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Article

A Self-Regulating Feed-Forward Circuit Controlling *C. elegans* Egg-Laying Behavior

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Summary

Background: Egg laying in *Caenorhabditis elegans* has been well studied at the genetic and behavioral levels. However, the neural basis of egg-laying behavior is still not well understood; in particular, the roles of specific neurons and the functional nature of the synaptic connections in the egg-laying circuit remain uncharacterized.

Results: We have used in vivo neuroimaging and laser surgery to address these questions in intact, behaving animals. We have found that the HSN neurons play a central role in driving egg-laying behavior through direct excitation of the vulval muscles and VC motor neurons. The VC neurons play a dual role in the egg-laying circuit, exciting the vulval muscles while feedback-inhibiting the HSNs. Interestingly, the HSNs are active in the absence of synaptic input, suggesting that egg laying may be controlled through modulation of autonomous HSN activity. Indeed, body touch appears to inhibit egg laying, in part by interfering with HSN calcium oscillations.

Conclusions: The egg-laying motor circuit comprises a simple three-component system combining feed-forward excitation and feedback inhibition. This microcircuit motif is common in the *C. elegans* nervous system, as well as in the mammalian cortex; thus, understanding its functional properties in *C. elegans* may provide insight into its computational role in more complex brains.

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Introduction

How cells in a neural circuit interact with each other and with sensory inputs to give rise to a behavioral output is a fundamental question in neuroscience. Recent studies have focused on possible circuit mechanisms in a range of neurological abnormalities, including drug addiction and Parkinson's disease [1]; however, the complexity of the neural circuits involved in these disorders has prevented full elucidation of their underlying neural mechanisms. Several simpler neural circuits have been characterized at the cellular level, including the crustacean stomatogastric ganglion [2–4] and the forward-swimming circuits in the leech [5] and lamprey [6–8]. However, none of these model systems is genetically accessible, limiting any study of the molecular machinery that may be conserved among different species and important in human disease. Recent advances in noninvasive laser surgery and in vivo imaging of neural activity in a genetically tractable model organism, the nematode *C. elegans* [9–11], make integrated studies of the genetic, cellular, and circuit-level processes governing behavior possible.

Among the anatomically simplest neural circuits in *C. elegans* is the one involved in the control of egg-laying behavior. Individual egg-laying events occur through contraction of the vulval muscles, which causes transient opening of the vulva and allows eggs to be expelled. The anatomical structure of the egg-laying circuit [12] is relatively simple, composed of three critical cell types: the vulval muscles (vm1 and vm2) and two sets of hermaphrodite-specific motor neurons (two HSNs and six VCs) (Figure 1A and 1B). Both the HSNs and the VCs (in particular the vulval-proximal VC4 and VC5 neurons) make neuromuscular synapses with the vm2 vulval muscles, and the HSNs make many neuron-to-neuron synapses with the VCs. Laser-ablation studies have revealed that the vm2 vulval muscles are essential for egg laying (M. Stern, personal communication). In addition, ablation of the HSNs severely decreases egg laying, though egg laying still occurs at a low frequency [13, 14]. The role of the VCs is more ambiguous, given that ablation of the VCs has a weak egg-laying-constitutive phenotype on its own [15] but enhances the egg-laying defect of animals lacking the HSNs [16]. In addition, recent studies [15] found evidence that the cholinergic VC neuron may inhibit HSN activity extrasynaptically through a muscarinic acetylcholine receptor, GAR-2.

Both classes of motor neurons are notable for expression of multiple neurotransmitters and neuromodulators. The HSNs synthesize both acetylcholine [17] and serotonin [18], as well as several neuropeptides [19, 20]. The VCs are cholinergic [17] and express at least one RF-amide neuropeptide [19]. Both acetylcholine and serotonin have been found to have both stimulatory and inhibitory effects on egg laying [21], making study of them by classical genetic methods quite complicated.

Like most *C. elegans* behaviors, egg laying is regulated by a diverse set of environmental cues. Egg-laying events occur in a specific temporal pattern [16, 22], in which eggs are laid in bursts separated by inactive periods averaging 20 min in

