Cellular/Molecular

Activation of EGL-47, a $G\alpha_o$-Coupled Receptor, Inhibits Function of Hermaphrodite-Specific Motor Neurons to Regulate *Caenorhabditis elegans* Egg-Laying Behavior

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Caenorhabditis elegans **egg-laying behavior is inhibited by neurotransmitter signaling through the neural G-protein $G\alpha_o$ and serves as a model for analyzing $G\alpha_o$ signaling. Mutations that alter egg-laying frequency have identified genes encoding a number of signaling proteins that act with $G\alpha_o$, but the receptors that activate $G\alpha_o$ remain mostly uncharacterized. To further analyze $G\alpha_o$ signaling, we cloned the *egl-47* gene, which was identified by two dominant mutations that severely inhibit egg laying. *egl-47* encodes two orphan G-protein-coupled receptor isoforms, which share all seven transmembrane domains but have different extracellular N termini. Both dominant mutations change the same alanine to valine in the sixth transmembrane domain, resulting in constitutively activated receptors. Deletion of the *egl-47* gene caused no detectable egg-laying defects, suggesting that EGL-47 functions redundantly, or it inhibits egg laying under specific circumstances as yet unidentified. Using promoter::green fluorescent protein transgenes, we found that EGL-47 is expressed in a number of neurons, including the hermaphrodite-specific neurons (HSNs) that innervate the egg-laying muscles to stimulate contraction. Transgenic expression of constitutively active EGL-47 or constitutively active $G\alpha_o$ specifically in the HSNs was sufficient to inhibit egg-laying behavior. Our results suggest that EGL-47 regulates egg laying by activating $G\alpha_o$ in the HSN motor neurons to inhibit their activity. Because several neurotransmitters act through $G\alpha_o$ to inhibit HSN function, it appears that loss of any one receptor, such as EGL-47, causes only mild defects. $G\alpha_o$ apparently integrates signaling from multiple receptors in the HSNs, including EGL-47, to set the frequency of egg-laying behavior.**

Key words: **EGL-47; egg-laying behavior;** ***Caenorhabditis elegans*****; G-protein-coupled receptor; $G\alpha_o$; HSN neuron**

Introduction

$G\alpha_o$ is an abundant neural G-protein in humans and mediates signaling by many neurotransmitter receptors, but the mechanism and ultimate effects of $G\alpha_o$ signaling are not understood. *Caenorhabditis elegans* egg-laying behavior is inhibited by neurotransmission through $G\alpha_o$ and serves as a model for studying $G\alpha_o$ signaling. Eggs are laid when the hermaphrodite-specific neurons (HSNs) release serotonin to stimulate contraction of egg-laying muscles (Desai et al., 1988). Serotonin may additionally feedback inhibit HSN activity by signaling through $G\alpha_o$ to terminate an episode of egg laying, although the serotonin autoreceptor(s) responsible for this effect have not been identified (Shyn et al., 2003). The ventral type C (VC) neurons, which synapse onto both the HSN processes and the egg-laying muscles, inhibit egg laying by releasing acetylcholine (White et al., 1986; Bany et al., 2003). This acetylcholine is hypothesized to signal through $G\alpha_o$ in the HSN presynaptic termini to inhibit neurotransmitter release, thus blocking egg laying. GAR-2, a G-protein-coupled receptor on the HSNs, mediates a portion of the effects of acetylcholine on egg laying; additional unidentified acetylcholine receptors must also inhibit HSN function (Bany et al., 2003). Both serotonin and acetylcholine are thus thought to act through $G\alpha_o$ to presynaptically inhibit HSNs. $G\alpha_o$ is also expressed in egg-laying muscles and may have additional functions in these cells (Mendel et al., 1995; Ségalat et al., 1995; Shyn et al., 2003).

The mechanism of neurotransmitter signaling in the egg-laying system has been investigated via genetic screens for mutants with altered rates of egg laying (Trent et al., 1983; Desai and Horvitz, 1989). Some of these mutants simply have developmental defects in the HSNs, VCs, or egg-laying muscle cells. Other mutants are anatomically normal but have defects in neurotransmitter signaling. These have been used to identify components of the $G\alpha_o$ signaling pathway. For example, mutations that result in hyperactive egg-laying behavior have identified the $G\alpha_o$ ortholog GOA-1 and the regulator of G-protein signaling (RGS) protein EAT-16 (Mendel et al., 1995; Ségalat et al., 1995; Hajdu-Cronin et al., 1999). Mutations that reduce egg-laying behavior cause the egg-laying-defective (Egl) phenotype and have identified the RGS protein EGL-10, the $G\alpha_q$ protein EGL-30, and the PLCβ protein EGL-8, a downstream effector of $G\alpha_q$ (Brundage et al., 1996;

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Koelle and Horvitz, 1996; Lackner et al., 1999). Studies of these mutants have demonstrated that $G\alpha_o$ signaling is antagonized by $G\alpha_q$ and that each G-protein is under RGS control.

Overall, genetic studies of egg laying suggest that $G\alpha_o$ directs presynaptic inhibition in the HSNs. However, this idea remains based primarily on indirect evidence because it has not been shown that $G\alpha_o$ activity in the HSNs is sufficient to inhibit HSN function, and the receptors that activate $G\alpha_o$ in the HSNs remain mostly unknown. We have addressed these issues by cloning and characterizing *egl-47*, a gene identified by mutations that block egg laying. Our analysis shows that EGL-47 is a G-protein-coupled receptor found in the HSNs, which can activate GOA-1 in the HSNs to inhibit neurotransmitter release.

Materials and Methods

Nematode culture. The wild-type strain was Bristol N2. Worms were cultured at 20°C under standard conditions, and double- and triple-mutant strains were generated using standard genetic techniques (Brenner, 1974).

Unlaid egg assay. Unlaid eggs were quantified as described (Koelle and Horvitz, 1996). The staged adults used in all assays were obtained by collecting late fourth larval stage (L4) animals and culturing at 20°C for 30 hr. In the case of the experiment shown in Figure 5, animals were aged for 40 hr after L4 to allow expression from the cell-specific promoters, which become active only at the L4–adult transition. For each strain analyzed, at least 30 staged adults were individually dissolved in 1.2% sodium hypochlorite, and their eggs, which survived because of their protective eggshells, were counted.

Pharmacological assays. Individual staged adult animals were placed in 50 μl of M9 buffer (Brenner, 1974), or M9 containing 7.5 mg/ml serotonin or 0.5 mg/ml fluoxetine. After 30 min, the number of released eggs was counted. N2 animals typically laid 0 eggs in M9 buffer, 3–8 eggs in serotonin, and 5–8 eggs in fluoxetine. *egl-47(dm)* animals and Egl transgenic lines typically laid 0 eggs in M9, >15 eggs in serotonin, and 0 eggs in fluoxetine. Egl animals release a large number of eggs in serotonin because they have many unlaid eggs at the start of the assay.

Mapping of egl-47. Triple-mutant *unc-42(e270) egl-47(n1081dm) sqt-3(sc63ts)* hermaphrodites were crossed with polymorphism-containing Hawaiian CB4856 males. Two hundred eighty F2 Sqt-non-Unc animals were isolated: these carried recombination events in the 1.75 map units between *unc-42* and *sqt-3*. Recombinant chromosomes were homozygosed, and restriction digests of PCR products for nine polymorphic sites were used to determine whether Bristol or Hawaiian DNA was present, thereby determining the positions of recombination events. The presence or absence of the *egl-47(n1081dm)* mutation was determined by scoring for the Egl phenotype.

