Cellular/Molecular

Multiple Subthreshold GPCR Signals Combined by the G-Proteins $G\alpha_q$ and $G\alpha_s$ Activate the *Caenorhabditis elegans* Egg-Laying Muscles

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Individual neurons or muscle cells express many G-protein-coupled receptors (GPCRs) for neurotransmitters and neuropeptides, yet it remains unclear how cells integrate multiple GPCR signals that all must activate the same few G-proteins. We analyzed this issue in the *Caenorhabditis elegans* egg-laying system, where multiple GPCRs on muscle cells promote contraction and egg laying. We genetically manipulated individual GPCRs and G-proteins specifically in these muscle cells within intact animals and then measured egg laying and muscle calcium activity. Two serotonin GPCRs on the muscle cells, $G\alpha_q$-coupled SER-1 and $G\alpha_s$-coupled SER-7, together promote egg laying in response to serotonin. We found that signals produced by either SER-1/$G\alpha_q$ or SER-7/$G\alpha_s$ alone have little effect, but these two subthreshold signals combine to activate egg laying. We then transgenically expressed natural or designer GPCRs in the muscle cells and found that their subthreshold signals can also combine to induce muscle activity. However, artificially inducing strong signaling through just one of these GPCRs can be sufficient to induce egg laying. Knocking down $G\alpha_q$ and $G\alpha_s$ in the egg-laying muscle cells induced egg-laying defects that were stronger than those of a SER-1/SER-7 double knockout, indicating that additional endogenous GPCRs also activate the muscle cells. These results show that in the egg-laying muscles multiple GPCRs for serotonin and other signals each produce weak effects that individually do not result in strong behavioral outcomes. However, they combine to produce sufficient levels of $G\alpha_q$ and $G\alpha_s$ signaling to promote muscle activity and egg laying.

Key words: **C. elegans; G-protein; GPCR; neuroscience; serotonin**

Significance Statement

How can neurons and other cells gather multiple independent pieces of information from the soup of chemical signals in their environment and compute an appropriate response? Most cells express >20 GPCRs that each receive one signal and transmit that information through three main types of G-proteins. We analyzed how this machinery generates responses by studying the egg-laying system of *C. elegans*, where serotonin and multiple other signals act through GPCRs on the egg-laying muscles to promote muscle activity and egg laying. We found that individual GPCRs within an intact animal each generate effects too weak to activate egg laying. However, combined signaling from multiple GPCR types reaches a threshold capable of activating the muscle cells.

Introduction

Individual neuron or muscle cells can express many different G-protein coupled receptors (GPCRs), which in turn act through just three main types of heterotrimeric G-proteins, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$ (Kaur et al., 2017; Smith et al., 2019; Jiang et al., 2022). Multiple chemical signals impinge on individual cells within the body, and signaling through multiple GPCRs integrates this complex information to produce appropriate responses. How this occurs remains largely unclear and is the focus of this study.

Evidence for the widespread use of multiple GPCRs on individual cells comes from studies across different cell types and organisms. Single-cell transcriptomics on primary cultures of mouse smooth muscle cells and endothelial cells indicate that individual cells express ~20 GPCRs on average (Kaur et al.,

Received Dec. 14, 2022; revised Mar. 21, 2023; accepted Apr. 7, 2023.

Author contributions: A.C.O. and M.R.K. designed research; A.C.O., A.M.B., and N.T.M.C. performed research; A.S. contributed unpublished reagents/analytic tools; A.C.O. and M.R.K. analyzed data; and A.C.O. and M.R.K. wrote the paper.

This work was supported by National Institutes of Health (NIH) Grant NS036918. We thank the Yale Center for Advanced Light Microscopy Facility for assistance with confocal microscopy, funded by NIH Shared Instrument Grant S10 OD023598. Some strains were provided by the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs Grant P40 OD010440, and the Mitani laboratory, Japan National BioResource Project.

The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.2301-22.2023

2017). Even when only 29 of the >100 neuropeptide receptor genes are analyzed, a typical neuron expresses multiple such receptors (Smith et al., 2019). Vertebrate mast cells use at least 16 different GPCRs to respond to various neurotransmitters and neuropeptides (Xu et al., 2020). Pyramidal neurons can express up to five different subtypes of serotonin receptors, including two different $G\alpha_q$-coupled $5HT_2$ receptor subtypes and the $G\alpha_s$-coupled $5HT_4$ receptor (Feng et al., 2001). Signaling through each of these serotonin receptors can increase the excitability of target neurons (Rasmussen and Aghajanian, 1990; Lopez et al., 2021). The logic of using multiple GPCRs for the same ligand in parallel on the same target cells remains unclear.

Neural circuits of invertebrates that consist of only a small number of cells provide model systems in which one can tease out how multiple GPCRs function together on individual cells. For example, in the crustacean somatogastric circuit, indirect evidence suggests many different neurotransmitters and neuropeptides modulate activity of individual neurons (Marder and Bucher, 2007). In this study, we focus on the *Caenorhabditis elegans* egg-laying circuit, where we recently found that the muscle cells that execute egg laying express at least five types of $G\alpha_q$-coupled and $G\alpha_s$-coupled neurotransmitter GPCRs (Fernandez et al., 2020).

The *C. elegans* system provides the genetic tools to manipulate individual GPCRs and G-proteins specifically within the egg-laying muscle cells inside of intact, behaving animals such that all other endogenous GPCRs and signals affecting these same cells remain in place. Thus, the mechanisms by which multiple GPCR signals are integrated can be investigated in a physiological setting as opposed to an artificially simplified system such as cell culture in which GPCR signaling has often been investigated in the past.

In the *C. elegans* egg-laying circuit, schematized in Figure 1*A*, the hermaphrodite-specific neurons (HSNs) and ventral type C (VC) motor neurons synapse onto the egg-laying muscles. The HSNs release both serotonin and a neuropeptide named NLP-3 to induce activity of the VCs and contraction of the egg-laying muscles, resulting in egg laying (Collins and Koelle, 2013; Brewer et al., 2019). There are 16 egg-laying muscle cells in total, four each of four types, which are the um1 and um2 uterine muscle cell types, as well as vm1 and vm2 vulval muscle cell types. The um1, um2, and vm2 muscle cells each coexpress the two serotonin receptors SER-1 and SER-7 that contribute to inducing egg laying (Fernandez et al., 2020). SER-1 is a $G\alpha_q$-coupled receptor, whereas SER-7 couples to $G\alpha_s$ (Hamdan et al., 1999; Hobson et al., 2003; Carnell et al., 2005; Dempsey et al., 2005; Carre-Pierrat et al., 2006; Hobson et al., 2006). The vm1 muscle cells as well as the VC4 and VC5 neurons each express SER-7 but not SER-1 (Fernandez et al., 2020).

In this study we genetically manipulated GPCRs and G-proteins in specific cells of the *C. elegans* egg-laying circuit within intact animals to discover how endogenous signals act through multiple GPCRs on the same cells to alter the behavioral output of the circuit. We found that multiple, individually weak GPCR signals are combined by the G-proteins $G\alpha_q$ and $G\alpha_s$ to activate the egg-laying muscles.

Materials and Methods

Strains and culture. A complete list of the *C. elegans* strains and transgenes used in this article is in Table 1. Tables 2 and 3 detail the transgenes carried in these strains and how they were constructed. *C. elegans* were maintained at 20°C on standard nematode growth media (NGM), seeded with OP50 strain of *Escherichia coli* as their food source. Mutants and animals carrying chromosomally integrated transgenes were backcrossed 2–10× to N2 (wild type) to generate clean genetic backgrounds, as indicated in Table 1. New strains were constructed using standard genetic cross procedures, and genotypes were confirmed by PCR genotyping or sequencing. Extrachromosomal array transgenic strains were generated through microinjection. Phenotypes were typically scored in animals from greater than or equal to five independent transgenic lines, and at least one independent line has been frozen for storage.

Molecular biology. The construction of plasmids used in this manuscript is described in Table 4.

Egg-laying muscle-specific RNAi. Transgenic animals with egg-laying muscle-specific RNAi were created as described in Esposito et al., (2007). PCR was used to fuse one amplicon containing the egg-laying muscle-specific *unc-103e* promoter with a second amplicon containing an exon-rich region of the gene to be targeted by RNAi. To increase the yield of the fusion PCR product, NEBuilder HiFi DNA Assembly Mix (New England BioLabs) was used to fuse the promotor fragment to the exon-rich gene fragment before nested PCR. Two fusion PCR products for each gene of interest were injected into *C. elegans*, one expressing sense RNA and the other antisense RNA. The sense and antisense RNA strands expressed anneal in the muscle cells to form the double-stranded RNA (dsRNA) that induces RNAi. Because of the highly similar sequences of $G\alpha_q$ and $G\alpha_s$, care was taken to choose dissimilar regions of the genes encoding $G\alpha_q$ and $G\alpha_s$ to target with RNAi. The regions chosen had no more than 14 bp of contiguous sequence identity. Fifty to 100 ng/µl of fusion PCR product expressing sense RNA and 50–100 ng/µl of fusion PCR product expressing antisense RNA were injected into *sid-1 (qt9) V; lin-15(n765ts) X* animals along with 10 ng/µl pCFJ90 (pharyngeal mCherry coinjection marker), 50 ng/µl pL15EK [*lin-15(+)* coinjection marker], and 25 ng/µl DH5alpha genomic DNA digested with BamHI/HindIII. The *sid-1(qt9)* mutation kept the RNAi cell specific by preventing cell-to-cell spreading of the RNAi via systemic RNAi. Table 3 details the construction of the fusion PCR products, including the exact concentrations injected for each DNA and the PCR primer sequences used to amplify the *unc-103e* promoter region and each exon-rich gene region that was targeted by RNAi. For knockdown of the G-proteins, mCherry was also expressed in the egg-laying muscles to demonstrate that the G-protein knockdown did not interfere with muscle development (Extended Data Fig. 5-1*A*,*B*). Expression of mCherry in the egg-laying muscles of $G\alpha_q$ and $G\alpha_s$ RNAi knockdown animals did not affect the number of eggs retained in the uterus (Extended Data Fig. 5-1*C*).

Calcium imaging. Animals were staged as late-stage (L4) larvae and recorded 24 h later. Freely behaving animals were mounted between a glass coverslip and an ~1 cm^2 chunk from an NGM plate containing OP50 food for imaging as previously described (Collins and Koelle, 2013; Collins et al., 2016; Ravi et al., 2018a). A brightfield and two fluorescence channels (for the green GCaMP calcium sensor and a control red mCherry protein) were recorded with a 20× air objective using a Zeiss LSM 880 microscope. Recordings were collected at ~16 fps at 256 × 256 pixels, 16 bit resolution, for 1 h. Three 1 h recordings were collected for each genotype studied. As previously described (Brewer et al., 2019), calcium imaging was recorded in both the vm1 and vm2 vulval muscles simultaneously, and ratiometric analysis of the calcium recordings was performed in Volocity software (PerkinElmer) to generate traces of calcium transients. As described in Brewer et al. (2019), a video of each peak was examined and scored as vm1 only or vm1 + vm2.

Confocal imaging. Animals were mounted on microscope slides with 2% agarose pads containing 120 mM OptiPrep (Sigma-Aldrich) to reduce refractive index mismatch (Boothe et al., 2017) and a 22 × 22–1 microscope cover glass (Thermo Fisher Scientific) was placed on top of the agarose pad. Animals were anesthetized using a drop of 150 mM sodium azide (Sigma-Aldrich) with 120 mM OptiPrep. *Z*-stack confocal images of *C. elegans* staged 24 h post-L4 were taken on a Zeiss LSM 880 microscope using a 40× water-immersion objective lens.

Serotonin-induced egg laying on NGM plates. This assay was adapted from the work of Hobson et al. (2006). NGM plates containing 26 mM serotonin creatine sulfate monohydrate (catalog #H7752-5G, Sigma-