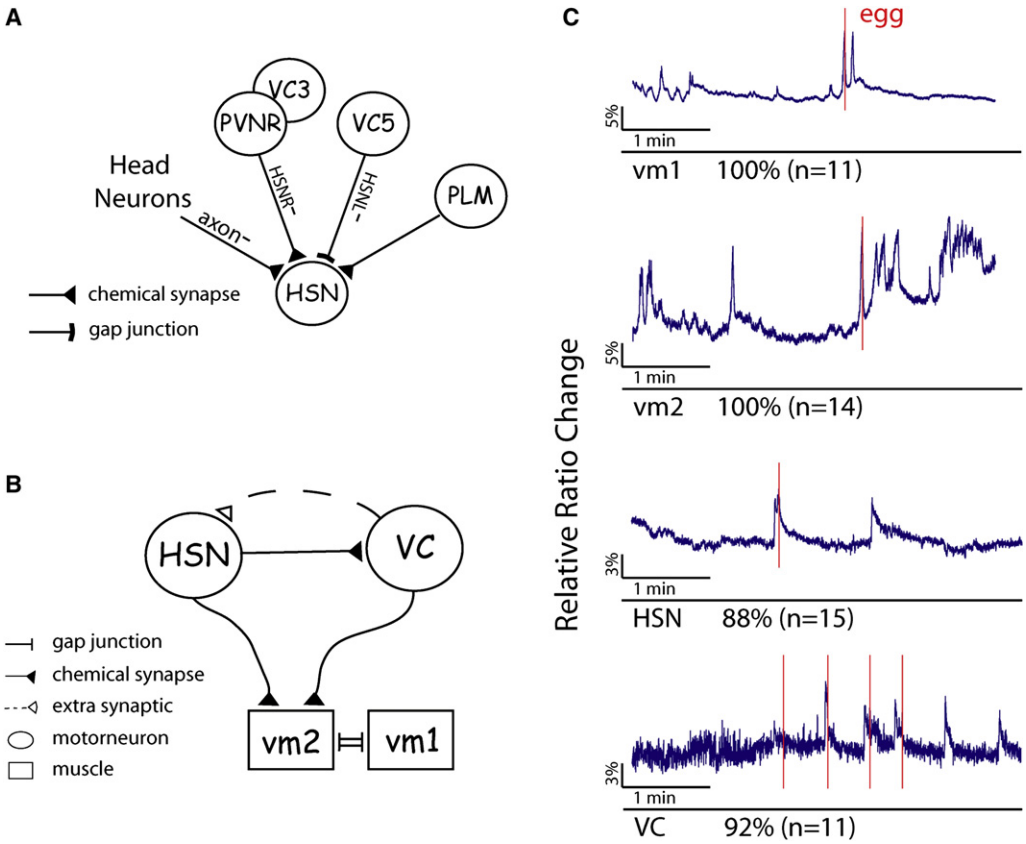

Figure 1. Egg Laying is Temporally Correlated with Motor-Neuron and Muscle Activity

(A and B) Structure of the egg-laying circuit. Synaptic-connectivity information is derived from White et al. [12].

(C) Egg-laying events are coupled with the neural activities in vm2, vm1, HSN, and VC cells. The blue lines are time-lapse cameleon signals, the relative change in its YFP/CFP ratio, which indicates the relative intracellular calcium concentrations. The percentage of active traces and the total number of recordings for the indicated cell are shown. Red lines indicate the times that egg-laying events occurred. Scale bars for cameleon signals are as indicated.

duration. When food is less abundant, these inactive periods become much longer, an effect that requires neuropeptides encoded by the *flp-1* gene [23]. Other genes affecting the modulation of egg laying by food have been identified [24, 25], but the neural basis for this regulation is not well understood. Vibrational stimulation also inhibits egg laying; this response has been shown to require the ALM and PLM touch-receptor neurons [26].

Although the putative components of the egg-laying circuitry have been identified, fundamental questions remain about how this circuit controls egg-laying behavior. For example, it is not known whether the synapses onto and between the egg-laying motor neurons are excitatory or inhibitory. Likewise, the functional significance of these connections in the generation of egg-laying behavior is not well understood. Here, we describe a functional characterization of the neural circuit regulating *C. elegans* egg-laying behavior using in vivo calcium imaging and femtosecond laser ablation. By combining visible-light microscopy with in vivo calcium imaging, we were able to correlate cellular activity with egg-laying behavior and use genetic and pharmacological manipulations to study the roles of individual neurons and neurotransmitters in the control of egg laying. In this way, we have been able to define the neural mechanisms by which the core egg-laying circuit generates behavior.

Results

Temporal Correlation between Motor-Neuron Activity and Egg-Laying Behavior

To obtain more information about the roles of individual muscles and neurons in the egg-laying circuit, we imaged their activities in behaving animals. In a previous study [27], we imaged the vulval muscle and egg-laying motor-neuron activity in intact, immobilized worms; however, the conditions under which these experiments were performed were not permissive for egg laying. We determined that if animals were immobilized on low osmolarity medium, they laid eggs efficiently under our neuroimaging conditions. To study egg-laying behavior and neural activity simultaneously, we also developed a technique of combined fluorescence and visible-light microscopy, which allowed us to identify within a single video frame when egg-laying events occurred and to correlate these events with calcium transients detected by cameleon expressed in egg-laying neurons or muscle cells (Figure 1C).

We first investigated how egg-laying events correlated with the activities of each component of the egg-laying circuit. We observed that all egg-laying events occurred rapidly, within a single 100 ms frame (Figure S1, available online); thus, for our purposes, each egg-laying event could be regarded as an instantaneous event. In these experiments, we observed that egg-laying

