Systems/Circuits

Cellular Expression and Functional Roles of All 26 Neurotransmitter GPCRs in the *C. elegans* Egg-Laying Circuit

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Maps of the synapses made and neurotransmitters released by all neurons in model systems, such as *Caenorhabditis elegans* have left still unresolved how neural circuits integrate and respond to neurotransmitter signals. Using the egg-laying circuit of *C. elegans* as a model, we mapped which cells express each of the 26 neurotransmitter GPCRs of this organism and also genetically analyzed the functions of all 26 GPCRs. We found that individual neurons express many distinct receptors, epithelial cells often express neurotransmitter receptors, and receptors are often positioned to receive extrasynaptic signals. Receptor knockouts reveal few egg-laying defects under standard laboratory conditions, suggesting that the receptors function redundantly or regulate egg-laying only in specific conditions; however, increasing receptor signaling through overexpression more efficiently reveals receptor functions. This map of neurotransmitter GPCR expression and function in the egg-laying circuit provides a model for understanding GPCR signaling in other neural circuits.

Key words: **C. elegans; egg-laying circuit; GPCR; metabotropic receptor; neural circuit**

Significance Statement

Neurotransmitters signal through GPCRs to modulate activity of neurons, and changes in such signaling can underlie conditions such as depression and Parkinson's disease. To determine how neurotransmitter GPCRs together help regulate function of a neural circuit, we analyzed the simple egg-laying circuit in the model organism *C. elegans*. We identified all the cells that express every neurotransmitter GPCR and genetically analyzed how each GPCR affects the behavior the circuit produces. We found that many neurotransmitter GPCRs are expressed in each neuron, that neurons also appear to use these receptors to communicate with other cell types, and that GPCRs appear to often act redundantly or only under specific conditions to regulate circuit function.

Introduction

Analysis of small neural circuits, such as those of genetically tractable model organisms, has the potential to uncover principles that generalize to circuits of more complex nervous systems (Bargmann and Marder, 2013). Deeply understanding a neural circuit might involve mapping the synaptic connections between all its cells, identifying the neurotransmitters released by each neuron, identifying the neurotransmitter receptors present on each cell, and defining how signaling through each receptor affects the circuit's function. This depth of understanding has not yet been achieved for any circuit.

A major challenge is to understand how neurotransmitters control neural circuit function by acting as neuromodulators through GPCRs (Marder, 2012). Each neurotransmitter signals through multiple GPCRs: serotonin, for example, has 12 different GPCRs expressed in human brain that can couple to different G proteins to produce different effects (McCorvy and Roth, 2015). Neurotransmitters bind GPCRs with high affinity,

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allowing response to neurotransmitters released at a distance, an effect known as extrasynaptic or volume transmission (Fuxe et al., 2005, 2012; Agnati et al., 2006). Thus, mapping the synaptic connections between the neurons in a circuit is insufficient to understand the GPCR signaling that may occur between them.

Caenorhabditis elegans provides an opportunity to define how neural circuits use neurotransmitter GPCR signaling because: (1) a complete neural connectome delineates ~7000 synaptic connections between all 302 neurons in this organism (Albertson and Thomson, 1976; White et al., 1986; Xu et al., 2013); (2) a complete neurotransmitter map shows which small-molecule neurotransmitter is released by each neuron (Serrano-Saiz et al., 2013; Pereira et al., 2015; Gendrel et al., 2016); (3) *C. elegans* has ~26 homologs of human neurotransmitter GPCRs, five of which remain "orphan" receptors without assigned ligands, whereas the other 21 have been assigned as receptors for one of seven neurotransmitters (serotonin, dopamine, octopamine, tyramine, acetylcholine, GABA, and glutamate); and (4) gene knockouts for all neurotransmitter GPCRs are available (Koelle, 2018).

In this study, we analyzed the expression and functions of all 26 neurotransmitter GPCRs in the neural circuit that controls egg laying in *C. elegans*. This circuit serves as a model for neural G protein signaling since mutations that alter signaling by the neural G proteins $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ strongly affect the frequency of egg laying (W. F. Schafer, 2006; Koelle, 2018). Also, the neurotransmitters serotonin, tyramine, octopamine, and dopamine, which act through GPCRs, have all been shown to functionally affect egg laying (Horvitz et al., 1982; W. R. Schafer and Kenyon, 1995; Hapiak et al., 2009; Vidal-Gadea et al., 2012; Collins et al., 2016; Nagashima et al., 2016). However, it remains to be determined how specific receptors for these neurotransmitters alter activity of the egg-laying circuit. Our analysis reveals features of neurotransmitter signaling that may generalize beyond the egg-laying circuit, and provides a path toward understanding how neurotransmitter signaling within a model neural circuit generates its dynamic pattern of activity to control a behavior.

Materials and Methods

Some *C. elegans* strains generated in this work will be made available via the *Caenorhabditis* Genetics Center. Further information and requests for other reagents and resources should be directed to M.R.K. (michael.koelle@yale.edu).

Strains and culture

C. elegans strains used in this study were maintained at 20°C on standard nematode growth media seeded with OP50 strain of *Escherichia coli* as their food source.

Null alleles for neurotransmitter receptors and biosynthetic enzymes were obtained through the *Caenorhabditis* Genetics Center, the Japanese National BioResource Project, or as otherwise indicated. Null alleles were backcrossed 2-10× to N2 (WT), as indicated. New strains were constructed using standard procedures, and genotypes were confirmed by PCR or sequencing. Extrachromosomal array transgenic strains for neurotransmitter GPCRs were generated through microinjection, and at least one independent line was kept. Extrachromosomal strains for neurotransmitter GPCRs were chromosomally integrated through UV/TMP mutagenesis, and at least one integrant was kept. Integrated strains were backcrossed 2-4× to N2 (WT) as indicated. Young adult hermaphrodite animals were used for confocal microscopy. For egg-laying assays, animals were staged 40 h after L4. A complete list of all of the *C. elegans* strains used in this work is found in Extended Data Figure 2-1.

Recombineered fosmids

Recombineered transgenes were received from TransgeneOme (https://transgeneome.mpi-cbg.de) in EPI300. Clones were recovered and DNA was isolated as described by Sarov et al. (2012) with some modifications. Nonclonal cell populations carrying the constructs were streaked onto triple antibiotic plates (15 μg/ml chloramphenicol, 100 μg/ml streptomycin, and 50 μg/ml cloNAT), and individual colonies were inoculated for 14-16 h in LB broth with 1× Fosmid Autoinduction solution and the antibiotics. A subset of the transgenes (see below) do not contain the cloNAT resistance marker; therefore, only chloramphenicol and streptomycin were used for their growth. Recombineered fosmid DNA was isolated using QIAGEN Plasmid Mini Kits. The purified DNA was run on 0.4% agarose gels to determine whether the recombineered fosmid was at the correct size. The GFP coding region of each fosmid was also sequenced to determine whether the GFP tag was inserted at the correct position.

Microinjections

Recombineered transgenes were injected at 20–100 ng/μl into *unc-119 (ed3)* animals, or into *lin-15(n765ts)* animals along with a *lin-15* rescuing plasmid (pL15EK) at 50 ng/μL, plus 25 ng/μl of *E. coli* DH5α genomic DNA that had been digested to an average length of ~6 kb. Strains carrying recombineered transgenes are listed in Extended Data Figure 2-1.

Chromosomal integrations

Extrachromosomal transgenic strains were chromosomally integrated following UV/TMP mutagenesis. At least 300 F1 brightly GFP-positive animals were picked to individual plates. Thirty or more of the best of these F1 plates were selected based on the presence of F2 progeny that were > 75% nonmultivulva (i.e., positive for the *lin-15* marker on the transgene) and brightly GFP-positive. For each selected F1 plate, 6 F2 animals were placed on single plates. F2 homozygous integrants were identified as giving rise to true-breeding 100% non-Muv and 100% GFP-positive strains. This procedure resulted in 1-7 independent integrants per transgene.

Transgenic reporter mCherry strains

All mCherry marker strains used in our study are chromosomally integrated. The pBR1 plasmid for *ida-1::mCherry* (Ravi et al., 2018) was injected at 20 ng/μl with 50 ng/μl pL15EK plus 25 ng/μl of *E. coli* DH5α genomic DNA digested to an average length of ~6 kb into *lin-15(n765ts)* animals to generate extrachromosomal lines and chromosomally integrated. The *ida-1::mCherry* transgenic strain was used for cell identifications of the hermaphrodite specific neuron (HSN), uv1s, ventral cord Type C neuron 4 (VC4), and VC5 of the *C. elegans* egg-laying circuit. Independent integrants for *unc-103e::mCherry* previously generated in our laboratory (Collins and Koelle, 2013) were used for identification of the vulval (vm1 and vm2) and uterine (um1 and um2) muscles. The *ajm-1::mCherry* integrated strain was received from the Gottschalk laboratory. It labels the apical borders of epithelial cells and was used to identify the uterine toroid (ut1-4) cells, uv3 uterine ventral cells, vulval toroid (vt) cells, spermatheca-uterine (sp-ut) valve, spermatheca, dorsal uterine cell (du), and uterine seam (utse).

GPCR::GFP transgenic strains

C-terminal GFP constructs. Nineteen C-terminal GFP constructs listed in Extended Data Figure 1-2 were recombineered fosmid clones with GFP coding sequences inserted in the last coding exon of a receptor gene, between the codon for the C-terminal amino acid and the stop codon, as described by Sarov et al. (2012). Thirteen of these were constructed by Sarov et al. (2012), and details are available at the TransgeneOme web site (https://transgeneome.mpi-cbg.de). Five additional constructs (SER-1, SER-7, GAR-3, DOP-5, and DOP-6) were recombineered in an analogous fashion, except that for these constructs the *unc-119* and cloNAT-resistant markers were not inserted into the final clone. The GFP reporter for *gbb-2* was recombineered in a similar fashion, except that GFP coding sequences were inserted in the middle of exon 14, such that GFP is inserted C-terminal to the amino acid Arg716. The most 3′ coding exons of the *gbb-2* gene have not been confidently determined, so this GFP insertion site was chosen as being 3′ to the coding sequences for the last transmembrane domain yet still confidently within the *gbb-2* coding region.

SL2::NLS::GFP constructs. Microinjection of four C-terminal GFP transgenes (*dop-1::gfp*, *dop-4::gfp*, *gar-2::gfp*, and *F35H10.10::GFP*) resulted in GFP expression that was either weak or strong but localized to neural processes. In either case, cell bodies were not labeled with GFP adequately to allow identification of GFP-positive cells. Using fosmid genomic clones (Extended Data Fig. 1-2), these four receptor genes were recombineered to insert a an SL2 trans-splicing signal followed by GFP coding sequences directly after the GPCR gene stop codon, following the protocol of Tursun et al. (2009). The resulting transgenes express a single primary transcript that is processed into two separate mRNAs: one expressing the GPCR and the other expressing soluble GFP, allowing us to visualize GFP in the cell bodies.