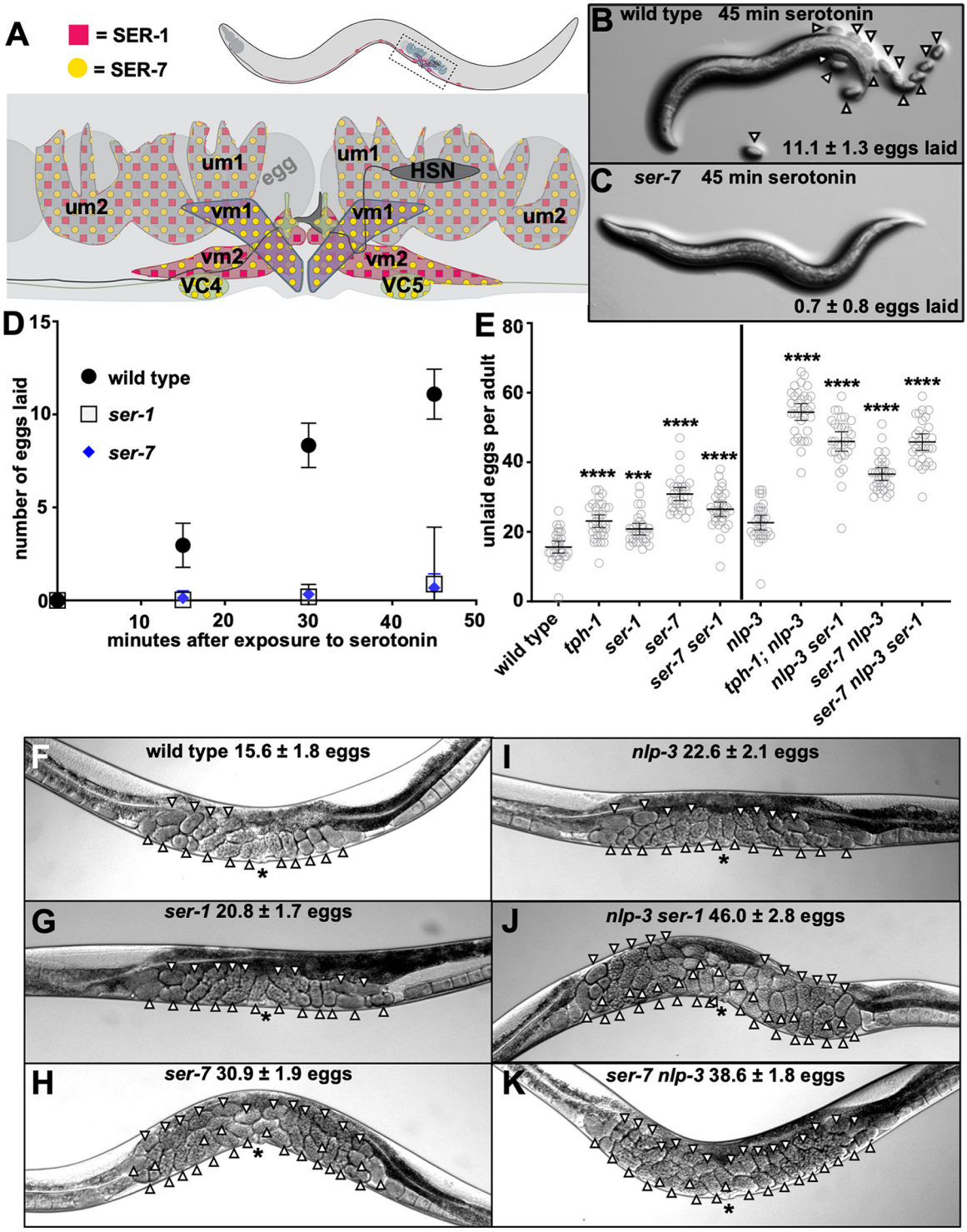


Figure 1. The serotonin receptors SER-1 and SER-7 are coexpressed on cells of the egg-laying circuit and loss of either blocks the ability of serotonin to stimulate egg laying. ***A***, Schematic of the *C. elegans* egg-laying system. Yellow circles denote cells that express SER-1, and pink squares denote cells that express SER-7. The HSN neurons and the vm1, vm2, um1, and um2 muscle cells each occur in left/right pairs, but only the cells on the left side of the animal are shown in this schematic. VC4 and VC5 are single neurons. ***B***, ***C***, Serotonin-induced egg-laying assays for wild-type or *ser-7* knock-out worms. Worms were photographed 45 min after being placed on plates containing 26 mM serotonin. Serotonin partially paralyzed the worms so that they remained adjacent to their laid eggs, which are indicated by arrowheads (***B***). The average number of laid eggs for each genotype in this assay is shown (***C***). ***D***. Results of a time course using the same assay illustrated in ***B*** and ***C*** for wild-type, *ser-1*, and *ser-7* null mutant animals. The assay was repeated with 10 worms/plate at least three times per genotype. ***E***, Average number of unlaid eggs per adult worm, $n \geq 30$ for each genotype. Each circle represents the number of unlaid eggs in a single worm. Genotypes left of the vertical black line are statistically compared with wild-type control animals using ordinary one-way ANOVA analysis ($F_{(4,148)} = 39.99$, $p < 0.0001$). Dunnett's multiple comparisons test was used; ***$p = 0.0004$, ****$p < 0.0001$. Genotypes on the right of the vertical line are statistically compared with *nlp-3* single-mutant control animals using ordinary one-way ANOVA analysis ($F_{(4,148)} = 114.3$ $p < 0.0001$). Dunnett's multiple comparisons test was used; ****$p < 0.0001$ for these comparisons. ***F–K***, Photographs of individual worms illustrating the accumulation of unlaid eggs (indicated by white arrowheads) in some of the genotypes analyzed in ***E***. The vulval slit is indicated by an asterisk (*). Photographs of individual worms for the remaining genotypes are shown in Extended Data Figure 1-2. The average number of unlaid eggs for each genotype is also indicated. All measurements are given with 95% confidence intervals.

Table 1. *C. elegans* strains used in this work

Strain	Genotype	Features	Number of outcrosses to the wild type	Used in figures	Source
N2	Wild type			1, 6, Ext. 1-1	Brenner, 1974
MT15434	*tph-1(mg280) II*			1, Ext. 1-2	Schumacher et al., 2012
DA1814	*ser-1(ok345) X*		10X	1, 6	Caenorhabditis Genetics Center (deposited by Leon Avery)
LX1984	*ser-7(gk414345) X*		6x	1, 6	Fernandez et al., 2020
LX2455	*ser-7(gk414345)X ser-1(ok345) X*		*ser-1* 12x, *ser-7* 7x	1, Ext. 1-2	This study
LX1978	*nlp-3(tm3023) X*		8x	1, Ext. 1-1	Brewer et al., 2019
LX2366	*tph-1(mg280) II; nlp-3(tm3023) X*		*tph-1* 1x, *nlp-3* 9x	1, Ext. 1-2	Brewer et al., 2019
LX2372	*nlp-3(tm3023) X ser-1(ok345) X*		*ser-1* 11x, *nlp-3* 9x	1	This study
LX2371	*ser-7(gk414345) X nlp-3(tm3023) X*		*ser-7* 7x, *nlp-3* 9x	1	This study
LX2456	*ser-7(gk414345) X nlp-3(tm3023) X ser-1(ok345) X*		*ser-7* 8x, *nlp-3* 10x, *ser-1* 13x	1, Ext. 1-2	This study
LX2467	*ser-5(ok3087) I*		6X outcrossed	Ext. 1-1	This study
LX2498	*ser-5(ok3087) I; nlp-3(tm3023) X*		*ser-5* 8x, *nlp-3* 9x	Ext. 1-1	This study
LX1919	*vsIs165 IV; lite-1(ce314) X lin-15(n765ts) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 6x	2, 3	Collins et al., 2016
LX2504	*tph-1(mg280) II; vsIs165 IV; lite-1(ce314) X*	GCaMP5/mCherry in vulval muscles	*tph-1* 2x, *vsIs165* 7x, *lite-1* 2x	2, 3	This study
LX2500	*vsIs165 IV; lite-1(ce314) X ser-1(ok345) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 7x, *lite-1* 2x, *ser-1* 11x	2, 3	This study
LX2501	*vsIs165 IV; ser-7(gk414345) X lite-1(ce314) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 7x, *ser-7* 8x, *lite-1* 2x	2, 3	This study
LX2495	*vsIs165 IV; lite-1(ce314) X nlp-3(tm3023) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 7x, *nlp-3* 9x, *lite-1* 2x	2, 3	This study
LX2553	*tph-1(mg280) II; vsIs165 IV; lite-1(ce314) X nlp-3(tm3023) X*	GCaMP5/mCherry in vulval muscles	*tph-1* 3x, *vsIs165* 7x, *lite-1* 2x, *nlp-3* 10x	2, 3	This study
LX2557	*vsIs165 IV; lite-1(ce314) X nlp-3(tm3023) X ser-1(ok345) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 7x, *lite-1* 2x, *nlp-3* 10x, *ser-1* 12x	2, 3	This study
LX2558	*vsIs165 IV ; ser-7(gk414345) X lite-1(ce314) X nlp-3(tm3023) X*	GCaMP5/mCherry in vulval muscles	*vsIs165* 7x, *lite-1* 2x, *ser-7* 9x, *nlp-3* 10x	2, 3	This study
LX2799	*sid-1(qt9) V; lin-15(n765ts) X; vsIs237; vsEx981*	Anti-*ser-7* RNAi in egg-laying muscles, SER-7::GFP	*sid-1* 3x, *vsIs237* 2x	4	This study
LX2835	*sid-1(qt9) V; lin-15(n765ts)X; vsIs237; vsEx1011*	Anti-*ser-1* RNAi in egg-laying muscles, SER-7::GFP	*sid-1* 3x, *vsIs237* 2x	4	This study
LX2804	*sid-1(qt9) V; lin-15(n765ts) X; vsEx986*	Anti-*gfp* RNAi in egg-laying muscles, used for a negative control	*sid-1* 2x	4, 5	This study
LX2806	*sid-1(qt9) V; lin-15(n765ts) X; vsEx988*	Anti-*ser-1* RNAi in egg-laying muscles	*sid-1* 2x	4, 5	This study
LX2807	*sid-1(qt9) V; lin-15(n765ts) X; vsEx989*	Anti-*ser-7* RNAi in egg-laying muscles	*sid-1* 2x	4, 5	This study
LX2827	*sid-1(qt9) V; lin-15(n765ts) X; vsEx1003*	Anti-*egl-30* ($G\alpha_q$) RNAi in the egg-laying muscles		5	This study
LX2828	*vsEx1004sid-1(qt9) V; lin-15(n765ts) X; vsEx1004*	Anti *gsa-1* ($G\alpha_s$) RNAi in the egg-laying muscles		5	This study
LX2821	*sid-1(qt9) V; lin-15(n765ts) X; vsEx997*	Anti-*egl-30* ($G\alpha_q$) and anti-*gsa-1* ($G\alpha s$) RNAi in egg-laying muscles	*sid-1* 2x	5, Ext. 5-1	This study
LX2096	*lin-15(n765) X; vsIs191*	mCherry in vulval muscles	*vsIs191* not outcrossed	Ext. 5-1	Collins et al., 2016
LX2822	*sid-1(qt9) V; lin-15(n765ts) X; vsEx998*	mCherry in vulval muscles, anti-*egl-30* ($G\alpha_q$) and anti-*gsa-1* ($G\alpha_s$) RNAi in egg-laying muscles	*sid-1* 2x	Ext. 5-1	This study
LX2819	*ser-1(ok345) X; vsIs238*	Overexpressed SER-7::GFP translational fusion	*ser-1* 11x, *vsIs238* 2x	6	This study
LX2820	*ser-7(gk414345) X; vsIs244*	Overexpressed SER-1::GFP translational fusion is overexpressed	*ser-7* 7x, *vsIs244* 4x	6	This study
LX2867	*ser-7(gk414345) X lite-1(ce314) X lin-15(n765ts) X; vsEx1042*	Empty vector control for photoactivatable adenylyl cyclase in egg-laying muscles	*ser-7* 9x, *lite-1* 2x	7	This study
LX2868	*ser-7(gk414345) X lite-1(ce314) X lin-15(n765ts) X; vsEx1043*	Photoactivatable adenylyl cyclase in egg-laying muscles	*ser-7* 9x, *lite-1* 2x	7	This study
LX2837	*ser-7(gk414345) X; vsEx1013*	Empty vector control for the $G\alpha_q$ DREADD in egg-laying muscles	*ser-7* 6x	7, 8	This study
LX2831	*ser-7(gk414345) X; vsEx1007*	$G\alpha_q$ DREADD in egg-laying muscles	*ser-7* 6x	7, 8	This study
LX2830	*ser-1(ok345) X; vsEx1006*	$G\alpha_q$ DREADD in egg-laying muscles	*ser-1* 10x	8	This study
LX2836	*ser-1(ok345) X; vsEx1012*	Empty vector control for the $G\alpha_q$ DREADD in egg-laying muscles	*ser-1* 10x	8	This study

Ext., Extended Data Figure. X= times.

Aldrich) were poured and seeded with OP50 1 d before assay. Animals were staged as late L4 larvae for assay 24 h later. At time 0 of the assay, 5–10 worms were placed on the serotonin plates, spaced in a manner that made it unlikely they would be able to crawl near each other before being paralyzed by the serotonin. Serotonin-induced paralysis, which resulted in the worms remaining adjacent to the eggs they laid during the time course, made it was possible to attribute the number of eggs laid to each individual worm.

Serotonin- or CNO-induced egg laying in M9 buffer. Animals were staged as late L4 larvae for assay 24 h later. Serotonin creatine sulfate monohydrate (catalog #H7752-5G, Sigma-Aldrich) and clozapine *N*-oxide dihydrochloride (CNO; catalog #6329, Tocris Bioscience) were dissolved to desired concentrations in M9 buffer. Ten microliter drops of serotonin, CNO, or a combination of the two were placed on the lid of a 96-well plate. At time 0 a single worm was placed in each drop of drugged buffer, and after a specific incubation period the number of eggs by each worm laid was counted under a dissecting microscope. The incubation period was 60 min, and for all other experiments it was 30 min (see Fig. 8*A*–*C*).

