Serotonin and neuropeptides are both released by the HSN command neuron to initiate *C. elegans* egg laying

Short title: Serotonin and a neuropeptide cotransmitter in *C. elegans*

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Abstract

Neurons typically release both a small molecule neurotransmitter and one or more neuropeptides, but how these two types of signal from the same neuron might act together remains largely obscure. For example, serotonergic neurons in mammalian brain express the neuropeptide Substance P, but it is unclear how serotonin signaling might be modulated by a coreleased neuropeptide. We studied this issue in *C. elegans*, in which all serotonergic neurons express the neuropeptide NLP-3. The serotonergic Hermaphrodite Specific Neurons (HSNs) are command motor neurons within the egg-laying circuit that have previously been shown to release serotonin to initiate egg-laying behavior. We found that egg-laying defects in animals lacking serotonin were far milder than in animals lacking HSNs, suggesting that HSNs must release other signal(s) in addition to serotonin to stimulate egg laying. While null mutants for *nlp-3* had only mild egg-laying defects, animals lacking both serotonin and NLP-3 had severe defects, like those of animals lacking HSNs. Optogenetic activation of HSNs induced egg laying in wild-type animals, or in mutant animals lacking either serotonin or NLP-3, but failed to induce egg laying in animals lacking both. We recorded calcium activity in the egg-laying muscles of animals lacking either serotonin, NLP-3, or both. The single mutants, and to a greater extent the double mutant, showed muscle activity that was uncoordinated and unable to expel eggs, such that the vm2 muscles cells that are direct postsynaptic targets of the HSN failed to contract simultaneously with other egg-laying muscle cells. Our results show that

39 the HSN neurons use serotonin and the neuropeptide NLP-3 as partially
40 redundant cotransmitters that together stimulate and coordinate activity of the
41 target cells onto which they are released.

42

Author Summary

Activity of the brain results from neurons communicating with each other using chemical signals. A typical neuron releases two kinds of chemical signals: a neurotransmitter, such as serotonin, and one or more small proteins, called neuropeptides. For example, many human brain neurons that release serotonin, a neurotransmitter thought to be involved in depression, also release the neuropeptide Substance P. Neuroscientists have typically studied the effects of neurotransmitters and neuropeptides separately, without considering how a neuron might use the two types of signals together. Here we analyzed how specific neurons in the model organism *C. elegans* use both serotonin and a neuropeptide together. The Hermaphrodite Specific Neurons (HSNs) activate a small group of neurons and muscles to generate egg-laying behavior. Killing the HSNs resulted in animals unable to lay eggs, but we found that eliminating either serotonin or the neuropeptide resulted in HSNs that still remained able to activate egg laying. However, eliminating both serotonin and the neuropeptide resulted in HSNs unable to activate coordinated contractions of the egg-laying muscles. Our results show that in a living animal, serotonin acts in concert with a coreleased neuropeptide to carry out its functions.

Introduction

Drugs that selectively manipulate serotonin signaling are widely used to treat depression and other psychiatric disorders, yet these drugs are often ineffective, and no specific molecular defects in serotonin signaling have been identified as the cause of these disorders (1). This situation suggests there is more to understand about the basic science of serotonin signaling that could help explain the cause of many psychiatric disorders. One feature of serotonin signaling in the mammalian brain that remains poorly understood is that serotonin neurons appear to also release a specific neuropeptide, Substance P (2–6). Of the ~80 billion neurons in the human brain, only about 100,000 make serotonin: their cell bodies are concentrated in the raphe nuclei of the brain stem, but extend axons throughout the brain that release serotonin to influence many brain functions (2,7,8). Several methods have been used to measure the proportion of serotonin neurons that express Substance P in the various raphe subnuclei of human or rat brain, with results suggesting that from 25% to nearly all serotonin neurons also express substance P (2,5,9,10). The apparent co-release of serotonin and Substance P from the same neurons is just one instance of the broad but poorly studied phenomenon of cotransmission by small-molecule neurotransmitters and neuropeptides (11–14). It remains unclear how exactly coreleased serotonin and neuropeptide might functionally interact. Clinical studies of Substance P antagonists showed that they, like selective serotonin reuptake inhibitors, can have significant anti-depressant activity (15–17). One study in the brain stem

respiratory circuit indicated that serotonin and substance P each independently stimulate activity of the circuit (10), but the complexity of mammalian brain circuits makes precise analysis of such effects difficult.

Small neural circuits of invertebrates provide the potential for more precise analysis of the functional effects of cotransmission. In such circuits, every cell can be identified, the small molecule neurotransmitter and neuropeptide content of each cell can be determined, and the functional effects of each signal can potentially be characterized. An elegant body of work on small circuits from crustaceans has used pharmacological and electrophysiological methods to analyze the functional effects of cotransmitters (11). However, the nature of these experimental systems typically requires that the isolated circuit be studied after dissection from the animal.

C. elegans provides the opportunity to use powerful genetic methods to functionally analyze cotransmission within small circuits of intact, behaving animals. Thus mutations and transgenes can manipulate neurotransmitters, neuropeptides, and their receptors, optogenetic methods can manipulate activity of presynaptic cells, and the functional consequences of all these manipulations can be read out in behavioral effects and using genetically-encoded calcium indicators to measure activity of postsynaptic cells. So far, there has been limited use of this approach to study cotransmission (18,19). One such study examined an olfactory neuron that releases glutamate to evoke a behavioral response to

105 odor. This neuron coreleases a neuropeptide to activate a feedback loop to
106 dampen activity of the olfactory neuron on specific timescales (18).

107 Here, we have applied the genetic toolbox described above to analyze the
108 functional consequences of cotransmission by serotonin and a neuropeptide
109 within a well-characterized small circuit of *C. elegans*. The *C. elegans* egg-laying
110 circuit contains three neuron types that release neurotransmitters onto egg-laying
111 muscles and each other to generate ~two minute active phases, during which
112 rhythmic circuit activity induces egg-laying behavior, that alternate with ~20
113 inactive phases, during which the circuit is largely silent and no eggs are laid
114 (20). The two serotonergic hermaphrodite specific neurons (HSNs) serve as the
115 command neurons (21) within this circuit in that 1) worms lacking HSNs are egg-
116 laying defective (22); 2) optogenetic activation of HSNs is sufficient to induce
117 activity of the circuit that mimics a spontaneous active phase (23–25); 3) no other
118 cells in the circuit have these properties (25). We show here that the HSNs use a
119 combination of serotonin and a neuropeptide to induce the coordinated circuit
120 activity of egg-laying active phases.

Results

The serotonergic HSN egg-laying neurons remain largely functional without serotonin

The small circuit that initiates egg-laying behavior is schematized in Fig 1A. The serotonergic Hermaphrodite-Specific Neurons (HSNs), along with the cholinergic Ventral Cord type C neurons (VCs), synapse onto the type 2 vulval muscles (vm2), which are electrically coupled by gap junctions to the type 1 vulval muscles (vm1) and contract with them to expel eggs (20). Loss of the HSNs results in a severe egg-laying defect: a mutation in *egl-1* causes death of the HSNs and results in animals that continue to make eggs but rarely lay them (26), resulting in the striking phenotype of adult worms distended with accumulated unlaid eggs (Fig 1B and 1C). Because addition of exogenous serotonin to worm culture media is sufficient to induce egg laying, even in worms lacking HSNs (22), it has been suggested that HSNs induce circuit activity simply by releasing serotonin, sensitizing the egg-laying muscles to activation by the acetylcholine released by other motorneurons of the circuit (25,27).

Contrary to this model, we found that animals lacking serotonin (Fig 1D) had only mild egg-laying defects. The *tph-1* gene encodes the serotonin biosynthetic enzyme tryptophan hydroxylase, and animals with a *tph-1* null mutation have no serotonin detectable by anti-serotonin antibodies or HPLC analysis (28,29). *tph-1* mutant animals had only mild egg-laying defects (~18 unlaid eggs), appearing more similar to wild type (~12 unlaid eggs) than they did

to *egl-1* mutants lacking HSNs (~47 unlaid eggs; Fig 1B-1D). This result, along with previous pharmacological, genetic, and behavioral studies of the function of serotonin in egg laying (30), are consistent with the idea that serotonin release can only partially explain how the HSNs initiate egg laying.

To determine definitively if serotonin is required for the HSNs to stimulate egg laying, we optogenetically stimulated the HSNs of animals either wild-type for *tph-1* or deleted for the *tph-1* gene (Fig 1E). Animals with channelrhodopsin (ChR2) expressed in the HSNs and that are wild-type for *tph-1* have been shown previously to lay eggs within a few seconds of exposure to blue light, but only if the required ChR2 cofactor all-trans retinal (ATR) is supplied to the worms (23,24). We found that upon optogenetic activation of HSNs, *tph-1* mutant animals laid a number of eggs statistically indistinguishable from the number laid by control animals wild-type for *tph*-1 (Fig 1E). Thus the HSNs do not require serotonin to stimulate egg-laying behavior.