Transcript analysis. The cDNA clone yk1065f10, encoding EGL-47B (GenBank accession number NM_073148), was obtained from Dr. Yuji Kohara (National Institute of Genetics of Japan). RNA was prepared from mixed stage worms and analyzed by the 5′ rapid amplification of cDNA ends (RACE) technique (Frohman et al., 1988), identifying the transcript encoding EGL-47A (GenBank accession number AY532645).

egl-47(n1081dm) *transformation.* Genomic DNA was prepared was from *n1081dm* mutants and used for long-range PCR using the GeneAmp XL PCR kit (PerkinElmer Life Sciences, Emeryville, CA). PCR products were purified using QIAEX II (Qiagen, Hilden, Germany) and injected into *lin-15(765ts)* animals at 1 ng/μl along with the *lin-15*-rescuing plasmid pL15EK at 50 ng/μl.

egl-47 *gene knock-out.* The *egl-47(vs81)* deletion mutation was identified by the method of Liu et al. (1999) using a PCR screen of DNA from a frozen *C. elegans* mutant library representing the progeny of 460,000 trimethylpsoralen-mutagenized animals. Deletion mutants were backcrossed four times to wild-type N2 animals to produce a clean genetic background. *egl-47(vs81)* is a 2501 bp deletion of sequences whose limits are TCATTTT TGTAGCGAGACAG . . . GCTCAACTGGTATGATGACT.

Egg-laying behavior of egl-47 *knock-out.* Egg-laying assays were performed according to the following references: inhibition by aldicarb (Bany et al., 2003), inhibition by octopamine (Tsalik et al., 2003), inhibition by dopamine or chronic serotonin (Schafer and Kenyon, 1995), response to starvation and refeeding (Dong et al., 2000), inhibition by vibration (Sawin, 1996), inhibition by liquid medium and stimulation by acute serotonin (Trent et al., 1983), and stimulation by fluoxetine (Weinshenker et al., 1995).

Wild-type and mutant egl-47 *transgenes.* Genomic DNA containing the coding sequences and 4851 bp 5′ of the first A-isoform exon and 733 bp of 3′ untranslated region were subcloned into pBluescript (Stratagene, La Jolla, CA) to make pJM5. Additional constructs contained one or two mutations introduced into pJM5. Single-nucleotide additions caused frameshifts in the second A-specific exon, first B-specific exon, or first common region exon. We also engineered the missense mutation found in *n1081(dm).* Transgenic strains were generated by injecting 10 ng/μl pJKL449.1 (*myo-2–gfp* coinjection marker; a gift from A. Fire, Carnegie Institute of Washington, Washington, DC), with either 50 ng/μl test construct and 20 ng/μl pBluescript or 2 ng/μl of test construct and 68 ng/μl pBluescript, into *egl-47(vs81)* deletion animals. We analyzed at least five independent transgenic lines for each injection and used only green fluorescent protein (GFP)-positive animals in the unlaid egg assay. Because extrachromosomal transgenes used are randomly lost during development to produce genetically mosaic animals, transgenes that induced the Egl phenotype did not always do so in 100% of animals within a line. An individual animal was defined as Egl if it contained at least 30 eggs. Any line with >20% Egl animals was defined as an Egl line. Transgenes that failed to produce Egl lines by this definition never produced >2% Egl animals.

GFP transgenes. A GFP reporter plasmid was constructed by inserting a fragment from cosmid C50H2 into pPD95.77 (a gift from A. Fire). This construct contained the promoter region and 5′ coding sequences of *egl-47*, such that the first common exon was fused in frame to the coding sequence for GFP. The construct contained the same promoter region as the *egl-47* expression transgenes. Single-nucleotide additions to either the second A- or B-specific exons resulted in frameshift mutations to block expression of one isoform, thus generating isoform-specific expression patterns.

HSN and egg-laying muscle promoter transgenes. Based on the work of Sze et al. (2002), we generated two vectors to drive cell-specific expression. pNSM (pJM51A) contains a 377 bp fragment of the *tph-1* promoter inserted into the *Hin*dIII--*Sph*I site of pPD49.26 (a gift from A. Fire). When the GFP coding sequence was inserted between the *Kpn*I and *Sac*I sites of pJM51A, the resulting construct gave robust expression in the two NSM neurons: no other expression was detected. pNSM/HSN (pJM66A) contains a 3124 bp fragment of the *tph-1* promoter inserted into the *Sph*I site of pPD49.26. When the GFP coding sequence was inserted between the *Kpn*I and *Sac*I sites of pJM66A, the resulting plasmid gave robust GFP expression in the two NSMs and the two HSN neurons: no other expression was detected. To drive expression in egg-laying muscles, we used the pELM promoter, derived from pBH34.21 (Harfe and Fire, 1998), which contains a multimer of the *Nde*I-box enhancer in front of a minimal *pes-10* promoter. pNSM and pNSM/HSN, and pELM were used to drive cDNAs encoding EGL-47B(gf) and GOA-1(Q205L). The EGL-47B cDNA clone yk1065f10 was modified to contain the gain-of-function mutation. pNSM::cDNA, pNSM/HSN::cDNA, and pELM::cDNA constructs were coinjected with the corresponding GFP constructs and the *lin-15*-rescuing plasmid pL15EK (50 ng/μl) into *lin-15(n765ts)* animals. Staged non-Muv adults were used in the unlaid egg assay.

Fluorescent visualization of HSN morphology. Transgenic worms containing pNSM/HSN (pJM60A) were paralyzed in a drop of 2 mM levamisole atop a thin layer of 3% agarose on a glass slide. For each strain analyzed, the morphology of the HSNs in 10 worms was examined using a Bio-Rad (Hercules, CA) MRC 1024 confocal microscope.

Results

egl-47 dominant mutants have a defect in HSN neuron function

egl-47 was originally identified by two dominant mutations that cause a phenotype associated with a defect in HSN neuron activity (Desai and Horvitz, 1989). This phenotype has four charac-

teristics: (1) animals accumulate unlaid eggs, suggesting that egg-laying behavior fails to occur; (2) animals can be stimulated to lay eggs by treatment with exogenous serotonin, demonstrating that the egg-laying muscles are functional and that the egg-laying defect is thus likely to result from lack of HSN function; (3) serotonin reuptake inhibitors, such as imipramine and fluoxetine, fail to stimulate egg laying, suggesting that the HSN neurons fail to release serotonin, whose action would otherwise be potentiated by these drugs; and (4) the HSN neurons appear morphologically normal, suggesting that they have functional rather than developmental defects.