Optogenetic activation of photoactivatable adenylyl cyclase to induce egg laying. Animals were staged as late L4 larvae for assay 24 h later. A photoactivatable adenylyl cyclase (PAC) from *Beggiatoa sp* (amplified from pET28a-ec_bPAC, a gift from Peter Hegemann; plasmid #28135, Addgene) or empty vector control was transgenically expressed in the egg-laying muscles of *C. elegans* with a *lite-1 (ce314)* background. Worms were kept in foil-covered boxes and maintained quickly under

Table 2. *C. elegans* transgenes used in this study

Transgene	Function	DNA microinjected or transgene integrated	Source
vsIs165	GCaMP5/mCherry in vulval muscles (*unc-103e* promoter)	Integrant of *vsEx739*	Collins et al., 2016
vsIs191	mCherry in vulval muscles	Integrant of *vsEx780*	Collins et al., 2016
vsIs237	SER-7::GFP	Integrant of *vsEx870*	Fernandez et al., 2020
vsIs238	SER-7::GFP translational fusion.	Integrant of *vsEx870*	Fernandez et al., 2020
vsIs244	SER-1::GFP translational fusion.	Integrant of *vsEx880*	Fernandez et al., 2020
vsEx739	GCaMP5/mCherry in vulval muscles (*unc-103e* promoter)	pKMC274B at 15 ng/µl and pKMC257 at 2 ng/µl along with 50 ng/µl pL15EK.	Collins et al., 2016
vsEx780	mCherry in vulval muscles	pKMC257 at 50 ng/µl, pL15EK at 50 ng/µl	Collins et al., 2016
vsEx870	SER-7::GFP translational fusion.	*ser-7::gfp* fosmid at 60 ng/µl, pL15EK at 50 ng/µl, DH5alpha genomic DNA (digested with BamHI and Hind III) at 25 ng/µl.	Fernandez et al., 2020
vsEx880	SER-1::GFP translational fusion.	*ser-1::gfp* fosmid at 60 ng/µl and pL15EK at 50 ng/µl, DH5alpha genomic DNA (digested with BamHI and Hind III) at 25 ng/µl.	Fernandez et al., 2020
vsEx981	*ser-7* RNAi in egg-laying muscles (*unc-103e* promoter)	sense RNAi 100 ng/µl, antisense RNAi 100 ng/µl pCFJ90 10 ng/µl pL15EK 50 ng/µl DH5apha genomic DNA (digested with BamHI and Hind III) 25 ng/µl	This study
vsEx986	Anti-*gfp* RNAi in egg-laying muscles (*unc-103e* promoter)	Sense RNAi strand at 100 ng/µl, anti-sense RNAi strand at 100 ng/µl, 10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA digested with BamHI/HindIII	This study
vsEx988	Anti-*ser-1* RNAi in egg-laying muscles (*unc-103e* promoter)	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA digested with BamHI/HindIII Sense RNAi strand at 100 ng/µl , anti-sense RNAi strand at 100 ng/µl	This study
vsEx989	Anti-*ser-7* RNAi in egg-laying muscles (*unc-103e* promoter)	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA digested with BamHI/HindIII sense RNAi strand at 100 ng/µl , anti-sense RNAi strand at 100 ng/µl	This study
vsEx997	Anti-*egl-30* ($G\alpha_q$) and anti-*gsa-1*($G\alpha_s$) RNAi in egg-laying muscles	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA (digested with BamHI/HindIII), Sense *egl-30* RNAi strand at 50 ng/µl, anti-sense *egl-30* RNAi strand at 50 ng/µl, sense *gsa-1* RNAi strand at 50 ng/µl, anti-sense *gsa-1* RNAi strand at 50 ng/µl	This study
vsEx998	mCherry in egg-laying muscles, anti-*egl-30* ($G\alpha_q$) and anti-*gsa-1* ($G\alpha_q$) RNAi in egg-laying muscles	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA (digested with BamHI/HindIII), Sense *egl-30* RNAi strand at 50 ng/µl, anti-sense *egl-30* RNAi strand at 50 ng/µl, sense *gsa-1* RNAi strand at 50 ng/µl, anti-sense *gsa-1* RNAi strand at 50 ng/µl, pKMC257 at 50 ng/µl	This study
vsEx1003	Anti-*egl-30* ($G\alpha_q$) RNAi in the egg-laying muscles	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and HindIII), sense RNAi strand at 50 ng/µl, anti-sense RNAi strand at 50 ng/µl	This study
vsEx1004	Anti gsa-1 ($G\alpha_s$) RNAi in the egg-laying muscles	10 ng/µl pCFJ90, 50 ng/µl pL15EK, 25 ng/µl DH5alpha genomic DNA (digested with BamHI/HindIII), Sense RNAi strand at 50 ng/µl, anti-sense RNAi strand at 50 ng/µl	This study
vsEx1006	Empty vector control for the $G\alpha_q$ DREADD	10 ng/µl pCFJ90, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and Hind III), 50 ng/µl pAO60	This study
vsEx1007	$G\alpha_q$ DREADD in egg-laying muscles	10 ng/µl pCFJ90, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and Hind III), 50 ng/µl pAO67	This study
vsEx1011	*ser-1* RNAi in egg-laying muscles (*unc-103e* promoter)	Sense RNAi strand at 100 ng/µl, anti-sense RNAi at 100 ng/µl, pCFJ90 at 10 ng/µl pL15EK at 50 ng/µl DH5apha genomic DNA (digested with BamHI and Hind III) at 25 ng/µl	This study
vsEx1012	$G\alpha_q$ DREADD in egg-laying muscles	10 ng/µl pCFJ90, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and Hind III), 50 ng/µl pAO67	This study
vsEx1013	Empty vector control for the $G\alpha_q$ DREADD	10 ng/µl pCFJ90, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and Hind III), 50 ng/µl pAO60	This study
vsEx1042	Empty vector control for photoactivatable adenylyl cyclase in egg-laying muscles	50 ng/µl pAO75, 50 ng/µl pkmc257, 25 ng/µl DH5alpha genomic DNA (digested with BamHI and Hind III), 50 ng/µl pL15EK	This study
vsEx1043	Photoactivatable adenylyl cyclase in egg-laying muscles	50 ng/µl pAO76, 50 ng/µl pkmc257, 25 ng/µl DH5apha genomic DNA (digested with Bam HI and Hind III), 50 ng/µl pL15EK	This study

dim light to avoid premature activation of the PAC. Twenty-four hours before the experiment, single L4 worms were transferred to a new NGM plate containing OP50 and returned to the dark. Both the PAC and empty vector control used the *unc-103e* promoter and were coinjected with *unc-103ep::mCherry*. On the day of the experiment only animals with visible mCherry in their vulva muscles were selected to be assayed. A Leica M165FC microscope equipped with GFP filter set and a digital camera was used to record the experiment. The exposure settings of the camera were adjusted so the activation of the blue emission light of the GFP filters set would be visible on screen. At time 0 the worm was illuminated with 18.2 mW/cm^2 of 470 $\pm$ 20 nm blue light from the GFP filter set of the microscope. The number of eggs laid during 1 min of blue light illumination was recorded.

Quantification of unlaid eggs. Animals were staged as L4 larva 30 h before assay. Quantitation of unlaid eggs was performed as described in Chase and Koelle (2004).

Statistical analysis. In the figures, error bars shown in all graphs represent 95% confidence intervals. All statistical analysis was analyzed using GraphPad Prism version 9.5.1 software.

Results

Both $G\alpha_q$-coupled and $G\alpha_s$-coupled receptors are required for exogenous and endogenous serotonin to stimulate egg laying

We began our study of how multiple GPCRs activate a cell by analyzing how two serotonin receptors act together to promote *C. elegans* egg laying. Application of exogenous serotonin causes wild-type worms to quickly initiate egg laying (Fig. 1*B*,*D*). We reproduced previous studies (Carnell et al., 2005; Dempsey et al., 2005; Hobson et al., 2006) showing that $G\alpha_q$-coupled SER-1 and $G\alpha_s$-coupled SER-7 are each required for such serotonin-induced egg laying (Fig. 1*B*–*D*). Animals carrying null alleles of the *ser-1* (Fig. 1*D*) or *ser-7* (Fig. 1*C*,*D*) genes each showed severely reduced egg laying in response to exogenous serotonin. Although one might have expected these coexpressed receptors to function redundantly, resulting in weak defects when knocking out one or the other, the surprisingly strong defects seen in the single

Table 3. DNA amplicons used to generate egg-laying muscle-specific RNAi

Gene targeted	Allele	Construction
ser-7	*vsEx981*	*unc-103e promoter::ser-7* RNAi Antisense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser7anti. Amplify *ser-7* cDNA from pAO57 with primer cDNAampF and primer cDNAampR. After annealing the two fragments, use primer unc103nest and primer ser7nestAnt as nested primers. *unc-103e* promoter::ser-7 RNAi Sense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser7sens. Amplify *ser-7* cDNA from pAO57 with primer cDNAampF and primer cDNAampR. After annealing the two fragments use primer unc103nest and primer ser7nestSen as nested primers. Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncser7anti GGCTGTCTCATCCTACTTTCACCTaccaccaccaccacaaccac uncser7sens CCATATGGTACCGTCGACGCTAGCaccaccaccaccacaaccac cDNAampF GCTAGCGTCGACGGTACCAT cDNAampR AGGTGAAAGTAGGATGAGACAGC unc103nest TGACCATGATTACGCCAAGC ser7nestAnt GCCCGTGCAGTCAACATATC ser7nestSen ACTTGCTTCGTGACTGTCATTG
gfp	*vsEx986*	*unc-103e promoter::gfp* RNAi Antisense amplicon amplify unc-103e promoter from pAO60 with Punc103RNAi and uncgfpAnt2 (67°C, extend for 1 min 21 s, 2665 bp). Amplify *gfp* cDNA from pPD128.110 use with gfpcDNAf and gfpcDNAr (64°C, extend for 40 s, 661 bp.) After using NEBuilder to fuse the two fragments use unc103nest and gfpnestAnti as nested primers (63°C, extend for 1 min 37 s, 3233 bp). *unc-103e promoter::gfp* RNAi Sense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncgfpsen2 (67°C, extend for 1 min 21 s, 2665 bp). Amplify *gfp* cDNA from pPD128.110 use with primer gfpcDNAf and primer gfpcDNAr (64°C, extend for 40 s. 661 bp.) After using NEBuilder to fuse the two fragments use primer unc103nest and primer gfpnestSen as nested primers (anneal at 61°C, extend for 1 min 37 s, 3157 bp). Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncgfpAnt2 caccctctccactgacagaaaaccaccaccaccacaaccac uncgfpsen2 gctgggattacacatggcatggaaccaccaccaccacaaccac gfpcDNAf TCCATGCCATGTGTAATCCCAGC gfpcDNAr TTTCTGTCAGTGGAGAGGGTG unc103nest TGACCATGATTACGCCAAGC gfpnestAnti GCTGTTACAAACTCAAGAAGGACC gfpnestSen GGCCAACACTTGTCACTAC
ser-1	*vsEx988*	*unc-103e promoter::ser-1* RNAi antisense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser1anti (67°C, 1 min 21 s, 2709 bp). Amplify *ser-1* sense cDNA from ser-1 fragA cDNA from twist bioscience with primer cDNAampF and primer ser1fragAR2 (66°C 40 s, ~1100 bp). After annealing the two fragments use primer unc103nest and primer ser1nestAnt as nested primers (63°C, extend for 1 min 52 s, 3632 bp). *unc-103e promoter::ser-1* RNAi Sense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser1sen2 (67°C and extend for 1 min 21 s, 2665 bp). Amplify *ser-1* cDNA from ser-1 fragA cDNA from Twist Bioscience with primer cDNAampF and primer ser1fragAR2(66°C 40 s, ~1100 bp) . After annealing the two fragments use primer unc103nest and primer ser1nestSen as nested primers (anneal at 63°C, extend for 1 min 52 s, 3733 bp). Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncser1anti CCCGTGTGCTCGCAGTCGTGTTTGaccaccaccaccacaaccac uncser1sen2 CGCTGTGTGAGAAGAGTTCAATGaccaccaccaccacaaccac cDNAampF GCTAGCGTCGACGGTACCAT ser1fragAR2 AACACGACTGCGAGCACAC unc103nest TGACCATGATTACGCCAAGC ser1nestAnt GCAGTTGATCCCGACGATG ser1nestSen GGTCGCCTTGTGCTCATTTG
ser-7	*vsEx989*	*unc-103e promoter::ser-7 RNAi* Antisense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser7anti. Amplify *ser-7* cDNA from pAO57 with primer cDNAampF and primer cDNAampR. After annealing the two fragments use primer unc103nest and primer ser7nestAnt as nested primers. *unc-103e promoter::ser-7* RNAi Sense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncser7sens. Amplify *ser-7* cDNA from pAO57 with primer cDNAampF and primer cDNAampR. After annealing the two fragments use primer unc103nest and primer ser7nestSen as nested primers. Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncser7anti GGCTGTCTCATCCTACTTTCACCTaccaccaccaccacaaccac uncser7sens CCATATGGTACCGTCGACGCTAGCaccaccaccaccacaaccac cDNAampF GCTAGCGTCGACGGTACCAT cDNAampR AGGTGAAAGTAGGATGAGACAGC unc103nest TGACCATGATTACGCCAAGC ser7nestAnt GCCCGTGCAGTCAACATATC ser7nestSen ACTTGCTTCGTGACTGTCATTG
egl-30 and *gsa-1*	*vsEx997*	See *egl-30* and *gsa-1* below for the protocols for preparing RNAi targeting those genes.
egl-30 and *gsa-1*	*vsEx998*	See *egl-30* and *gsa-1* below for the protocols for preparing RNAi targeting those genes.
egl-30 ($G\alpha_q$)	*vsEx1003*	*unc-103e promoter::egl-30* RNAi Antisense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncegl30An2 (67°C, extend for 1 min 21 s, 2705 bp). Amplify *egl-30* cDNA from pAO46 with primer egl30cDNAf and primer egl30cDNAr (anneal 63°C at extend for 15 s, 427 bp). After using NEBuilder to fuse the two fragments use primer unc103nest and primer egl30nestA as nested primers (anneal at 63°C extend for 1 min 30 s, 2977 bp). *unc-103e promoter::egl-30* RNAi Sense amplicon amplify Punc-103e from pAO60 with primer Punc103RNAi and primer uncegl30Sn2 (anneal at 67°C, extend for 1 min 21 s, 2707 bp). Amplify *egl-30* cDNA from pAO46 use with primer egl30cDNAf and primer egl30cDNAr (anneal 63°C at extend

(*Table continues*.)