The neuropeptide gene *nlp-3* stimulates egg laying, and loss of both *nlp-3* and serotonin together severely reduces egg laying

Our results, along with previous studies (30–33), lead to the hypothesis that the HSN releases a cotransmitter that allows the HSNs to stimulate egg laying without serotonin. We thought that this cotransmitter could be one or more neuropeptides encoded by the five neuropeptide genes previously shown to be expressed in the HSNs (34–36). To test this idea we created five types of

transgenic worm strains, each overexpressing one of these neuropeptide genes. Each strain we created carried an extrachromosomal transgene containing multiple copies of a ~45 kb *C. elegans* genomic DNA clone containing one neuropeptide gene. Overexpressing a neuropeptide gene can result in a gain-of-function phenotype caused by an increase in the normal signaling effects of the encoded neuropeptides (19,37). For a neuropeptide that induces egg laying, we expected overexpression to cause an increase in the frequency of egg-laying behavior.

We found that overexpressing the *nlp-3* neuropeptide gene resulted in a dramatic increase in the frequency of egg-laying behavior. An increased rate of egg-laying behavior results in an increased number of eggs being laid at early stages of development, since the eggs have little time to develop inside the mother before they are laid (38). More than 80% of the eggs laid by worms carrying the high-copy *nlp-3* transgene were laid at early stages of development, compared to about 5% for control animals not overexpressing any neuropeptide (Fig 2A). We saw no such phenotype for worms overexpressing any of the other four neuropeptide genes (Fig 2A).

We obtained *nlp-3* null mutant animals in which the *nlp-3* gene is deleted. Unlike the *egl-1* mutants lacking HSNs that accumulate ~47 unlaid eggs (Fig 1C), *nlp-3* null mutants accumulated only ~19 unlaid eggs (Fig 2B), and thus were more similar to the wild type (Fig 1B) or *tph-1* mutant worms lacking serotonin (Fig 1D). However, when we made *tph-1; nlp-3* double mutant so that the HSNs

lacked both serotonin and NLP-3 neuropeptides, the adult animals were distended with ~42 unlaid eggs and thus showed a severe egg-laying defect similar to that of *egl-1* animals. We obtained a second, independent deletion mutant for *nlp-3* and observed the same mild defect in the single mutant and the same severe defect in the double mutant with *tph-1* (Fig 2D).

The HSN egg-laying neurons use both serotonin and NLP-3 neuropeptides to stimulate egg laying

The above experiments demonstrated that serotonin and NLP-3 stimulate egg laying but did not examine if they do so by being released from the HSN neurons. Previous studies demonstrated that HSNs contain serotonin, and it was inferred from indirect evidence that HSNs can release serotonin to stimulate egg laying (22,39,40), although our results presented in Fig 1 show that HSNs do not require serotonin to stimulate egg laying. We used the *nlp-3* promoter to drive GFP expression and saw, as previously reported (41), that *nlp-3* is expressed in the HSN neurons but no other cells of the egg-laying circuit (Fig 3A), consistent with the hypothesis that NLP-3 neuropeptides are released from HSNs to stimulate egg laying.

To directly test what combination of transmitters the HSNs use to stimulate egg laying, we generated animals that express ChR-2::YFP in the HSN neurons (Fig 3B) and that were wild-type for *tph-1* and *nlp-3* (controls), or that carried null mutations in *tph-1*, *nlp-3*, or both. We then tested whether optogenetic stimulation

of the HSNs could induce egg laying. Both the control animals and null mutants for *tph-1* laid eggs readily upon ChR2 activation, with no statistically significant differences in the number of eggs laid (Fig 3C), or in several other measures of the egg-laying behavior induced (e.g. time to first egg laid, time to last egg laid, Fig S2A-S2C). However, whereas all wild-type and *tph-1* animals tested laid eggs upon ChR2 activation, 7/20 *nlp-3* mutant animals failed to lay any eggs, and the 13/20 that did lay eggs laid fewer on average than did the wild-type or *tph-1* animals. No eggs were laid by any *tph-1; nlp-3* double mutant animals (Fig 3C). Therefore, we conclude that the HSN neurons release both serotonin and NLP-3 peptides to stimulate egg laying, either signal alone is sufficient to stimulate at least some egg laying, and when lacking both signals the HSNs have no detectable ability to stimulate the behavior.

Serotonin and NLP-3 can each stimulate egg laying in the absence of the other

To further investigate the relationship between serotonin and NLP-3 in activating egg-laying behavior, we performed additional experiments to test if either of these transmitters is required to allow the other to stimulate egg laying. For serotonin stimulation of egg laying, we used a standard assay (22) in which worms were placed in microtiter well containing plain buffer or buffer containing serotonin, and the number of eggs laid in 60 minutes was counted. We saw, as observed previously (39,42–44), that exogenous serotonin stimulates egg laying

231 in wild-type animals, but not in animals deleted for the serotonin receptor gene
232 *ser-1* (Fig 4A). Null mutants for *nlp-3* were stimulated by serotonin to lay eggs at
233 the same rate as were the wild-type controls, demonstrating that NLP-3 is not
234 required for serotonin to stimulate egg laying.

235 We used a converse experiment to test if NLP-3 could stimulate egg-
236 laying in the absence of serotonin. We generated *C. elegans* transgenes that
237 overexpressed *nlp-3* by containing multiple copies of *nlp-3* genomic DNA, and
238 control transgenes that did not overexpress *nlp-3*. In a strain background wild-
239 type for *tph-1*, we observed (Fig 4B), as we had seen previously in an analogous
240 experiment (Fig 2A), that overexpression of *nlp-3* resulted in hyperactive egg
241 laying as evidenced by a high percentage of early-stage eggs laid. When we
242 carried out this same experiment in a *tph-1* null mutant, *nlp-3* overexpression
243 also resulted in hyperactive egg laying, albeit at a modestly reduced level (Fig
244 4B). Thus serotonin is not required to allow *nlp-3* overexpression to induce egg
245 laying.

246

247 **Serotonin and NLP-3 together cause the HSN postsynaptic targets, the**
248 **vm2 muscle cells, to contract coordinately with other egg-laying muscles**

249 To understand the functional effects of the HSN cotransmitters, we
250 recorded Ca^{2+} activity of the vulval muscle cells, which are postsynaptic targets
251 of the HSNs, in animals that were wild-type, lacked serotonin, lacked NLP-3, or
252 lacked both. We thus coexpressed the Ca^{2+}-sensitive green fluorescent protein

GCaMP5 and the Ca^{2+}-insensitive red fluorescent protein mCherry in the vm2 muscle cells, the direct postsynaptic targets of the HSNs, and also in the vm1 muscle cells, which are gap-junctioned to vm2 and have been thought to contract with vm2 to expel eggs (45). Using methods we previously developed (25,46,47), we carried out ratiometric fluorescence imaging of intact animals to measure Ca^{2+} transients under conditions that allow egg-laying behavior to proceed as it does in standard lab culture, such that in wild-type animals, ~2 minute egg-laying active phases occur about every 20 minutes. Each animal was recorded for one hour. Fig 5A shows traces of Ca^{2+} transients recorded for the entire ensemble of vm1 and vm2 cells together for three animals of each of the following genotypes: wild-type, *tph-1* and *nlp-3* single mutants, the *tph-1;nlp-3* double mutant, and *egl-1* animals lacking HSNs.

We observed frequent vulval muscle activity in all genotypes, with each genotype showing hundreds of Ca^{2+} transients over the three hours recorded. However, in the wild-type less than 10% of the vulval muscle Ca^{2}+ transients resulted in egg release, and even fewer successful egg-laying events occurred in *tph-1* mutants lacking serotonin (*tph-1*) or in *nlp-3* mutants (30 eggs released over three hours for the wild type, compared to 15 for *tph-1* and 9 for *nlp-3*). Activity in animals lacking both serotonin and NLP-3 neuropeptides (*tph-1; nlp-3)* or lacking HSNs (*egl-1*) was actually more frequent than in the wild type, but very rarely produced successful egg release (each genotype released just two eggs in the three hours recorded).

To identify the differences between vulval muscle contractions that did or did not release eggs, we adjusted how we collected images during Ca^{2+} recordings. Previously-published Ca^{2+} imaging of the vulval muscles used images focused at the center of the group of two vm1 and two vm2 muscles found on either the left or right side of the animal, and the resulting images showed Ca^{2+} activity that was usually focused at the most dorsal tip of this group of muscles, but that could not be assigned to individual muscle cells (25,46,47). By focusing more laterally on either the left or right set of vulval muscles, we could more clearly resolve individual vm1 and vm2 cells and determine which of the four muscle cells within the set were active during any given Ca2+ transient detected (Fig 5B). All of the data presented in Fig 5 and Fig S3 results from use of this more lateral focus.

The large majority of the muscle activity we observed in every genotype examined occurred exclusively in one both of the vm1s imaged, with no concurrent activity detected in the vm2s (Fig 5C). In the wild-type, 12% of Ca2+ transients involved both vm1 and both vm2 cells imaged, and we refer to such events as "coordinated". We never observed an event in any genotype in which a Ca^{2+} transient occurred exclusively in vm2 cell(s) without accompanying activity in vm1 cell(s). In the wild-type, coordinated vulval muscle contractions occurred exclusively within active phases, the ~2 minute intervals during which eggs were laid and that contained frequent vulval muscle transients (Fig 5A). All 30 egg release events observed in the wild type occurred during one of the 51

297 coordinated vulval muscle contractions we saw during the three hours of
298 recordings analyzed. Thus it appears that coordinated contraction of all the vulval
299 muscle cells is necessary for efficient egg release.