We analyzed the *egl-47* phenotype in detail. We found that the two dominant (dm) *egl-47* mutants were phenotypically indistinguishable. *egl-47(n1081dm)* animals accumulated 44 ± 4 unlaid eggs, and *egl-47(n1082dm)* animals accumulated 44 ± 2 eggs, whereas the wild-type accumulated only 14 ± 1 eggs (Fig. 1*A*,*B*,*F*). The accumulation of eggs in *egl-47(dm)* animals was similar to that seen in *egl-1(dm)* mutant animals, which completely lack HSN neurons because of a developmental defect (Trent et al., 1983). Both *egl-47* alleles were fully dominant because heterozygotes were indistinguishable from homozygotes (Fig. 1*F*). We verified that both mutants laid eggs in response to serotonin but not to fluoxetine (data not shown). Finally, we developed a GFP reporter that enabled us to analyze the fine details of HSN morphology in living animals. HSN cell bodies are normally located slightly posterior to the vulva and extend a single process that has a small branch near the vulva and continues anteriorly toward the nerve ring in the head (Fig. 1*D*). HSN morphology in *egl-47(n1081dm)* animals was within the variability seen for the wild type (Fig. 1*D*,*E*).

egl-47 mutants thus meet all four criteria outlined above, suggesting that they have severe defects in HSN function but normal HSN development. Similarly, null mutants for *egl-10*, the RGS protein that inactivates the G-protein GOA-1, also have the same four characteristics (Koelle and Horvitz, 1996). This similarity suggests that *egl-47* dominant mutations, like *egl-10* null mutations, might result in increased GOA-1 signaling. *egl-10* mutants show a variety of behavioral defects, such as sluggish locomotion, that are attributable to increased GOA-1 signaling in cells outside the egg-laying system (Koelle and Horvitz, 1996). In contrast, we have not detected locomotion defects in *egl-47* mutants, suggesting that EGL-47 function may be restricted to the egg-laying system.

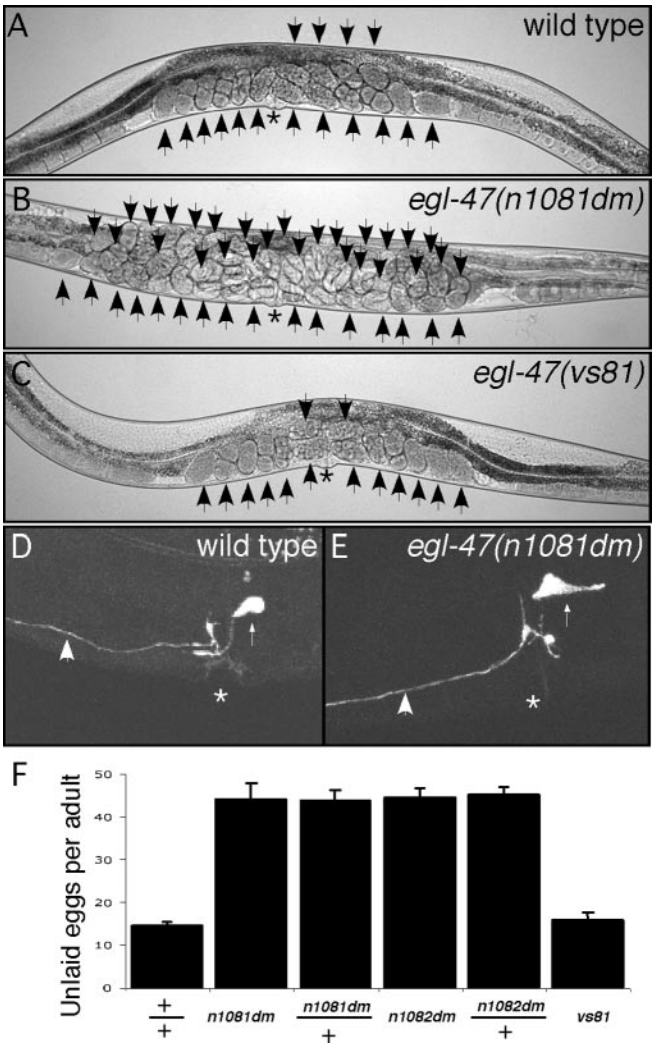


Figure 1. Characteristics of adult *egl-47* mutants. *A*, Wild-type hermaphrodite. *B*, *egl-47(n1081dm)* gain-of-function mutant. *C*, *egl-47(vs81)* null mutant. Arrows indicate unlaid eggs; asterisks indicate the vulvas. *n1081dm* animals rarely lay eggs, causing an accumulation of unlaid eggs. *D*, *E*, HSN morphology in a wild-type and *n1081dm* hermaphrodite revealed by GFP fluorescence. High-magnification micrographs of the vulval region are shown. Large arrows indicate HSN processes; small arrows indicate the HSN cell bodies; asterisks indicate the vulvas. HSN morphology was not affected by *egl-47(n1081dm)*. *F*, Number of unlaid eggs retained in wild-type or mutant strains. $n \geq 30$ for all measurements. *n1081dm* and *n1082dm* mutations caused animals to fill with eggs and were fully dominant, as heterozygous and homozygous animals accumulated numbers of unlaid eggs that were not statistically different ($p > 0.1$, Student's *t* test). The *vs81* null mutant was not statistically distinguishable from the wild type.

egl-47 encodes two orphan G-protein-coupled receptors

We set out to molecularly identify the *egl-47* gene. *egl-47* had previously been mapped to a 403 kb interval between *unc-42* and *egl-3* on chromosome V (Desai and Horvitz, 1989). We identified single-nucleotide polymorphisms in this interval and used them for high-resolution mapping. *egl-47* mapped to the 97 kb interval between polymorphisms *vsP4* and *vsP8*. Seven recombinants within this interval did not separate *egl-47* from two polymorphisms, *vsP5* and *vsP7,* suggesting that *egl-47* lies very close to these polymorphisms (Fig. 2*A*). We used transgenes to identify the gene in this region corresponding to *egl-47*. Because *egl-47* mutations are dominant, we expected that transforming wild-type animals with a genomic clone containing the mutant *egl-47* gene would induce the Egl phenotype. We used DNA from *n1081dm* animals as a template to generate long-range PCR products. Transgenic animals with the PCR product containing predicted open reading frame C50H2.2 phenocopied *egl-47(dm)* mutants: they were full of eggs, laid eggs in response to exogenous serotonin, and did not lay eggs in response to fluoxetine (data not shown). The C50H2.2 coding region was sequenced for both *egl-47(dm)* alleles. Both had the same mutation, changing an alanine to a valine (Fig. 2*C*). Thus, genetic mapping, transformation, and sequencing confirmed C50H2.2 as *egl-47*.

To analyze the *egl-47* transcripts we obtained an *egl-47* cDNA, which was full-length because it included an SL1 trans-spliced leader at its 5′ end and a poly(A) tract at its 3′ end. Using the RACE technique, we found that *egl-47* produces two types of transcripts, one corresponding to the cDNA and the other containing different 5′ exons as well as an SL1 leader (Fig. 2*B*). EGL-47 therefore appears to use two different promoters to generate transcripts with two different 5′ ends. These transcripts encode two protein isoforms (EGL-47A and EGL-47B) with different N-terminal sequences and the same C-terminal sequence.