Table 3 Continued

Gene targeted	Allele	Construction
		for 15 s, 427 bp). After using NEBuilder to fuse the two fragments use primer unc103nest and primer egl30nestS as nested primers (anneal at 63°C, extend for 1 min 30 s, 2977 bp). Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncegl30An2 caaccgagcaggacattctgaccaccaccaccacaaccac uncegl30Sn2 catcgctcgtatcatagactggaccaccaccaccacaaccac egl30cDNAf TATAGGGAGACCGGCAGATC egl30cDNAr CAGAATGTCCTGCTCGGTTG unc103nest TGACCATGATTACGCCAAGC egl30nestA CCAGTCTATGATACGAGCGATG egl30nestS cagatagtctggcaccgcca
gsa-1 ($G\alpha_s$)	*vsEx1004*	*unc-103e promoter::gsa-1* RNAi Antisense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncegsa1Ant (anneal at 67°C, extend for 1 min 21 s, 2710 bp). Amplify *gsa-1* cDNA from pAO47 use with primer gsa1cDNAf and primer gsa1cDNAr (anneal at 63 bp, extend for 15 s, 489 bp) . After using NEBuilder to fuse the two fragments use primer unc103nest and primer gsa1nestA as nested primers (anneal at 63°C, extend for 1 min 30 s, 2953 bp). *unc-103e promoter::gsa-1* RNAi Sense amplicon amplify *unc-103e* promoter from pAO60 with primer Punc103RNAi and primer uncgsa1Sen (anneal at 67°C, extend for 1 min 21 s, 2710 bp). Amplify *gsa-1* cDNA from pAO47 use with primer gsa1cDNAf and primer gsa1cDNAr (anneal at 63 bp, extend for 15 s, 489 bp) . After using NEBuilder to fuse the two fragments use primer unc103nest and primer gsa1nestS as nested primers (anneal at 63°C, extend for 1 min 30 s, 2820 bp). Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncegsa1Ant acgttgtgcgacagaacaactacgaaccaccaccaccacaaccac uncgsa1Sen aagtgagaatgtgatcgtagaactcaccaccaccaccacaaccac gsa1cDNAf TCAGTGAGCGAGGAAGCAAC gsa1cDNAr catgacacgacaccgaagg unc103nest TGACCATGATTACGCCAAGC gsa1nestA TGCGGGAGTTGAGACACAAG gsa1nestS GATCGTAGTTGTTCTGTCGCAC
ser-1	*vsEx1011*	*unc-103e promoter::ser-1* RNAi Antisense amplicon Amplify *unc-103e promoter* from pAO60 with primer Punc103RNAi and primer uncser1anti (67°C, 1 min 21 s, 2709 bp). Amplify *ser-1* cDNA from ser-1 fragA cDNA from Twist Bioscience with primer cDNAampF and primer ser1fragAR2 (66°C 40 s, ~1100 bp). After annealing the two fragments use primer unc103nest and primer ser1nestAnt as nested primers (63°C, extend for 1 min 52 s, 3632 bp). *unc-103e promoter::ser-1* RNAi Sense amplicon Amplify *unc-103e promoter* from pAO60 with primer Punc103RNAi and primer uncser1sen2 (67°C and extend for 1 min 21 s, 2665 bp). Amplify *ser-1* cDNA from ser-1 fragA cDNA from Twist Bioscience with primer cDNAampF and primer ser1fragAR2 (66°C 40 s, ~1100 bp) . After annealing the two fragments use primer unc103nest and primer ser1nestSen as nested primers (anneal at 63°C extend for 1 min 52 s, 3733 bp). Punc103RNAi TCCGGCTCGTATGTTGTGTGG uncser1anti CCCGTGTGCTCGCAGTCGTGTTTGaccaccaccaccacaaccac uncser1sen2 CGCTGTGTGAGAAGAGTTCAATGaccaccaccaccacaaccac cDNAampF GCTAGCGTCGACGGTACCAT ser1fragAR2 AACACGACTGCGAGCACAC unc103nest TGACCATGATTACGCCAAGC ser1nestAnt GCAGTTGATCCCGACGATG ser1nestSen GGTCGCCTTGTGCTCATTTG

Transgenic animals with egg-laying muscle-specific RNAi were created using the method described in Esposito et al. (2007). Fusion PCR was used to create two amplicons that express sense and antisense RNA targeting the gene of interest. RNA expression was driven from the egg-laying muscle-specific *unc-103e* promoter. The *unc-103e* promoter was amplified from the pAO60 plasmid. cDNA targeting the gene of interest was amplified from a plasmid or from linear dsDNA synthesized by Twist Bioscience.

receptor mutants prompted us to analyze in depth how SER-1 and SER-7 function together.

A third serotonin receptor, SER-5, has been reported to weakly promote serotonin-induced egg laying in certain genetic backgrounds (Hapiak et al., 2009). Because this effect is so weak (Extended Data Fig. 1-1), and SER-5 expression in the egg-laying system is reported as either weak and variable (Hapiak et al., 2009) or undetectable (Fernandez et al., 2020), this study excludes SER-5 from further analysis.

To reveal how SER-1 and SER-7 receptors mediate the ability of endogenously released serotonin to induce egg laying, we measured the accumulation of unlaid eggs in *ser-1* and *ser-7* null mutant animals. Because *C. elegans* continues to produce eggs even when it cannot lay them, the accumulation of unlaid eggs serves as a convenient measure of defects in egg-laying behavior (Chase and Koelle, 2004). Endogenous serotonin is coreleased from the HSN neurons with NLP-3 neuropeptides, and these two signals act semiredundantly to stimulate egg laying (Brewer et al., 2019). Therefore, the functional role of serotonin in the egg-laying system is best revealed in an *nlp-3* null mutant background; with NLP-3 removed, endogenous serotonin is the strongest remaining signal that stimulates egg laying, and mutations that perturb serotonin signaling thus show much stronger effects on egg laying. This effect is seen in the egg accumulation assays shown in Figure 1, *E–K*, and Extended Data Figure 1-2. Knocking out *tph-1*, the tryptophan hydroxylase enzyme responsible for synthesizing endogenous serotonin (Sze et al., 2000), or knocking out *ser-1* or *ser-7* individually or together, caused only moderate egg-laying defects as seen by accumulation of ~20–30 unlaid eggs (Fig. 1*E–H*; Extended Data Fig. 1-2*A*,*C*). Knocking out *nlp-3* alone, like knocking out serotonin signaling alone, also caused only a modest egg-laying defect in which animals retained 22.6 ± 2.1 unlaid eggs (Fig. 1*E*,*I*; Extended Data Fig. 1-2*A*). However, in a *tph-1; nlp-3* double mutant, the worms developed a far more severe

Table 4. Plasmids used in this work

Plasmid	Features	Construction	Source
pAO46	*egl-30* RNAi expressed from the L4440 RNAi feeding vector backbone	Amplified *egl-30* genomic DNA from lysed *C. elegans* with primer egl30RNAiF1 (aagatgACTAGTCCAGTCTATGATACGAGCGATG) and cloned into L4440 with Spe I and Xho I	This study
pAO57	Expresses partial *ser-7* cDNA from a *gar-2* promoter	Used Gibson cloning to insert a partial segment of *ser-7* cDNA into pAO54. The *ser-7* sequence is from 5′-ATGGCCCGTGCAGTCAACAT to 3′ ACGAAGCAAGTGACGTCTAG	This study
pAO60	Empty vector control for the experiment that expressed Gα_q DREADD in egg-laying muscles	Amplified *unc-103e* promoter from pKMC189 using primers Punc103eF (CAAGCTTGCATGCCTGCAGGgtgcatgcctattttatatttacaatatt)and Punc103eR (CCCGGGGATCCTCTAGAGTCGAaccaccaccaccacaacc), then Gibson cloned into MCS I area of pAO51. pAO51 was prepared for Gibson cloning with primers pAO51mcsIR (CCTGCAGGCATGCAAGCT) and pAO51mcsIF (TCGACTCTAGAGGATCCCCG).	This study
pAO67	Expresses Gα_q DREADD in egg-laying muscles	Amplified *cegar-3Dq*, which is the *gar-3* Gα_q DREADD, from pSP110, using primers pAO67F1 tggctagcgtcgacggtaccatATGCAGTCCTCTTCGTTGGG and pAO67R1 ggtgaaagtaggatgagacagcCTAGTTGCGTCGGACATATCCCT (anneal at 65°C extend for 2 min 33 s (~5100 bp), then Gibson cloned into pAO60. pAO60 was amplified with pAO5xfwd gctgtctcatcctactttcacc and pAO5xrev atggtaccgtcgacgctagc [anneal at 63°C, extend for 3 min 32 s (~7073 bp)].	This study
pAO75	Empty vector control for the experiment that expressed photoactivatable adenylyl cyclase	Used primers pAO75F(TCGACGGTACCATCGGCCGCTGTCATCAGATCG) and pAO75R (GACAGCGGCCGATGGTACCGTCGACGCTAGC) to amplify from pAO70, removing *gsa-1Q208L-SL2-mCherry*. Gibson clone to recircularize	This study
pAO76	Expresses photoactivatable adenylyl cyclase in egg-laying muscles	Amplified the backbone from pAO70 with pAO74F2 (cggccgctgtcatcagatc) and pAO75R1 (ATGGTACCGTCGACGCTAGCCAAGG) anneal at 66°C and extend for 3 min, amplifed the bPAC plus homology arms from pET28a-ec_bPAC with pAO76F1a (GCTAGCGTCGACGGTACCATatgatgaaacgcctggtgtat) and pAO76R1a (CGATCTGATGACAGCGGCCGttatttatcgttttccagggtctgac) anneal at 63 C extend for 1 min 5 s; assembled with NEBuilder.	This study
pCFJ90	Expresses mCherry in pharyngeal muscles from the *myo-2* promoter.		Frøkjaer-Jensen et al., 2008
pKMC257	Expresses mCherry in egg-laying muscles	Amplified *unc-103e* promoter and ligated into pKMC190 using SphI/XmaI	Collins and Koelle, 2013
pKMC274B	Expresses GCaMP5 in egg-laying muscles	Amplified GCaMP5 from pCMV-GCaMP5 and ligated into pKMC189 with NheI/NcoI	Collins et al., 2016
pL15EK	Coinjection marker, rescues *lin-15*	The 11 kb Eag1/Nru1rescuing fragment of cosmid C29B12 was cloned into pBSKS with Eag1/Nru1.	Clark et al., 1994

egg-laying defect and became bloated with 54.4 ± 2.4 unlaid eggs (Fig. 1*E*; Extended Data Fig. 1-2*B*).

The above-described results allow us to interpret measurements of animals carrying null mutations for *ser-1* and/or *ser-7* in the *nlp-3* null mutant background. Such animals showed egg-laying defects almost as strong as the defects of the *tph-1; nlp-3* double mutant that completely lacks both serotonin and NLP-3 (Fig. 1*E*,*J*,*K*; Extended Data Fig. 1-2). In the wild-type or *nlp-3* null mutant backgrounds, knocking out both SER-1 and SER-7 resulted in a defect not much more severe than knocking out either serotonin receptor alone.

Together these data indicate that serotonin signals through both a Gα_q-coupled receptor, SER-1, and a Gα_s-coupled receptor, SER-7, to initiate egg laying in *C. elegans*. Although these receptors are coexpressed on most muscle cells in the egg-laying system, surprisingly, loss of either the Gα_q-coupled SER-1 or Gα_s-coupled SER-7 receptor resulted in what appeared to be an almost complete loss of the ability of exogenous serotonin to stimulate egg laying and severely disrupted egg laying in response to endogenous serotonin.

The SER-1 and SER-7 serotonin receptors are each required for endogenous serotonin to coordinate calcium transients in the vm1 and vm2 vulval muscles

We next sought to determine how signaling through Gα_q-coupled SER-1 and Gα_s-coupled SER-7 induces egg laying in *C. elegans*. The expression of serotonin receptors in each cell of the egg-laying system has been characterized, and this work showed that both SER-1 and SER-7 are coexpressed on the vm2 vulval muscles, whereas SER-7 but not SER-1 is expressed on the vm1 vulval muscles (Fernandez et al., 2020). Egg laying only occurs during simultaneous vm1 and vm2 calcium transients, which drive coordinated contraction of these vulval muscle cells to release eggs (Brewer et al., 2019). We hypothesized that SER-1 and SER-7 are the receptors through which serotonin signals to generate simultaneous vm1 and vm2 calcium transients.

We note here that the full set of signals that activate the vm1 and the vm2 vulval muscles remains the subject of ongoing investigations. Issues regarding these muscles that remain currently unresolved include the following: (1) Multiple GPCRs in addition to serotonin receptors are found on the vulval muscles (Fernandez et al., 2020); (2) among these is likely the NLP-3 receptor because the neuropeptide NLP-3 acts with serotonin to promote vulval muscle activity, but the GPCR receptor for NLP-3 remains to be characterized (Brewer et al., 2019); (3) some simultaneous vm1/vm2 muscle transients occur even in the absence of both serotonin and NLP-3, suggesting that additional endogenous signals also promote activity of these muscles (Brewer et al., 2019); and (4) there are gap junctions between the vm1 and vm2 muscle cells that may allow excitation to spread from one muscle type to the other (White et al., 1986).

To test our hypothesis that SER-1 and SER-7 promote the simultaneous vm1/vm2 calcium transients that result in egg laying, we recorded calcium transients in the vulval muscles of *C. elegans* carrying *ser-1* or *ser-7* null mutations. We expressed the calcium reporter GCaMP5 in the egg-laying muscles and performed 1 h optical recordings of these muscles within freely behaving animals as previously described (Collins and Koelle, 2013; Brewer et al., 2019).