300 Mutants lacking serotonin, NLP-3, or both continued to show vm1 Ca2+
301 transients at a rate similar to or even greater than seen in the wild-type, but a
302 decreased portion of these events were accompanied by vm2 Ca2+ transients to
303 produce coordinated events (Fig 5C). This decrease in coordinated events was
304 modest in *tph-1* and *nlp-3* single mutants, but severe in the *tph-1; nlp-3* double
305 mutant and in *egl-1* animals lacking HSNs. In the mutants, as in the wild-type,
306 egg release occurred almost only during coordinated events that included both
307 vm1 and vm2 activity (Fig S3): we only observed one exceptional egg-release
308 event that occurred in an *egl-1* animal during a vm1-only contraction. Thus the
309 loss of vm2 activity in the mutants correlated strongly with the loss of successful
310 egg laying. Thus, while vm1 calcium activity occurs in each genotype we
311 observed, only wild-type animals, in which the HSN neurons were able to release
312 both serotonin and NLP-3 neuropeptides onto vm2 cells, were able to frequently
313 trigger coordinated activity in both vm1 and vm2 and efficiently lay eggs.
314

Discussion

HSN command neurons release serotonin and NLP-3 neuropeptides to activate and coordinate activity of the egg-laying circuit

Our results show that serotonin and NLP-3 released by the HSNs induces activity of the vm2 muscle cells and coordinates their activity with that of the vm1 muscles to productively release eggs. In wild-type animals, the egg-laying circuit alternates between ~20 minute inactive states during which no eggs are laid, and ~2-3 minute active states during which a few eggs are laid. Previous work (21,42,43) as well as results in this study show that there are occasional vm "twitch" Ca^{2+} transients during the inactive state that do not release eggs, while there are robust, rhythmic vm transients during the active state, some of which release eggs. There are a total of four vm1 and four vm2 cells, with Fig. 1 diagramming just the two of each type on the left side of the animal. Gap junctions are present among the vm1 and vm2 cells found either anterior or posterior to the vulva, but there are no gap junctions between the anterior and posterior sets of vm cells (45). A previous study (48) showed that egg-laying events always coincide with Ca^{2+} transients that occur simultaneously in both anterior and posterior vm cells, while animals lacking HSNs fail to show such anterior/posterior vm coordination, potentially explaining the lack of efficient egg laying in animals lacking HSNs. It was assumed in such previous studies that the gap junctions between vm1 and vm2 muscle cells anterior or posterior on either

side of the vulva would efficiently electrically couple these cells so that they would contract as unit. In this study, we increased the spatial resolution of our Ca^{2+} imaging and found that this assumption was incorrect. We observed that the vm twitch Ca^{2+} transients seen in the inactive state occur in some or all of the vm1 cells but are not detected in the vm2 cells. During the active state in wild-type animals, a subset of the rhythmic vm transients seen happen simultaneously in all vm1 and vm2 cells, and it is in turn a subset these "coordinated" events that result in egg release. Animals lacking the HSNs or the HSN-released signals continue to have vm1 Ca^{2+} transients, but show a profound loss of vm2 Ca^{2+} transients. Thus the HSNs and their signals are not necessary for vm1 activity, which apparently are stimulated by other source(s), but the HSNs are critical for stimulating vm2 activity so that all vm cells can contract coordinately to productively release eggs.

The anatomy of the egg-laying circuit helps explain how the vm1 and vm2 muscle cells are activated. Figure 6 diagrams the synapses and gap junctions among cells in the circuit as determined by serial section reconstruction of electron micrographs (EM) (45). The HSNs synapse onto the vm2 muscles, as do the cholinergic VC neurons. In past studies, HSN and VC were considered to be the primary motor neurons that stimulate the vm muscles, but our results show that the vm1 cells, which are not synaptic targets of HSN or VC neurons, are activated independently of vm2. What cells and signals then excite the vm1 muscles?

We hypothesize that the VA and VB motor neurons are the source of excitation of the vm1 muscles, and also the central pattern generators that produce rhythmic activity of the egg-laying muscles. EM reconstruction (45) shows that the vm1 cells receive synapses from the cholinergic VA7 and VB6 motor neurons, although these synapses have been disregarded in the past because they are much smaller than are the VC and HSN synapses onto vm2. VA and VB are ventral cord motor neuron classes that synaptically release acetylcholine onto the body wall muscles to coordinate the body bends of locomotion. Rhythmic behaviors such as locomotion generally require central pattern generator (CPG) neurons that are the source of their rhythmicity (49). VA and VB are rhythmically active and serve as CPGs that produce the pattern of body bends during locomotion (50). During the active state, egg laying is a repetitive behavior (27), and there must be a connection between the CPG for locomotion and the egg-laying circuit because repetitive vm activity is phased with the body bends of locomotion (25,46). Cholinergic activation of vm1 by VA/VB neurons would neatly explain both the source of vm1 activity and the source of the rhythmicity of vm activity. Previous work showed that vm muscles are apparently stimulated at a specific phase of every body bend, even during the inactive state, since mutations in specific K^+ channels that normally reduce excitability of the vm cells result in increased rhythmic vm contractions, phased with body bends, during both the inactive and active egg-laying states. VA/VB neurons are active at a specific phase of each body bend (50) regardless of

whether animals are in the inactive or active state of the egg-laying system, and thus their pattern of activity exactly matches the pattern of activity inferred for the previously unknown source of vm activity.

We found that HSN neurons release serotonin and NLP-3 to initiate the egg-laying active state. Prevoius work showed that optogenetic activation of HSNs is sufficient to stimulate egg laying (24), and induces a pattern of activity in the VC neurons and in vm muscles reminiscent of activity seen during spontaneous active states (25). In this work, we found that optogenetic stimulation of HSNs lacking either serotonin or NLP-3 can still induce egg laying, but loss of both eliminates any egg laying response. Therefore, it appears that serotonin and NPL-3 together allow the HSNs to induce the active state of the egg-laying circuit. Examining spontaneous activity of the egg-laying circuit (i.e. without optogenetic activation of HSNs), we see that elimination of serotonin, NLP-3, both, or even killing the HSNs with an *egl-1* mutation does not eliminate egg-laying circuit activity. Indeed, vm Ca^{2+} activity remains high in these animals, and even includes activity in clusters, a property of the spontaneous active state seen in the wild type. However, the vm activity in animals lacking the HSN or its signals is uncoordinated, with Ca^{2+} transients mainly seen in vm1 cells only. Coordinated events with simultaneous Ca^{2+} transients in all vm cells that result in egg release occur on occasion, but these tend to be isolated events, as opposed to the groups of several coordinated egg-laying contractions that tend to occur within a 2-3 minute active phase in the wild type. The vm1-only activity seen in

mutants lacking the HSN or its signals could arise simply from direct release of acetylcholine onto vm1 cells by the VA/VB neurons. We also know from our previous work that it depends on an unknown signal released when the uterus contains unlaid eggs (25): the bloating of the uterus with excess unlaid eggs in animals lacking the HSNs or its signals may increase the uterus signal to result in the high levels of vm1-only activity seen in such animals.

How do HSNs use serotonin to produce vm2 contractions and the egg-laying active phase? The HSN and VC neurons make synapses onto muscle arms that project from all vm2 muscle cells, including those both anterior and posterior to the vulva. This anatomy ideally positions HSN and VC to stimulate vm2 contractions, and to do so such that both the anterior and posterior sets of vm cells contract simultaneously to productively release eggs. Previous studies showed that serotonin stimulates egg laying via the SER-1, SER-7, and SER-5 G protein coupled receptors (GPCRs), with potentially additional help from the MOD-1 serotonin-gated ion channel (51). Promoter::GFP transgenes for the *ser-1*, *ser-7*, and *ser-5* receptor genes show expression in the vm cells (32,42,44,51,52). The transgenic animals carrying these GFP reporters have not been examined carefully to determine which receptors are expressed in vm1 versus vm2 cells, but the published images suggest that both vm1 and vm2 express one or more of these GPCRs. No expression of *mod-1*::GFP has yet been seen in the egg-laying circuit (53). Thus, the anatomy of the egg-laying circuit and serotonin receptor expression patterns suggest that HSN-release

424 serotonin is released at synapses directly onto vm2 cells to activate these
425 muscles via one or more GPCRs, and additionally activates vm1 cells
426 extrasynaptically via one or more GPCRs. As a neuromodulator, serotonin could
427 increase the excitability of vm1 cells and thus their depolarization upon release of
428 acetylcholine onto vm1 by the VA/VB neurons. Serotonin could similarly act on
429 vm2 cells to increase their response to acetylcholine released onto vm2 by the
430 VC neurons, and/or their ability to respond to depolarization via gap junctions
431 from vm1 cells.