The deduced amino acid sequences contain seven predicted

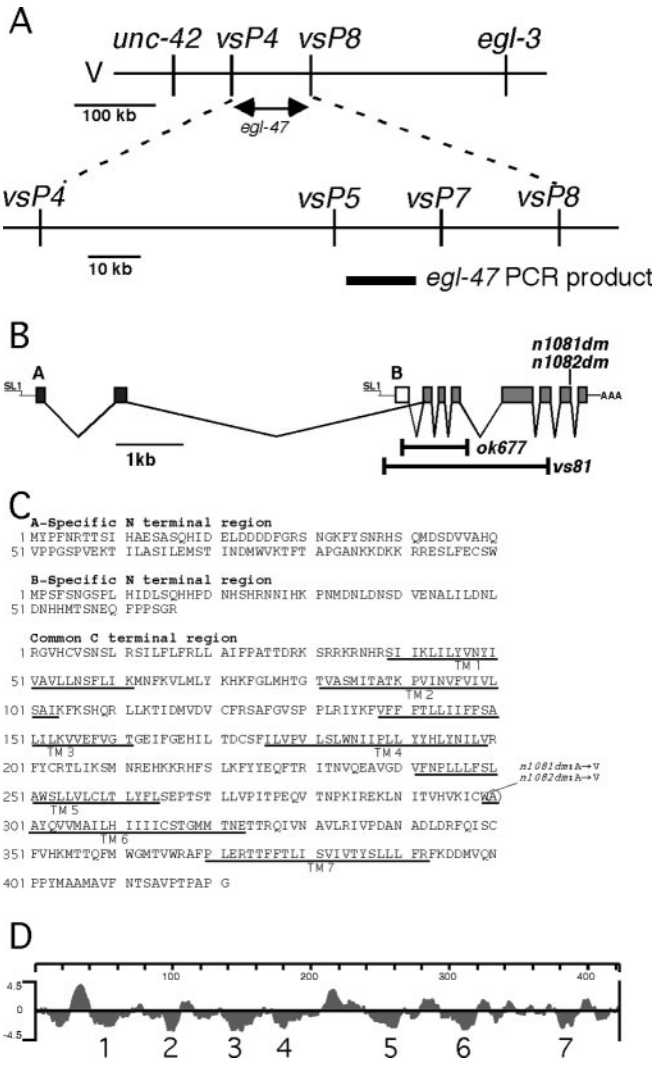


Figure 2. *egl-47* mapping, exon structure, protein sequence, and hydrophilicity plot. *A*, Mapping and cloning strategy for *egl-47*. The top line schematizes the genetic positioning of *egl-47* between single-nucleotide polymorphisms *vsP4* and *vsP8*. The bottom line shows additional polymorphisms that were not separated from *egl-47* by seven recombination events within this interval. The thick bar under the bottom line represents a PCR product that contains *egl-47*. When amplified from the *n1081dm* gain-of-function mutant and transformed into wild-type animals, this PCR product induced the *egl-47* egg-laying defect. *B*, *egl-47* gene structure and locations of mutations. Black boxes represent A-specific exons; the white box represents the B-specific exon; the gray boxes indicate exons common to both isoforms. The SL1 trans-splice leaders and poly(A) tail found at the ends of the transcripts are indicated. Bars indicate deleted regions of probable null alleles *vs81* and *ok677*. *C*, Protein sequence of the A-specific region, B-specific region, and common region. Predicted transmembrane domains are underlined. The circle indicates the amino acid residue altered in dominant mutants *n1081dm* and *n1082dm*. *D*, Kyte–Doolittle hydrophilicity plot of the common region. Seven predicted transmembrane domains are indicated.

transmembrane domains encoded by the exons common to both isoforms (Fig. 2*C*,*D*), suggesting that EGL-47A and EGL-47B are G-protein-coupled receptors (GPCRs). The fact that an alanine to valine change in the sixth transmembrane domain is a dominant mutation in EGL-47 is consistent with the hypothesis that EGL-47 proteins are GPCRs because similar mutations cause constitutive activity in known GPCRs (Parnot et al., 2002). Ligand binding often occurs in the transmembrane domains of GPCRs, but the extracellular N termini can also contribute to ligand binding (Bockaert and Pin, 1999); thus, the different N termini of EGL-47A and EGL-47B could cause these isoforms to bind different ligands. GPCR sequences are highly diverse, but many can be assigned to subfamilies based on sequence similarity (Remm and Sonnhammer, 2000). Comparison of EGL-47 proteins to other GPCRs, however, shows that they cannot be clearly assigned to any subfamily. We performed a basic local alignment search tool (BLAST) search with the EGL-47 common region and identified the *Drosophila* orphan receptor Gr39a, a putative gustatory receptor (Clyne et al., 2000), as the most similar known sequence. No *C. elegans* receptors with high similarity to EGL-47 were identified. Thus, sequence analysis does not suggest which ligand(s) might activate EGL-47A and EGL-47B.

Our genetic and molecular analyses of *egl-47* suggest a model in which the EGL-47 proteins function as receptors to activate the G-protein GOA-1. First, *egl-47(dm)* mutations block egg laying in a manner similar to that of mutations that increase GOA-1 signaling. Second, the EGL-47 proteins have the seven transmembrane domains characteristic of GPCRs, proteins that directly activate G-proteins. Third, the *egl-47(dm)* mutations are similar to mutations in other GPCRs that cause constitutive (ligand-independent) activation of G-proteins. Based on these lines of evidence, we hereafter refer to the EGL-47 proteins as orphan G-protein-coupled receptors. Definitive proof that EGL-47 proteins are receptors that directly activate GOA-1 would require expressing the EGL-47 proteins and showing that they stimulate GTP binding by GOA-1. However, we have so far been unable to achieve expression of EGL-47 protein in cultured cells for this purpose.

egl-47 null animals have no obvious phenotypic defects

We generated *egl-47* null mutants to determine their phenotype. We screened a library of mutagenized animals to obtain deletion alleles of *egl-47*. We recovered one allele (*vs81*) from this library that had a 2501 bp deletion. The deletion removed the B-specific exon and five of the seven common exons (Fig. 2*B*). *vs81* is thus a presumptive *egl-47* null allele. *egl-47(vs81)* mutants appeared normal (Fig. 1*C*) and had wild-type levels of unlaid eggs (Fig. 1*F*). *ok677*, a deletion allele obtained from the *C. elegans* knock-out consortium, also appeared to be wild type. Wild-type animals alter their egg laying in response to multiple external factors, including the availability of food (Dong et al., 2000), the presence of vibration (Sawin, 1996), and immersion in liquid (Trent et al., 1983). In addition, treatment with several neurotransmitters or drugs can alter egg laying, including fluoxetine (Weinshenker et al., 1995), acute serotonin (Trent et al., 1983), chronic serotonin (Schafer and Kenyon, 1995), aldicarb (Bany et al., 2003), octopamine (Tsalik et al., 2003), and dopamine (Schafer and Kenyon, 1995). We tested the ability of *vs81* animals to alter egg laying under all of these conditions and found *vs81* animals were able to properly regulate egg laying in response to all of the conditions tested (Table 1). Although we failed to detect a defect in null mutants, EGL-47 may have subtle or redundant functions in egg laying. Another possibility is that EGL-47 is required for regulation of egg laying in response to yet another environmental condition that remains to be discovered.