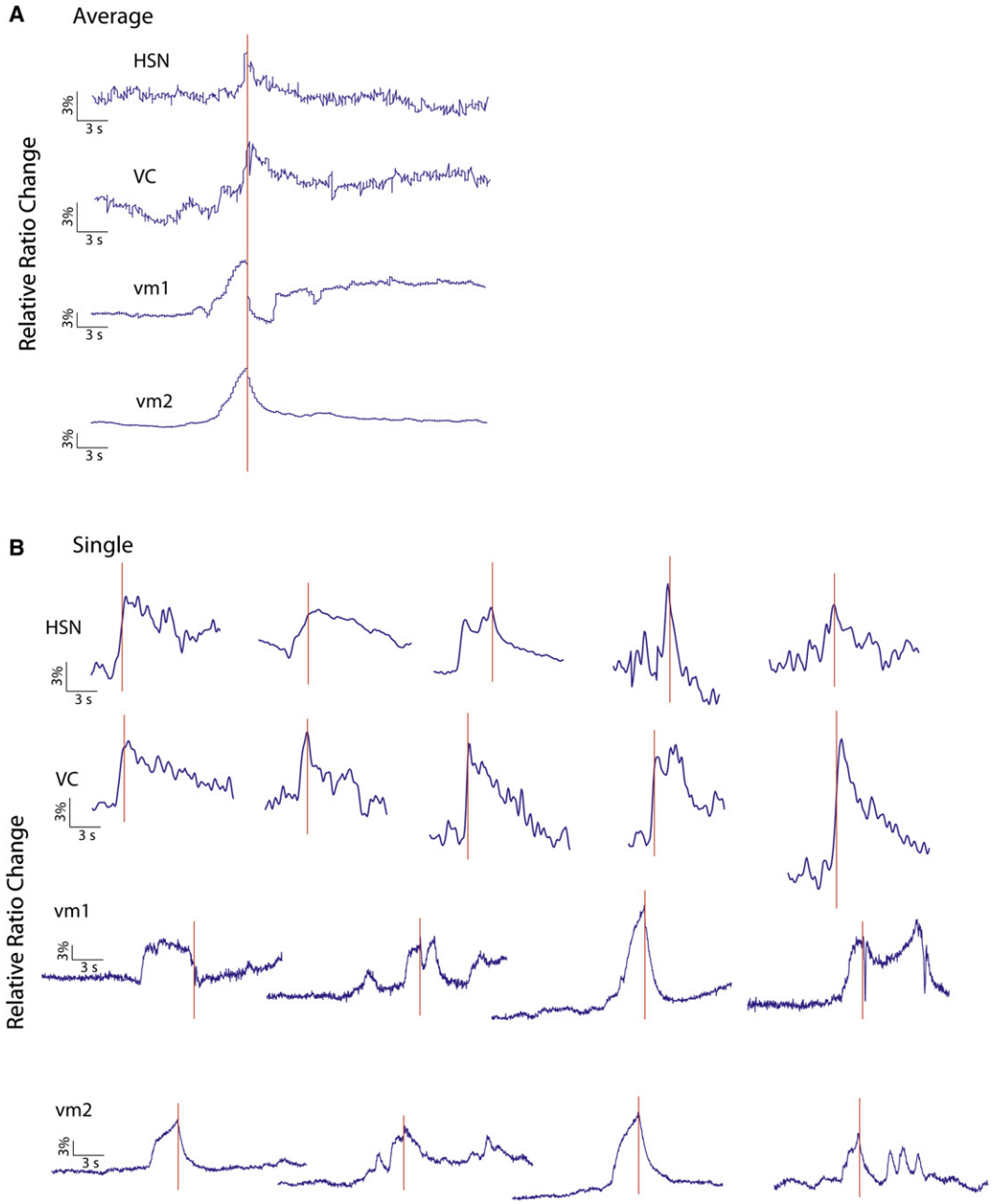

Figure 2. Timing of Motor-Neuron and Vulval-Muscle Calcium Transients Relative to Egg Laying

(A) Shown are averaged calcium recordings of HSN, VC, vm1, and vm2 cells phase-locked to egg-laying events. The onset of calcium transients in all cells clearly precedes egg laying. Interestingly, vulval-muscle calcium influx often occurs before the neuronal calcium transient, suggesting that although neuronal activity may be important for evoking egg laying, it is not essential for vulval-muscle calcium transients.

(B) Sample individual calcium traces around egg-laying events. In vulval-muscle cells, an egg-laying event can happen during a single calcium spike (3rd in the vm1 and vm2 rows) or any time during a prolonged excitation (1st, 2nd, and 4th in the rows). In contrast, egg-laying events tend to be associated with single spikes in the HSN and VC motor neurons. Scale bars are indicated.

events were temporally correlated with Ca^{2+} spikes in both vm1 (n = 11/11 egg-laying events) and vm2 (n = 14/14 egg-laying events) vulval muscles (Figures 1 and 2). Most egg-laying events occurred after the onset of a calcium surge but before the calcium trace began returning to baseline. Calcium spikes in the vulval muscles can occur singly or in clusters (Figure 2), and egg laying can coincide either with single calcium spikes or at any time during cluster-spiking activity (Figure 2). Although egg laying was never observed without a calcium spike in vulval muscles, many vulval-muscle calcium transients that were not associated with egg-laying events or even detectable muscle contraction were observed (data not shown). There was no obvious difference in the dynamics of calcium spikes that successfully resulted in egg laying compared to those that did not, and the functional significance of vulval-muscle calcium transients not associated with egg laying is not known (see Discussion).

In addition, we also observed a strong temporal correlation between egg-laying events and calcium transients in both the