432 How do HSNs use NLP-3 neuropeptides to produce vm2 contractions and
433 the egg-laying active phase? Hypotheses for the action of NLP-3 are more
434 speculative than are those for the action of serotonin, as NLP-3 receptors have
435 not yet been identified, and we thus do not yet know which cells express these
436 receptors. NLP-3 might act on the vm cells just as does serotonin, so that these
437 two signals would activate the egg-laying circuit in the same manner. However, a
438 more interesting hypothesis is that NLP-3 is the signal that the HSNs use to
439 activate the VC neurons. Previous studies show that the VC neurons are
440 essentially silent during the egg-laying inactive state, but become rhythmically
441 active during the egg-laying active state, and that optogenetically activating the
442 HSN neurons is sufficient to induce such activity of the VC neurons (52).
443 Although the expression pattern of every known serotonin receptor in *C. elegans*
444 has been described, none have so far been seen expressed on the VC neurons.
445 Therefore, an attractive model is that NLP-3 is the HSN-released signal that acts

446 on the VC neurons to induce their activity. Active VC neurons would then release
447 acetylcholine directly onto the vm2 muscles. In this way, HSN-released serotonin
448 and NLP-3 would each have an independent mechanism for inducing vm2
449 activity. The possible targets of signaling by serotonin and NLP-3 outlined in the
450 model described above are depicted in Figure 6. This model is consistent with
451 our experimental observations that HSN-released serotonin and NLP-3 can each
452 independently induce egg laying.

453

454 **Co-release of small molecule neurotransmitters and neuropeptides is a**
455 **widespread phenomenon**

456 The apparent co-release of serotonin and NLP-3 from the HSN neurons is
457 just one instance of the broad but poorly-studied phenomenon of co-transmission
458 by small-molecule neurotransmitters and neuropeptides. Neurons typically
459 release one (or more rarely more than one) small molecule neurotransmitter from
460 small synaptic vesicles (SSVs) (54,55), and release neuropeptides from large
461 dense-core vesicles (LDCVs) (11,56). Certain small-molecule neurotransmitters,
462 including serotonin, can also be found in LDCVs (54,55). SSVs and LCDVs can
463 be localized in different parts of the cell and released by different mechanisms
464 (55,57).

465 Most neurons release both small-molecule neurotransmitters and
466 neuropeptides. This issue has been analyzed in greatest detail within the *C.*

467 *elegans* nervous system. There are 118 neuron types in *C. elegans*
468 hermaphrodites, and 107 of them express one or more of the seven known small
469 molecule neurotransmitters found in this organism (58). At least 95 *C. elegans*
470 neuropeptide genes have been described, including 23 FLP genes encoding
471 FMRFamide-related peptides, 32 NLP genes encoding neuropeptide-like
472 proteins, and 40 INS genes encoding insulin-like peptides. Promoter::GFP fusion
473 transgenes have been generated for all 95 of these neuropeptide genes to
474 analyze their expression patterns. The individual neurons expressing each FLP
475 gene were identified, and >50% of *C. elegans* neurons express at least one FLP
476 peptide gene (35). The individual cells expressing each NLP and INS gene have
477 not yet been identified, but images of the expression patterns show that the large
478 majority of these peptide genes are expressed complex subsets of neurons
479 (34,59). Thus, we can infer that the typical neuron in *C. elegans* releases one
480 small-molecule neurotransmitter, and one or more type of neuropeptide.
481 Similarly, the presence of both small molecule neurotransmitters and
482 neuropeptides within the same individual neurons is widespread in both
483 *Drosophila* (60,61) and in mammals (62).

484 The functional consequences of a neuron releasing two different types of
485 signaling molecules have been difficult to study with precision in the complex
486 circuits of the mammalian brain, but this issue has been the focus of many
487 studies of small neural circuits in invertebrate model organisms (11,63). In such
488 small circuits, individual presynaptic neurons that co-release a small molecule

neurotransmitter and neuropeptides can be identified, and the functional effects of each signal can be measured by bath application of neurotransmitter agonists/antagonists and/or neuropeptides, followed by measurements of circuit activity using electrophysiological methods. Such work has led to a rich set of findings, and many different schemes for the use of co-transmission within circuits (11,64). However, the limitations of these studies include that bath application of signaling molecules does not always mimic the effects of their release from neurons (11). Further, the electrophysiological recordings used require dissecting neural circuits out of the animal, replacing their extracellular fluid with an artificial solution, eliminating the movements that motor circuits normally induce and that provide proprioceptive feedback to these circuits, and impaling the recorded neurons with electrodes that dialyze their intracellular fluid, all of which may affect circuit function. The genetic approaches for analyzing co-transmission described in this work provides a useful complement to electrophysiological studies, as they permit us to manipulate endogenous signaling molecules with mutations and transgenes, to record circuit activity using genetically-encoded calcium indicators, and to manipulate neural activity using optogenetics, all within intact, freely-behaving animals. We are aware of just one previous study that focused on co-transmission using this combination of genetic approaches (18). In this pioneering study, an odor was shown to cause a *C. elegans* sensory neuron to release glutamate to act via ionotropic receptors on specific interneurons that further regulate a complex and incompletely

511 understood motor circuit to evoke a behavioral response to the odor. The same
512 sensory neuron also releases a neuropeptide that acts via a G protein coupled
513 receptor on a different interneuron to cause it to in turn release a second
514 neuropeptide back onto the sensory neuron, limiting activity of the sensory
515 neuron and the timescale of the behavioral response to the odor.

516 Our studies of co-transmission focus on the *C. elegans* egg-laying circuit
517 because its anatomical simplicity holds the promise that all the cells and
518 signaling events that control this circuit can be defined, something that has not
519 yet been accomplished for any neural circuit. We discovered that serotonin and
520 NLP-3 peptides released from the HSN command neurons have parallel and
521 partially redundant effects to activate coordinated, rhythmic contraction of the
522 egg-laying muscles. This finding may be analogous to results of some previous
523 studies of co-transmission, in which the two co-released signals act convergently
524 to increase activity the same target cells. The most relevant such example is in
525 the mammalian brain respiratory circuit, where co-release of serotonin and the
526 neuropeptide Substance P have parallel effects promoting rhythmic circuit activity
527 (10). It will be interesting to determine just how mechanistically analogous these
528 two cases of serotonin/neuropeptide co-transmission actually are, and whether
529 the action of serotonin within the *C. elegans* egg-laying circuit will provide a
530 model for the detailed workings of serotonin within neural circuits of the human
531 brain.
532

Methods

C. elegans strains

C. elegans strains were cultured at 20°C on NGM agar plates with *E. coli* strain OP50 as a food source (66). All strains were derived from the Bristol N2 wild-type strain. Genetic crosses and generation of transgenic strains were by standard methods (67,68). A list of strains, mutants, and transgenes used in this study can be found in Table 1.

Gene deletion strains were for *nlp-3 (69)*, *tph-1 (70)*, and *ser-1* (42)were outcrossed four to ten times to the wild-type strain, as was the strain carrying an additional, previously unpublished *nlp-3* deletion allele, *tm3023*, which we obtained from the Japanese National Bioresource Project. It carries a 354 bp deletion that removes sequences flanked by the sequences GTCTGGACGGAAAGATCGTT…CGTGAGACTAGAAGTCCAC. Each gene deletion used removes a portion or all of the promoter and/or coding sequences of the corresponding gene such that no functional gene product is expected. The genotypes for all strains constructed using these deletions were verified by agarose gel analysis of PCR amplification products from the corresponding genes.

553

554 **Table 1. Strains used in this study.**

Strain	Feature	Genotype	Figures
N2	Bristol strain	Wild type	1,2
MT2059	Lacks HSN neurons	*egl-1(n986dm)* V	1,2
MT15434	Lacks serotonin	*tph-1(mg280)* II	1,2
LX1836	*egl-6*::ChR2::YFP	*wzIs30 IV; lite-1(ce314) lin-15(n765ts) X*	1,3
MT8189	Strain for transgene production	*lin-15(n765ts) X*	2
LX1954	*nlp-3* overexpressor	*lin-15(n765ts)* X ; *vsEx748*	2
LX1955	nlp-8 overexpressor	*lin-15(n765ts)* X ; *vsEx749*	2
LX1956	*nlp-15* overexpressor	*lin-15(n765ts)* X ; *vsEx750*	2
LX1957	*flp-5* overexpressor	*lin-15(n765ts)* X ; *vsEx751*	2
LX1981	*flp-19* overexpressor	*lin-15(n765ts)* X ; *vsEx757*	2
LX1978	*nlp-3* null mutant	*nlp-3(tm3023)* X	2,4
LX2366	double mutant	*tph-1(mg280)* II; *nlp-3(tm3023)* X	2
LX2388	*nlp-3* null mutant	*nlp-3(n4897)* X	2
LX2389	double mutant	*tph-1(mg280)* II; *nlp-3(n4897)* X	2
LX1836		*wzIs30* IV; *lite-1(ce314) lin-15(n765ts)* X	3
LX1832	mate to LX1836 for Fig 3C "control"	*lite-1(ce314) lin-15(n765ts)* X	3
LX1837		*tph-1(mg280)* II; *wzIs30* IV; *lite-1(ce314) lin-15(n765ts)* X	3
LX2335	*mate to LX1837 for Fig 3C "tph-1"*	*tph-1(mg280)* II; *lite-1(ce314) lin-15(n765ts)* X	3
LX2367		*wzIs30* IV; *lite-1(ce314) nlp-3(tm3023) lin-15(n765ts)* X	3
LX2364	mate to LX2367 for Fig 3C *"nlp-3"*	*lite-1(ce314) nlp-3(tm3023) lin-15(n765ts)* X	3
LX2368		*tph-1(mg280)* II; *wzIs30* IV; *lite-1(ce314) nlp-3(tm3023) lin-15(n765ts)* X	3
LX2365	mate to LX2368 for Fig 3C *"tph-1; nlp-3"*	*tph-1(mg280)* II; *lite-1(ce314) nlp-3(tm3023) lin-15(n765ts)* X	3
DA1814	Serotonin receptor 1 deletion	*ser-1(ok345)* X	4
LX2392	"control" in Fig 4B	*lin-15(nt65ts)* X; *vsEx885*	4
LX2394	"*nlp-3* ox" in Fig 4B	*lin-15(nt65ts)* X; *vsEx887*	4
LX2393	"control in tph-1" in Fig 4B	*tph-1(mg280)* II; *lin-15(nt65ts)* X; *vsEx886*	4
LX2395	"*nlp-3* ox in *tph-1*" in Fig 4B	*tph-1(mg280)* II; *lin-15(nt65ts)* X; *vsEx888*	4

555

556 **Egg-laying behavioral assays**

557 Quantitation of unlaid eggs in adult animals and percentage of early-stage eggs
558 laid was done as described in (71), using adult animals 30 hours after staging as
559 late L4 larvae.