Overexpression of wild-type EGL-47 blocks egg laying

We used transgenes to overexpress EGL-47 in *C. elegans* to determine the effects of increasing EGL-47 signaling. In general, overexpression of GPCRs can lead to increased response to ligands as well as constitutive signaling by these receptors (Chen et al., 2000). We constructed a plasmid containing a fragment of genomic DNA including the entire wild-type *egl-47* gene. To overexpress EGL-47, we injected a solution containing a high

Table 1. Egg-laying analysis of *egl-47* null animals

Egg-laying behavior assayed[a]	Test conditions	Wild-type measurement	*egl-47(vs81)* measurement
Response to starvation and refeeding[b]	Starved/starved	6.0 ± 4.0	6.0 ± 4.0
	Fed/fed	64.5 ± 1.5	62.0 ± 4.0
	Starved/fed	50.0 ± 15.0	64.5 ± 15.5
Inhibition by vibration[c]	No vibration	33.2 ± 4.2	24.6 ± 4.5
	Vibration	0.0 ± 0.0	0.0 ± 0.0
Inhibition in liquid medium[d]		0.0 ± 0.0	0.0 ± 0.0
Stimulation by fluoxetine[d]	0.5 mg/ml	8.5 ± 0.9	9.3 ± 0.9
Stimulation by acute serotonin[d]	7.5 mg/ml	3.8 ± 1.2	3.7 ± 1.2
Inhibition by chronic serotonin[e]	0 mg/ml	0.0 ± 0.0	0.0 ± 0.0
	3 mg/ml	92.1 ± 3.7	87.5 ± 4.6
Inhibition by aldicarb[f]	0 μM	90.7 ± 9.0	94.0 ± 8.3
	10 μM	74.3 ± 7.7	81.4 ± 4.3
Inhibition by octopamine[g]	0 mg/ml	18.8 ± 1.3	20.2 ± 2.8
	16 mg/ml	2.0 ± 1.3	0.8 ± 0.4
Inhibition by dopamine[h]	0 mg/ml	55.8 ± 11.7	54.2 ± 7.5
	6 mg/ml	7.7 ± 4.2	7.6 ± 4.5

[a]References for assays are given in Materials and Methods. Errors represent SEM. None of the differences between wild type and *vs81* are statistically significant.

[b]Ten animals were preconditioned for 2 hr on plates containing (to feed the animals) or not containing (to starve them) bacteria. Animals were then moved to assay plates with or without food, and the number of eggs laid in 30 min was counted. $n = 2$.

[c]Eggs laid by five animals in 20 min on a plate with or without vibration. $n = 5$.

[d]Eggs laid by one animal in 30 min in M9 liquid containing the indicated drug. $n = 10$.

[e]Percentage of egg-laying-defective animals after 15 hr on a plate containing the indicated amounts of serotonin. $n \geq 50$.

[f]Eggs laid by 10 animals in 60 min on a plate containing the indicated amounts of aldicarb. $n \geq 3$.

[g]Eggs laid by two animals in 60 min on a plate containing the indicated amounts of octopamine. $n = 5$.

[h]Eggs laid by 10 animals in 45 min on a plate containing the indicated amounts of dopamine. $n = 6$.

concentration (50 ng/μl) of this plasmid into worms to generate transgenic lines carrying high-copy tandem arrays of the plasmid. All five transgenic lines produced a significant number of Egl animals (Fig. 3*B*, row 1) that laid eggs in response to serotonin but not fluoxetine, phenocopying the genomic *egl-47(dm)* mutants. We modified the plasmid by inserting a stop codon into the first common exon (Fig. 3*A*, C stop) to prevent expression of either isoform of EGL-47. When injected at a high concentration, none of the five lines produced were Egl (Fig. 3*B*, row 2). Thus, EGL-47 protein expression is required to induce the Egl phenotype. To obtain lines with lower-copy transgenes and presumably lower EGL-47 expression, we injected a solution containing 2 ng/μl wild-type plasmid: this did not produce Egl lines (Fig. 3*B*, row 3). These experiments showed that an increase in EGL-47 signaling, induced by high-level expression of wild-type EGL-47, can cause an Egl phenotype. In addition, because *egl-47(dm)* mutations cause a similar Egl phenotype, these mutations appear to act by increasing the normal signaling function of EGL-47.

Mutant EGL-47 is more active than wild-type EGL-47

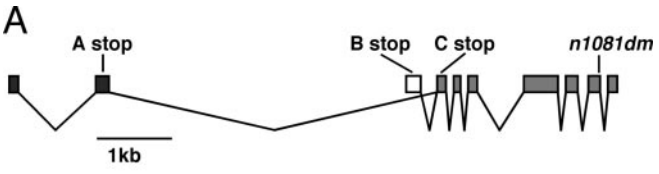
To further analyze the effect of the *egl-47(dm)* mutation on protein function, we modified the *egl-47* genomic clone to carry the *n1081dm* mutation (Fig. 3*A*). When injected at a low concentration, this modified clone conferred the Egl phenotype in four of six lines (Fig. 3*B*, row 4). Protein expression is required for this effect because insertion of a stop codon into the first common exon prevented the generation of any Egl lines (Fig. 3*B*, row 5). Because the wild-type clone did not induce the Egl phenotype at a low concentration, these results suggest that EGL-47 protein with the alanine to valine gain-of-function mutation is more potent than wild-type EGL-47 protein. This is consistent with *n1081dm* being an activating mutation that causes constitutive EGL-47 activity. Because the mutant proteins result in a gain of function, we hereafter refer to them as EGL-47(gf).

EGL-47A(gf) or EGL-47B(gf) can inhibit egg laying

To determine which isoform of EGL-47 is responsible for regulating egg laying, we constructed isoform-specific expression plasmids. We modified the *egl-47(n1081dm)*-containing construct to have a stop codon in either an A- or B-specific exon, so that only one isoform could be produced (Fig. 3*A*). When injected at the low concentration of 2 ng/μl, each of these constructs made four of five lines Egl (Fig. 3*B*, rows 6, 7). These experiments indicate that either EGL-47A(gf) or EGL-47B(gf) can induce the Egl phenotype.

egl-47 is expressed in the HSNs and other neurons

To examine the expression pattern of *egl-47*, we fused an *egl-47* genomic fragment, containing the promoters for both *egl-47* transcripts as well as the coding sequences up to the first common exon, to the coding sequences for the GFP. This construct was further modified to contain a stop codon in either an A- or B-specific exon. This allowed visualization of the expression pattern specific for each isoform. We examined transgenic animals carrying these constructs by confocal fluorescent microscopy. EGL-47A::GFP was expressed in a small subset of neurons in the head (Fig. 4*A*), the HSN neurons (Fig. 4*B*), and the PVQ interneurons of the tail (Fig. 4*C*). In the fourth larval stage, EGL-47A::GFP was expressed in the vulval cells (Fig. 4*D*), but this was not detected in the adult. A similar pattern was observed for EGL-47B::GFP (Fig. 4*E–H*). The similar expression patterns of the two isoforms are consistent with those of the earlier transgenic experiments showing that either isoform can cause the Egl phenotype. The HSNs were the only cells in the adult egg-laying system to express EGL-47, suggesting that EGL-47 functions in the HSNs to inhibit egg laying. Expression outside of the egg-laying system was unexpected because the only obvious defect in *egl-47* mutants is in egg-laying behavior.