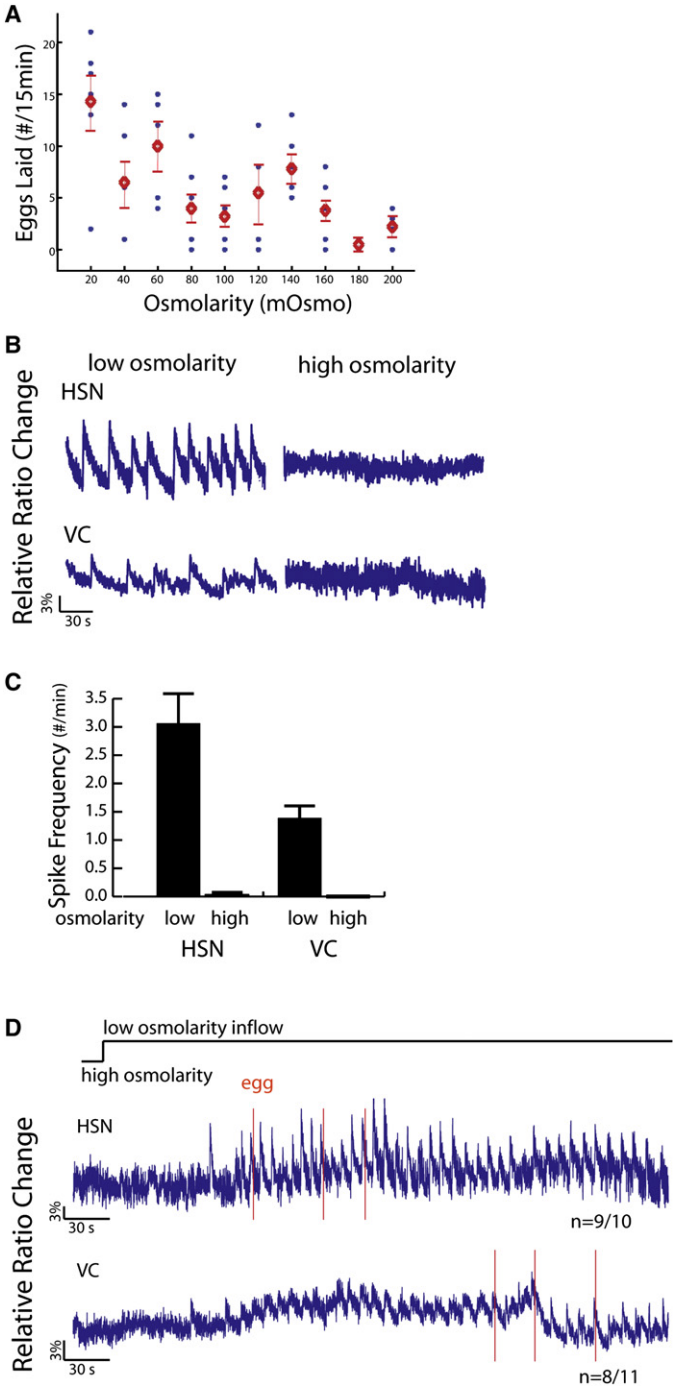

Figure 3. Osmolarity Affects Egg-Laying Behavior and Neural Activity

(A) Egg-laying rates in different osmolarity buffers made from sucrose. Each blue dot represents one worm; the red circles indicate the mean number of eggs laid. Standard errors of the mean (SEM) are shown as extended red lines.

(B and C) Responses of HSN and VC neurons to osmolarity. (B) shows representative traces of HSN and VC calcium of WT worms in extremely high (200 mOsm) or low (0 mOsm) osmolarity conditions, as measured by cameleon. In (C), the mean and the SEM of spike frequencies of multiply imaged animals are plotted for illustrating the HSN and VC responses to different osmolarities.

(D) The HSN and VC responses to acute low-osmotic shock. A low-osmolarity inflow was opened at 30 s after recording began. N represents the number of worms that showed similar responses, as illustrated here. All blue traces are cameleon signals representing relative intracellular calcium concentrations. Scale bars are as indicated.

HSN (n = 15/17 egg-laying events) and the VC (n = 11/12 egg-laying events) motor neurons (Figures 1 and 2). In general, neuronal calcium transients were more predictive of egg-laying events than were those in vulval muscles; for example, under moderately permissive conditions (150 mOsm), only 10% of vulval-muscle calcium transients were associated with egg-laying events, whereas the majority of calcium spikes in the HSN or the VC were associated with egg laying (52% and 68%, respectively; data not shown). The onset of the neuronal calcium transient preceded the egg-laying event by an average of approximately 1 s for both motor-neuron classes (Figure 2), suggesting that neural activity in both the HSNs and the VCs triggers egg-laying events, rather than the converse.

To further investigate the causal links between motor-neuron activity and egg laying, we imaged HSN and VC calcium transients in an *egl-15(n484)* mutant, in which vulval muscles fail to properly develop due to a defect in sex-myoblast migration. In these animals, both the HSN and the VC neurons still showed significant calcium activity under low osmolarity conditions (Figure S2), indicating that muscle contraction is not required for activation of either neuron class. Together, these results suggest that one or both of these motor-neuron classes, as well as the vulval muscles, are important for the generation of egg-laying events, most likely through fast excitatory neurotransmission.

Effects of Osmolarity on Egg Laying

We investigated in more detail how osmolarity affected egg-laying behavior and the egg-laying circuitry. By varying the concentrations of ions in the buffer individually and also in combinations, we found that it was the overall osmolarity of the solution, rather than the concentration of a particular ion, that affects the rate of egg laying (data not shown). Therefore, we tested dose responses of egg-laying behavior to different osmotic solutions made from sucrose (Figure 3A). These results showed a gradual, dose-dependent decrease in the egg-laying rate as the external osmolarity increased from 20 to 200 mOsm (M9 is 320 mOsm, and NGM plates are around 150–180 mOsm). When we performed calcium-imaging experiments in high or low osmolarity, we observed that in the HSN and the VC neurons, the calcium-spike frequencies were, likewise, high in low-osmolarity conditions and silent in high-osmolarity conditions (Figures 3B and 3C), indicating that osmolarity affects motor-neuron activity. We found that at low osmolarity, both HSN and VC neurons exhibited calcium spikes in a strikingly rhythmic pattern, with periods ranging from 6 to 12 s. We also analyzed the response of the cells in the circuit to an acute osmolarity change during one continuous 10 min Ca^{2+}-imaging recording and observed an activation of HSN and VC activity following the shift to low osmolarity (Figure 3D). Together, these results indicate that the regulatory effect of osmolarity on egg laying involves the control of motor-neuron activity.