Other constructs. Two GPCR::GFP transgenes could not be constructed by recombineering fosmids for technical reasons. The *ser-4::gfp* construct (Tsalik et al., 2003) is an integrated transgene that contains 4.1 kb of *ser-4* promoter region 5′ of the start codon and fuses GFP coding sequences at an internal exon 3.7 kb downstream of the start codon. The *mgl-2::gfp* plasmid was a gift from Oliver Hobert (Yemini et al., 2020). It contains 7.9 kb of the *mgl-2* promoter region 5′ upstream of the start codon followed by GFP coding sequences, which was injected to generate extrachromosomal lines and chromosomally integrated.

GCPR::GFP restriction fragments from recombineered fosmid clones. Microinjection of recombineered fosmid clones for five GPCR genes (*dop-3::gfp*; *tyra-2::gfp*; *tyra-3::gfp*; *mgl-3::gfp*; *dop-5::gfp*) resulted in lethality or an absence of strong GFP expression in the extrachromosomal transgenic lines. To overcome these issues, 1-3 μg of each recombineered transgene was digested with restriction enzymes to cut away other genes flanking the *gpcr::gfp* gene on the fosmid as described in Extended Data Figure 1-2. The digests were run on 0.4% agarose gels for >10 h to purify the large fragments carrying the *gpcr::gfp* genes and gel extracted using the QIAEX II Gel Extraction Kit (QIAGEN). The resulting product yielded a purified sample of 30 ng/μl, a portion of which was run on an analytical 0.4% agarose gel to determine whether product was at the expected size. To further confirm the correct fragment was purified, PCR primers were used to amplify the 5′ and 3′ flanking ends of the fragment and the products were analyzed on agarose gels. The purified restriction fragment transgenes were used for microinjections, resulting in satisfactory viability and GFP expression.

Confocal imaging

Animals were on mounted on 2% agarose pads containing 120 mM Optiprep (Sigma Millipore) to reduce refractive index mismatch (Boothe et al., 2017) on premium microscope slides Superfrost (Thermo Fisher Scientific), and a 22×22^{-1} microscope cover glass (Fisher Scientific) was placed on top of the agarose pad. Animals were anesthetized using a drop of 150 mM sodium azide (Sigma Millipore) with 120 mM Optiprep. *z*-stack confocal images of ~100-130 slices (each ~0.60 μm thick) were taken on an LSM 710 using a 40× objective lens (Carl Zeiss). For at least 10 individual animals for each double-labeled transgenic strain, images of the mid-body where the egg-laying circuit lies were taken. Images were visualized using Vaa3D (Peng et al., 2010).

Behavioral assays

For all egg-laying assays, unlaid eggs and early-staged eggs laid were quantified at 40 h past the late L4 stage (Waggoner et al., 1998; Chase et al., 2004; Collins and Koelle, 2013; Brewer et al., 2019).

Statistical analyses

Data graphs were made using Prism 7.0 software (GraphPad). Scatter plots show individual data points (dots), mean (horizontal line), and error bars indicate 95% CIs for the mean. *N* values are indicated in the figure legends and sample size for behavioral assays followed previous studies (Waggoner et al., 1998; Chase et al., 2004; Collins and Koelle, 2013; Brewer et al., 2019). Statistical significance was tested using one-way ANOVA with Bonferroni correction for multiple comparisons for the unlaid egg assay and Fisher's exact test for the early-staged egg-laying assay. For the unlaid egg assays of neurotransmitter GPCR overexpressors and biosynthetic enzyme KOs, Tukey's test was used to determine statistical significance. For the early-staged egg-laying assay, the Wilson-Brown method was used to determine the 95% CI for binomial data. $p \geq 0.05$ was considered not significant. $p < 0.05$ was considered significant.

Data and software availability

All data, including confocal images, are available on request as electronic files.

Results and Discussion

GFP reporter strains to identify cells expressing each neurotransmitter GPCR

Our analysis includes 21 neurotransmitter GPCRs for monoamines and classical neurotransmitters, plus five "orphan" GPCRs (Fig. 1*A*). Expression patterns for 13 of these have been previously analyzed (Cho et al., 2000; Lee et al., 2000; Tsalik et al., 2003; Chase et al., 2004; Rex et al., 2004; Carnell et al., 2005; Dempsey et al., 2005; Carre-Pierrat et al., 2006; Suo et al., 2006; Wragg et al., 2007; Hapiak et al., 2009; Plummer, 2011; Gürel et al., 2012; Yemini et al., 2020) using GPCR gene promoter fragments of a few kilobases to drive GFP expression. However, recent work shows that obtaining accurate expression patterns often requires larger GFP reporter transgenes (Serrano-Saiz et al., 2013; Pereira et al., 2015; Gendrel et al., 2016) that include the full gene of interest and up to ~20 kb of flanking genomic DNA on either side (Tursun et al., 2009).

Therefore, we sought to generate large GFP reporter transgenes for the 26 neurotransmitter GPCR genes (Fig. 1; see Extended Data Figs. 1-1, 1-2). Thirteen were already constructed (Sarov et al., 2012), and we generated seven more in a similar fashion. These "C-terminal::GFP" reporters express a GPCR with GFP fused at or very close to the C-terminus of the receptor (Fig. 1*B*). For a few receptors, these C-terminal::GFP fusion proteins were strongly localized to neural processes and excluded from the cell bodies, making it difficult to identify the GFP-expressing neurons. So, for four such receptors (DOP-1, DOP-4, GAR-2, and F35H10.10), we generated "SL2::GFP" reporters (Fig. 1*B*), which produce a single primary RNA transcript that is trans-spliced to produce separate GPCR and GFP mRNAs, so that the GFP produced fills out the cells that express the GPCR gene (Tursun et al., 2009). For the remaining two receptors (MGL-2 and SER-4), technical issues precluded use of large fosmid-based reporters, so we analyzed expression of smaller promoter::GFP reporters that had been previously generated (Tsalik et al., 2003; Gürel et al., 2012; Yemini et al., 2020).

The 26 GPCR::GFP reporter transgenes were each transformed into *C. elegans* to produce high-copy, chromosomally integrated transgenes. Such transgenes are expressed in the same cells as the corresponding endogenous genes (Serrano-Saiz et al., 2013; Pereira et al., 2015; Gendrel et al., 2016), but at high enough levels that the GFP fluorescence produced is sufficient to identify even cells that normally express a GPCR gene at low levels.

Cells of the egg-laying system

We first examined animals carrying transgenes that express mCherry in specific cells of the egg-laying system to generate accurate diagrams of its anatomy (Fig. 2).

Neurons of the egg-laying system were labeled by *ida-1::mCherry* (Fig. 2*B*,*C*). These neurons are as follows: (1) the HSNs, which promote egg laying by releasing serotonin and neuropeptides (Waggoner et al., 1998; Shyn et al., 2003; Hapiak et al., 2009; Emtage et al., 2012; Brewer et al., 2019); (2) the uv1s, which inhibit egg laying by releasing tyramine and neuropeptides

A

G Protein Coupled Receptor	Neurotransmitter	Category
SER-1, SER-4, SER-5, SER-7	Serotonin	Monoamine
DOP-1, DOP-2, DOP-3, DOP-4	Dopamine	
OCTR-1, SER-3, SER-6	Octopamine	
SER-2, TYRA-2, TYRA-3	Tyramine	
GAR-1, GAR-2, GAR-3	Acetylcholine	Classical
GBB-1, GBB-2	GABA	
MGL-1, MGL-2, MGL-3	Glutamate	
ADOR-1, F35H10.10, DOP-5, DOP-6	Unknown	Orphan

B

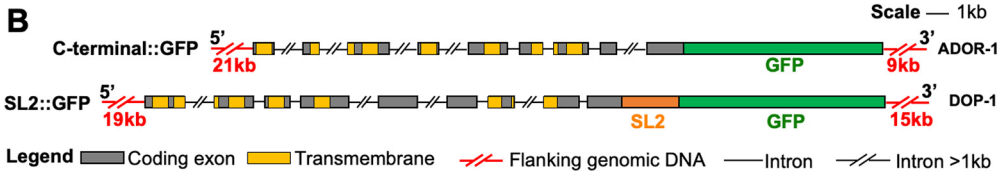


Figure 1. GFP reporter transgenes for 26 neurotransmitter GPCRs. ***A***, Small-molecule neurotransmitter GPCRs in *C. elegans*. ***B***, Schematics of GFP reporters used in this study. C-terminal::GFP constructs are illustrated by the reporter for the orphan receptor ADOR-1. The construct provides 21 kb of promoter region DNA 5′ to the coding exons and 9 kb of 3′ DNA. GFP coding sequences were inserted into the last ADOR-1 coding exon so that the transgene expresses the receptor with GFP fused to its C-terminus. SL2::GFP constructs are illustrated by the dopamine receptor DOP-1 reporter. This reporter is similar to that for ADOR-1, except that an SL2 trans-splicing signal followed by GFP coding sequences were inserted immediately downstream of the stop codon so that this reporter coexpresses the receptor and GFP as separate proteins. Analogous schematics of all 26 receptor::GFP reporter transgenes are found in Extended Data Figure 1-1 and described in Extended Data Figure 1-2.

(Alkema et al., 2005; Collins et al., 2016; Banerjee et al., 2017); and (3) VC4/VC5, which release acetylcholine onto the vm2 egg-laying muscles (Duerr et al., 2001; Collins et al., 2016).

We refer to the four uv1 cells in this work as "neurons" despite the fact that they are not on the canonical list of 302 neurons established by White et al. (1986). The uv1 (uterine-vulval type 1) cells were originally assumed to be epithelial cells that simply help form the structure connecting the uterus to the vulva through which eggs are laid (Newman et al., 1996). However, a body of evidence now shows that the uv1s regulate egg-laying behavior by acting as neurosecretory cells: (1) the uv1 cells express neuronal markers, including synaptotagmin, syntaxin, and UNC-13 (Jose et al., 2007) as well as *rab-3* (our unpublished observations); (2) the uv1 cells release the neurotransmitter tyramine to inhibit egg laying (Alkema et al., 2005; Collins et al., 2016); (3) the uv1 cells also release the neuropeptides NLP-7 and FLP-11 to inhibit egg laying (Banerjee et al., 2017); and (4) the uv1 cells show calcium transients at a specific time after each egg is laid (Collins et al., 2016). Thus, the uv1 cells have properties analogous to those of the more widely studied NSM cells, which are also neurosecretory cells that make no classical synapses (Nelson and Colón-Ramos, 2013), and which are included in the list of 302 canonical neurons (White et al., 1986).