As controls for the serotonin receptor mutant recordings, we first recorded egg-laying muscle calcium activity in wild-

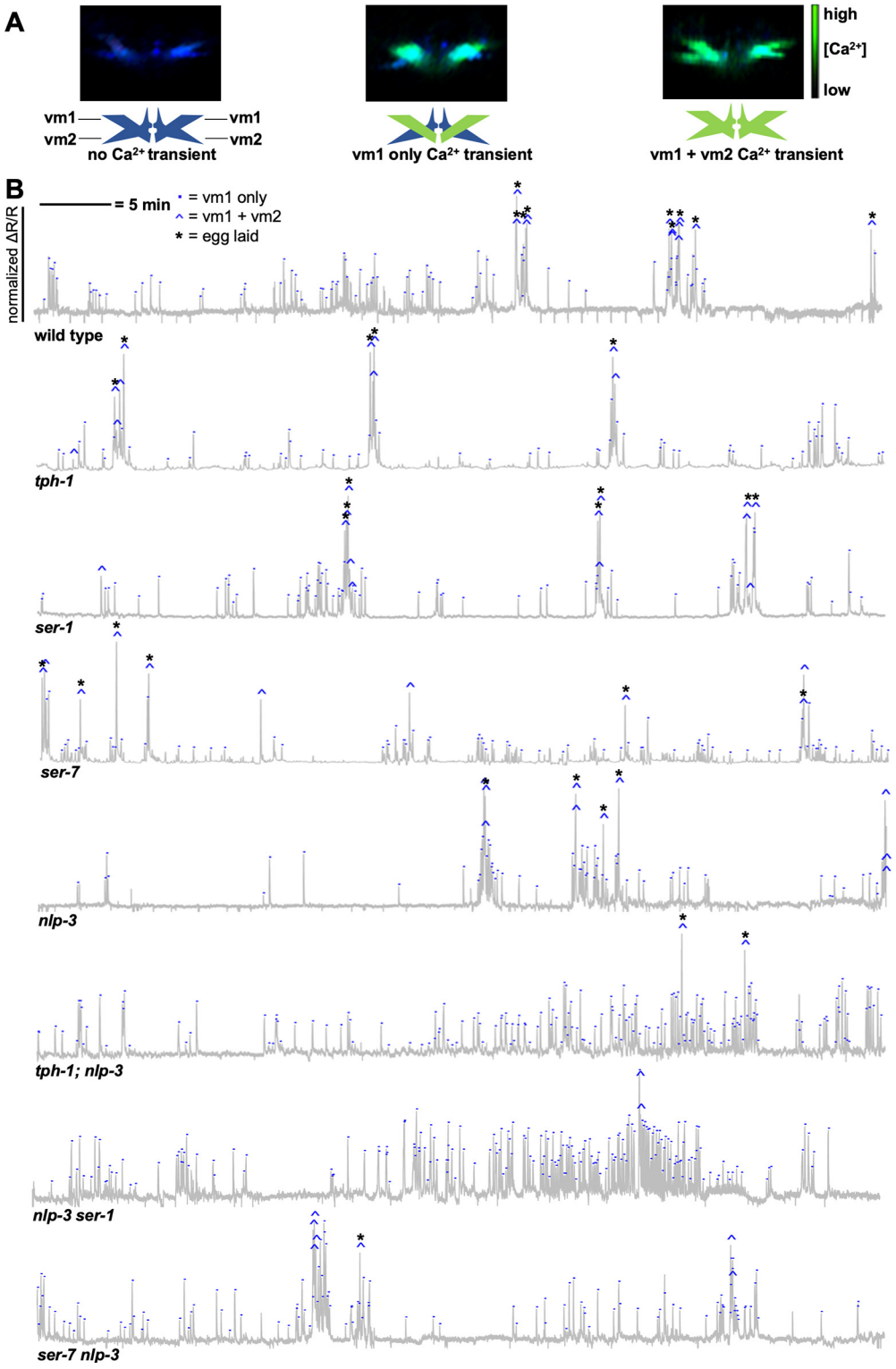


Figure 2. Serotonin signals through SER-1 and SER-7 to coordinate vm1 and vm2 vulval muscle calcium transients. ***A***, Representative still frames from ratiometric calcium imaging of 1 h video recordings of vulval muscles depicting no calcium transient (left), a vm1-only calcium transient (center), and a simultaneous vm1 + vm2 calcium transient (right). The mCherry channel is rendered in blue, and the GCaMP channel is superimposed in green, with intensity rendered by ranging from transparent (low calcium) to bright green (high calcium). ***B***, Representative calcium traces representing 1 h recordings of changes in the GCaMP5/mCherry ratio (ΔR/R) in the vulval muscles of individual worms. Each trace is of a representative animal from the genotype indicated. Five such recordings were captured of wild-type animals, and three were captured for all other genotypes. All traces for each genotype are shown in Extended Data Figure 2-1. Vertical scales have been normalized to the highest peak height within each trace. Each calcium peak was manually scored as vm1 only, marked with a blue dot, or simultaneous vm1 + vm2, marked with a blue caret (^), and transients associated with release of one or more eggs are indicated by asterisks (*).

type animals as well as in *tph-1* and/or *nlp-3* null mutant animals. Wild-type animals showed two different types of calcium transients in their vulval muscles, 1) vm1-only transients restricted to the vm1 muscles and 2) vm1 + vm2 transients that occurred simultaneously in both the vm1 and vm2 muscles (Fig. 2*A*). We never observed a vm2 transient to occur in the absence of a vm1 transient. Wild-type worms had vm1-only transients distributed throughout the entire 1 h recordings (Fig. 2*B*; Extended Data Fig. 2-1). In contrast, vm1 + vm2 transients tended to occur in clusters, known as egg-laying active phases (Waggoner et al., 1998; Brewer et al., 2019), during which a subset of vm1 + vm2 transients was accompanied by release of one or more eggs (Fig. 2*B*; Extended Data Fig. 2-1). In the wild type, ~17% of the total vulval muscle calcium transients were vm1 + vm2 transients (Fig. 3). When *tph-1* (i.e., serotonin) or *nlp-3* were knocked out, there was a modest reduction in the percentage of vm1 + vm2 transients (Fig. 3*B*) that correlated with the modest egg laying defects in these mutants (Fig. 1*E*,*I*; Extended Data Fig. 2-1*A*). Knocking out *tph-1* and *nlp-3* together resulted in both an increase in the number of vm1-only transients and a reduction in the number of vm1 + vm2 transients, which combined to produce a significant reduction in the percentage of total transients that were of the vm1 + vm2 type (Fig. 2*B*; Extended Data Fig. 2-1; Fig. 3). The reduction in the percentage of vm1 + vm2 transients correlated with the strong egg-laying defect in the *tph-1; nlp-3* double mutant (Fig. 1*E*; Extended Data Fig. 2-1*B*). Our recordings in these control genotypes reproduced the findings of Brewer et al. (2019) and confirmed that signaling by serotonin and NLP-3 neuropeptides together lead to the simultaneous activity of the vm1 and vm2 vulval muscles that drives egg laying.

Next, we examined the effects of null mutations for SER-1 and SER-7. Single mutants for *ser-1* or *ser-7* each showed a modest reduction in the percentage of vm1 + vm2 transients (Fig. 2*B*; Extended Data Fig. 2-1; Fig. 3), which is likely responsible for the modest reduction in egg laying seen in these mutants (Fig. 1*E*,*G*,*H*). Crossing the *ser-1* or *ser-7* serotonin receptor null mutants into the *nlp-3* null mutant background isolated serotonin signaling through the remaining serotonin receptor as the remaining driver of egg laying. Both the *nlp-3 ser-1* and *ser-7 nlp-3* double mutants showed a strong reduction in the percentage of vm1 + vm2 transients (Fig. 2*B*; Extended Data Fig. 2-1; Fig. 3), which correlated with the strong egg-laying defects seen in these double mutants (Fig. 1*E*,*J*,*K*). Indeed, for the *nlp-3 ser-1* double mutant, the defects in egg laying and in the percentage of vm1 + vm2 transients were as strong as those of the *tph-1; nlp-3* double mutant (Figs. 1*E*, 3*B*). The defects in vm1 + vm2 transients in the *ser-7 nlp-3* double mutant were also severe but slightly less so than those of the *tph-1; nlp-3* double mutant (Fig. 3).

Together, these data show that endogenous serotonin signals through both the Gα_q-coupled SER-1 and Gα_s-coupled SER-7 receptors to coordinate simultaneous vm1 and vm2 vulval muscle transients and thus egg laying. Knocking out either receptor appears to severely reduce the ability of endogenously released serotonin to activate the vm2 egg-laying muscles.

The SER-1 and SER-7 receptors are required on the egg-laying muscles for serotonin to stimulate egg laying

We sought to determine if the SER-1 and SER-7 serotonin receptors are required on the egg-laying muscles themselves to allow serotonin to initiate egg laying. Applying the method developed by Esposito et al. (2007), we used RNAi to cell specifically knock down genes in the egg-laying muscles. We used the *unc-103e* promoter to drive expression of dsRNA transcripts specifically in the egg-laying muscles of worms. To ensure that the RNAi effect would remain restricted to the egg-laying muscle cells, these experiments were done in null mutants for SID-1, a double-stranded RNA channel that can allow RNAi to spread from cell to cell (Winston et al., 2002).

We tested the ability of our RNAi system to knock down gene expression specifically in the egg-laying muscles by using transgenic animals in which a 12 kb *ser-7* promoter drives expression of the SER-7 receptor fused to the green fluorescent protein (SER-7::GFP). Thus SER-7::GFP is expressed in the cells that normally express SER-7, including the egg-laying muscle cells and the VC neurons of the egg-laying system (Fig. 1*A*) as well as a set of head neurons (Fig. 4*A*). In these animals, when we used the egg-laying muscle-specific promoter to express *ser-7* dsRNA, there was a dramatic loss of SER-7::GFP fluorescence in the egg-laying muscles of 22/25 of worms examined (Fig. 4*B*,*D*) but no noticeable loss of SER-7::GFP from head neurons (Fig. 4*B*) or from the VC neurons (Fig. 4*D*), which lie immediately adjacent to the egg-laying muscles. This knockdown was also gene specific as expression of a control dsRNA rather than *ser-7* dsRNA did

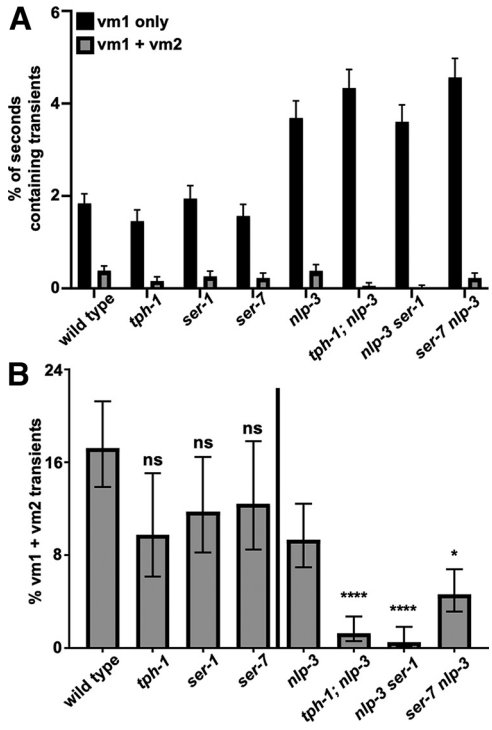


Figure 3. SER-1 and SER-7 are each required for serotonin to signal with NLP-3 to properly induce simultaneous vm1 + vm2 egg-laying muscle contractions. ***A***, Percentage of seconds during 1 h recordings that contained either vm1-only or simultaneous vm1 + vm2 calcium transients, averaged from five wild-type recordings and three recordings for each mutant genotype. (***B***) Percentage of total calcium transients that were of the vm1 + vm2 type. Genotypes to the left of the vertical black line were statistically compared with the wild type, and genotypes to the right of the vertical black line were statistically compared with *nlp-3*. The comparisons were performed using Fisher's exact test with two-sided *p* values, which were adjusted using the Bonferroni correction for multiple comparisons. ns, Not significant with $p > 0.05$, *$p = 0.0135$, ****$p < 0.0001$. Comparing *tph-1; nlp-3* with *nlp-3 ser-1* gave $p = 0.3049$ (not significant), whereas comparing *tph-1; nlp-3* with *ser-7 nlp-3* gave $p = 0.0044$. Wild type, $n = 5$; *tph-1*, $n = 3$; *ser-1*, $n = 3$; *ser-7*, $n = 3$; *nlp-3*, $n = 3$; *tph-1; nlp-3*, $n = 3$; *nlp-3 ser-1*, $n = 3$; *ser-7 nlp-3*, $n = 3$. All measurements are given with 95% confidence intervals.

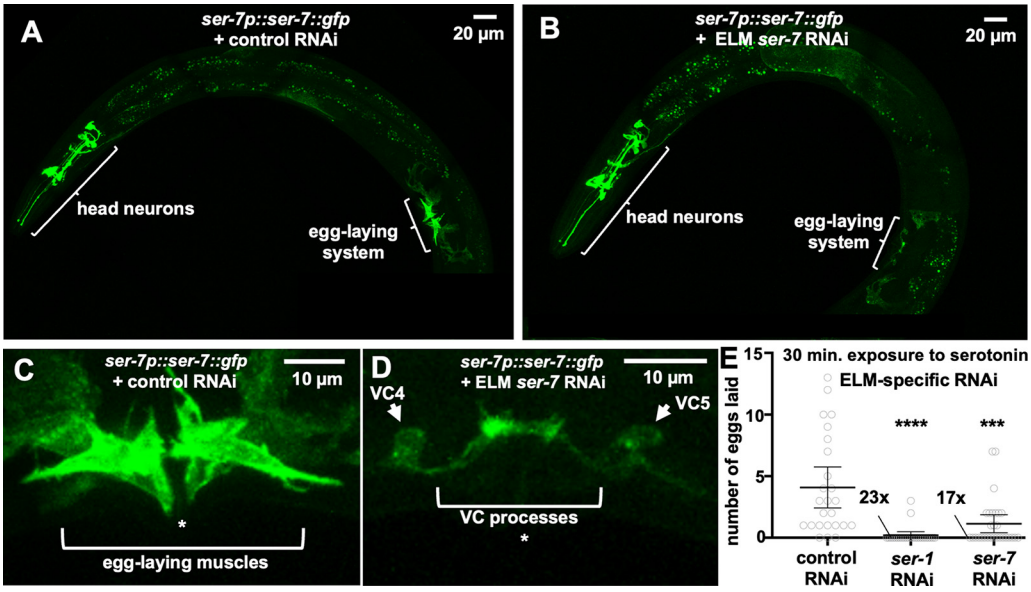


Figure 4. SER-1 and SER-7 are required on the egg-laying muscles for serotonin to stimulate egg laying. ***A***, Image of a transgenic animal in which the *ser-7* promoter (*ser-7p*) drives expression of the SER-7 receptor fused to GFP. GFP fluorescence is seen in a set of head neurons and in the muscles and VC neurons of the egg-laying system. As a negative control, this animal also expresses dsRNA for *ser-1* using an egg-laying muscle-specific promoter (ELM), which fails to knock down expression of the *ser-7::gfp* transcript, and thus it does not affect GFP fluorescence. ***B***, Image of an animal from the same *ser-7p::ser-7::gfp* strain, but with *ser-7* dsRNA expressed in the egg-laying muscles. Absence of GFP fluorescence in the egg-laying muscles alongside the continued presence of fluorescence in the head neurons indicates successful cell-specific knockdown of *ser-7::gfp*. ***C***, ***D***, Close-up images of the egg-laying system of the same animals shown in ***A*** and ***B***. Knockdown of GFP fluorescence in the egg-laying muscles in ***D*** reveals the fainter GFP fluorescence of the neighboring VC neurons that was obscured in ***C***. Asterisks (*) denote location of the vulval slit. ***E***, Eggs laid per animal after 30 min of exposure to exogenous serotonin on NGM plates for the indicated genotypes. Each circle represents the number of eggs laid by an individual worm. RNAi was induced specifically in the egg-laying muscles. *ser-1* RNAi or *ser-7* RNAi was compared with control RNAi using a Kruskal–Wallis test with Dunn's multiple comparisons test (Kruskal–Wallis statistic = 31.64, $p < 0.0001$); $n \geq 25$, ****$p < 0.0001$, ***$p = 0.0005$. When many measurements of zero are clustered on the horizontal axis, the number of such data points is indicated. All measurements are given with 95% confidence intervals.

not result in loss of SER-7::GFP fluorescence (Fig. 4*A*,*C*). We note, however, that our RNAi transgenes may not result in complete knockdown of gene expression, and thus the results described below may reflect partial rather than complete knockdown of gene expression in the egg-laying muscles.