We also analyzed the effect of osmolarity on vulval-muscle activity. Like the HSNs and the VCs, the vm1 and vm2 muscles showed higher activity in low-osmolarity media (Figures S3A and S3B), as expected from the higher rate of egg laying under those conditions. However, even in high-osmolarity conditions, under which the motor neurons were nearly silent, we observed significant activity in the vulval muscles (Figure S3).

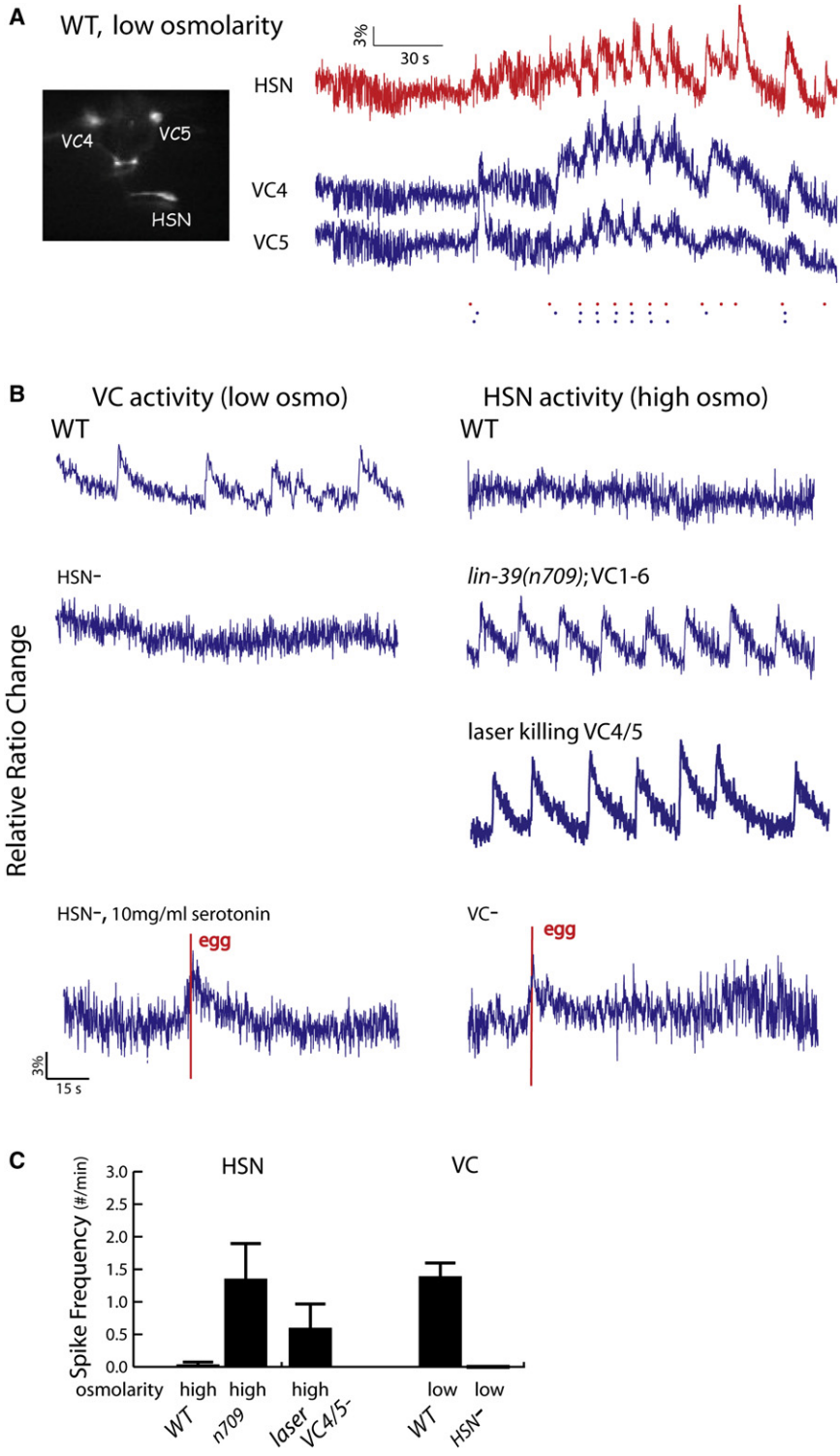

Figure 4. Functional Connectivity between the HSN and VC Motor Neurons

(A) Both the HSN and VC activities are simultaneously captured with the help of a worm-rotating device [28]. Shown are intracellular calcium traces for HSN (red trace) and for VC4 and VC5 (blue traces), as measured by cameleon. Dots indicate the correlated spikes.