Muscle cells of the egg-laying system were labeled by *unc-103e::mCherry*, which is expressed in the vm1 and vm2 vulval muscle cells and was used as a landmark to identify the um1 and um2 uterine muscle cells (Fig. 2*D*,*E*). Eggs are laid when vm1 and vm2 open the vulva and um1 and um2 muscle cells squeeze eggs out of the uterus through the vulva (Waggoner et al., 2000; Kim et al., 2001; Bany et al., 2003; Ringstad and Horvitz, 2008; Collins et al., 2016). There are also body wall muscle cells surrounding the egg-laying system (not shown in Fig. 2 but referred to as bwm in later figures).

Other cells of the egg-laying system were identified using *ajm-1::mCherry*, which labels the junctions between cells of the uterus and vulva (Fig. 2*F*,*G*). Eggs are fertilized in the spermatheca (sp) and enter the uterus via the spermatheca-uterine (sp-ut) valve. The uterus itself comprises the ut1-ut4 uterine toroid cells and du cells that together form a tube to hold eggs. Eggs pass from the uterus into the vulva through a junction comprising the uterine seam (utse) and uterine-ventral (uv1 and uv3) cells, and the vulva itself comprises several types of vulval toroid (vt) cells (Schindler and Sherwood, 2013; Ghosh and Sternberg, 2014; Ecsedi et al., 2015).

Analyzing GFP expression by neurotransmitter GPCR::GFP transgenes

We sought to (1) accurately identify every cell of the egg-laying system that expresses any of the 26 neurotransmitter GPCR::GFP transgenes; (2) qualitatively record the GFP expression levels for each reporter in each cell, which may reflect expression levels of the endogenous receptors; and (3) record animal-to-animal variability in GFP expression levels from these reporters, which may reflect variability in expression of the endogenous receptors. Thus, we crossed each of the 26 neurotransmitter GPCR::GFP transgenes into the mCherry marker strains and collected 3D confocal images for at least 10 young adult animals per double-labeled strain. In total, images of 809 animals were analyzed.

The process for identifying GFP-expressing cells and scoring GFP levels is illustrated for the octopamine receptor OCTR-1 (Fig. 3; Movie 1). Each image was analyzed using 3D rotations, cuts through the image volumes, and mCherry markers to identify internal features. The strongest GFP-expressing cells in a strain were scored as +++, those with weaker but easily detectable GFP as ++, and those with GFP just above background as +.

For reporters that expressed a receptor with GFP fused to its C-terminus, the fusion proteins were sometimes subcellularly localized. An example is seen in Figure 3*B*, where OCTR-1::GFP clusters within the ut2 uterine toroid cells. It is unclear whether such subcellular localization might reflect a similar localization of the corresponding endogenous receptors; thus, we chose not to interpret such subcellular localization in this work.

Neurotransmitter GPCR expression in the *C. elegans* egg-laying system

Figure 4 shows 2D image renderings illustrating a subset of findings, whereas Extended Data Figure 4-1 shows a larger set of images for each of the 26 neurotransmitter receptors. Figure 5

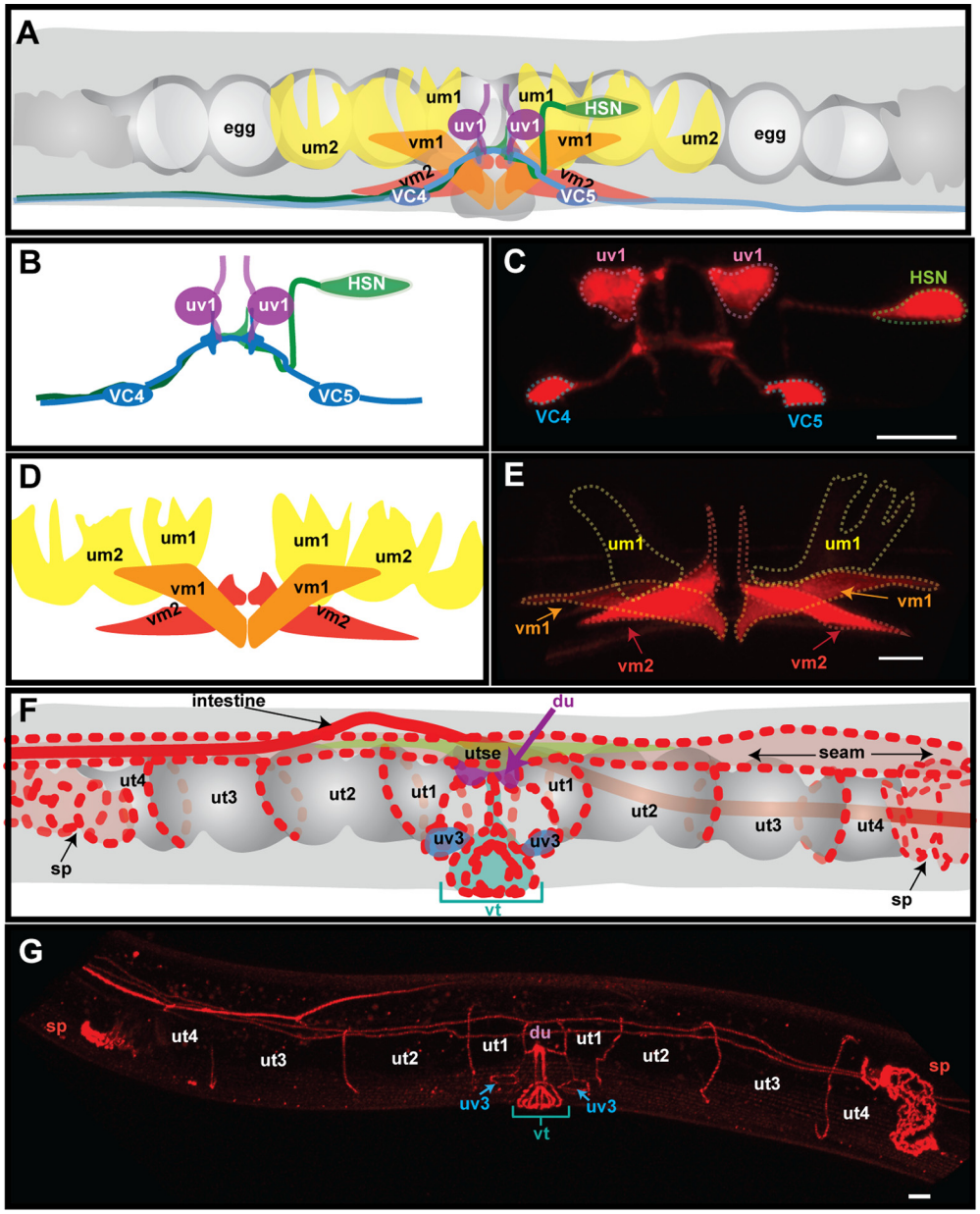


Figure 2. Anatomy of the egg-laying system visualized with mCherry markers. ***A***, Schematic of the *C. elegans* egg-laying system. Neuron and muscle cells (colored and labeled with cell names) are overlaid on the uterus (gray) containing eggs (white). VC4 and VC5 neurons are at the ventral midline. All other cells depicted are on the left side of the animal, and their equivalents on the right side of the bilaterally symmetric anatomy are not shown. ***B***, Schematic of the egg-laying neurons. ***C***, Confocal image of the egg-laying neurons labeled by the *ida-1::mCherry* marker. Dashed lines outline the cell bodies. ***D***, Schematic of the egg-laying muscles. ***E***, Confocal image of the egg-laying muscles labeled by the *unc-103e::mCherry* marker. um1 (outlined) and um2 (not indicated) are rarely and faintly labeled by *unc-103e::mCherry*; but even so, labeling of vm1/vm2 cells provides landmarks to help identify um1 and um2. ***F***, Schematic of cells other than neurons and muscles of the egg-laying system. Red dashed or solid lines indicate cell junctions labeled by the *ajm-1::mCherry* marker. In addition to cells of the egg-laying system, the *ajm-1::mCherry* marker also labels junctions of seam and intestinal cells**.** ***G***, Confocal image of the egg-laying system labeled by the *ajm-1::mCherry* marker. The following conventions are used in this and other figures in this work. Scale bars, 10 μm. Anterior is left and ventral down. du, Dorsal uterine cell; sp, spermatheca; um, uterine muscle cell; ut, uterine toroid cell; utse, uterine seam cell; uv, uterine ventral cell; vm, vulval muscle cell; vt, vulval toroid cells. All *C. elegans* strains used in this work are described in Extended Data Figure 2-1.

summarizes all the findings from all 809 images analyzed. In total, our results show 139 cases in which a neurotransmitter GPCR is expressed in a specific cell type of the egg-laying system.

Although 13 of the 26 neurotransmitter GPCRs were previously reported to be expressed in the egg-laying system, the vast majority of the expression we found was not previously reported (Extended Data Fig. 5-1*A*). In 20 instances, we reproduced a published report of a receptor being expressed in a particular cell type; but in 119 additional instances, we made a novel observation of a receptor being expressed in a cell type. In eight instances, a published case of a receptor being expressed in a cell type was not reproduced in our data (Extended Data Fig. 5-1*A*).