B

	Mutation(s) in transgene	Proteins encoded by transgene	[DNA] injected (ng/μl)	Egl lines
1	None	A,B	50	5/5
2	C stop	None	50	0/5
3	None	A,B	2	0/5
4	*n1081dm*	A(gf),B(gf)	2	4/6
5	C stop,*n1081dm*	None	2	0/5
6	A stop,*n1081dm*	B(gf)	2	4/5
7	B stop,*n1081dm*	A(gf)	2	4/5

Figure 3. Diagram and effects on egg laying of wild-type and mutant *egl-47* transgenes. *A*, Diagram of *egl-47* transgenes. Stop codons are shown that were included in certain transgenes so that one, both, or neither of the isoforms could be expressed. Also shown is the *n1081dm* gain-of-function (gf) mutation, included in some transgenes. The *egl-47* coding exons are indicated by boxes; A-specific exons are black; the B-specific exon is white; common exons are gray. In addition to the coding regions shown, the transgenes contained 4.8 kb of 5′ promoter and 0.7 kb of 3′ untranslated region DNA. *B*, Effects of the transgenes. Indicated are the mutations included in individual transgenes, concentrations of DNA injected, and the number of Egl lines produced. Lines were defined as Egl if >20% of adults contained at least 30 eggs. When EGL-47 activity was increased, by overexpression or by mutation, egg laying was inhibited. Either isoform was capable of inhibiting egg laying.

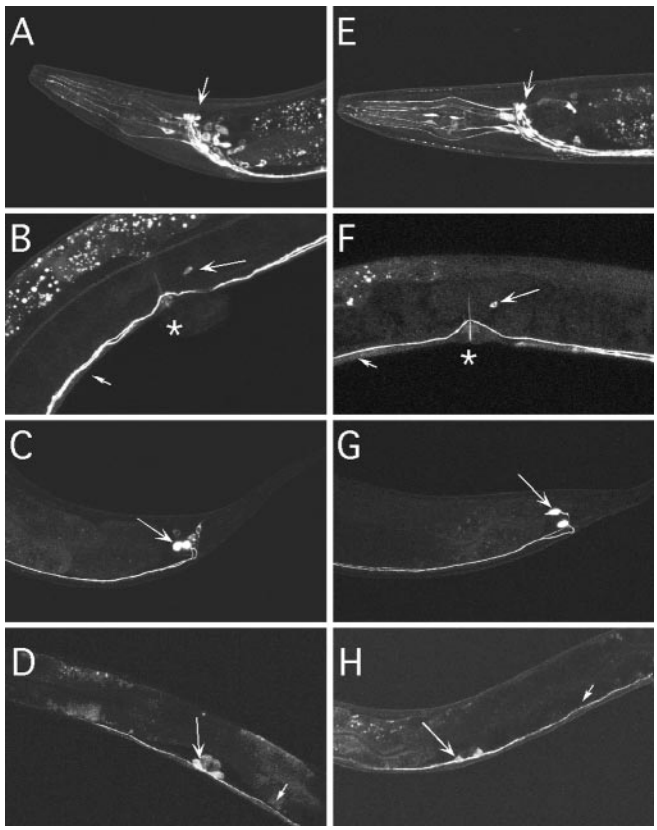


Figure 4. Expression patterns of *egl-47::gfp* reporter transgenes. *A–D*, GFP expression driven from the *egl-47* isoform A promoter. *E–H*, GFP expression driven from the *egl-47* isoform B promoter. *A, E*, Head region. Arrows point to fluorescent processes of the nerve ring flanked by a number of associated cell bodies. *B, F*, Midbody region. Small arrows indicate fluorescent neural processes of the ventral cord; large arrows indicate the HSN cell bodies; asterisks indicate the vulvas. *C, G*, Tail region. Arrows indicate the PVQ neural cell bodies, which send processes anteriorly along the ventral nerve cord. *D, H*, Midbody region of animals in the fourth larval stage. Large arrows indicate fluorescent vulval cells; small arrows indicate fluorescent neural processes of the ventral cord. EGL-47 isoforms A and B have similar expression patterns. In the adult, the HSNs are the only cells of the egg-laying system that express *egl-47*.

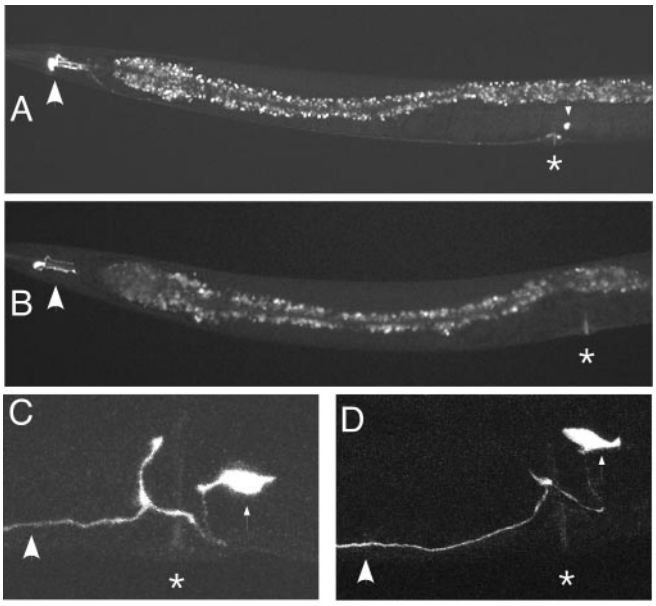
EGL-47(gf) expressed in the HSNs can induce the Egl phenotype

Because the *egl-47(dm)* phenotype suggested a functional defect in the HSNs, and the expression pattern described above demonstrates EGL-47 was expressed in the HSNs, we tested whether activation of EGL-47 specifically in the HSNs was sufficient to induce the Egl phenotype. For this purpose, we expressed a cDNA for EGL-47B(gf) in the HSNs and determined whether this conferred the Egl phenotype on wild-type animals. Because there was no completely HSN-specific promoter available, we drove cDNA expression using pNSM/HSN, a promoter that expresses only in the HSNs and the NSMs (Fig. 5*A*). The NSMs are a pair of neurons in the head with no known role in egg laying. To rule out effects attributable to NSM expression, we also used pNSM, a modified version of pNSM/HSN that lacks HSN expression but retains NSM expression (Fig. 5*B*). Expression of EGL-47B(gf) from pNSM/HSN caused the Egl phenotype in five of seven lines (Fig. 5*E,* row 1). Egl animals were similar to the *egl-47(dm)* mutants in that they laid eggs in response to serotonin but not fluoxetine. They were also similar to *egl-47(dm)* mutants in that they had normal HSN morphology, as visualized by coexpression of GFP in the HSNs (Fig. 5*C*). No Egl lines were produced when EGL-47B(gf) was expressed from pNSM (Fig. 5*E,* row 2), when only GFP was expressed from pNSM (Fig. 5*E,* row 3), or when GFP was expressed from pNSM/HSN (Fig. 5*E,* row 4). Thus, we conclude EGL-47 activity in the HSNs is sufficient to induce the Egl phenotype associated with *egl-47(dm)* mutants.