(B) Effects of genetic ablations of HSN or VC neurons on the activity of the remaining motor-neuron class. HSN- refers to animals carrying an *egl-1* mutation, which causes inappropriate HSN apoptosis in hermaphrodites. The *lin-39* mutation leads to the absence of all VC neurons; the vulval-proximal VCs (VC4 and VC5) were eliminated by laser ablation.

(C) Histogram showing the mean and SEM of the spike frequencies in WT, HSN-, and VC- animals, as described in (B). Loss of VC neurons leads to significantly higher constitutive activity in the HSNs, ($p < 0.5$ for *lin-39* and for VC4/5 ablation; Mann-Whitney rank test), indicating that the VCs normally inhibit the HSNs. Loss of HSN neurons leads to significantly reduced VC activity ($p < 0.05$; Mann-Whitney rank test), indicating that the HSNs normally excite the VCs.

Interestingly, there was a significant difference in the degree to which the activity of individual vm2 muscle cells was correlated under different osmolarity conditions. In low-osmolarity solution, the vm2 showed a 52% within-type correlation ($p < 0.001$) and the vm1s showed an 80% within-type correlation ($p < 0.001$); in contrast, in high-osmolarity solution, the vm2s showed a 15% correlation ($p > 0.05$) and the vm1s showed a 59% correlation ($p < 0.001$). The differences in the vm2s' within-type correlations suggest that the neural activity specifically observed under low-osmolarity conditions may serve to coordinate vm2 activity. In contrast, this neural activity may be less important for preserving within-type correlation of the vm1s, which are connected to each other through gap junctions but do not receive direct synaptic input [12].

The Functional Nature of Connections between the HSNs and the VCs

Because HSN and VC activities were both temporally correlated with egg-laying behavior, and because the HSNs direct synaptic output to the VCs, we reasoned that HSN activity might likewise be correlated with VC activity. To investigate this possibility directly, we used a slide-rotating device [28] to bring both HSNs and VCs into the same focal plane and image their activity simultaneously. HSN and VC showed a 70% ($p < 0.001$) correlation in the timing of their calcium transients (Figure 4A), suggesting that the HSNs might excite the VCs to drive their activity or, alternatively, that the VCs might excite the HSNs. With our temporal resolution of 0.1 s, we were unable to resolve the temporal order of HSN and VC spikes; the onset of HSN and VC spikes typically occurred simultaneously within one or two frames.

To investigate causal relationships between HSN and VC activities, we investigated how eliminating one class of motor neuron affected the activity of the other. We first analyzed *egl-1(n986)* mutants, in which the HSNs specifically and inappropriately undergo programmed cell death in hermaphrodites [29]. We observed that the VC neurons in *egl-1* worms were largely inactive, even under low-osmolarity conditions, when

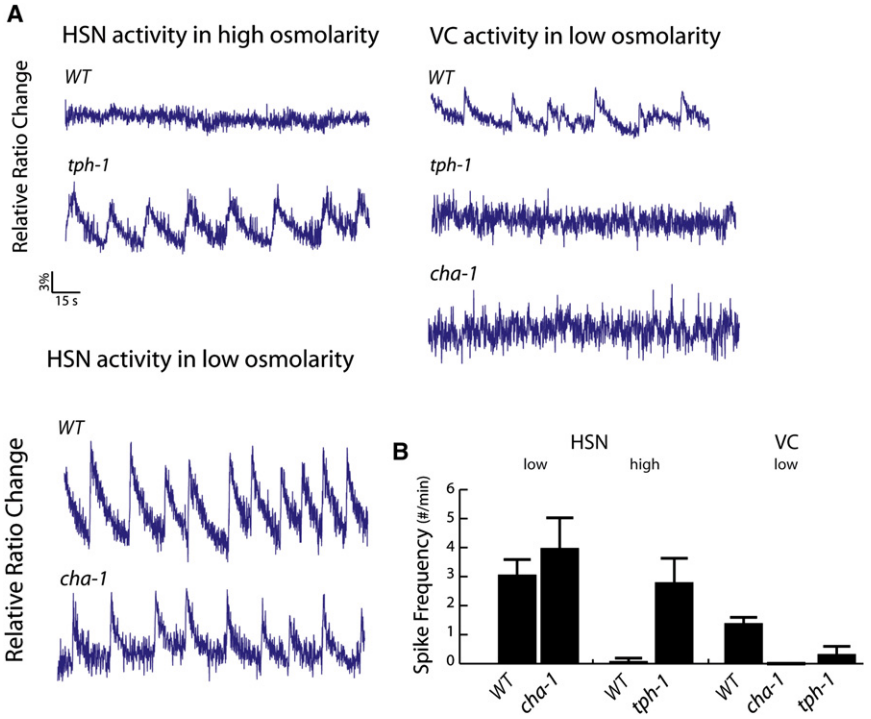

Figure 5. Effect of Serotonin and Acetylcholine on HSN and VC Activity

(A) Representative calcium traces of HSN and VC neuronal cell bodies in *tph-1* mutants (defective in serotonin synthesis) and *cha-1* mutants (conditionally defective in acetylcholine synthesis). *cha-1* animals were shifted to the restrictive temperature and recorded 7 min later.