While GFP reporters were often expressed strongly and reproducibly in a given cell type, we also observed cases of weak and/or variable GFP expression (Fig. 5; Extended Data Fig. 5-1*B*). This phenomenon is illustrated for *octr-1::gfp* expression in the HSN, VC4, and VC5 neurons in Extended Data Figure 3-1. We collected data for all detectable expression so that future studies can investigate what types of expression of a GPCR::GFP

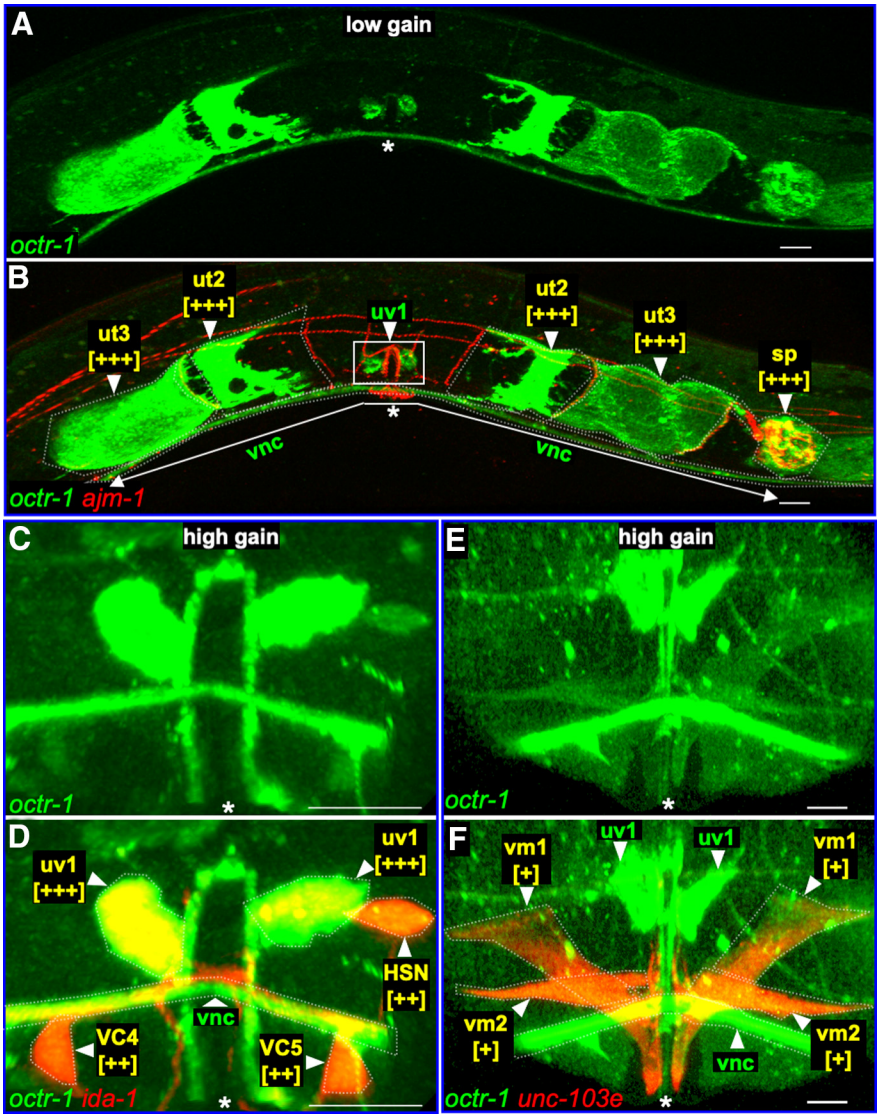


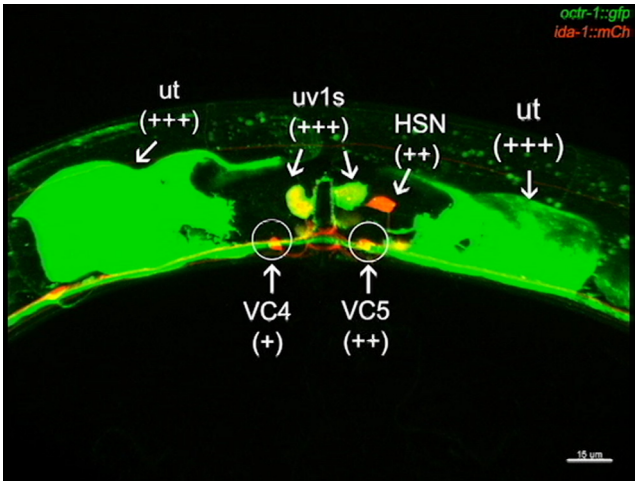
Figure 3. Scoring expression of a GFP reporter for the octopamine receptor OCTR-1 in cells of the egg-laying system. ***A***, ***B***, Two-dimensional renderings of a confocal image of the egg-laying system in an *octr-1::gfp; ajm-1::mCherry* young adult showing GFP (***A***) or GFP and mCherry fluorescence (***B***). The ut2 uterine toroid cells are outlined by the *ajm-1::mCherry* marker, allowing them to be identified as GFP-positive despite not being uniformly filled out by OCTR-1::GFP. Solid line with arrowheads labels the ventral nerve cord (vnc), a bundle of axons that run past the vulva but that are not known to affect egg laying. In these and other figure panels, white asterisks indicate position of the vulval opening, cells labeled with both GFP and mCherry are named with yellow text, cells labeled with GFP only are named with green text, the number of + symbols indicates the relative intensity of GFP labeling, and identified cells are outlined by faint dotted lines. ***B***, White box represents the region shown at higher magnification in ***C-F***. ***C***, ***D***, An *octr-1::gfp; ida-1::mCherry* animal with GFP fluorescence displayed at high gain to make ++ GFP labeling visible in the HSN and VC4/VC5 neurons. These neurons are double-labeled by *ida-1::mCherry* in ***D***. ***E***, ***F***, An *octr-1::gfp; unc-103e::mCherry* animal with GFP fluorescence displayed at high gain and at a different focal plane than in ***C***, ***D*** to visualize + GFP labeling in vm1 and vm2 egg-laying muscles. Movie 1 is a video that illustrates in more detail how *octr-1::gfp* strains were analyzed. Extended Data Figure 3-1 illustrates how animal-to-animal variations in GFP expression were analyzed.

reporter might reflect functional presence of the corresponding endogenous receptor.

We note that single-cell RNA sequencing (RNAseq) has the potential to map which cells express any gene, including the neurotransmitter GPCR genes we characterize here. Our data are far richer than the currently available RNAseq data for the egg-laying system. Our work scores expression in 19 cell types in the egg-laying system (Fig. 5) versus only four in the current RNAseq data (Taylor et al., 2020). As examples, we scored expression in vm1 and vm2 separately rather than aggregating all vulval muscle cell expression, and we included in our analysis cells other than neurons and muscles, which turn out to express many neurotransmitter GPCRs. Further, we measured variability in expression from animal to animal, and discovered weak expression below the detection threshold of current RNAseq data.

Individual cells express multiple neurotransmitter GPCRs

Individual cells expressed up to 20 different neurotransmitter GPCRs (Fig. 5). Neurons (examples in Fig. 4*A-H*) each expressed 2-7 receptors at strong, reproducible levels and 4-10 additional receptors at weak and/or variable levels (Fig. 5). Non-neuronal cells also expressed multiple neurotransmitter GPCRs strongly and reproducibly; but unlike neurons, these cells expressed only 0-3 additional receptors at weak and/or variable levels. Figure 4*I-P* shows examples of GPCR::GFP expression in muscles cells, whereas Figure 4*Q-V* shows examples of GPCR::GFP expression in non-neuronal, nonmuscle cells of the egg-laying system.



Movie 1. Video of methods used to score expression of a GFP reporter for the octopamine receptor OCTR-1 in cells of the egg-laying system. The video illustrates how images of the egg-laying system were analyzed in three dimensions to identify GFP-expressing cells and score GFP expression levels. [View online]

The multiple receptors expressed on a single cell often couple to different G proteins (Fig. 5*B*). The *C. elegans* G proteins $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ are each expressed in most or all neurons plus some muscle and other cells (Koelle, 2018). Receptors that couple to $G\alpha_q$ and $G\alpha_s$ cooperate to activate neurons and muscles, whereas receptors that couple to $G\alpha_o$ oppose such activation (for review, see Koelle, 2018). Therefore, our results suggest that individual cells of the egg-laying system have the ability to synthesize information received from multiple neurotransmitters and to compute an appropriate resulting level of activity by integrating signaling from multiple receptors and G proteins.

Neurotransmitter GPCRs are often positioned to receive extrasynaptic signals

As expected, neurotransmitter receptors were often expressed on cells that receive synapses from neurons releasing their activating neurotransmitter. More interestingly, receptors were also expressed on cells that do not receive any synapses at which the corresponding neurotransmitter is released (Fig. 5*C*).

Serotonin receptors illustrate expression in cells positioned to receive synaptic versus extrasynaptic signals. In the egg-laying circuit, HSN neurons release serotonin to stimulate egg laying at synapses onto both the vm2 muscle cells and VC neurons (White et al., 1986; Collins and Koelle, 2013; Brewer et al., 2019). We observed that serotonin receptors are expressed, as expected, on these vm2 and VC postsynaptic cells (Fig. 5). If we restrict our analysis only to strongly expressed receptors, vm2 cells express three serotonin receptors: the SER-1 and SER-7 receptors that stimulate egg laying by coupling to $G\alpha_q$ and $G\alpha_s$, respectively, and the SER-4 receptor that inhibits egg laying by coupling to $G\alpha_o$ (Cho et al., 2000; Carnell et al., 2005; Dempsey et al., 2005; Carre-Pierrat et al., 2006; Hapiak et al., 2009; Gürel et al., 2012). The VC neurons express SER-7, which could mediate the ability of the HSN to excite the VCs (Collins et al., 2016). Finally, the HSN variably expresses SER-7, which might serve as an autoreceptor to allow the HSN to excite itself. The above instances of serotonin receptor expression all could mediate synaptic serotonin signaling.

Remarkably, our data show seven additional instances in which serotonin receptors are strongly and reproducibly expressed by cells of the egg-laying system that do not receive synapses from the HSN or any other serotonergic neurons (Fig. 5). For example, the vm1 and um1/2 egg-laying muscle cells, which contract with the vm2 muscle cells to cause egg laying, all express serotonin receptors. Therefore, serotonin released by HSN may travel extrasynaptically to the vm1 and um1/2 cells to signal through serotonin receptors on these cells to help induce their activity and egg-laying events.

Acetylcholine, GABA, and glutamate, like serotonin, each have GPCRs expressed on cells onto which these neurotransmitters are synaptically released. In addition, acetylcholine, GABA, and glutamate each have GPCRs expressed on neurons, muscles, and/or epithelial cells of the uterus that could receive these signals extrasynaptically (Fig. 5*A*,*C*).

No cells of the egg-laying system receive synapses from neurons releasing dopamine, octopamine, or tyramine, yet receptors for each are expressed in the egg-laying system (Fig. 5). For example, each of the four dopamine receptors DOP-1 through DOP-4 is strongly and reproducibly expressed in at least one cell type of the egg-laying circuit (Fig. 5*A*). Indeed, endogenous dopamine has been shown to promote egg laying (Nagashima et al., 2016), and exogenous dopamine has been reported to both promote and inhibit egg-laying (W. R. Schafer and Kenyon, 1995; Vidal-Gadea et al., 2012). Similarly, application of octopamine and tyramine has been shown to inhibit egg laying (Horvitz et al., 1982; Alkema et al., 2005; Collins et al., 2016); and as in the case of dopamine, these effects may occur via extrasynaptic signaling through the receptors for these neurotransmitters we found expressed in the egg-laying system.