560

561 **Optogenetic assays**

562 HSN neurons were optogenetically activated in animals carrying the *wzIs30*
563 transgene, which expresses a Channelrhodopsin-2::yellow fluorescent protein
564 (ChR2::YFP) fusion in the HSN and a few other neurons unrelated to the egg-
565 laying circuit from the *egl-6a* promoter (72,73). *wzIs30* also carries a *lin-15*
566 marker plasmid that rescues the multivulva phenotype of *lin-15* mutant animals.
567 All animals used in optogenetic assay were also mutant for the *lite-1* gene to
568 eliminate an endogenous response of *C. elegans* to blue light. The *wzIs30*
569 transgene was homozygous for the experiment shown in Fig 1E, but we noticed
570 that the homozygous transgene caused developmental defects in the HSNs of
571 some animals (Fig S1) that resulted in these animals being egg-laying defective.
572 Therefore, for the experiment in Fig 1E, we examined the animals prior to
573 optogenetic stimulation and discarded the small percentage of animals that were
574 visibly egg-laying defective. The experiment shown in Fig 3C was carried out
575 such that all animals were *wzIs30*/+ heterozygotes, which we found had
576 morphologically normal HSNs (Fig S1). First we constructed the strains indicated
577 in Table 1 that were homozygous for *wzIs30* and also homozygous for the other

mutations required by the experiment. We generated males of each of these strains, and mated them to corresponding strains that were genetically identical except that they lacked *wzIs30*. The cross progeny, identified by the presence of YFP-labeling, thus were heterozygous for *wzIs30* but homozygous for all other mutations used in the experiment.

ChR2 expressing strains were grown in the presence or absence of the ChR2 cofactor all-trans retinal (ATR). ATR was prepared at 100 mM in 100% ethanol and stored at 20° C. To prepare NGM plates for behavior analysis, ATR was diluted to 0.4 mM with warmed cultures of OP50 bacteria in B Broth, and 200 ml of culture was seeded onto each 60 mm NGM plate. The plates were allowed to grow for 24 hr at 25–37 °C, after which late L4 worms were staged onto prepared plates for behavioral assays 24 hr later. To initiate an assay, the shutter was opened to initiate exposure to blue light simultaneously with a recording (Flea 3, 0.3 Megapixel, FireWire CCD camera, Point Grey Research) and shutter opening on a EL6000 metal halide light source generating 10 mW/cm^2 of 470 ± 20 nm blue light via a EGFP filter set mounted on a Leica M165FC stereomicroscope.

Molecular biology and transgenes

For overexpression of neuropeptide genes (Fig 2A), fosmid genomic clones including individual neuropeptide genes were selected from the *C. elegans* fosmid library (74,75). The fosmids used for four neuropeptide genes were: *nlp-*

600 *3*, WRM0633dC06; *nlp-8*, WRM0614aB10; *nlp-15*, WRM066cH12; *flp-5*,
601 WRM0622aF03. For overexpression of a fifth neuropeptide gene, *flp-19*, we
602 instead PCR amplified genomic DNA containing the 746 bp *flp-19* coding region
603 along with 5015 bp upstream and 746 bp downstream. Multicopy
604 extrachromosomal transgenes were generated for each neuropeptide gene by
605 microinjection (reference), using the fosmid or PCR product at 50 ng/μl along with
606 the *lin-15* rescuing plasmid pL15EK at 50 ng/μl into *lin-15(n765ts*) mutant
607 animals. Negative controls were injected with pL15EK without any neuropeptide
608 gene. Five independent transgenic overexpressor lines were generated for each
609 injection and Fig 2A shows data averaged from these. Table 1 lists one
610 representative overexpressor strain for each neuropeptide gene.

611 For determine the effects of overexpressing *nlp-3* in animals lacking
612 serotonin (Fig 4B), either a ~5 kb PCR product containing the *nlp-3* gene (primers
613 used were 5'-accaagctaatcaaattttgtcaccg-3' and 5'-gcaatacaaccaatccctttttcatctc-
614 3') or as a control, *E. coli* genomic DNA digested to an average size of ~5 kb,
615 was injected at 10 ng/μl along with 50 ng/μl of the *lin-15* rescuing plasmid
616 pL15EK into either *lin-15* or *tph-1; lin-15* animals, and transgenic lines were
617 identified by rescue of the *lin-15* phenotype. Five independent transgenic lines
618 were established for each injection, and the early stage egg assay (76) was
619 carried out on 50 eggs per line (250 eggs total per condition tested). One
620 representative line for each condition is listed in Table 1.
621

622 **Ratiometric Calcium Imaging**

623 Freely-behaving animals were mounted between a glass coverslip and chunked
624 section of an NGM plate for imaging as described (25,46,47) and recorded with a
625 20X Plan-Apochromat objective (0.8 NA) using a Zeiss LSM 710 Duo LIVE head
626 set to record two channels. Recordings were collected at 20 fps at 256 x 256
627 pixel, 16 bit resolution, for 1 hour. The stage and focus were adjusted manually to
628 keep the egg-laying system in view and focused during recording periods. Care
629 was taken to find a lateral focus that included as much of the vm1s and vm2s as
630 possible. Ratiometric analysis for Ca^{2+} recordings was performed in Volocity
631 (version 5, PerkinElmer). A ratio channel was calculated from GCaMP5 (GFP)
632 and mCherry fluorescence channels. Volocity was also used to identify the vulval
633 muscles using size and intensity parameters that varied over a small range
634 based on individual animals. Any misidentified objects were manually excluded
635 prior to final analysis. The lowest 10% of the GCaMP5/mCherry ratio values were
636 averaged to establish a $\Delta R/R$ baseline using a custom Matlab script. This script
637 also identifies the peak of a transient based on identifying a change in
638 prominence that was typically 0.25 ΔR/R over the preceding second, but this was
639 adjusted based on the smoothness of the data for individual animals. With the
640 experimenter blinded to the genotype of the animals being scored, video of each
641 peak was observed in the ratio channel to determine whether the indicated
642 activity was restricted to vm1 or present in both vm1 and vm2, and whether an
643 egg was laid. We scored a transient as vm1-only if it was clear in the ratio

644 channel that there was a difference of more than 50% of maximum activity
645 between the vm1s and the adjacent regions where vm2 cells were located.
646
647 **Statistical Methods**
648 Statistical analyses were performed using GraphPad Prism for Mac OS X v. 7.0a.
649 95% confidence intervals were determined and 1- or 2-way ANOVA with multiple
650 comparisons were performed to determine statistical significance. For egg stage
651 assays, we used the Wilson-Brown method for determining the 95% confidence
652 intervals for binomial data.

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656

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Figure Legends

Fig 1. Serotonin is not required for the HSN to stimulate egg laying. A) Schematic of the *C. elegans* egg-laying circuit, adapted from (25). HSN and VC motorneurons synapse onto vm2 vulval muscles, which along with vm1 muscles contract to open the vulva and release eggs. Only the left HSN and vm cells are shown – equivalent cells are also found on the right side of the animal. The uv1 neuroendocrine cells that inhibit the circuit are not shown. **B-D)** Images of representative animals of the indicated genotypes, showing the average number of unlaid eggs +/- 95% confidence intervals, n=30. Arrowheads indicate individual unlaid eggs. Asterisks indicate the location of the vulva. **E)** Average number of eggs laid during 60 seconds of blue light exposure by animals expressing ChR2 in the HSNs. Both control and *tph-1* animals also have *lite-1(ce314)* mutations that eliminates a locomotion response to blue light (77). Black bars indicate animals that were grown for a generation in the presence of ChR2's required cofactor all-trans retinal (ATR). White bars indicate negative control animals grown in the absence of ATR. Error bars, 95% confidence intervals, n=20, n.s., no statistically significant difference, ∅, no egg laying observed.