(B) Histogram of calcium-spike frequencies (mean/SEM) in HSN and VC neurons. Serotonin-deficient animals showed elevated HSN activity and silenced VC activity, and acetylcholine-deficient animals showed reduced VC activity (all $p < 0.05$, according to the Mann-Whitney rank test).

the VCs are normally hyperactive (Figures 3B and 3C), suggesting that the HSNs are required for efficient activation of the VCs. We also analyzed *lin-39(n709)* mutant animals [30], in which the VC neurons undergo inappropriate cell death. Surprisingly, in these animals, the HSN neurons showed increased activity compared to those of wild-type (WT) animals, even in high-osmolarity conditions, when the HSNs are normally hypoactive (Figures 4B and 4C). We also found that laser ablation of VC4 and VC5 neurons caused hyperactivity in HSN calcium dynamics (Figures 4B and 4C). These data suggest that the VCs inhibit, rather than excite, the HSNs.

The *egl-1* and *lin-39* mutant strains also allowed us to determine whether neuromuscular excitation in the egg-laying circuit is mediated by the HSNs, the VCs, or both. We observed that in *lin-39* mutant animals, which lack VC neurons, egg-laying events were still strongly correlated with HSN activity (Figure 3B), suggesting that the synapses from HSN to vulval muscles are excitatory and capable of inducing egg-laying events. Performing the analogous experiment in *egl-1* worms was difficult, because these animals have severely defective egg-laying behavior and nearly silent VCs under all conditions. To surmount this problem, we imaged VCs in *egl-1* worms in the presence of exogenous serotonin, which is known to stimulate egg laying in the absence of the HSN neurons. In the presence of serotonin, VC activity was partially restored, and this activity was temporally coupled with egg-laying events ($p < 0.001$), with egg laying following the onset of the calcium transient by an average of 1.4 s (Figure 4B). Together with the previous finding that exogenous serotonin does not stimulate egg-laying events if both the HSNs and the VCs are absent [16], our results suggest that the VCs directly excite the vulval muscles to induce egg-laying events.

Effects of Serotonin and Acetylcholine on the Egg-Laying Circuit

Next, we investigated the roles of serotonin and acetylcholine, two neurotransmitters that are expressed in the HSN and VC motor neurons. Previous neuroimaging studies indicated that exogenous serotonin inhibits HSN activity [27]. To further investigate the role of serotonin, we analyzed a mutant defective in the essential serotonin biosynthetic gene *tph-1*. We observed that *tph-1(mg280)* null-mutant worms (defective in tryptophan hydroxylase) [31] showed hyperactivity in the HSN neurons (Figure 5A), which correlated with egg-laying events (data not shown), and reduced activity in VCs (Figure 5A and 5B). Together with our previous finding (Figure 4B) that exogenous serotonin stimulates VC activity in HSN-deficient *egl-1* mutants, these results support the hypothesis that serotonin directly enhances VC activity. Serotonin may, therefore, inhibit HSN activity either directly or, alternatively, indirectly by promoting the VCs' inhibitory effect on HSN.

To investigate the role of acetylcholine in the egg-laying circuit, we used a temperature-sensitive allele of the choline acetyltransferase gene, *cha-1(y226)*, which upon transfer from 15°C to 21°C rapidly undergoes a coiled paralysis characteristic of a strong defect in cholinergic neurotransmission. When we imaged neural activities in the egg-laying circuit approximately 7 min after a temperature shift from 15°C to 21°C, we observed significant activity in the HSNs but no activity in the VCs (Figures 5A and 5B). Moreover, under these conditions, egg-laying behavior was strongly reduced (0.5 ± 0.4 eggs per 15 min, compared to 12.8 ± 1.3 for WT). These results suggest that acetylcholine is necessary for excitation of the VCs and triggering of egg-laying events by the HSNs. Interestingly, calcium transients were still observed in both the vm1 and vm2 vulval muscles in temperature-shifted *cha-1* mutants. In the vm1 cells, these transients were frequent and highly synchronous (Figures S4A and S4C), whereas in the vm2s, we found lower-frequency transients that were, nevertheless, highly synchronous (Figures S4B and S4C). It is worth noting that these ACh-independent calcium transients do not lead to egg laying. Together, these results suggest that ACh and 5-HT may function as cotransmitters at egg-laying neuromuscular synapses, with 5-HT acting through a GPCR to modulate muscle activity and ACh acting through a nicotinic receptor to evoke individual egg-laying events.

Gentle Body Touch Transiently Inhibits HSN Activity

We were then interested in investigating the neural mechanisms by which egg-laying behavior might be controlled by sensory stimuli. It was shown previously that mechanical

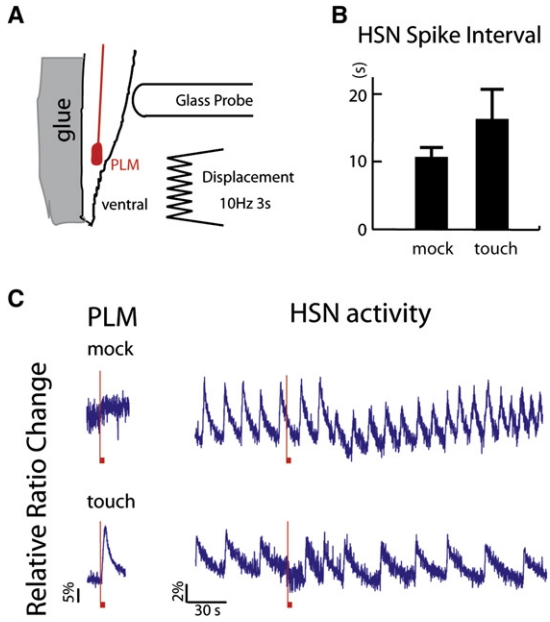

Figure 6. The PLM Posterior Mechanoreceptor Neuron Transiently Inhibits HSN

(A) Diagram of the protocol for mechanical stimulation of PLM mechanoreceptor neurons. During the stimulus, the probe was moved against the animal's skin in the indicated position for 3 s.