Neurotransmitter GPCRs are expressed by cells other than neurons and muscles

In addition to being expressed in neurons and muscles (Fig. 4*A*-*P*), GPCR::GFP reporters were also frequently expressed on other cell types not previously known to respond to neurotransmitters (Figs. 4*Q*-*V*, 5*A*,*B*). Such cells include the uterine toroid epithelial cells ut1-ut4 that form a tube that holds unlaid eggs (Fig. 2*F*,*G*), the vulval toroid (vt) epithelial cells that form the opening through which eggs are laid (Fig. 2*F*,*G*), and the spermatheca that holds sperm and through which eggs pass to be fertilized (Fig. 2*F*,*G*).

The presence of neurotransmitter receptors on epithelial cells has precedent in mammals, as serotonin receptors are found on epithelial/endothelial cells of the gut (Berger et al., 2009; Hoffman et al., 2012) and blood vessels (Ullmer et al., 1995; Berger et al., 2009). The presence of receptors on the uterine toroid epithelial cells may indicate that these ut cells receive neurotransmitter signals to aid in pushing eggs out of the uterus (Newman et al., 1996). Neurotransmitter receptors on the spermatheca may regulate its stretch and contraction before ovulation (Wirshing and Cram, 2017), particularly since downstream effectors of the G protein $G\alpha_q$ have been shown to be involved in ovulation (Clandinin et al., 1998).

Knockout mutants suggest neurotransmitter GPCRs function redundantly and/or under specific conditions

We analyzed knockout mutants for each of the receptors for changes in the frequency of egg-laying behavior. The mutations used are all deletions that remove large portions of the receptor coding regions and that are predicted to result in a lack of any functional receptor expression. One assay of egg-laying behavior counts unlaid eggs in a worm (Fig. 6*A*). A WT worm both produces and lays eggs, accumulating ~18 unlaid eggs in its uterus at steady state. Mutants with a hyperactive or inactive

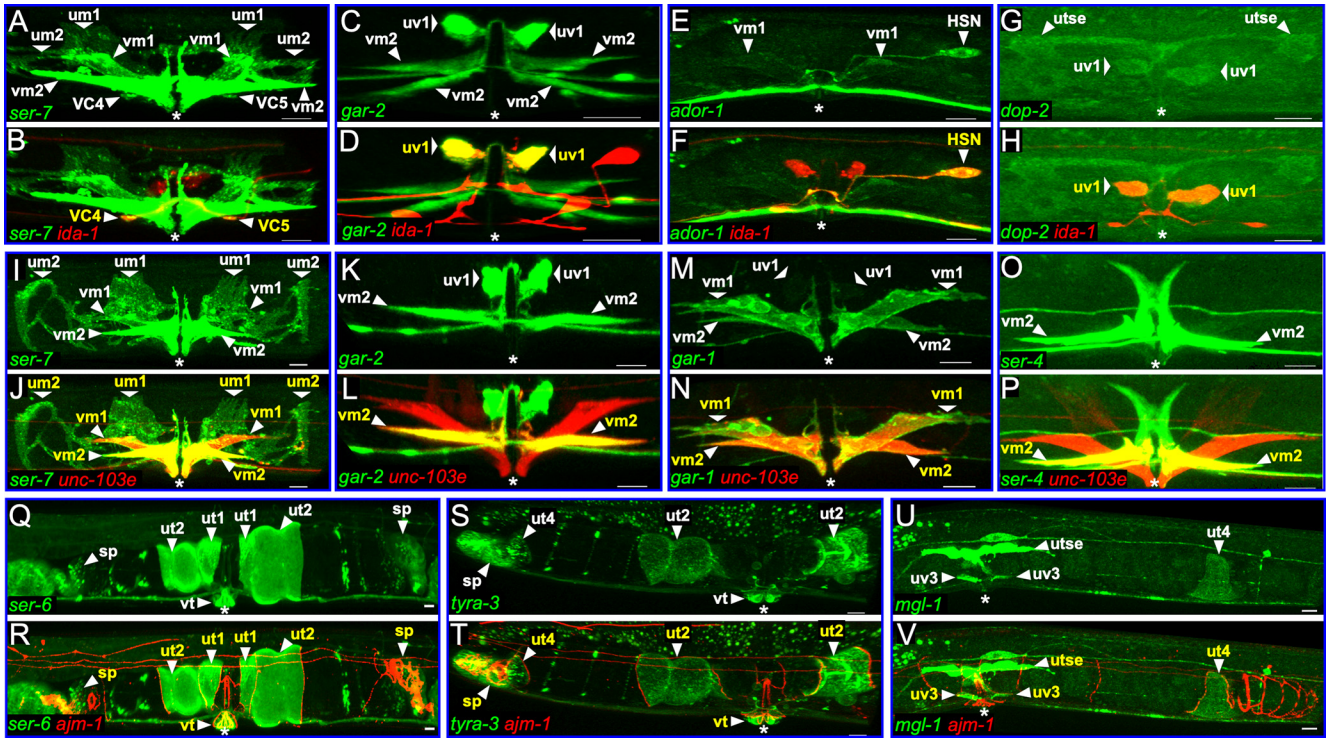


Figure 4. Examples of neurotransmitter GPCR::GFP expression in neuronal, muscle, and other cells of the egg-laying system. ***A-H***, Examples of GPCR::GFP expression in neurons of the egg-laying system. Top panel of each image pair shows reporter GFP reporter fluorescence for the receptor named in green, with all GFP-labeled cells of the egg-laying system indicated by white cell names and arrowheads. Bottom, GFP plus the *ida-1::mCherry* fluorescence used to confirm identity of egg-laying neurons, with only the double-labeled neurons indicated by yellow names and white arrowheads. ***I-P***, Examples of GPCR::GFP expression in muscle cells of the egg-laying system. Images are labeled as in ***A-H***, but bottom panels in each pair show the *unc-103e::mCherry* fluorescence used to confirm identity of muscle cells. mCherry labeling of um1 is not visible in ***J***. ***Q-V***, Examples of GPCR::GFP expression in cells of the egg-laying system other than neurons and muscles. Images are labeled as in ***A-H***, but bottom panels in each pair show *ajm-1::mCherry* fluorescence. sp, ut, utse, uv3, and vt cells are identified by their outlines visualized with *ajm-1::mCherry*. Extended Data Figure 4-1 presents examples of GPCR::GFP expression for all 26 neurotransmitter GPCRs.

egg-laying circuit continue to produce eggs but accumulate fewer or more unlaid eggs, respectively (Chase et al., 2004). A second assay of egg-laying behavior scores the developmental stage of just-laid eggs. Eggs in WT animals undergo multiple rounds of cell divisions while they wait in the uterus to be laid, but eggs of hyperactive egg-layers are laid shortly after being fertilized and often at earlier stages of development, with fewer than eight cells (Fig. 6*A*).

Single KOs of neurotransmitter GPCRs showed at most modest changes in the number of unlaid eggs accumulated (Fig. 6*B*). While the *dop-4(ok1321)* null mutant used in Figure 6*B* appeared WT for egg laying, a second independent allele of *dop-4* showed a possible defect (Extended Data Fig. 6-1*A*). However, we were unable to rescue the egg-laying defect of this second mutant with a WT *dop-4* transgene (data not shown), suggesting that its egg-laying defect was an artifact of the genetic background and not because of loss of *dop-4*. Twelve GPCR KOs accumulated significantly fewer unlaid eggs than did the WT (Fig. 6*B*); however, only two of these laid significantly more early-stage eggs than did the WT control, suggesting that the other 10 may have accumulated fewer unlaid eggs simply because of decreased egg production, not a change in egg laying. Even the two that laid significant early-stage eggs did so at levels far below the 50% early-stage egg threshold considered biologically significant (Bany et al., 2003). In summary, loss of no single neurotransmitter causes strong defects in the standard egg-laying assays used in Figure 6.

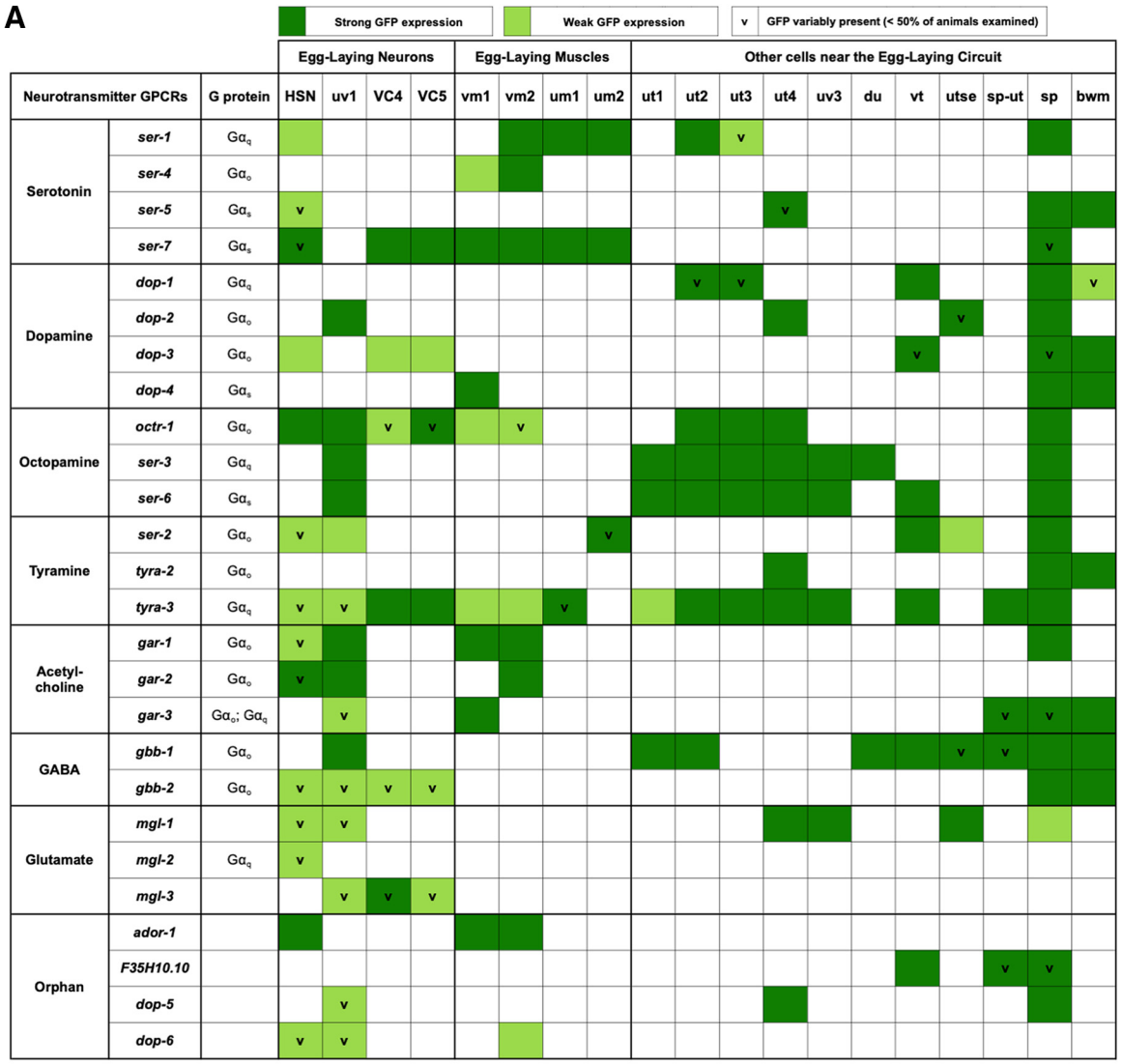
Because the *goa-1* mutant for the G protein Gα_o has a strong hyperactive egg-laying phenotype (Fig. 6), we hypothesized that the effects of knockouts for receptors that signal through this G protein were masked by functional redundancy. Thus, we crossed together Knockout mutations for Gα_o-coupled neurotransmitter GPCRs to see whether this would reveal a strong hyperactive egg-laying phenotype. We generated a series of combination knockouts, from double knockouts to a quintuple knockout, but none of these revealed a hyperactive egg-laying phenotype approaching the strength of the *goa-1* mutant (Fig. 6*D*), nor did they show an abnormal accumulation of unlaid eggs (Extended Data Fig. 6-1*B*). One possibility is that Gα_o signals through even more Gα_o-coupled receptors, such as neuropeptide receptors (Ringstad and Horvitz, 2008), which would need to be knocked out to phenocopy the Gα_o mutant.

