiophot epifluorescence microscope, and images were acquired with a Hamamatsu Orca charge-coupled device camera and OpenLabs image acquisition software.

EGL-6 expression in *X. laevis* oocytes. *egl*-6 cDNAs were flanked with *EcoR*I sites by PCR and cloned into the pGEMHE vector[49]. *egl-6*, GIRK1 and GIRK4 cRNAs were prepared using the mMessage Machine kit (Ambion). *X. laevis* oocytes were injected with 50 ng of *egl-6* receptor sense cRNA and 0.5 ng of GIRK1 and GIRK4 sense cRNA. Injected oocytes were incubated at 18 °C in ND96 medium (96 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.6) for 2–5 d before recording.

Whole-cell current recordings were made using the two-electrode voltage-clamp technique at a holding potential of –80 mV as described[27]. The recording chamber was continuously perfused with ND96 medium. To assay activation of GIRK channels, we equilibrated oocytes in high-K^+ medium (96 mM KCl and 2.5 mM NaCl instead of 2.5 mM KCl and 96 mM NaCl) to reverse the K^+ gradient and measured inward currents before, during and after addition of test peptide. Data were acquired with Clampex 8.0 software (Molecular Devices) and analyzed offline with Clampfit (Molecular Devices). All experiments using *X. laevis* oocytes were done according to guidelines of the Committee on Animal Care at MIT. Peptides were synthesized by the MIT Biopolymers Laboratory.

Behavioral assays and neuron ablation. Transgenic lines were scored as egg-laying defective if young adults (24 h after late L4) had, on average, greater than 30 eggs *in utero*. To score the developmental stages of newly laid eggs, young adults were transferred to fresh nematode growth medium plates with bacteria, five worms per plate, for 1 h at room temperature and then removed. Operated worms were assayed individually, and a single operated worm was assayed two and four times.

Eggs on the plate were examined using a high-power dissecting microscope and categorized as described in **Figure 2**.

Distributions of the developmental stages of eggs laid by worms of different genotypes were analyzed with the Wilcoxon Mann-Whitney rank-sum test, a nonparametric test of statistical significance, as implemented in the Coin package of the R statistical analysis program[50]. The results of statistical tests of significance can be found in **Supplementary Table 1**. Egg-laying defects of many strains were also independently quantified by measuring the number of retained eggs in staged adults as previously described[19], and we observed similar interactions between receptor- and ligand-encoding genes, receptor- and G protein–encoding genes, and genes in the *egl-6* signaling pathway and acetylcholine biosynthesis genes (data not shown).

Laser microsurgeries were performed on L2-stage larvae as described[51]. Cell identifications were made on the basis of nuclear position and cell morphology. A *gcy-33::gfp* transgene was used in some experiments to identify BAG neurons (see **Supplementary Methods**).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank Y. Kohara for *flp-10* and *flp-17* cDNAs; N. Dascal for GIRK1 and GIRK4 expression constructs; J. Rand for *unc-17 cha-1* strains; J. Moresco and M. Koelle for *tph-1* promoter constructs; A. Fire for expression vectors; A. Hellman, S. McGonagle, B. Castor and N. An for technical assistance; N. Abe and R. O'Hagan for help with *Xenopus* oocyte electrophysiology; J. Chung for help with data analysis; and B. Galvin and A. Saffer for critical reading of the manuscript. N.R. received support from the Howard Hughes Medical Institute, the Life Sciences Research Foundation and the Medical Foundation. H.R.H. is a David H. Koch Professor of Biology and an

Investigator of the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grant GM24663.

AUTHOR CONTRIBUTIONS

N.R. performed all experiments. N.R. and H.R.H. designed the experiments and wrote the manuscript.