We used the egg-laying muscle-specific RNAi system to knock down either *ser-1* or *ser-7* in the *C. elegans* egg-laying muscles and then tested the ability of exogenous serotonin to induce egg laying. In controls in which neither receptor was knocked down, 22/25 animals laid one or more eggs in response to exogenous serotonin over 30 min. In contrast, almost none of the *ser-1* knock-down animals and less than half of the *ser-7* knock-down animals laid any eggs after serotonin treatment, and for both receptor knockdowns the average number of eggs laid was significantly reduced (Fig. 4*E*). To test whether *ser-1* and *ser-7* are also required in the egg-laying muscles for endogenous serotonin to stimulate egg laying, we used the same *ser-1* or *ser-7* egg-laying muscle-specific knockdown strains but did not treat with exogenous serotonin and instead simply measured the accumulation of unlaid eggs in adult animals. We saw significant increases in unlaid eggs accumulated for both the *ser-1* and *ser-7* knockdowns (Extended Data Fig. 4-1). The mildness of these increases was expected as whole-animal null mutants for these receptors have similarly mild effects (Fig. 1*E*).

These cell-specific RNAi results show that both SER-1 and SER-7 signaling is required in the egg-laying muscles for serotonin to properly induce egg laying. This finding prompted us to broaden our analysis to understand how signaling by these two GPCRs might be integrated with signaling by additional GPCRs on the same muscle cells that also act through the same G-proteins.

$G\alpha_q$ and $G\alpha_s$ signaling is required in the egg-laying muscles to combine endogenous GPCR signals that stimulate egg laying

We next investigated whether the G-proteins through which SER-1 and SER-7 signal, $G\alpha_q$ and $G\alpha_s$, respectively, are necessary in the egg-laying muscles for proper egg laying in response to the entire spectrum of endogenous signals within the animal. We used our RNAi system to knock down the genes for $G\alpha_q$ and $G\alpha_s$ specifically in the egg-laying muscles and measured the accumulation of unlaid eggs. We note that in these experiments, the animals were not treated with serotonin or any other drug, so egg laying occurs in response to the normal endogenous signals that promote egg laying. These signals include serotonin, NLP-3 neuropeptides, as well as additional neurotransmitters signaling to $G\alpha_q$- and $G\alpha_s$-coupled GPCRs, such as the dopamine receptor DOP-4, the tyramine receptor TYRA-3, and the metabotropic acetylcholine receptor GAR-3, which are expressed on the egg-laying muscles (Fernandez et al., 2020).

We found that RNAi knockdown of either $G\alpha_q$ or $G\alpha_s$ in the egg-laying muscles had moderate effects on the accumulation of unlaid eggs (Fig. 5). The defects seen in these single $G\alpha$ knockdowns indicate that each $G\alpha$ protein acts in the egg-laying muscles to promote egg laying. The mildness of these defects is difficult to interpret because we are not certain of the extent to which RNAi reduced the levels of the $G\alpha$ proteins.

The most important result of this experiment was that knocking down both $G\alpha$ proteins together caused a stronger egg-laying defect (39.5 ± 4.2 unlaid eggs; Fig. 5) than the egg-laying defects observed in animals with complete knockouts of both *ser-1* and *ser-7* (26.5 ± 2.1 unlaid eggs; Fig. 5*A*) or than in animals with a *tph-1* knockout that completely eliminates endogenous serotonin (23 ± 1.8 unlaid eggs; Fig. 5*A*). The strong defect in the $G\alpha_q$/$G\alpha_s$ double knockdown was not the result of developmental

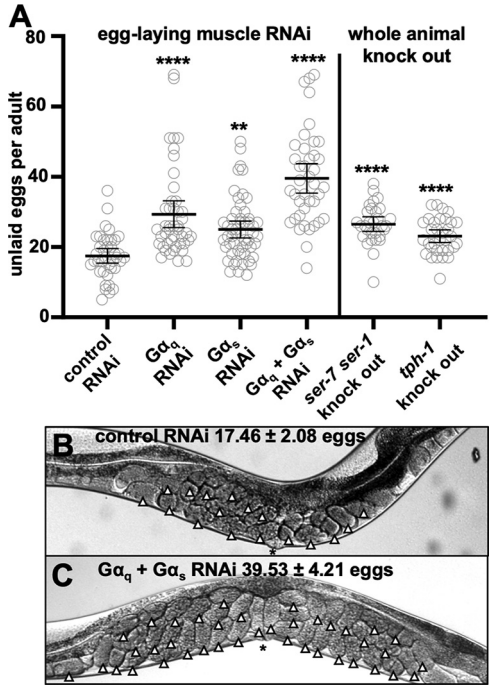


Figure 5. $G\alpha_q$ and $G\alpha_s$ signaling is necessary in the egg-laying muscles to drive proper egg laying. Egg-laying muscle-specific RNAi was used to knock down $G\alpha$ proteins, and the resulting accumulation of unlaid eggs was measured. Anti-*gfp* RNAi was used as a negative control. ***A***, Average number of unlaid eggs per adult worm, $n \geq 30$ for each genotype. Each circle represents the number of unlaid eggs in a single worm. Genotypes to the left of the line were statistically compared with control RNAi using ordinary one-way ANOVA analysis ($F_{(3,174)} = 30.33$ $p < 0.0001$). Dunnett's multiple comparisons test was used. The two lanes to the right of the line are replotted data from Figure 1*E* showing whole animal knockouts of *ser-7 ser-1* and *tph-1*, which were statistically compared with $G\alpha_q + G\alpha_s$ RNAi using ordinary one-way ANOVA analysis ($F_{(2,100)} = 32.70$, $p < 0.0001$). Dunnett's multiple comparisons test was used; $^{**}p = 0.0025$, $^{****}p < 0.0001$. ***B***, ***C***, Photographs of worms of the indicated genotypes, with unlaid eggs indicated by white arrowheads. The vulval slit is indicated by an asterisk (*). All measurements are given with 95% confidence intervals.

defects in the egg-laying muscles as (1) the egg-laying muscle-specific *unc-103e* promoter used to express dsRNA for these gene knockdowns only turns on in the egg-laying muscles as the muscle cells are completing their terminal differentiation (Ravi et al., 2018b) and (2) we labeled the egg-laying muscles with a fluorescent protein in $G\alpha_q/G\alpha_s$ double knockdown animals and saw no visible morphologic defects in these muscle cells (Extended Data Fig. 5-1). Thus, it appears that in adult egg-laying muscles, there must be additional endogenous signals in addition to serotonin that generate $G\alpha_q$ and $G\alpha_s$ activity to stimulate egg laying, as might be expected given the multiple additional $G\alpha_q$- and $G\alpha_q$-coupled receptors expressed on these muscles (Fernandez et al., 2020). We conclude that normal levels of egg-laying activity result from $G\alpha_q$ and $G\alpha_s$ acting in the egg-laying muscles to combine signals from SER-1, SER-7, plus additional non-serotonin GPCRs.

Overexpressed $G\alpha_q$-coupled SER-1 is sufficient to allow serotonin to induce egg laying in the absence of $G\alpha_s$-coupled SER-7

Results presented above show that knocking out or knocking down either the $G\alpha_q$-coupled SER-1 or the $G\alpha_s$-coupled SER-7 serotonin receptors result in severe defects in the ability of serotonin to induce egg laying. In some cases, the defects observed were as strong as those caused by knocking out both SER-1 and SER-7 at the same time or as strong as those seen when completely eliminating serotonin with a *tph-1* null mutation (Fig. 1*E*). These results raise the question of whether serotonin absolutely requires both $G\alpha_q$ signaling and $G\alpha_s$ signaling to induce egg laying or whether these two $G\alpha$ signaling pathways might rather combine to induce egg laying in a more nuanced fashion. Thus, we designed several different experiments to determine whether increasing the strength of just one of the two pathways could induce egg laying in the absence of the other pathway.

The first method was to simply overexpress one serotonin GPCR by increasing the copy number of the GPCR gene. Previous genetic studies have shown that overexpression can increase the normal functions of a GPCR in a manner that is suppressed by knocking out the endogenous ligand for that GPCR (Ringstad and Horvitz, 2008; Harris et al., 2010; Brewer et al., 2019; Fernandez et al., 2020), suggesting that the overexpressed GPCR is activated by its endogenous ligand to signal at a higher level than would the endogenous levels of the GPCR. Indeed, overexpressing SER-1 in *C. elegans* was shown to increase egg laying in a manner completely dependent on endogenous serotonin (Fernandez et al., 2020).

To overexpress serotonin receptors, we used chromosomally integrated transgenes that carry multiple copies of the complete *ser-1* or *ser-7* genes, including their own promoters, resulting in overexpression of these genes in the same cells that normally express them while not ectopically expressing them in cells where they are not endogenously found (Fernandez et al., 2020). We tested the ability of exogenous serotonin to induce egg laying in animals overexpressing one serotonin receptor while also carrying a deletion mutation for the other serotonin receptor. Our results are graphed in Figure 6*A*, and the design and logic of this experiment are schematized in Figure 6*B–F*. We found that animals overexpressing *ser-7* in a *ser-1*-null background were not able to lay eggs in response to exogenous serotonin, similar to animals that simply lacked *ser-1*. However, animals overexpressing *ser-1* in a *ser-7*-null background did lay eggs in response to exogenous serotonin, unlike animals that simply lacked *ser-7*.

These results show that although SER-1/$G\alpha_q$ signaling is normally not sufficient to allow serotonin to induce egg laying in the absence of SER-7/$G\alpha_s$ signaling, artificially increasing SER-1 expression levels overcomes this limitation. It is difficult to interpret the negative result from the converse experiment, in which overexpressed SER-7 failed to induce egg laying in the absence of SER-1. It could be that the transgene used in our SER-7 overexpression experiment may have not increased SER-7/$G\alpha_s$ signaling to a high enough level to induce egg laying in the absence of SER-1/$G\alpha_q$ signaling, or it could mean that strongly activating $G\alpha_s$ signaling is incapable of driving egg laying. Thus, we conducted additional experiments to resolve this uncertainty, and data shown below indicate that sufficiently strong $G\alpha_s$ signaling is able to drive egg laying.

Strong $G\alpha_q$ or $G\alpha_s$ signaling in the egg-laying muscles is sufficient to drive egg laying

We next designed experiments to artificially activate $G\alpha_q$ or $G\alpha_s$ signaling in the egg-laying muscles independently of manipulating SER-1 and SER-7 to further understand how these two $G\alpha$ proteins combine signals to activate the egg-laying muscle cells. A previous study developed a designer receptor exclusively activated by designer drugs (DREADD; Lee et al., 2014) to activate