Fig 2. The neuropeptide gene *nlp-3*, together with serotonin, stimulates egg laying. A) Overexpression of *nlp-3,* but not of four other neuropeptide genes, increased the rate of egg-laying behavior. Genomic clones for each

neuropeptide gene, or the coinjection marker alone (control), were injected into *C. elegans* to generate high-copy extrachromosomal transgenes. For each gene, 250 freshly laid eggs (50 from each of five independent transgenic lines) were examined and the percent laid at early stages of development (eight cells or fewer) was determined. Error bars, 95% confidence intervals. **B-C)** Representative images of *nlp-3* and *tph-1; nlp-3* animals showing the average number of unlaid eggs (n=30). **D)** Histogram of the average unlaid eggs for the strains indicated. Two independent deletion alleles of *nlp-3* were used. n≥30 for each strain; error bars, 95% confidence intervals. *tph-1* and the two independent *nlp-3* mutants accumulated significantly more eggs than did the wild type, and significantly fewer than did the *tph-1; nlp-3* double mutant strains ($p \leq 0.05$, Student's t-test). *egl-1* mutants were not significantly different from *tph-1; nlp-3(n4897*).

Fig 3. The HSNs require the *nlp-3* neuropeptide gene and *tph-1* to stimulate egg laying. A) *nlp-3* is expressed specifically in the HSNs. Vulval region of an adult animal carrying an *nlp-3*::GFP transgene, and a second transgene that expresses mCherry in the vulval muscles from the *unc-103e* promoter (46). **B)** The *egl-6p::*ChR2-YFP transgene is expressed specifically in the HSN. In A and B, asterisks indicate the vulva. Filled arrowheads the HSN cell body, and open arrowheads the HSN synapse onto the vulval muscles. **C)** Average number of eggs laid during 60 seconds of blue light exposure by animals expressing ChR2-YFP in the HSNs, and carrying the indicated null mutations in *tph-1* and/or *nlp-3*.

940 Control, animals wild type for *tph-1* and *nlp-3*. Black bars, animals grown in the
941 presence of ChR2's required cofactor all-trans retinal (ATR). White bars, animals
942 grown in the absence of ATR. All animals in this experiment were homozygous
943 for a *lite-1* mutation that abolished an endogenous *C. elegans* response to blue
944 light (78). Error bars, 95% confidence intervals, n=20. ∅, no egg laying was
945 observed.

946 **Fig 4. Serotonin and NLP-3 neuropeptides can stimulate egg laying in the**
947 **absence of each other. A)** Exogenous serotonin stimulates egg laying in wild
948 type and *nlp-3* animals. The number of eggs laid by 10 animals over 30 minutes
949 in plain buffer or buffer plus serotonin was measured, averaging 10 replicates per
950 genotype. The *ser-1* serotonin receptor null mutant is the negative control. **B)** *nlp-*
951 *3* overexpression stimulates egg laying even in the absence of serotonin.
952 Animals wild-type for *tph-1* or *tph-1* null mutants were injected with marker DNA
953 alone (control), or *nlp-3* genomic DNA plus marker DNA to overexpress *nlp-3*
954 (*nlp-3* ox). In each case, five independent transgenic lines were produced, and
955 50 freshly laid eggs per line (250 eggs total per condition) were examined to
956 determine their developmental stages. Error bars, 95% confidence intervals.

957

958 **Fig. 5. Vulval muscle activity, but not egg release, occurs frequently in**
959 **mutants lacking serotonin, NLP-3, or both. A)** Graphs of Ca^{2+} transients
960 showing ΔR/R of GCaMP5/mCherry signal recorded over 1 hour for three

different animals per genotype. ▼indicates a calcium transient associated with an egg-laying event. “2x” indicates that two eggs were laid nearly simultaneously during the same calcium transient. Scale bar, 10 minutes. Vertical scales have been normalized to depict comparable peak heights in all animals shown. **B)** Still frames from ratiometric recordings. The mCherry channel is rendered in blue. The mCherry channel is rendered in green, with a scale of intensity ranging from transparent, low intensity to greener high intensity. Schematics are shown below the images to distinguish the muscle types and indicate where activity appears to occur. **C)** A graph of the number and proportion of calcium transients occurring in the vm1s only compared to those appearing to occur in both vm1 and vm2 for each genotype. Error bars show 95% confidence intervals for the proportion that would result from an infinite number of observations.

Fig 6. Model depicting signaling events that activate the egg-laying circuit. Solid arrows, synaptic signaling; dashed arrow, extrasynaptic signaling; and bar, gap junctions. vm1 and vm2 cells are known to express multiple serotonin receptor isoforms that each mediate activation of egg laying. The signaling of serotonin onto VC neurons (arrow 3) remains hypothetical since no serotonin receptors have yet been described as expressed on these neurons. The possible

982 direct signaling of NLP-3 depicted onto vm1 (arrow 2), vm2 (arrow 2), or and/or
983 VC (arrow 3) are also hypothetical: NLP-3 receptors have yet to be identified, and
984 it thus remains unknown which cell(s) of the circuit express them.

985

986

Supporting Information Captions

Fig S1. Animals homozygous for the *egl-6*::ChR2-YFP transgene have visibly defective HSNs. A-B) Vulval region of an adult homozygote for the *egl-6p*::ChR2::YFP transgene. Abnormal HSN morphology can be seen by comparing to normal HSN morphology in Fig. S1B. Asterisk, location of the vulva. B) Vulval region of an adult heterozygote for the *egl-6p*::ChR2::YFP transgene. This is the same image seen in Fig 3B, repeated here for comparison to Fig S1A.

Fig S2. A *tph-1* null mutation does not detectably affect egg laying upon optogenetic activation of HSNs. Measurements of egg-laying upon blue light stimulation of egg-laying in *egl-6p*::ChR2-YFP/+ animals that were controls (wild type for *tph-1* and *nlp-3*) or that carried null mutations in *tph-1* or *nlp-3*. All animals also had a *lite-1(ce314)* mutation that eliminates endogenous responses to blue light. **A)** Average time from onset of blue light simulation to first egg laid, measured as in (73). There was no significant difference between control and *tph-1* animals, but *nlp-3* animals initiated egg laying more slowly and with higher animal-to-animal variability, and 7/20 nlp-3 animals tested failed to lay any eggs. Center line is the mean, error bars are 95% confidence intervals. n.s., no significant difference, *, $p<0.033$, ***, $p<0.001$. **B)** Cumulative distribution plot of the time to last egg laid during the 60 second blue light illumination experiment.

One Gaussian curve fit both control and *tph-1* data for last egg laid during the assay. **C) Plots of raw dating showing the time point of** each egg laid by each genotype. Each of 20 animals tested per genotype is represented by a vertical column, with each point indicating the time after the onset of blue light illumination when an individual egg was laid Empty columns indicate that no eggs were laid. Two or three horizontally adjacent points indicate eggs laid simultaneously within the 0.05 sec time resolution of our video recording.

Fig S3. Egg-laying events are associated with coordinated vm1 + vm2 Ca^{2+} transients. The number and proportion of either vm1 or vm1+vm2 coordinated transients that are associated with egg-laying events. One exceptional egg-laying event in an *egl-1* animal occurred after a vm1-only transient, while all 57 others occurred during coordinated vm1+vm2 events. Error bars are 95% confidence intervals for the results expected if an infinite sample size was used.