Published online at http://www.nature.com/natureneuroscience/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E. & Evans, P.D. Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science* **216**, 1012–1014 (1982).
2. Trent, C., Tsung, N. & Horvitz, H.R. Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **104**, 619–647 (1983).
3. White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **314**, 1–340 (1986).
4. Rand, J.B. & Nonet, M.L. Neurotransmitter assignments for specific neurons. in *C. elegans II* (eds. Riddle, D.L., Blumenthal, T., Meyer, B.J. & Priess, J.R.) 1049–1052 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1997).
5. Duerr, J.S., Gaskin, J. & Rand, J.B. Identified neurons in *C. elegans* coexpress vesicular transporters for acetylcholine and monoamines. *Am. J. Physiol. Cell Physiol.* **280**, C1616–C1622 (2001).
6. Waggoner, L.E., Zhou, G.T., Schafer, R.W. & Schafer, W.R. Control of alternative behavioral states by serotonin in *Caenorhabditis elegans*. *Neuron* **21**, 203–214 (1998).
7. Bany, I.A., Dong, M.Q. & Koelle, M.R. Genetic and cellular basis for acetylcholine inhibition of *Caenorhabditis elegans* egg-laying behavior. *J. Neurosci.* **23**, 8060–8069 (2003).
8. Mendel, J.E. *et al.* Participation of the protein G_o in multiple aspects of behavior in *C. elegans*. *Science* **267**, 1652–1655 (1995).
9. Segalat, L., Elkes, D.A. & Kaplan, J.M. Modulation of serotonin-controlled behaviors by G_o in *Caenorhabditis elegans*. *Science* **267**, 1648–1651 (1995).
10. Brundage, L. *et al.* Mutations in a *C. elegans* $G_q$alpha gene disrupt movement, egg laying, and viability. *Neuron* **16**, 999–1009 (1996).
11. Lackner, M.R., Nurrish, S.J. & Kaplan, J.M. Facilitation of synaptic transmission by EGL-30 $G_q$alpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. *Neuron* **24**, 335–346 (1999).
12. Miller, K.G., Emerson, M.D. & Rand, J.B. $G_o\alpha$ and diacylglycerol kinase negatively regulate the $G_q\alpha$ pathway in *C. elegans*. *Neuron* **24**, 323–333 (1999).
13. Nurrish, S., Segalat, L. & Kaplan, J.M. Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. *Neuron* **24**, 231–242 (1999).
14. Carnell, L., Illi, J., Hong, S.W. & McIntire, S.L. The G-protein-coupled serotonin receptor SER-1 regulates egg laying and male mating behaviors in *Caenorhabditis elegans*. *J. Neurosci.* **25**, 10671–10681 (2005).
15. Hobson, R.J. *et al.* SER-7, a *Caenorhabditis elegans* 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. *Genetics* **172**, 159–169 (2006).
16. Shyn, S.I., Kerr, R. & Schafer, W.R. Serotonin and G_o modulate functional states of neurons and muscles controlling *C. elegans* egg-laying behavior. *Curr. Biol.* **13**, 1910–1915 (2003).
17. Kass, J., Jacob, T.J., Kim, P. & Kaplan, J.M. The EGL-3 prohormone convertase regulates mechanosensory responses of *Caenorhabditis elegans*. *J. Neurosci.* **21**, 9265–9272 (2001).
18. Jacob, T.C. & Kaplan, J.M. The EGL-21 carboxypeptidase E facilitates acetylcholine release at *Caenorhabditis elegans* neuromuscular junctions. *J. Neurosci.* **23**, 2122–2130 (2003).
19. Moresco, J.J. & Koelle, M.R. Activation of EGL-47, a Galpha(o)-coupled receptor, inhibits function of hermaphrodite-specific motor neurons to regulate *Caenorhabditis elegans* egg-laying behavior. *J. Neurosci.* **24**, 8522–8530 (2004).
20. Gether, U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev.* **21**, 90–113 (2000).
21. Sze, J.Y., Victor, M., Loer, C., Shi, Y. & Ruvkun, G. Food and metabolic signaling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* **403**, 560–564 (2000).
22. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
23. Cazzamali, G. & Grimmelikhuijzen, C.J. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl. Acad. Sci. USA* **99**, 12073–12078 (2002).
24. Meeusen, T. *et al.* Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl. Acad. Sci. USA* **99**, 15363–15368 (2002).
25. Li, C. The ever-expanding neuropeptide gene families in the nematode *Caenorhabditis elegans*. *Parasitology* **131** Suppl: S109–S127 (2005).