Activated GOA-1 expressed in the HSNs can induce the Egl phenotype

What G-protein is activated by EGL-47 to inhibit egg laying? The $G\alpha_o$ homolog GOA-1 is expressed in all neurons, including the HSNs. Null mutants for GOA-1 are hyperactive for egg laying, suggesting that this G-protein inhibits egg laying (Mendel et al., 1995; Ségalat et al., 1995). Deletion mutants for every $G\alpha$ gene of *C. elegans* have been analyzed, and GOA-1 was the only $G\alpha$ protein found to inhibit egg laying, although other G-proteins might also affect egg laying but could have escaped detection because of redundancy (Jansen et al., 1999). These observations suggest GOA-1 to be the G-protein activated by EGL-47 receptors in the HSNs to inhibit egg laying. As one test of this idea, we examined the phenotype of animals carrying a *goa-1* null mutation and the *egl-47(dm)* mutation. We found *goa-1; egl-47(dm)* double mutants were similar to *goa-1* single mutants: both strains laid eggs so frequently that they retained an average of fewer than three unlaid eggs per adult, compared with the 14 ± 1 unlaid eggs seen in the wild type or the 44 ± 4 unlaid eggs seen in *egl-47(dm)* single mutants. Thus, GOA-1 is required for the EGL-47(gf) protein to inhibit egg laying, consistent with EGL-47 signaling through GOA-1.

To determine whether GOA-1 activation in the HSNs is sufficient to inhibit egg laying, we expressed a constitutively active mutant of GOA-1 in the HSNs. We used the pNSM and pNSM/HSN promoters to express GOA-1(Q205L), a GTPase-deficient mutant in which the glutamine at residue 205 is replaced with a



E

	Promoter	Proteins encoded by transgene	[DNA] injected (ng/μl)	Egl lines
1	pNSM/HSN	GFP	50	5/7
	pNSM/HSN	EGL-47B(gf)	50	
2	pNSM	GFP	50	0/5
	pNSM	EGL-47B(gf)	50	
3	pNSM	GFP	100	0/5
4	pNSM/HSN	GFP	100	0/5
5	pNSM/HSN	GFP	50	5/7
	pNSM/HSN	GOA-1(Q205L)	50	
6	pNSM	GFP	50	0/5
	pNSM	GOA-1(Q205L)	50	
7	pELM	GFP	20	0/5
8	pELM	GFP	10	0/5
	pELM	GOA-1(Q205L)	10	

Figure 5. Effects of expression of EGL-47B(gf) or activated GOA-1 in the HSNs and egg-laying muscles. *A*, Adult with GFP expression driven from the pNSM/HSN promoter. The large arrowhead indicates the fluorescent NSMs; the small arrowhead indicates the fluorescent HSN cell body; the asterisk indicates the position of the vulva. The HSN process can be seen passing the vulva and extending to the head. The intestine, a large structure running the length of the body, shows prominent punctate autofluorescence but does not express GFP. *B*, Adult with GFP expression driven by the pNSM promoter. The large arrowhead indicates the fluorescent NSMs. No HSN expression is evident. *C*, *D*, HSN morphology in animals expressing EGL-47B(gf) (*C*) or activated GOA-1(*D*) in the HSNs, revealed by GFP fluorescence. High-magnification micrographs of the vulval region are shown. The large arrowheads indicate HSN processes; small arrowheads indicate the HSN cell bodies; asterisks indicate the vulvas. HSN morphology appeared unaffected by expression of either EGL-47B(gf) or activated GOA-1 (compare with Fig. 1*D*). *E*, Effects of expression of EGL-47B(gf) or activated GOA-1 in specific cells of the egg-laying system. Promoters pNSM or pNSM/HSN were used to drive expression of cDNAs for EGL-47B(gf) or activated GOA-1 (carrying the Q205L mutation), as indicated. In addition, promoter pELM was used to express GOA-1(Q205L) in egg-laying muscles. In every case, GFP was coexpressed to visualize cell morphology, as shown in *A–D*. As negative controls, GFP was expressed alone, i.e., without EGL-47(gf) or GOA-1(Q205L). At least five independent lines were assayed for each experiment. Lines were defined as Egl if >20% of adults contained at least 30 eggs. Activation of EGL-47 in the HSNs or activation of GOA-1 in the HSNs inhibited egg laying similarly, suggesting that EGL-47 activates GOA-1 in the HSNs to inhibit egg laying.

leucine (Mendel et al., 1995). GOA-1(Q205L) expressed in the NSMs and the HSNs caused five of seven lines to be Egl (Fig. 5*E*, row 5). In the negative control, GOA-1(Q205L) expressed in the NSMs did not generate any Egl lines (Fig. 5*E*, row 6). The Egl animals were stimulated to lay eggs in response to serotonin but not to fluoxetine (data not shown), phenocopying the *egl-47(dm)* mutants. They were also similar to *egl-47(dm)* mutants in that they had normal HSN morphology, as visualized by coexpression of GFP in the HSNs (Fig. 5*D*).

Previous work has shown that GOA-1 is expressed in the egg-laying muscles and may function in these cells to inhibit egg laying (Mendel et al., 1995; Ségalat et al., 1995, Shyn et al., 2003). Thus, we also tested whether expression of GOA-1(Q205L) in the egg-laying muscles affected egg laying. The pELM promoter (Harfe and Fire, 1998) was used to drive expression of GOA-1(Q205L) and/or GFP in all 16 egg-laying muscles. This resulted in very bright GFP fluorescence in the muscles, but did not cause animals to be Egl (Fig. 5, rows 7, 8). Although this negative result does not rule out a function for GOA-1 in the egg-laying muscles, it suggests that the predominant site at which GOA-1 inhibits egg laying may be in the HSN neurons, rather than in the egg-laying muscles.

In summary, activation of GOA-1 in the HSNs was sufficient to inhibit egg laying. Because expressing EGL-47B(gf) in the HSNs was similarly sufficient to inhibit egg laying, these results are consistent with the idea that the normal function of EGL-47 is to signal through GOA-1 in the HSNs to regulate egg laying.

Discussion

Activated EGL-47 prevents egg laying by activating GOA-1 to inhibit neurotransmitter release from the HSNs

We suggest a model in which activation of EGL-47 receptor in the HSN motor neurons activates the $G\alpha_o$ protein GOA-1 to inhibit neurotransmitter release, thereby inhibiting egg laying. This is supported by the following: (1) activating EGL-47 blocks egg laying; (2) serotonin release from the HSNs in *egl-47(dm)* animals is reduced because serotonin reuptake inhibitors have no effect on egg laying; (3) EGL-47 contains seven transmembrane domains and is thus a putative G-protein-coupled receptor; (4) the amino acid substitution found in *egl-47(dm)* mutants is consistent with causing constitutive receptor activation; (5) GFP reporters show EGL-47 is expressed in the HSNs; (6) expression of activated EGL-47 specifically in HSNs blocks egg laying, phenocopying *egl-47(dm)* mutants; (7) the effects of EGL-47 activation require the G-protein GOA-1; and (8) expression of activated GOA-1 specifically in HSNs blocks egg laying, phenocopying *egl-47(dm)* mutants.

Previous studies have suggested a mechanism by which GOA-1 can inhibit neurotransmitter release. GOA-1 signaling results in relocalization of the synaptic protein UNC-13 away from neurotransmitter release sites in certain ventral cord neurons (Nurrish et al., 1999). UNC-13 is required to prime synaptic vesicles for release and is recruited to membranes by binding diacylglycerol (Rosenmund et al., 2003). Genetic studies suggest that GOA-1 signaling reduced diacylglycerol levels, thereby releasing UNC-13 from presynaptic membranes and blocking neurotransmitter release (Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999).