Figure 1

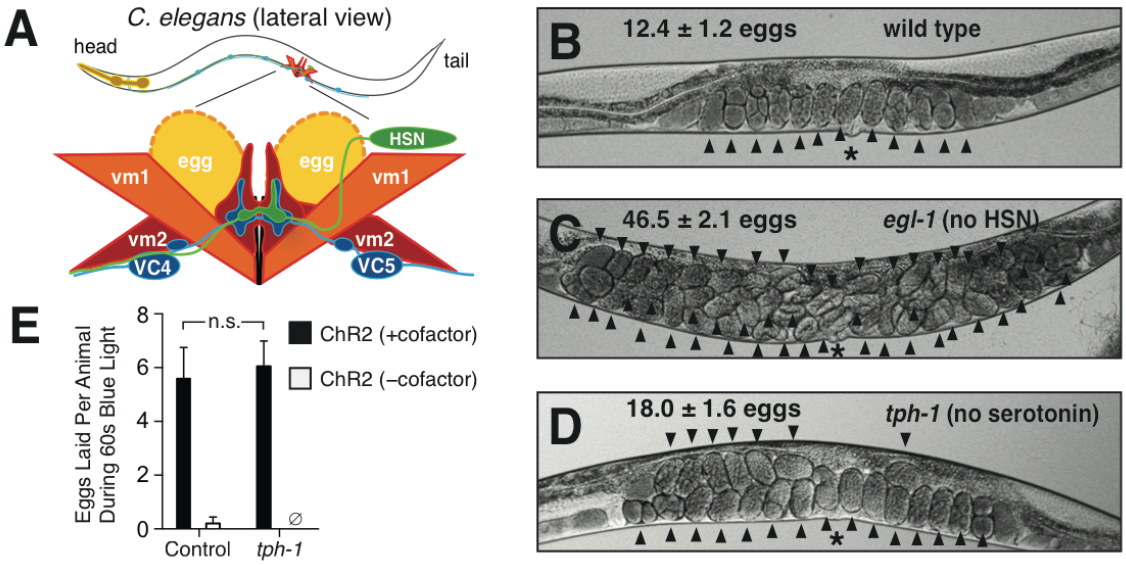

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Figure 2

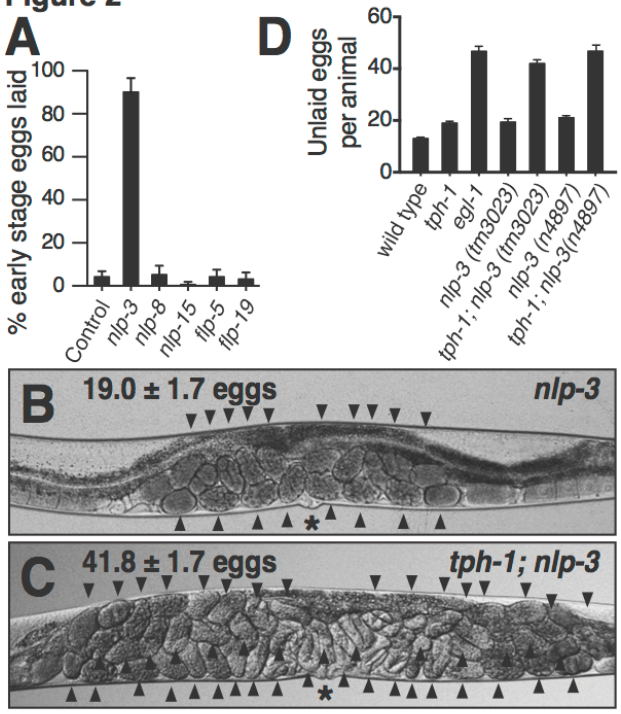

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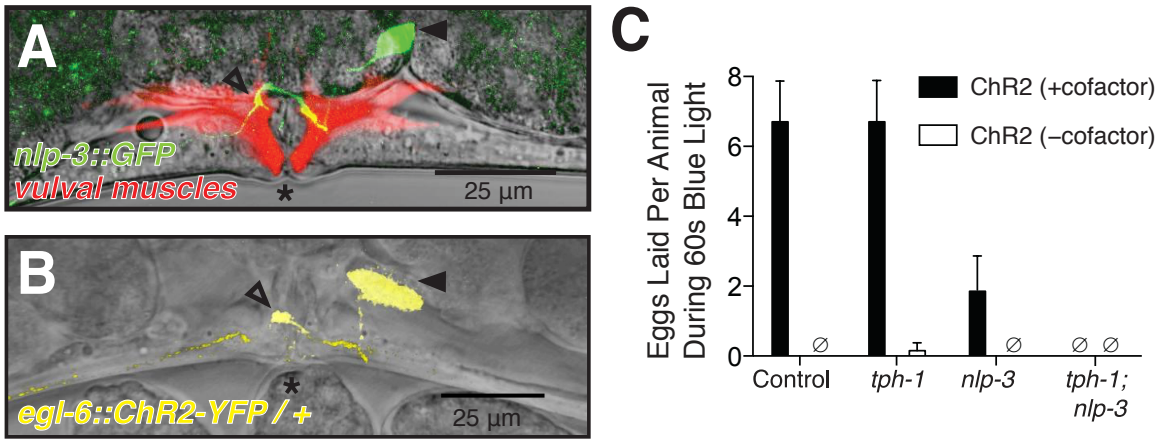
Figure 3



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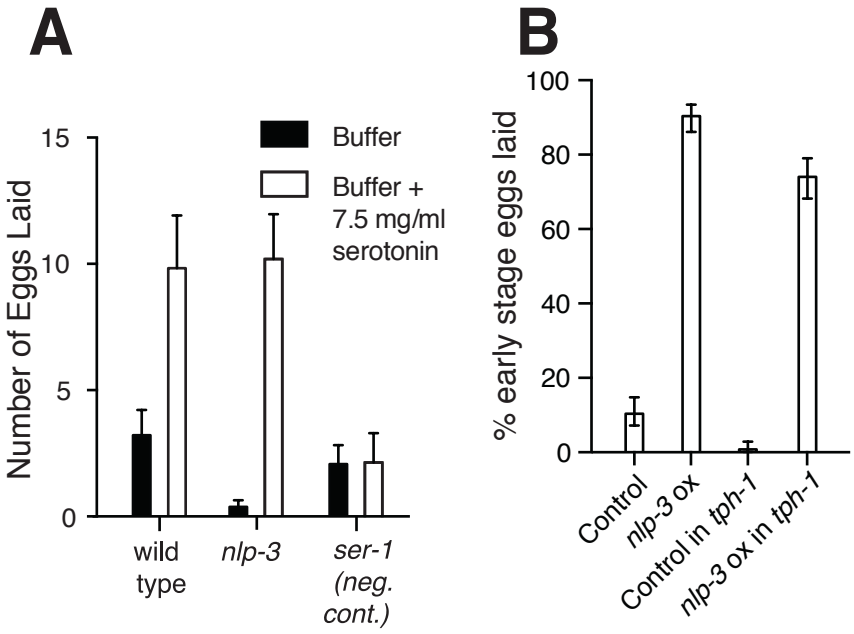

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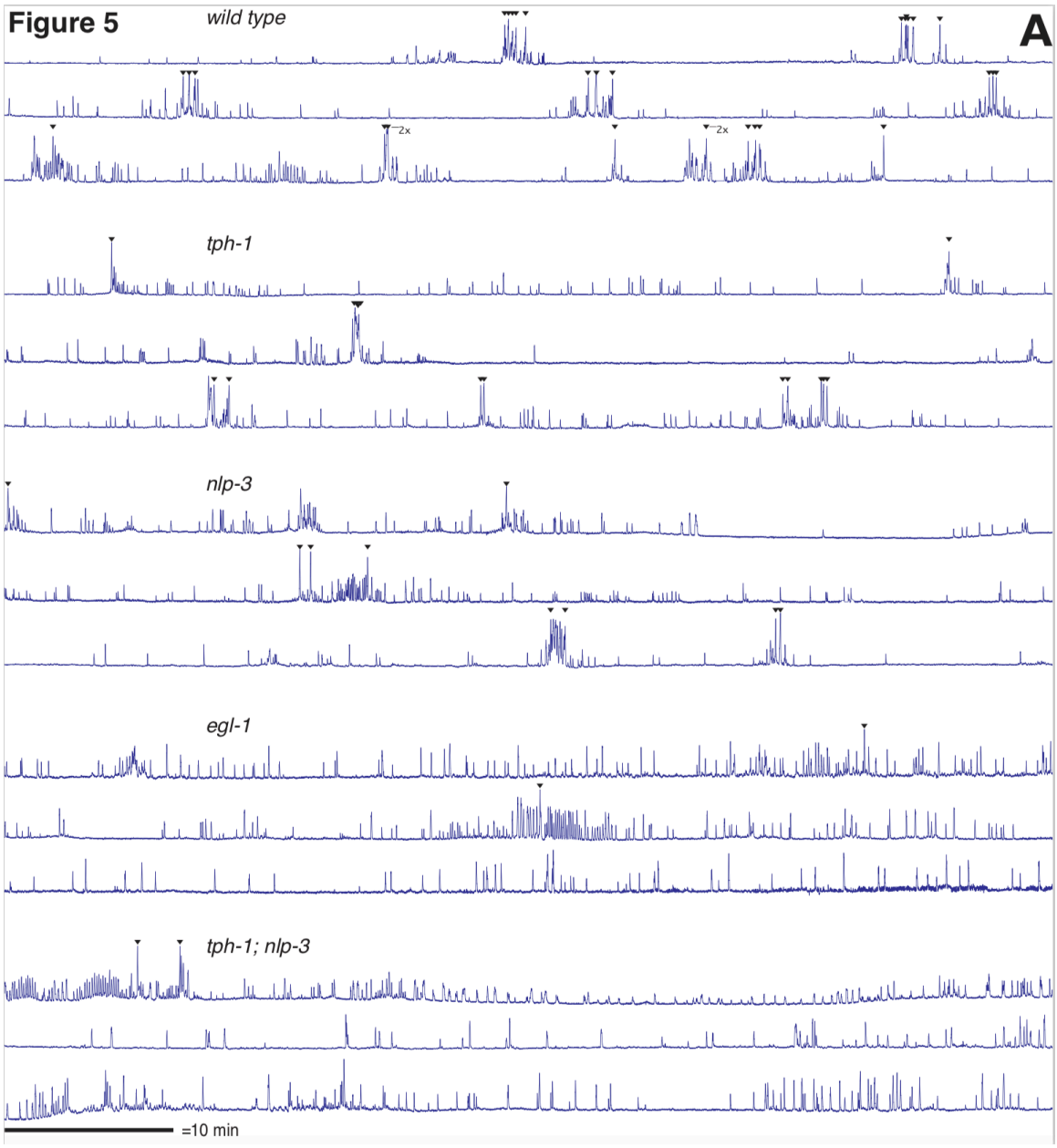