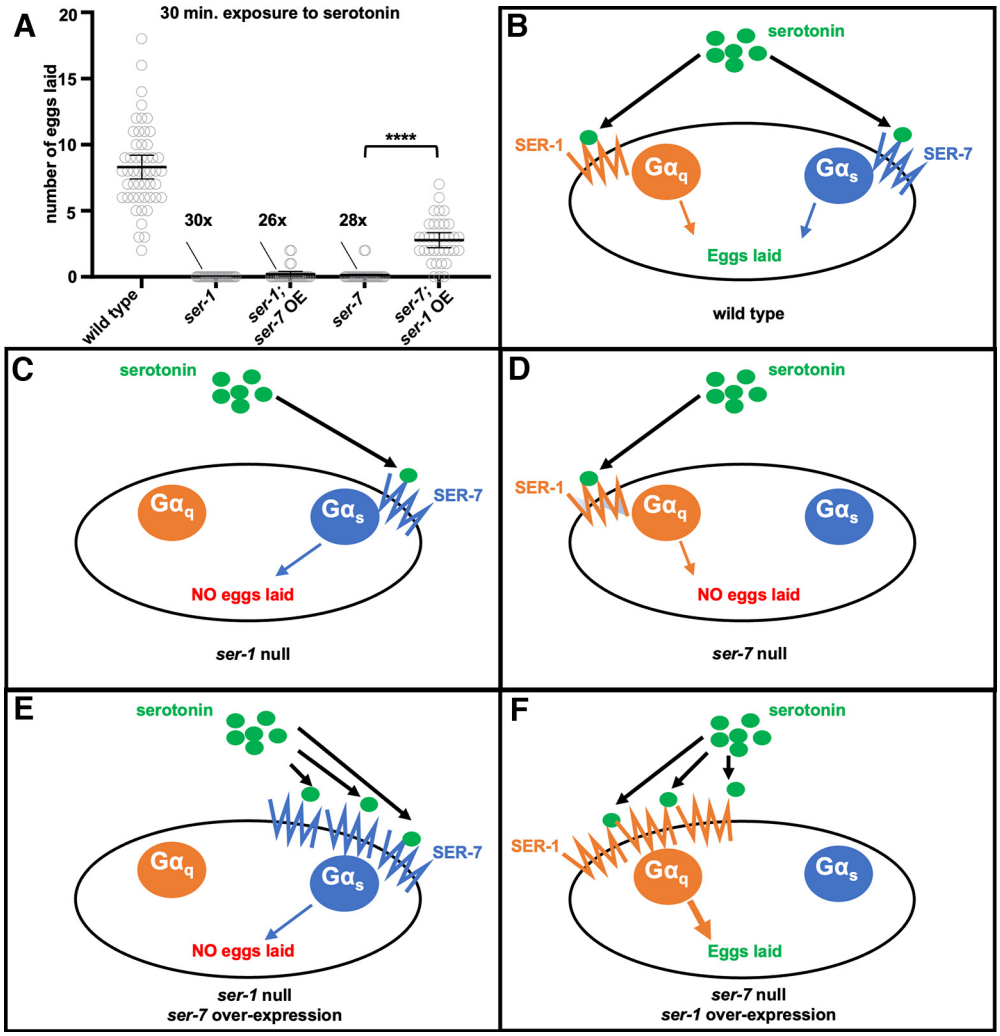


Figure 6. Overexpression of SER-1 is sufficient to allow serotonin to induce egg laying in the absence of SER-7. ***A***, Each circle represents the number of eggs laid per worm during a 30 min exposure to exogenous serotonin on NGM plates. Overexpression (OE) of the indicated receptor from a high-copy transgene that carries the entire receptor gene, including its promoter, so that the receptor is overexpressed in the same cells that normally express the endogenous receptor. For three genotypes, the number of animals that laid zero eggs are indicated as the many individual data points bunched on the horizontal axis are difficult to discern. *ser-7* was compared with *ser-7;ser-10E* using a Mann–Whitney test with a two-tailed *p* value. For the *ser-7* group, median was 0.0 and $n = 30$; for the *ser-7;ser-10E* group, the median was 3.0 and $n = 35$; the *U* value was 65; $n \geq 30$, ****$p < 0.0001$. ***B–F***, Schematic of the design and results of an experiment overexpressing SER-1 or SER-7 in the absence of the other receptor. In wild-type animals, serotonin signals through endogenous levels of SER-1 and SER-7 receptors to activate low levels of $G\alpha_q$ and $G\alpha_s$ signaling, respectively, that combine to induce egg laying (***B***). Signaling by endogenous SER-7/$G\alpha_s$ is not sufficient to induce egg laying in the absence of SER-1 (***C***). Signaling by endogenous SER-1/$G\alpha_q$ is not sufficient to induce egg laying in the absence of SER-7 (***D***). Overexpressed SER-7 receptor, which might be expected to increase $G\alpha_s$ signaling, is also not sufficient to allow serotonin induce egg laying in the absence of SER-1 (***E***). Overexpressed SER-1 receptor, which might be expected to increase $G\alpha_q$ signaling, is sufficient to allow serotonin to induce egg laying in the absence of SER-7 (***F***). All measurements are given with 95% confidence intervals.

$G\alpha_q$-signaling in *C. elegans* in response to the drug clozapine N-oxide (CNO) (Prömel et al., 2016). We acutely activated $G\alpha_q$ signaling specifically in the egg-laying muscles by transgenically expressing this designer $G\alpha_q$-coupled receptor using the egg-laying muscle-specific promoter and treating the worms with CNO. This induced egg laying (Fig. 7*A*,*B*). In contrast, worms carrying a control transgene were unable to lay eggs in response to CNO (Fig. 7*A*).

Next, we determined whether activating $G\alpha_s$ signaling in the egg-laying muscles was sufficient to drive egg laying. To date, there is no designer $G\alpha_s$-coupled receptor that is functional in *C. elegans* (Prömel et al., 2016), and we were unsuccessful in further attempts to design such a receptor (data not shown). $G\alpha_s$ signals by activating adenylyl cyclase, which in turn generates cAMP. A photoactivatable adenylyl cyclase (PAC) has been successfully used in the cholinergic neurons and body wall muscles of *C. elegans* to evoke changes in locomotion (Steuer Costa et al., 2017; Henss et al., 2022). We generated transgenic animals that express PAC in their egg-laying muscles and found that blue light activation of PAC was able to induce egg laying in these worms, whereas control worms carrying an empty vector transgene were unable to lay eggs in response to blue light (Fig. 7*C*,*D*; Movie 1, Movie 2). Thus, stimulating $G\alpha_s$ signaling in the egg-laying muscles with PAC is sufficient to induce egg laying, and the failure of overexpressed SER-7 to mediate serotonin-induced egg laying in Figure 6 was likely simply because of the inability of overexpressed SER-7 to produce enough $G\alpha_s$ signaling.

Together, these results demonstrate that activation of either the $G\alpha_q$ or $G\alpha_s$ pathways in the egg-laying muscles can be sufficient to induce egg laying and that these G-protein signals can originate from sources other than a serotonin receptor.

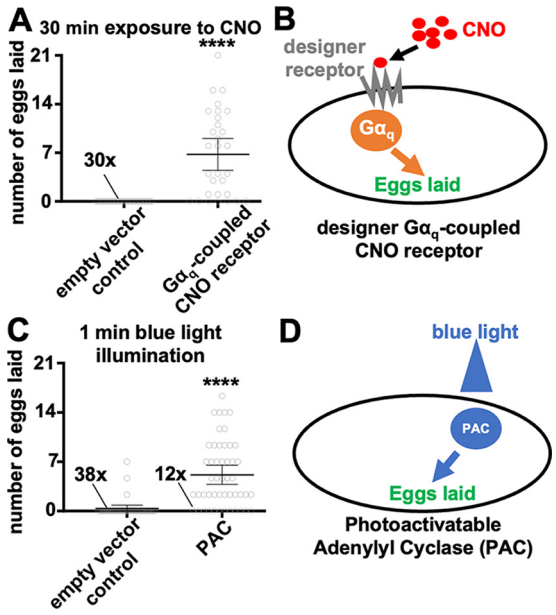
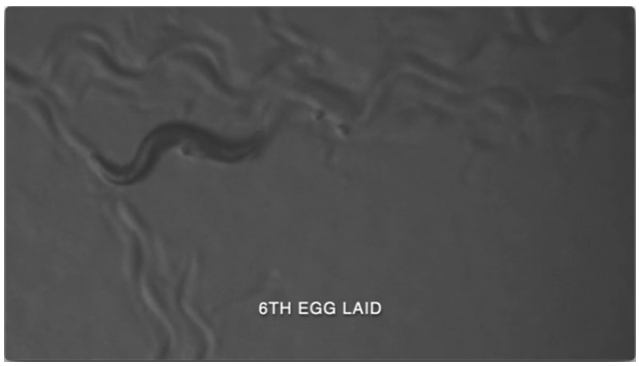
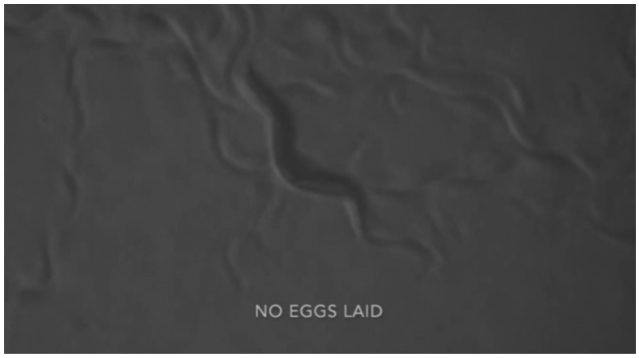


Figure 7. Signals from either the Gα_q or Gα_s pathways in the egg-laying muscles can be sufficient to drive egg laying. ***A***, Each circle represents the number of eggs laid per worm after 30 min of exposure to 10 mM CNO in worms expressing either the designer Gα_q-coupled CNO-responsive receptor in their egg-laying muscles or carrying a control empty vector transgene. The empty vector control was compared with the CNO receptor animals using a Mann–Whitney test with a two-tailed *p* value. For the empty vector control group, median was 0.0 and $n = 30$; for the CNO receptor group, median was 6.0 and $n = 30$; the *U* value was 105. ***B***, Schematic of the design of the experiment shown in ***A***. ***C***, Number of eggs laid per worm after 1 min of exposure to blue light in animals expressing PAC in the egg-laying muscles to induce the downstream effects of Gα_s signaling. The empty vector control was compared with the PAC animals using a Mann–Whitney test with a two-tailed *p* value. For the empty vector control group, median was 0.0 and $n = 38$; for the PAC group, median was 2.0 and $n = 49$, the *U* value was 287. ***D***, Schematic of the design of the experiment shown in ***C***. For both assays, $n \geq 30$; ****$p < 0.0001$. All measurements are given with 95% confidence intervals.



Movie 1. Optogenetic activation of PAC in the *C. elegans* egg-laying muscles is sufficient to induce egg laying. The experiment begins with a transgenic worm, expressing PAC in its egg-laying muscles, crawling on a standard laboratory nematode growth medium plate backlit by dim white light. Three seconds into the video, blue light illuminates the worm. Five seconds after blue light illumination the worm lays its first egg. Six eggs are laid within 29 s of blue light illumination. [View online]



Movie 2. Empty vector control animals do not lay eggs during blue light stimulation. The experiment begins with a transgenic worm, expressing an empty vector control in its egg-laying muscles, crawling on a standard laboratory nematode growth medium plate backlit by a dim white light. Five seconds into the video, blue light illuminates the worm. During 1 min of blue light illumination, the worm does not lay any eggs. [View online]

The combination of subthreshold signals from different Gα_q-coupled and Gα_s-coupled receptors in the egg-laying muscles is sufficient to drive egg laying

The results above demonstrate that artificially induced Gα_q or Gα_s signaling in the egg-laying muscles can be sufficient to induce egg laying. However, we also found that neither endogenous SER-1/Gα_q signaling alone nor endogenous SER-7/Gα_s signaling alone in these same egg-laying muscles is sufficient to drive egg laying; instead, both these endogenous signaling pathways must be active at the same time for serotonin to induce egg laying (Fig. 1*D*). To reconcile these findings, we hypothesized that the endogenous levels of SER-1/Gα_q and SER-7/Gα_s signaling are both subthreshold, that is, they occur at low levels that are not sufficient to properly activate egg laying on their own and together sum to reach a threshold necessary to activate egg laying.

To test this hypothesis, we replaced either SER-1 or SER-7 in the egg-laying muscles with a designer receptor that offered the opportunity to tune its signaling down to subthreshold levels. We then tested whether the subthreshold signaling through the remaining serotonin receptor plus subthreshold signaling through the designer receptor could combine to stimulate egg laying. Thus, in animals lacking either the SER-1 or SER-7 serotonin receptor, we transgenically expressed the Gα_q-coupled designer CNO receptor in the egg-laying muscles, as schematized in Figure 8*C*,*F*. To determine how to stimulate the CNO receptor at subthreshold levels, we treated each of the two strains with CNO over a range of concentrations (Fig. 8*A*,*D*). At sufficiently high concentrations, treatment with CNO alone was sufficient to induce egg laying in each strain, as expected based on our Figure 7, *A* and *B*, results. However, we identified concentrations of CNO for each strain below which CNO no longer stimulated egg laying (Fig. 8*A*,*D*), and we used these low CNO concentrations to stimulate subthreshold signaling by the designer receptor. The subthreshold CNO concentrations thus identified were different for the two strains, presumably because the different CNO receptor transgenes used in the two strains expressed the receptor at different levels. Note that since the strains used in this experiment lacked either SER-1 or SER-7, even 25 mM serotonin was not able, on its own, to induce egg laying through the remaining endogenous serotonin receptor (Figs. 1*D*, 8*B*,*E*).

Although neither serotonin nor subthreshold concentrations of CNO on their own could stimulate egg laying in the strains constructed for this experiment, we found that subthreshold signaling through designer Gα_q receptors could combine with either subthreshold serotonin signaling through endogenous SER-7/Gα_s receptors (Fig. 8*B*,*C*) or could combine with subthreshold serotonin signaling through endogenous SER-1/Gα_q receptors (Fig. 8*E*,*F*) to reach a threshold capable of activating egg laying.