26. Nathoo, A.N., Moeller, R.A., Westlund, B.A. & Hart, A.C. Identification of neuropeptide-like gene families in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **98**, 14000–14005 (2001).
27. Rogers, C. *et al.* Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat. Neurosci.* **6**, 1178–1185 (2003).
28. Kim, K. & Li, C. Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *J. Comp. Neurol.* **475**, 540–550 (2004).
29. Jin, Y., Jorgensen, E., Hartwieg, E. & Horvitz, H.R. The *Caenorhabditis elegans* gene *unc-25* encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *J. Neurosci.* **19**, 539–548 (1999).
30. Sulston, J., Dew, M. & Brenner, S. Dopaminergic neurons in the nematode *C. elegans*. *J. Comp. Neurol.* **163**, 215–226 (1975).
31. Lee, R.Y., Sawin, E.R., Chalfie, M., Horvitz, H.R. & Avery, L. EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in *Caenorhabditis elegans*. *J. Neurosci.* **19**, 159–167 (1999).
32. Alkema, M.J., Hunter-Ensor, M., Ringstad, N. & Horvitz, H.R. Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron* **46**, 247–260 (2005).
33. Alfonso, A., Grundahl, K., Duerr, J.S., Han, H.P. & Rand, J.B. The *Caenorhabditis elegans unc-17* gene: a putative vesicular acetylcholine transporter. *Science* **261**, 617–619 (1993).
34. Alfonso, A., Grundahl, K., McManus, J.R. & Rand, J.B. Cloning and characterization of the choline acetyltransferase structural gene (*cha-1*) from *C. elegans*. *J. Neurosci.* **14**, 2290–2300 (1994).
35. Rand, J.B. Genetic analysis of the *cha-1-unc-17* gene complex in *Caenorhabditis*. *Genetics* **122**, 73–80 (1989).
36. Culotti, J.G., Von Ehrenstein, G., Culotti, M.R. & Russell, R.L. A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans*. *Genetics* **97**, 281–305 (1981).
37. Ward, S., Thomson, N., White, J.G. & Brenner, S. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J. Comp. Neurol.* **160**, 313–337 (1975).
38. Ware, R.W., Clark, D., Crossland, K. & Russell, R.L. The nerve ring of the nematode *Caenorhabditis elegans*: sensory input and motor output. *J. Comp. Neurol.* **162**, 71–110 (1975).
39. Jose, A.M., Bany, I.A., Chase, D.L. & Koelle, M.R. A specific subset of transient receptor potential vanilloid-type channel subunits in *Caenorhabditis elegans* endocrine cells function as mixed heteromers to promote neurotransmitter release. *Genetics* **175**, 93–105 (2007).
40. Lee, Y.S. *et al.* Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. *J. Neurochem.* **75**, 1800–1809 (2000).
41. Hwang, J.M. *et al.* Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. *Receptors Channels* **6**, 415–424 (1999).
42. Lee, Y.S. *et al.* Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. *J. Neurochem.* **72**, 58–65 (1999).
43. Putrenko, I., Zakikhani, M. & Dent, J.A. A family of acetylcholine-gated chloride channel subunits in *Caenorhabditis elegans*. *J. Biol. Chem.* **280**, 6392–6398 (2005).
44. Weinshenker, D., Garriga, G. & Thomas, J.H. Genetic and pharmacological analysis of neurotransmitters controlling egg laying in *C. elegans*. *J. Neurosci.* **15**, 6975–6985 (1995).
45. Hallem, E.A. & Sternberg, P.W. Acute carbon dioxide avoidance in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **105**, 8038–8043 (2008).
46. Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H. & Plasterk, R.H. Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28**, 160–164 (2001).
47. Jansen, G., Hazendonk, E., Thijssen, K.L. & Plasterk, R.H. Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. *Nat. Genet.* **17**, 119–121 (1997).
48. Finney, M. & Ruvkun, G. The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**, 895–905 (1990).
49. Liman, E.R., Tytgat, J. & Hess, P. Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron* **9**, 861–871 (1992).
50. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051–07–0, URL http://www.R-project.org (2006).
51. Bargmann, C.I. & Avery, L. Laser killing of cells in *Caenorhabditis elegans*. *Methods Cell Biol.* **48**, 225–250 (1995).