Another interpretation of the lack of strong egg-laying defects in neurotransmitter GPCR knockouts is that we only assayed egg-laying behavior under standard laboratory growth conditions. Egg-laying behavior is known to be regulated by many different conditions, such as environmental CO_2 (Fenk and de Bono, 2015), the presence or absence of food (Dong et al., 2000), and whether the animal is in the roaming versus dwelling state of locomotion (Cermak et al., 2020). Specific neurotransmitter GPCR knockouts might be required to regulate egg laying in these or other specific circumstances.

Overexpression of neurotransmitter GPCRs reveals egg-laying defects

We next analyzed strains that overexpress individual neurotransmitter receptors, reasoning that this could result in gain-of-function phenotypes that might reveal receptor functions, even if individual receptors function redundantly with each other. Our high-copy

A



B

		HSN	uv1	VC4	VC5	vm1	vm2	um1	um2	ut1	ut2	ut3	ut4	uv3	du	vt	utse	sp-ut	sp	bwm
# of GPCRs	Strong	4	7	3	3	5	6	3	3	3	7	5	9	4	2	7	3	4	19	7
	Weak	10	8	3	3	3	3	0	0	1	0	1	0	0	0	0	1	0	1	1
	TOTAL	**14**	**15**	**6**	**6**	**8**	**9**	**3**	**3**	**4**	**7**	**6**	**9**	**4**	**2**	**7**	**4**	**4**	**20**	**8**
# of GPCRs coupled to: $G\alpha_o$	Strong	2	5	0	1	2	3	0	1	1	2	1	3	0	1	3	2	2	9	5
	Weak	4	3	3	2	2	1	0	0	0	0	0	0	0	0	0	1	0	0	0
	TOTAL	**6**	**8**	**3**	**3**	**4**	**4**	**0**	**1**	**1**	**2**	**1**	**3**	**0**	**1**	**3**	**3**	**2**	**9**	**5**
# of GPCRs coupled to: $G\alpha_q$	Strong	0	1	1	1	1	1	2	1	1	4	3	2	2	1	2	0	2	5	1
	Weak	3	2	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	1
	TOTAL	**3**	**3**	**1**	**1**	**2**	**2**	**2**	**1**	**2**	**4**	**4**	**2**	**2**	**1**	**2**	**0**	**2**	**5**	**2**
# of GPCRs coupled to: $G\alpha_s$	Strong	1	1	1	1	2	1	1	1	1	1	1	2	1	0	1	0	0	4	2
	Weak	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	TOTAL	**2**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**0**	**1**	**0**	**0**	**4**	**2**

C

	Egg-Laying Neurons and Muscles Expressing One or More Receptors	
Neurotransmitter	**Synaptic Target Cells**	**Extrasynaptic Target Cells**
Serotonin	***HSN*, VC4, VC5, vm2**	**vm1, um1, um2**
Dopamine	none	**HSN, uv1, VC4, VC5, vm1**
Octopamine	none	**HSN, uv1, *VC4*, *VC5*, vm1, *vm2***
Tyramine	none	***HSN*, uv1, VC4, VC5, vm1, vm2, *um1*, *um2***
Acetylcholine	***HSN*, vm1, vm2**	**uv1**
GABA	***HSN*, *VC4***	***VC5***
Glutamate	***HSN***	***uv1*, VC4, *VC5***

Figure 5. Summary of neurotransmitter GPCR::GFP expression in the *C. elegans* egg-laying system. ***A***, Summary of neurotransmitter GPCR::GFP expression by cells in and near the egg-laying system. Dark green represents GFP expression that was on average strong (scored as +++, among brightest of any cell in the animal) or easily detectable (++). Light green represents

GPCR::GFP transgenes should overexpress neurotransmitter receptors. The 20 C-terminal::GFP reporters overexpress a receptor with GFP fused to its C-terminus, while the four SL2::GFP reporters overexpress a receptor without any such modification (Extended Data Fig. 1-1). Two of our GPCR::GFP transgenes (for SER-4 and MGL-2) were constructed for technical reasons in a manner that would not cause receptor overexpression, so we did not analyze overexpression of these two receptors.

We piloted the neurotransmitter receptor overexpression strategy with the serotonin receptor SER-1, which had previously been reported to stimulate egg laying by acting partially redundantly with the serotonin receptor SER-7 (Hapiak et al., 2009). In the context of this experiment, we refer to the *ser-1::gfp* transgene as "*ser-1(oe)*" to indicate that the *ser-1* gene is overexpressed. The experimental strategy is schematized in Figure 7*A*; we expected SER-1 overexpression to increase serotonin stimulation of egg laying and result in hyperactive egg laying, and that knocking out serotonin biosynthesis would suppress this effect since the overexpressed SER-1 would no longer be activated by serotonin. As expected, the *ser-1(oe)* strain displayed a strong hyperactive egg-laying phenotype (Fig. 7*B*). The same phenotype was also observed in animals carrying an extrachromosomal *ser-1(oe)* transgene and in two independent chromosomal integrants for the *ser-1(oe)* transgene (Extended Data Fig. 7-1*B*), suggesting that the phenotype was not an artifact of chromosomal integration. The hyperactive egg-laying phenotype of *ser-1(oe)* was completely suppressed in animals lacking tryptophan hydroxylase enzyme (Fig. 7*B*), an enzyme required for serotonin biosynthesis (Sze et al., 2000). This control suggests that the effect of the high-copy *ser-1* transgene is because of the overexpressed SER-1 serotonin receptor mediating increased serotonin signaling. This control is crucial because the high copy transgenes we are using to overexpress neurotransmitter GPCRs each contain a ~40 kb stretch of *C. elegans* genomic DNA that often carries one or more flanking genes in addition to the GPCR gene, and these flanking genes are also overexpressed. Our results overexpressing *ser-1* were consistent with the previous data suggesting that SER-1 is one of two partially redundant receptors through which serotonin signals to stimulate egg laying (Hapiak et al., 2009).

We examined all 24 neurotransmitter GPCR overexpressor transgenes and found six more instances of strong effects on egg laying. We applied the same validation tests we used for *ser-1* overexpression to each of these 6 additional cases: we required that multiple independent overexpressor transgenes for a GPCR produce similar effects on egg laying, and that these effects are suppressed by a mutation that blocks synthesis or transport of the corresponding neurotransmitter. We found we could not validate two of these 6 cases: the GABA receptor GBB-1 and the octopamine receptor SER-3 each had one overexpressor transgene that resulted in changes to egg laying, but these phenotypes were not reproduced using other independent overexpressor transgenes (Extended Data Fig. 7-1*A*,*C*,*H*). However, the remaining 4 cases were validated, and each suggests novel functional effects of neurotransmitter signaling in the egg-laying system, as described below.

Overexpression of the glutamate GPCRs *mgl-1(oe)* and *mgl-3(oe)* caused strong accumulation of unlaid eggs (Fig. 7*C*). Extrachromosomal *mgl-1(oe)* and *mgl-3(oe)* transgenes also caused similar egg-laying defects (Extended Data Fig. 7-1*E*,*F*), and abolishing glutamate signaling by knocking out the vesicular glutamate transporter EAT-4 (Lee et al., 1999) completely suppressed the egg-laying defects of glutamate receptor overexpression (Fig. 7*D*,*E*). In the egg-laying system, *mgl-1* is weakly and variably expressed in uv1 and HSN neurons but shows strong and consistent expression in specific epithelial cells of the uterus, whereas *mgl-3* shows its strongest (albeit variable) expression in the VC4 neuron (Fig. 5). These glutamate receptors are also expressed outside the cells of the egg-laying system (Yemini et al., 2020), and it remains to be determined where they act to affect egg laying. Our results represent the first time glutamate signaling has been shown to affect *C. elegans* egg laying, and provide a starting point for future analysis of this issue.

Overexpression of the octopamine receptor SER-6 caused accumulation of unlaid eggs (Fig. 7*C*). We saw similar phenotypes using other extrachromosomal and chromosomally integrated *ser-6(oe)* transgenes (Extended Data Fig. 7-1*D*) and could suppress this egg-laying defect by knocking out the octopamine biosynthetic enzyme TBH-1 (Alkema et al., 2005) (Fig. 7*G*). Previous studies had shown that exogenous octopamine inhibits egg-laying behavior (Horvitz et al., 1982), but the mechanism and receptor(s) mediating this effect had remained unknown. SER-6, along with other octopamine receptors, is strongly expressed in the uv1 neuroendocrine cells and in the epithelial cells of the uterus (Fig. 5). To date, the uterine epithelial cells have not been implicated in regulation of egg laying. However, given that SER-6 is coupled to the excitatory G protein $G\alpha_s$, we hypothesize that SER-6 could activate the uv1s, which in turn inhibit egg laying (Jose et al., 2007; Banerjee et al., 2017).