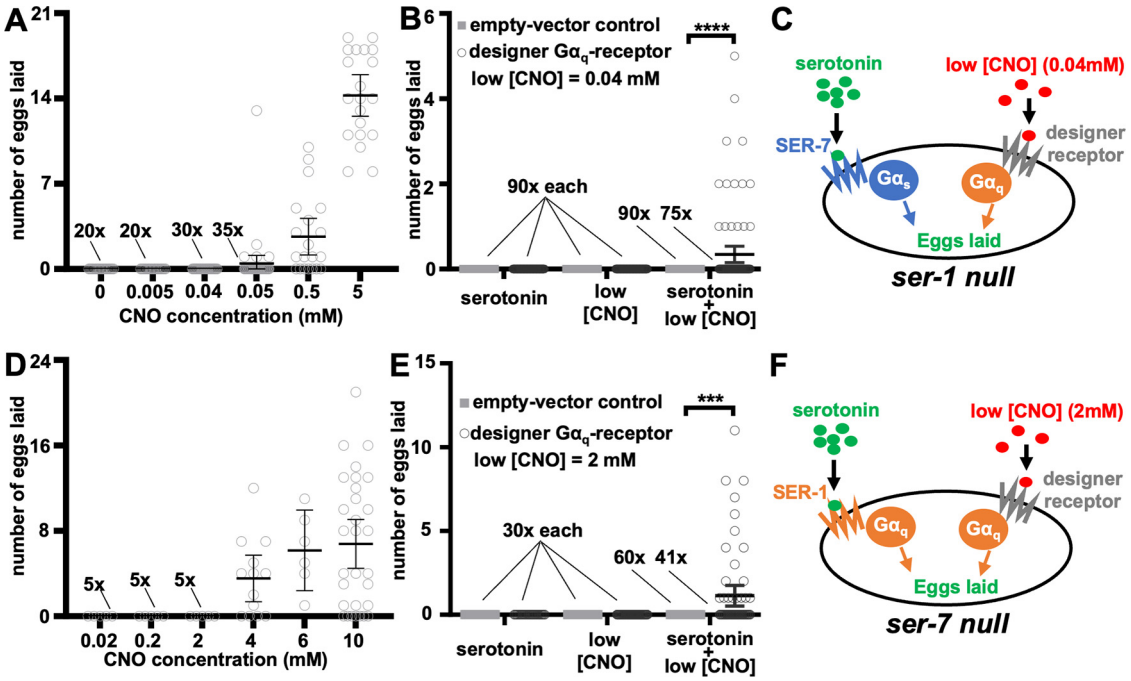


Figure 8. Combining subthreshold signals from Gα_q- and Gα_s-coupled receptors in the egg-laying muscles is sufficient to drive egg laying. ***A***, The number of eggs laid after 30 min by worms lacking *ser-1* and expressing the designer Gα_q-coupled CNO receptor in their egg-laying muscles, after being treated with a range of CNO concentrations. Each circle represents results from a single worm; $n \geq 20$ for each CNO concentration. ***B***, *ser-1* mutant animals, with or without expression of the CNO receptor in their egg-laying muscles, were exposed to either 25 mM exogenous serotonin, 0.04 mM CNO (low [CNO]), or both. Eggs laid after 30 min were counted; $n = 90$. This experiment was repeated on three separate occasions, and combined data from all repeats are shown. For each condition, the number of animals that laid zero eggs is indicated. Animals carrying empty vector were compared with animals carrying the designer receptor using the Mann–Whitney test with the Holm–Šídák method for multiple comparisons. For the serotonin + low [CNO], $U = 3375$, ****$p = 0.000097$. ***C***, Schematic of the design and results of the experiment shown in ***B***. ***D***, The number of eggs laid after 30 min by worms lacking *ser-7* and expressing the designer Gα_q-coupled CNO receptor in their egg-laying muscles after being treated with a range of CNO concentrations; $n \geq 5$ for each CNO concentration. ***E***, *ser-7* mutant animals, with or without expression of the CNO receptor in their egg-laying muscles, were exposed to either 25 mM exogenous serotonin, 2 mM CNO (low [CNO]), or both. Eggs laid after 30 min were counted; $n \geq 30$. This experiment was repeated on three separate occasions, and combined data from all repeats are shown. In each condition, the number of animals that laid zero eggs is indicated above the data point. Animals carrying empty vector were compared with animals carrying the designer receptor using the Mann–Whitney test with the Holm–Šídák method for multiple comparisons. For the serotonin + low [CNO], $U = 615.0$, ***$p = 0.001773$. ***F***, Schematic of the design and results of the experiment shown in ***E***. All measurements are given with 95% confidence intervals.

The behavioral responses induced were not as robust as seen when applying exogenous serotonin to wild-type animals, in which it is the endogenous SER-1/Gα_q and SER-7/Gα_s signals that combine to induce egg laying. A possible explanation for this is that endogenous SER-1/Gα_q and SER-7/Gα_s signals may be stronger than the signal produced from the Gα_q-coupled designer CNO receptor on treatment with low concentrations of CNO. However, our results replacing signaling by either serotonin receptor with subthreshold signaling by the designer CNO receptor support our model (Fig. 9) that egg laying results from multiple endogenous Gα_q- and Gα_s-coupled receptors, each producing subthreshold signals that combine to generate an overall level of signaling sufficient to result in wild-type egg-laying behavior.

Discussion

We found that multiple Gα_q- and Gα_s-coupled receptors signal in the *C. elegans* egg-laying muscles to induce coordinated contraction of these muscle cells and thus the laying of eggs. Signaling from endogenous levels of just one of these receptors alone is not strong enough to induce egg laying, but together the signals from multiple GPCRs on the same cells combine to reach a threshold that activates egg laying (Fig. 9). This study is perhaps the most detailed to date of how cells within an intact organism integrate signaling by multiple GPCRs to generate a concerted response to the complex mixture of endogenous chemical signals impinging on them. Such signal integration is a challenge faced by virtually all cells within multicellular organisms.

Multiple GPCRs signal through Gα_q and Gα_s to activate excitable cells

We found that knocking down both Gα_q and Gα_s in the egg-laying muscles resulted in a dramatic defect in egg laying, whereas loss of the SER-1 and SER-7 serotonin receptors that activate these G-proteins only had a modest effect. Therefore, serotonin appears to combine with other endogenous signals to generate sufficient Gα_q and Gα_s signaling in the egg-laying muscles to induce egg laying. Treating animals with a high concentration of exogenous serotonin is sufficient to induce egg laying, and, even in this artificial situation, both the SER-1 and SER-7 receptors must operate in parallel on the egg-laying muscles to mediate this effect, as loss of either receptor from the egg-laying muscles results in almost no response to exogenous serotonin. Artificially strong activation of a single type of Gα_q-coupled receptor on the egg-laying muscles (either overexpressed SER-1 or the designer CNO receptor) could induce egg laying. Additionally, activation of the Gα_s signaling pathway downstream of SER-7 with a photoactivatable adenylyl cyclase was sufficient to induce egg laying. Nonetheless, our results show that the normal situation in wild-type animals is that egg laying is induced by the combined signaling from multiple Gα_q- and Gα_s-coupled receptors.

What other signals in addition to serotonin might be acting through GPCRs on the egg-laying muscles to promote egg laying? The neuropeptide NLP-3 is coreleased with serotonin onto the egg-laying muscles to promote egg laying (Brewer et al., 2019), and the NLP-3 receptor, which has not yet been identified, is likely one of the additional GPCRs expressed on these muscles. Interestingly, animals lacking both serotonin and NLP-3 are still able to produce vulval muscle calcium transients and lay some eggs (Figs. 1*E*, 2; Extended Data Fig. 2-1). This indicates there are even more signals activating the egg-laying muscles. There are ~150 neuropeptide GPCRs in *C. elegans*, and single-cell RNA sequencing suggests most neurons and muscle cells express multiple neuropeptide receptors (Taylor et al., 2021). The egg-laying muscles also express multiple $G\alpha_q$- and $G\alpha_s$-coupled GPCRs for small molecule neurotransmitters (Fernandez et al., 2020). In addition to SER-1 and SER-7, these include the dopamine receptor DOP-4, the tyramine receptor TYRA-3, and the metabotropic acetylcholine receptor GAR-3. Just as for SER-1 and SER-7, knockouts for any one of these receptors have, at most, modest effects on the accumulation of unlaid eggs (Fernandez et al., 2020), consistent with the hypothesis that the G-proteins $G\alpha_q$ and $G\alpha_s$ integrate signals from a variety of GPCRs on the egg-laying muscles to maintain proper egg laying.

The strategy of multiple GPCRs combining signaling to induce strong effects appears to be a general feature of GPCR signaling in excitable cells within multicellular organisms. In *C. elegans*, forward genetic screens for mutants with behavioral defects resulting from disruption of G-protein signaling have been conducted for decades (Trent et al., 1983; Desai and Horvitz, 1989; Bargmann et al., 1993; Miller et al., 1996; Bany et al., 2003). However, mutants for GPCRs are almost absent from the results of these screens (for review, see Koelle, 2018). There is also a conspicuous paucity of GPCR mutations from genetic screens in *Drosophila* (Hanlon and Andrew, 2015). One possible explanation for these results could be that GPCR mutations are generally lethal; however, GPCR knock-out mutations in these model invertebrates are rarely, if ever, lethal and typically do not show any obvious behavioral defects (Fernandez et al., 2020). These results suggest that loss of a single neurotransmitter or neuropeptide GPCR rarely causes significant defects, although mutations in heterotrimeric G-proteins do show severe defects (Koelle, 2018). This paradox can be resolved if, as in the *C. elegans* egg laying system, multiple GPCRs typically combine their signaling to result in significant effects.

Studies of G-protein signaling in vertebrate cardiomyocytes (heart muscle cells) parallel our finding that multiple coexpressed GPCRs together regulate muscle contractility (Wang et al., 2018; Lymperopoulos et al., 2021). The GPCRs coexpressed on cardiomyocytes include four $G\alpha_s$- and $G\alpha_q$-coupled receptors that mediate signaling by epinephrine and norepinephrine (Bristow et al., 1986; McCloskey et al., 2003; O'Connell et al., 2003). Cardiomyocytes also express $G\alpha_q$-coupled receptors for the peptide hormones vasopressin (Xu and Gopalakrishnan, 1991) and angiotensin II (Meggs et al., 1993). Although it is clear that together these signals and receptors regulate heart muscle contractility and that each plays crucial roles mediating heart disease, it has remained unclear how they combine their effects within the intact organism to orchestrate proper control of heart muscle function.

How do $G\alpha_q$ and $G\alpha_s$ signals combine to modulate activity of excitable cells?

Previous studies suggested serotonin released by the HSNs acts directly on the egg-laying muscles to make these muscle cells more excitable, enabling other signals to directly depolarize the muscle cells and trigger the simultaneous vm1 + vm2 muscle cell contractions that release eggs (Collins and Koelle, 2013; Brewer et al., 2019; Kopchock et al., 2021). Serotonin enables contraction responses in the egg-laying muscles by acting via both the $G\alpha_q$-coupled SER-1 and $G\alpha_s$-coupled SER-7 receptors.

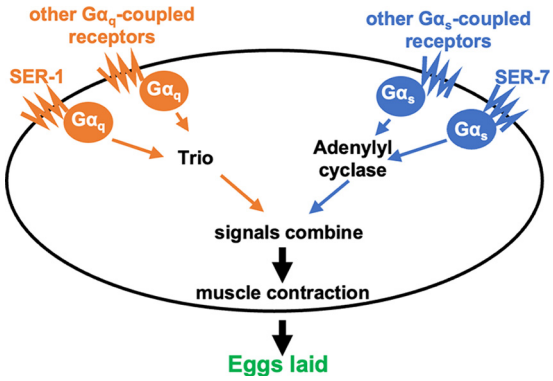


Figure 9. Model for the regulation of egg-laying muscle activity by multiple $G\alpha_q$- and $G\alpha_s$-coupled GPCRs. Each $G\alpha$ protein is activated in parallel by a serotonin receptor and additional GPCRs. $G\alpha_q$ directly activates the RhoGEF protein Trio, whereas $G\alpha_s$ directly activates adenylyl cyclase. The two signaling pathways intersect at a downstream point, yet to be determined, to promote muscle contraction, which drives egg laying. Individual GPCRs provide signaling too weak to result in a significant increase in egg laying, but combining signaling by multiple GPCRs through $G\alpha_q$, $G\alpha_s$, or both can result in a significant behavioral outcome.

Studies on the vertebrate heart muscle suggest mechanisms by which $G\alpha_q$ and $G\alpha_s$ signaling may together promote muscle contraction. $G\alpha_s$ is proposed to promote contraction by directly activating adenylyl cyclase to produce cAMP, which in turn binds and activates protein kinase A (PKA), causing PKA to phosphorylate and activate targets that promote contractility (for review, see Salazar et al., 2007). The proposed targets of PKA include the L-type Ca^{2+} channel, the ryanodine receptor, and the muscle filament protein troponin, with phosphorylation of each of these targets increasing Ca^{2+}-induced muscle contraction. $G\alpha_q$ signaling has complex effects on vertebrate heart function, including some that could combine with $G\alpha_s$ signaling to promote muscle contraction (Lin et al., 2001; McCloskey et al., 2003). First, $G\alpha_q$ activates its effector phospholipase C to ultimately lead to phosphorylation of $G\alpha_s$-coupled β-adrenergic receptors, altering their ability to regulate muscle contraction (Wang et al., 2018). Second, $G\alpha_q$ and $G\alpha_s$ signaling can collaborate to activate IP_3 receptors, which, like ryanodine receptors, are Ca^{2+} channels that release Ca^{2+} from internal stores to promote muscle contraction. In this mechanism, $G\alpha_q$ directly activates the enzyme phospholipase C (Smrcka et al., 1991; Taylor et al., 1991), which generates the second messenger IP_3 that directly binds and activates the IP_3 receptor. $G\alpha_s$ signaling, as noted above, activates the protein kinase PKA, which can phosphorylate and activate IP_3 receptors (Taylor, 2017).

The mechanism by which $G\alpha_q$ and $G\alpha_s$ signaling alters muscle and neuron function has been independently addressed through studies in *C. elegans* (Reynolds et al., 2005). In the egg-laying muscles, genetic studies show $G\alpha_q$ promotes contraction mainly not via phospholipase C (Dhakal et al., 2022), as suggested by vertebrate heart muscle studies (Salazar et al., 2007), but rather by activating the other major $G\alpha_q$ effector, the RhoGEF protein Trio, which in turn activates the small G-protein Rho (Chikumi et al., 2002; Lutz et al., 2005; 2007; Rojas et al., 2007; Williams et al., 2007). The different conclusions

reached in vertebrate heart versus *C. elegans* egg-laying muscles may reflect differences in how $G\alpha_q$ regulates these two types of muscles or rather could reflect the different experimental approaches used to study these two systems.

Conclusions

Combining signaling by multiple G-protein coupled receptors appears to be a universal mechanism used to modulate activity of neurons and muscle cells. How multiple GPCRs found on a single cell can meaningfully funnel their signaling through just a few types of $G\alpha$ proteins has long been a mystery. Our work shows that within an intact animal, multiple $G\alpha_q$- and $G\alpha_s$-coupled receptors coexpressed on the same cells each generate weak signals that individually have little effect but that sum together to produce enough signaling output to have an impact on behavior. This system allows a cell to gather multiple independent pieces of information from the complex soup of chemical signals in its environment and compute an appropriate response. In the case of the *C. elegans* egg-laying system, the multiple neurotransmitters and neuropeptides released by the egg-laying circuit are sensed to determine when conditions are right for the animal to lay an egg. More generally, this system for computing outcomes by integrating multiple inputs provides neurons and muscles with a vastly flexible mechanism for processing information.

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