What ligand activates EGL-47?

EGL-47A and EGL-47B, like the vast majority of GPCRs in both humans and model organisms, are orphan receptors whose activating ligand(s) remain unknown. Because each isoform has a

different extracellular N-terminal domain, it is possible that each isoform binds a different ligand. BLAST analysis showed EGL-47 has weak similarity to a putative *Drosophila* gustatory receptor. A role for gustatory receptors in regulating egg laying might be expected because egg-laying behavior is affected by food (Dong et al., 2000). However, we found that EGL-47 was highly expressed in nonchemosensory neurons, such as PVQ interneurons and HSN motor neurons, suggesting that the EGL-47 ligand is more likely a neurotransmitter produced in the worm rather than an external chemosensory signal.

Several neurotransmitters affect egg laying when applied exogenously to *C. elegans*, including serotonin (Trent et al., 1983), acetylcholine (Weinshenker et al., 1995; Waggoner et al., 1998; Bany et al., 2003), dopamine (Schafer and Kenyon, 1995), and octopamine (Horvitz et al., 1982). However, it is unlikely that any of these neurotransmitters activate EGL-47 because EGL-47 shows little sequence similarity to known biogenic amine or acetylcholine receptors. Neuropeptides are also candidate EGL-47 ligands. There are >200 putative neuropeptides in *C. elegans* (Li et al., 1999: Nathoo et al., 2001), but mutants for only two neuropeptide genes have been described (Nelson et al., 1998; Rogers et al., 2003). One of these mutants, *flp-1*, has an abnormal temporal pattern of egg laying and also does not properly turn off egg laying when removed from food, demonstrating that the FLP-1 peptide can modulate egg laying (Waggoner et al., 2000). However, EGL-47 is not likely to be an FLP-1 receptor because we found that *egl-47* null animals did properly turn off egg laying when removed from food.

GOA-1 integrates signals from multiple GPCRs in the HSNs to regulate egg laying

Multiple G-protein-coupled receptors on the HSNs appear to activate GOA-1 to inhibit neurotransmitter release. (Supplemental material, available at http://www.jneurosci.org, shows a model summarizing the signals thought to impinge on the HSNs.) Shyn et al. (2003) showed that an unidentified serotonin autoreceptor silences the HSNs, and this effect requires GOA-1. Thus, serotonin release from the HSNs into the neuromuscular junction causes contraction of the egg-laying muscles but also feeds back to inhibit HSN function. Bany et al. (2003) found acetylcholine released from the VC neurons also inhibits egg laying, and this effect is partially mediated by the G-protein-coupled acetylcholine receptor GAR-2, which is expressed on the HSNs (Bany et al., 2003). Our data, showing that activation of GOA-1 specifically in the HSNs is sufficient to inhibit egg laying, strengthens the models of Shyn et al. (2003) and Bany et al. (2003), in which serotonin and acetylcholine receptors inhibit egg laying by acting through GOA-1 to inhibit HSN activity. EGL-47A and EGL-47B are two additional receptors that use GOA-1 to regulate synaptic activity in the HSNs. EGL-47 may act as an HSN autoreceptor in a feedback mechanism similar to that suggested by Shyn et al. (2003) or to receive a signal from another neuron, as proposed for GAR-2 (Bany et al., 2003).

The multiple signals acting on the HSNs have all been demonstrated experimentally, but what biological purpose(s) do they serve? *C. elegans* regulates egg laying in multiple ways (Table 1), presumably to deposit its eggs in favorable circumstances. *C. elegans* also regulates egg laying so that clusters of eggs are laid approximately every 20 min (Waggoner et al., 1998), although any benefit of this clustering remains obscure. The egg-laying system thus requires a mechanism to integrate a number of factors to set a level of egg-laying activity. We hypothesize that GOA-1 activity in the HSN neurons serves as an integrator of multiple signals to set an appropriate level of HSN activity and thus egg-laying behavior. At the present time, we do not know which signals account for which aspect of egg-laying regulation. For example, the circumstances that lead to release of acetylcholine from the VC neurons or to the release of the EGL-47 ligand are not known. It has been speculated that feedback signaling by HSN-released serotonin could account for the clustering of egg-laying events (Shyn et al., 2003).

Why do EGL-47 null mutants, like null alleles of most GPCRs, have no obvious defects?

The *C. elegans* genome encodes up to 1000 G-protein-coupled receptors (Bargmann, 1998), making the GPCRs the largest family of proteins. However, genetic screens in *C. elegans* have identified only three GPCR genes: the odorant receptor ODR-10 (Sengupta et al., 1996), the neuropeptide receptor NPR-1 (de Bono and Bargmann, 1998), and EGL-47. Keating et al. (2003) used RNAi to knock down expression of 60 GPCRs predicted to bind neurotransmitters or neuropeptides and found only a small number with defects. Several mutagenesis screens have identified genes involved in G-protein control of egg laying (Trent et al., 1983; Desai and Horvitz 1989; Mendel et al., 1995; Robatzek and Thomas, 2000). These screens have yielded many mutations in G-proteins and other signaling proteins, but to date, EGL-47 is the only GPCR identified. Why are GPCR mutations so difficult to find? It was only possible to identify *egl-47* with gain-of-function mutations, and these were exceedingly rare. In fact, both gain-of-function alleles resulted in the very same amino acid substitution, suggesting that there may only be one point mutation that can hyperactivate EGL-47. It appears that loss-of-function mutations in GPCRs generally do not have obvious phenotypic defects. Consistent with this idea, deletion alleles of *egl-47* had no noticeable defects. The lack of defects in GPCR mutants could be attributed to redundancy. However, there is no other GPCR closely related to EGL-47, so we have no candidate to test for redundancy with EGL-47. Another hypothesis is that GPCRs like EGL-47 have subtle functions that are not obviously detected. For example, EGL-47 may inhibit egg laying in a special circumstance that we have not tested. As discussed above, EGL-47 is one of a number of GPCRs that all seem to act in the HSNs to signal through GOA-1 to inhibit egg laying. It appears that loss of any individual such receptor may cause only minor defects, whereas loss of GOA-1 or other shared downstream signaling components has dramatic effects.

EGL-47 is expressed in only a limited subset of neurons, just as that observed for other GPCRs (Troemel et al., 1995), and this is likely another factor contributing to the limited effects of GPCR mutations. We expect that the function of every neuron expressing EGL-47(gf) is inhibited as a result of overactivation of GOA-1. Even so, the only observed defect can be attributed to inactivation of just the HSN neurons. This is not entirely surprising. Many individual neurons in *C. elegans* can be ablated without causing obvious behavioral defects: such neurons in some cases have been shown to have subtle or redundant functions (Avery and Horvitz, 1989; Bargmann and Horvitz, 1991; McIntire et al., 1993). In the case of EGL-47, the neurons that most strongly expressed our *egl-47*::GFP reporter were the PVQ interneurons, and these are among the large set of *C. elegans* neurons for which no behavioral functions have been defined. We identified the EGL-47 receptor only because it acts in one neuron type (HSN) whose inactivation leads to a dramatic Egl defect and because powerful genetic screens for Egl mutants have allowed very rare gain-of-function mutations to be isolated.

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