Overexpression of the acetylcholine receptor GAR-1 similarly caused accumulation of unlaid eggs (Fig. 7*C*). We reproduced this defect with an extrachromosomal *gar-1(oe)* transgene (Extended Data Fig. 7-1*G*) but could not attempt to further validate this effect since a null allele for the acetylcholine biosynthetic enzyme is lethal (Rand, 1989). Inhibition of egg laying via GAR-1 signaling would be interesting as acetylcholine has been shown to both activate and inhibit egg laying (Bany et al., 2003; W. F. Schafer, 2006). GAR-1 is expressed principally in the uv1 neuroendocrine cells and the vm1/2 vulval muscles (Fig. 5). As a $G\alpha_o$-coupled receptor, GAR-1 is expected to reduce neural/muscle activity. Thus GAR-1 could activate egg laying by inhibiting the uv1 cells, which in turn inhibit egg laying, and GAR-1 could inhibit egg laying by inhibiting the vm1/2 muscles, which execute egg laying.

The final receptor that displayed a validated egg-laying defect when overexpressed was the tyramine receptor SER-2. The integrated *ser-2(oe)* transgene caused a mild retention of unlaid eggs (Fig. 7*C*) that was reproduced with an extrachromosomal *ser-2(oe)* transgene (Extended Data Fig. 7-1*I*) and suppressed when we knocked out the tyramine biosynthetic enzyme tyrosine decarboxylase (Alkema et al., 2005) (Fig. 7*F*). Tyramine was

←

weak GFP expression (+, just above background in at least 2 different animals). White represents cells in which GFP was not reproducibly observed. v, Variable (denotes cells in which GFP detected in more than two but less than half of animals observed). bwm, Body wall muscles; sp-ut, spermathecal-uterine valve. ***B***, Number of strongly and weakly expressed receptors in each cell type of the egg-laying system. Receptors with variable expression are included. Totals are shown broken down by the Gα protein thought to be activated by the receptors in each cell type when that information is known. ***C***, Neurons and muscles of the egg-laying system that express neurotransmitter GPCRs, indicating which cells have the corresponding neurotransmitter synaptically released onto them (synaptic target cells) or not (extrasynaptic target cells), based on the connectome (Albertson and Thomson, 1976; White et al., 1986; Xu et al., 2013) and neurotransmitter maps (Serrano-Saiz et al., 2013; Pereira et al., 2015; Gendrel et al., 2016). Cell names in dark/light green represent strong/weak receptor expression. Italics indicate that receptor expression is variable. Extended Data Figure 5-1 presents a more detailed summary of our GPCR::GFP expression results, including comparison with previously published work and details of variability in GFP expression.

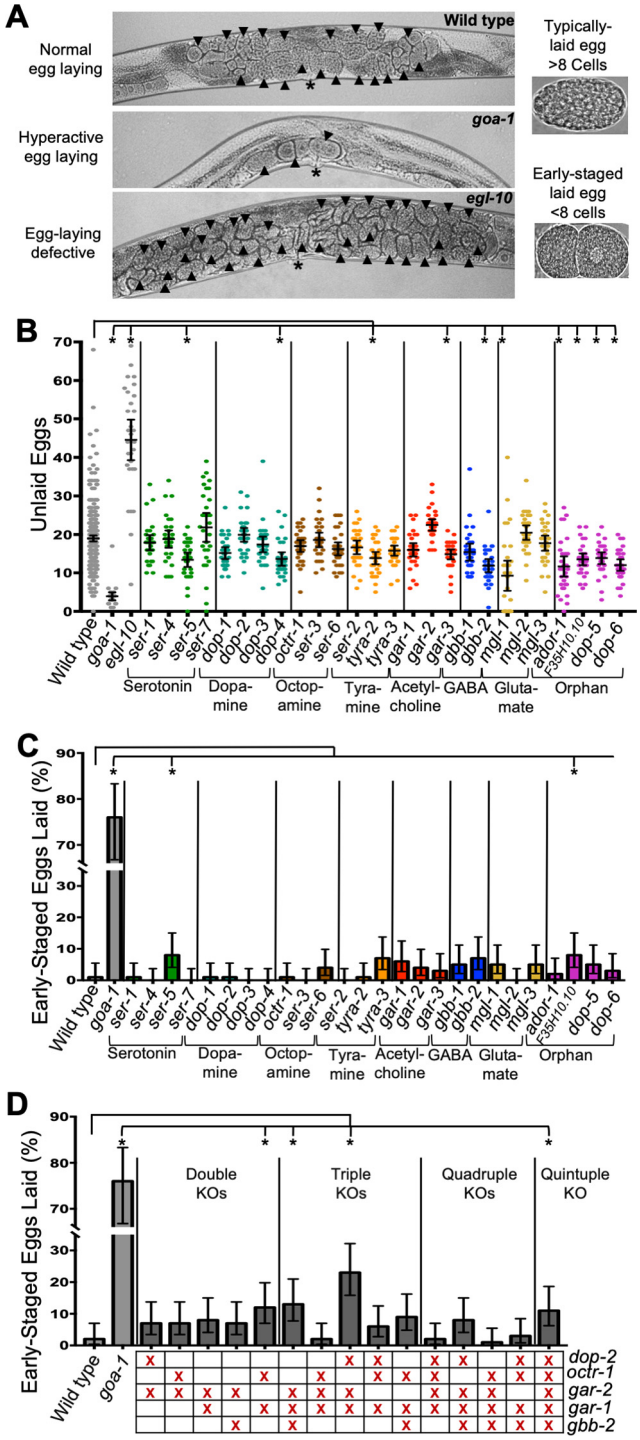


Figure 6. Neurotransmitter GPCRs knockouts rarely reveal egg-laying defects. ***A***, Micrographs of animals with egg-laying phenotypes. WT adults (top) typically accumulate 15-20 unlaid eggs, whereas hyperactive egg-laying mutants, such as like *goa-1* (second from top), accumulate fewer unlaid eggs and egg-laying defective mutants, such as *egl-10* (third from top), accumulate more unlaid eggs. Eggs from WT animals have developed to the ~100 cell stage by the time they are laid (top right), whereas eggs from hyperactive egg-laying mutants are laid earlier, often with fewer than eight cells (bottom right). ***B***, Number of unlaid eggs for neurotransmitter GPCR single knockout strains. *egl-10(md176)* and *goa-1 (n1134)* served as controls (these control data are replotted in Fig. 7*C* and Extended Data Fig. 6-1*B*). $n \geq 30$ for each strain. *Statistical significance ($p < 0.05$) compared with the WT using one-way ANOVA with Bonferroni correction for multiple comparisons. Significant p values were as follows: *goa-1* ($p < 0.0001$); *egl-10* ($p < 0.0001$); *ser-5* ($p = 0.0002$); *dop-4* ($p = 0.0004$); *tyra-2* ($p = 0.001$); *gar-3* ($p = 0.0021$); *gbb-1* ($p = 0.0253$); *gbb-2* ($p < 0.0001$); *mgl-1* ($p < 0.0001$); *ador-1* ($p < 0.0001$); *F35H10.10* ($p < 0.0001$); *dop-5* ($p = 0.0001$); and *dop-6* ($p < 0.0001$). Error bars indicate 95% CIs. Here and in other graphs in this work, different colors are used to plot data for receptors for different neurotransmitters, or for the orphan GPCR class. ***C***, Percent of early-stage eggs laid by neurotransmitter GPCR gene single knockout strains. ***C***, ***D***, *goa-1(n1134)* is used as a control hyperactive egg-laying mutant (these control data are replotted in Fig. 6*D* and Extended Data Fig. 7-1*A*). $n = 100$ eggs per strain. *Statistical significance ($p < 0.05$) compared with the WT using the Fisher's exact test. Significant p values were as follows: *goa-1* ($p < 0.00001$); *ser-5* ($p = 0.0349$); and *F35H10.10* ($p = 0.0349$). The Wilson-Brown method was used to determine the 95% CI for binomial data. ***D***, Percent of early-stage eggs laid by strains carrying combinations of KOs in genes encoding $G\alpha_o$-coupled neurotransmitter GPCRs. *goa-1(n1134)* is used as a control hyperactive egg-laying mutant. Red "X" identifies the receptors knocked out in a given strain. Statistical analysis was as in ***C***. The five significant p values (for measurements from left to right that are denoted with asterisks) were $p < 0.00001$, $p = 0.0101$, $p = 0.0055$, $p < 0.00001$, and $p = 0.0184$. Extended Data Figure 6-1 presents further analysis of neurotransmitter GPCR knockout strains.

previously known to inhibit egg laying (Alkema et al., 2005; Collins et al., 2016). SER-2 is weakly and/or variably expressed in neurons (HSN, uv1) and muscle cells (um2) of the egg-laying system, but strongly expressed in certain non-neuronal/non-muscle cells, such as epithelial cells of the vulva (Fig. 5). SER-2 is coupled to the inhibitory G protein $G\alpha_o$, and we hypothesize that it could inhibit egg laying by acting in the HSN and um2 cells that act to stimulate egg laying.

Rich and functionally robust neurotransmitter GPCR signaling controls neural circuit activity

This study provides, for the first-time, analysis of the expression and functions of every neurotransmitter GPCR in a model neural circuit. The *C. elegans* egg-laying circuit was already one of the most intensively studied small neural circuits, yet this study provides new insights into this circuit that may apply to neural circuits in general.

The first insight is that there may be far more neurotransmitter signaling within neural circuits than had been suggested by previous work. All cells in the egg-laying system express multiple neurotransmitter receptors, and each cell type expresses its own unique combination of up to 20 such receptors. Cells of the egg-laying circuit express receptors for neurotransmitters not previously implicated in control of egg laying, such as glutamate and GABA. Cells previously thought to have merely structural roles, such as the epithelial cells that make up the uterus and vulva, also express neurotransmitter receptors. Expression and function of neurotransmitter receptors in cells other than neurons and muscles have not been widely studied, yet our results suggest that this phenomenon may be widespread.

A second insight is that circuits may be remarkably robust systems because of an apparent high level of redundancy and compensation among neurotransmitter GPCRs. While mutations that affect downstream signaling by the neural G proteins $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ each show dramatic effects on egg laying (W. F. Schafer, 2006; Koelle, 2018), no single neurotransmitter GPCR receptor knockout causes significant defects. The receptor knockout strains we analyzed in our study are grossly healthy and move relatively normally (data not shown), suggesting that functional redundancy of neurotransmitter GPCRs extends beyond the egg-laying system. Another interpretation of the lack of obvious defects in GPCR knockouts is that GPCRs may function to regulate egg-laying only under specific circumstances that we did not test. An example of neurotransmitter GPCRs functioning to control egg-laying both redundantly and only in specific circumstances came to light very recently. Cermak et al.