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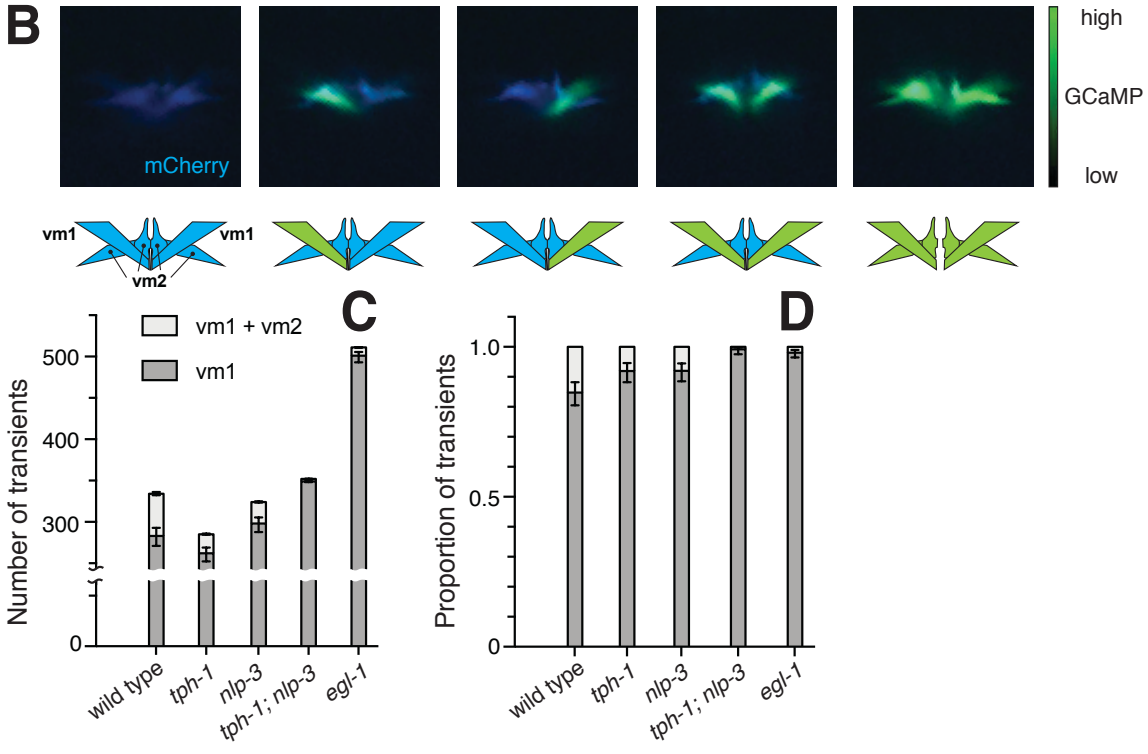

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Figure 6

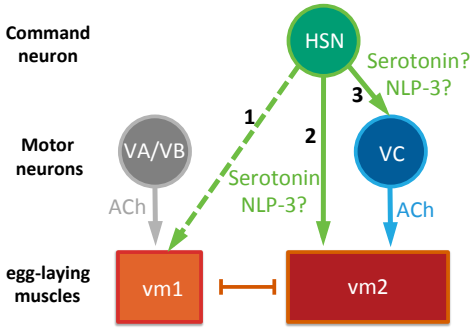
Command neuron
HSN
Serotonin?
NLP-3?
3
1
2
Motor neurons
VA/VB
VC
Serotonin
NLP-3?
ACh
ACh
egg-laying muscles
vm1
vm2

Figure S1

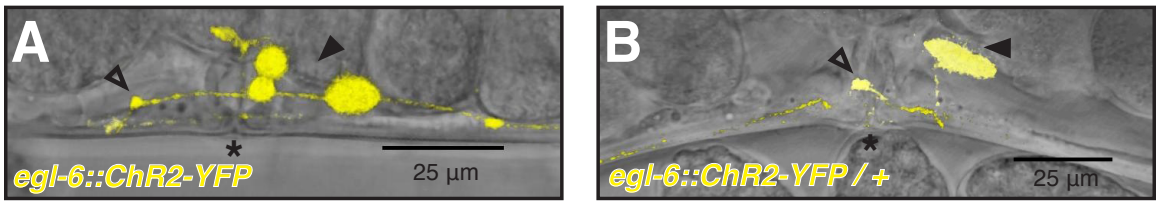

Figure S2

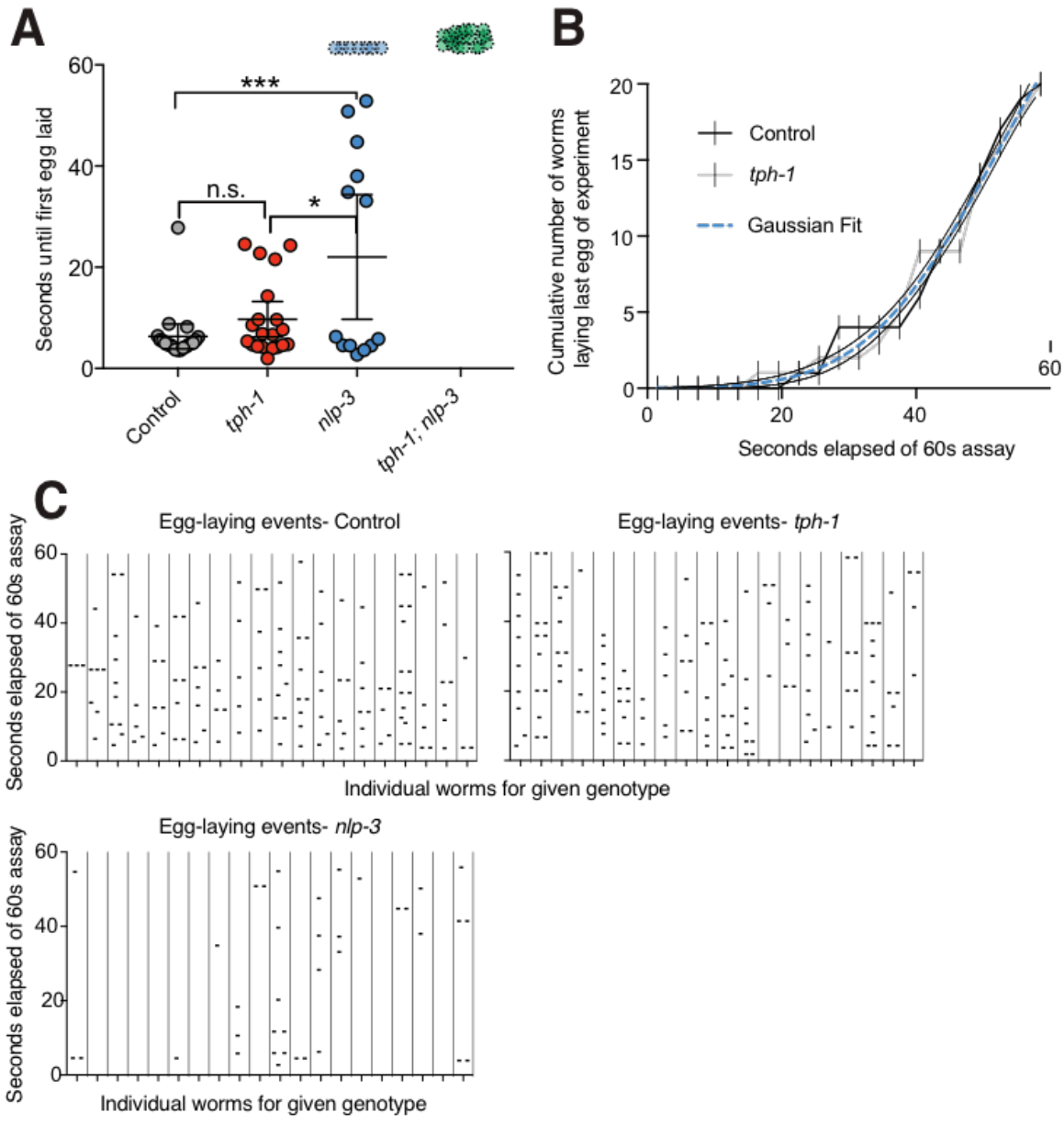

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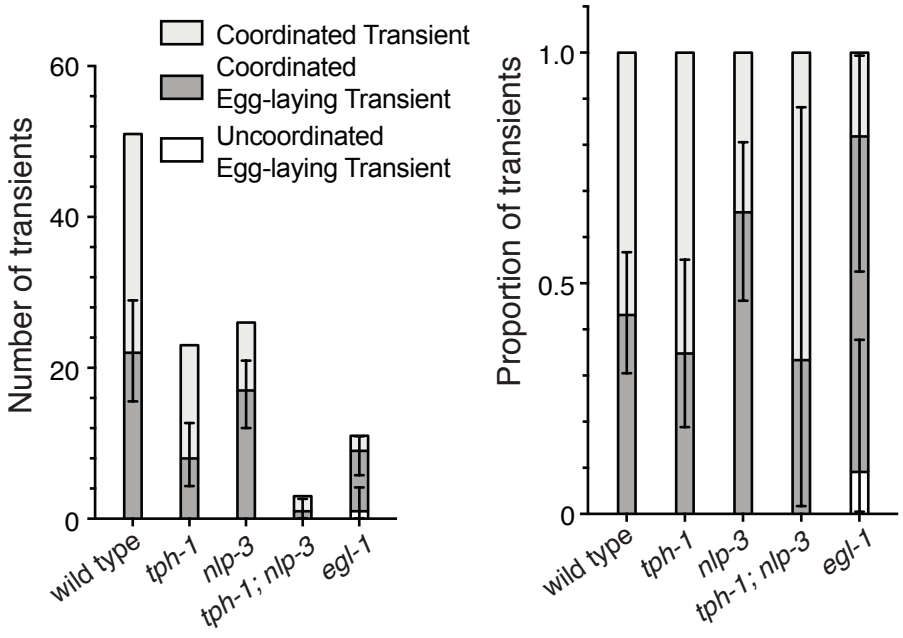
Figure S3



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