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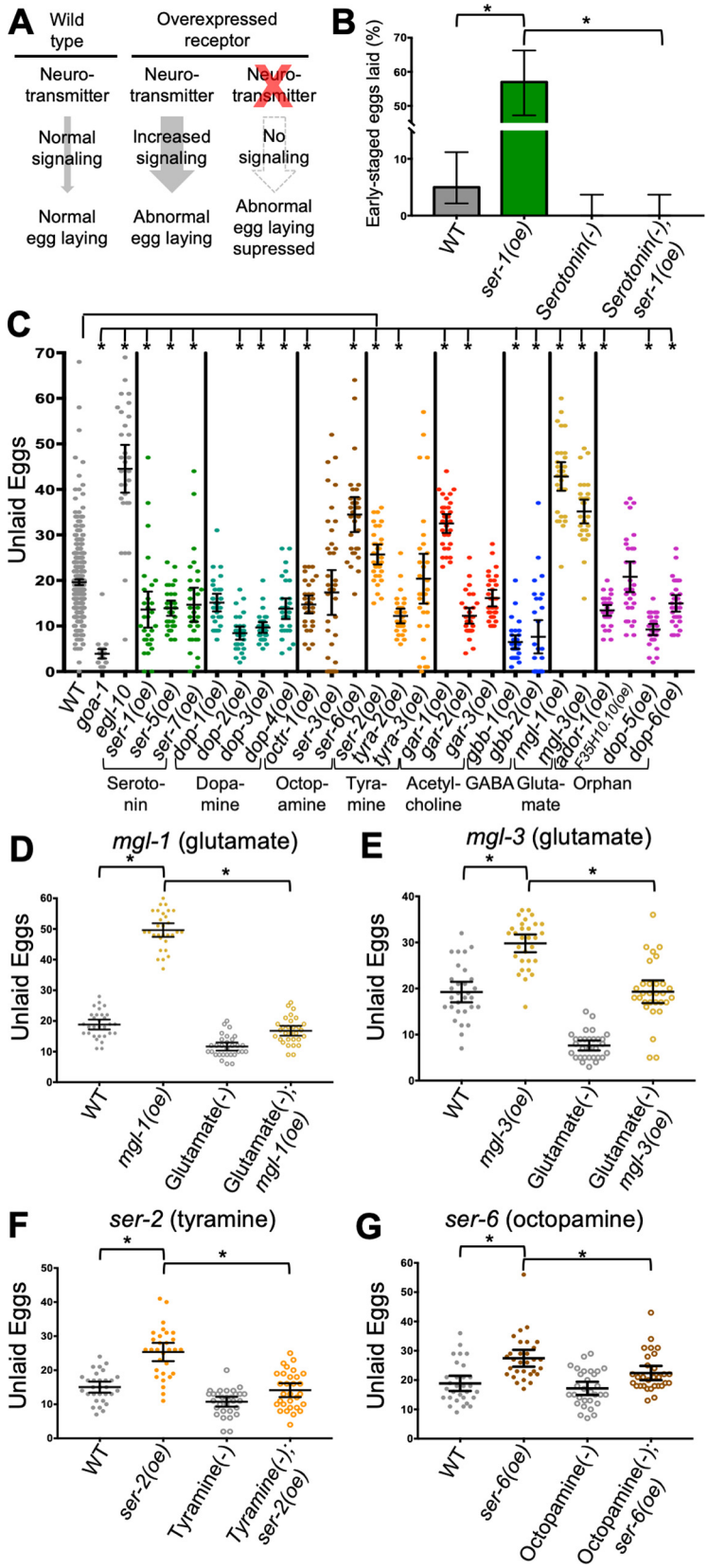

Figure 7. Neurotransmitter GPCR overexpressors reveal strong egg-laying defects. ***A***, Strategy to reveal and validate egg-laying defects caused by increased signaling through overexpressed neurotransmitter GPCRs. ***B***, Overexpression of the serotonin GPCR SER-1 (*ser-1(oe)*) causes hyperactive egg-laying that is suppressed by knocking out serotonin biosynthesis with a null mutant in the tryptophan hydroxylase enzyme (TPH-1). $n = 100$ eggs per strain. Error bars indicate 95% CIs determined by the Wilson-Brown method. *Statistical significance ($p < 0.05$) compared with the WT (Fisher's exact test). p values for the two asterisked comparisons were as follows: $p < 0.00001$ (left comparison) and $p < 0.00001$ (right comparison). ***C***, Number of unlaid eggs for neurotransmitter GPCR gene overexpressor strains. *egl-10(md176)* and *goa-1(n1134)* served as controls. $n \geq 30$ for each strain. Error bars indicate 95% CIs. *$p < 0.05$, compared with the WT (one-way ANOVA with Bonferroni correction for multiple comparisons). p values for the comparisons to the WT were as follows: *goa-1* ($p < 0.0001$); *egl-10* ($p < 0.0001$); *ser-1(oe)* ($p = 0.0011$); *ser-5(oe)* ($p = 0.0025$); *ser-7(oe)* ($p = 0.0189$); *dop-2(oe)* ($p < 0.0001$); *dop-3(oe)* ($p < 0.0001$); *dop-4(oe)* ($p = 0.0019$); *octr-1(oe)* ($p = 0.0223$); *ser-6(oe)* ($p < 0.0001$); *ser-2(oe)* ($p = 0.009$); *tyra-2(oe)* ($p < 0.0001$); *gar-1(oe)* ($p < 0.0001$); *gar-2(oe)* ($p < 0.0001$); *gbb-1(oe)* ($p < 0.0001$); *gbb-2(oe)* ($p < 0.0001$); *mgl-1(oe)* ($p < 0.0001$); *mgl-3(oe)* ($p < 0.0001$); *ador-1(oe)* ($p = 0.0006$); *dop-5(oe)* ($p < 0.0001$); and *dop-6(oe)* ($p = 0.0389$). ***D***, Overexpression of the glutamate GPCR MGL-1 causes defective egg laying that is suppressed by knocking out glutamate signaling with a mutation in the vesicular glutamate transporter EAT-4. ***D-G***, $n \geq 30$ for each strain. Error bars indicate 95% CIs. *$p < 0.05$ (one-way ANOVA with a Tukey's test to determine statistical significance for multiple comparisons). p values for the two asterisked comparisons in ***D*** were as follows: $p < 0.0001$ (left comparison) and $p < 0.0001$ (right comparison). ***E***, Overexpression of the glutamate GPCR MGL-3 causes defective egg laying that is suppressed by knocking out glutamate signaling with a mutation in the vesicular glutamate transporter EAT-4. p values for the two asterisked comparisons were as follows: $p < 0.0001$ (left comparison) and $p < 0.0001$ (right comparison). ***F***, Overexpression of the tyramine GPCR SER-2 causes defective egg-laying that is suppressed by knocking out the tyramine biosynthesis enzyme tyrosine decarboxylase (TDC-1). p values for the two asterisked comparisons were as follows: $p < 0.0001$ (left comparison) and $p < 0.0001$ (right comparison). ***G***, Overexpression of the octopamine GPCR SER-6 causes defective egg laying that is suppressed by knocking out the octopamine biosynthesis enzyme tyramine β-hydroxylase (TBH-1). p values for the two asterisked comparisons were as follows: $p < 0.0001$ (left comparison) and $p = 0.0257$ (right comparison). Extended Data Figure 7-1 presents additional validation tests of neurotransmitter GPCR overexpression phenotypes.

(2020) found that the DOP-2 and DOP-3 dopamine receptors function redundantly to promote egg laying, but only during the ~20% of the time worms in standard laboratory conditions spend in the "roaming" state of locomotion. We found both *dop-2* and *dop-3* were expressed in the egg-laying system, but we did not assay the *dop-2; dop-3* double mutant or assay animals specifically in the roaming state, so we could not detect the function of dopamine receptors in egg laying discovered by Cermak et al. (2020).

We found that overexpressing neurotransmitter GPCRs generate gain-of-function phenotypes that can reveal receptor functions despite the issue of redundancy. This strategy reveals novel potential functions of dopamine, octopamine, acetylcholine, GABA, and glutamate, and orphan receptors in controlling the egg-laying circuit. Receptor overexpression may similarly be useful in revealing redundant neurotransmitter signaling functions in other circuits.

Our results generate a rich set of hypotheses to direct future studies of the egg-laying circuit. For example, prior work has shown that egg laying is activated when the HSN releases a combination of serotonin and the neuropeptide NLP-3 (Brewer et al., 2019). These two signals act partially redundantly to turn on the egg-laying circuit, with serotonin itself acting through partially redundant receptors (Hobson et al., 2006; Xiao et al., 2006; Hapiak et al., 2009). The multiple layers of redundancy in this circuit activation step lead to circuit activity being highly robust, but they also complicate efforts to understand the mechanism of circuit activation. Our results show that the serotonin receptors that activate the circuit, principally SER-1 and SER-7, with SER-5 playing a lesser role (Hapiak et al., 2009), are each expressed in different overlapping sets of cells in the egg-laying system. SER-1 and SER-7 are both expressed in the vm2 muscle cells, whose serotonin activation is crucial for executing egg laying (Brewer et al., 2019). SER-7 is also expressed in the VC neurons, which synapse onto vm2. The HSNs activate the VCs, which fire every time an egg is laid (Collins et al., 2016), and our findings suggest the hypothesis that HSNs activate the VC neurons via the SER-7 serotonin receptor. We also found that the SER-1, SER-5, and SER-7 receptors are expressed on the HSNs themselves, suggesting the hypothesis that serotonin from the HSN could act in an autocrine feedback loop to maintain HSN activity. Prior work has shown that the uv1 cells release tyramine and a combination of neuropeptides (Collins et al., 2016; Banerjee et al., 2017), with these signals acting redundantly to inhibit the egg-laying circuit, and that tyramine itself acts via multiple redundant receptors. Our results show which cells of the circuit express each of the three tyramine receptors, setting the stage for studies to tease out the redundant mechanisms by which the circuit is inactivated.

The strategies and tools applied in this work for analyzing neurotransmitter signaling in the egg-laying circuit can be applied to any neural circuit in *C. elegans* or other organisms to generate sets of testable hypotheses for how signaling among the cells of a neural circuit control its activities and function.

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