(B) Average interval between stimulation (or mock stimulation) and the next calcium spike in the HSN. The time lag from the stimulus to the first HSN calcium spike was significantly longer in stimulated animals than in mock-stimulated animals, according to the Mann-Whitney rank sum test ($p < 0.05$).

(C) Representative traces of cell-body calcium for the PLM and HSN neurons during the mechanosensory-stimulation protocol. The red vertical bars indicate the beginning of the stimulations, and the boxes at the bottom indicate the time period of the stimulation.

vibrations, sensed by the body-touch mechanoreceptor neurons, inhibit egg laying [26]. Given that one of these neurons, the posterior touch receptor PLM, directly synapses onto the HSN cell body [12], we wondered whether activation of PLM might affect HSN activity. We stimulated the PLM by applying gentle touch stimulation to the posterior body region, which is in the receptive field of the PLM but not the other body-touch receptors [10], and imaged HSN activity (Figure 6A). As expected, PLM was immediately activated upon mechanical stimulation (Figure 6C). In contrast, although HSN showed significant activity before and after touch stimulation, closer analysis of the HSN recording revealed that there was a small but significant increase in the duration of the interval between calcium transients containing the mechanical stimulation compared to mock-stimulated animals ($p < 0.05$) (Figure 6B). These results indicate that the PLM transiently inhibits HSN activity, an effect that may contribute to the inhibition of egg-laying behavior by gentle body touch.

HSN Activity is Not Dependent on Presynaptic Excitation

The finding that mechanosensory inputs negatively regulate egg laying by inhibiting the HSNs raised the general question of how HSN activity is generated and controlled. In principle, the HSNs could be driven by excitatory inputs from presynaptic partners or from extrasynaptic neuromodulation. Alternatively, the HSNs could be autonomously active but modulated by neuromodulators and sensory cues. To investigate these possibilities, we first focused on whether the HSNs required extrinsic synaptic or extrasynaptic input for their activity. Most morphologically identified synapses to HSN occur within the nerve ring, a few (from PVNR, VC3, and VC5) occur on the neuronal processes near the vulva, and single synapses from mechanosensory neurons are made on the HSN cell body (Figures 1A and 7A) [12].

To determine the effect of the nerve-ring input on HSN activity, we severed the HSN axons using femtosecond laser ablation [11, 32, 33]. We observed that animals ablated between the nerve ring and the vulva (indicated as the point "a" in Figure 7A) showed mostly normal egg-laying behavior (Figure 7B), suggesting that the HSNs were still active in these animals. We also ablated either the HSNL cell body, which should eliminate the input from PVNR and VC3, or the HSNR cell body, which should eliminate the input from VC5. These ablated animals laid eggs normally, suggesting that neither of the PVNL, VC3, or VC5 neurons was necessary to drive HSN activity (Figure 7B). Finally, we directly imaged HSN activity in animals with severed axons. For these experiments, the HSN axons of animals expressing cameleon in the HSNs were cut between the cell body and the vulva (indicated as point "b" in Figure 7A), eliminating all synapses to and from the HSNs except for the PLM synapses (already inferred to be inhibitory). When calcium transients in the HSN cell body were imaged under permissive low-osmolarity conditions, we observed normal oscillatory activity patterns in both operated and intact animals (Figures 7C and 7E). Taken together, these results suggest that HSN activity does not require activation by excitatory presynaptic input.

Neuropeptides Are Not Essential for HSN Activity

Next, we investigated whether HSN activity is dependent on neuroendocrine or extrasynaptic signaling. *C. elegans* uses at least four monoamine neuromodulators (serotonin, dopamine, octopamine, and tyramine), as well as diverse neuropeptides [20, 34–36]. To assess the possible roles of these molecules in HSN activation, we first imaged HSN calcium transients in an *unc-31* null mutant, which is specifically defective in dense-core vesicle release [37, 38]. The HSNs showed robust and rhythmic activity in these mutant animals (Figures 7D and 7E), indicating that neuropeptides and monoamines are not essential for HSN activity. We also analyzed a deletion allele of *egl-3*, which encodes a proprotein convertase required for the processing of all known *C. elegans* neuropeptides [39–41], and a null allele of *egl-21*, a carboxypeptidase also known to be important for neuropeptide processing [42]. Again, HSN cell bodies in these animals showed robust and rhythmic calcium transients (Figures 7D and 7E), suggesting that neuropeptides are not necessary for HSN activity. We had shown previously that a severe loss-of-function allele of *unc-13*, functioning in fast synaptic vesicle release, likewise does not compromise HSN activity [43]. Taken together, these results suggest that HSN activity does not require direct neuroendocrine or extrasynaptic input and are consistent with the possibility that the HSNs are autonomously active.

Discussion

Our imaging studies have allowed us to understand more precisely the roles of specific neurons in the egg-laying circuit. As expected, the neurons most critically important for egg laying are the HSN motor neurons. We observed that HSN activity is highly correlated with egg laying, even when the VCs have been eliminated, indicating that HSN activity directly evokes individual egg-laying events. The HSNs are also highly

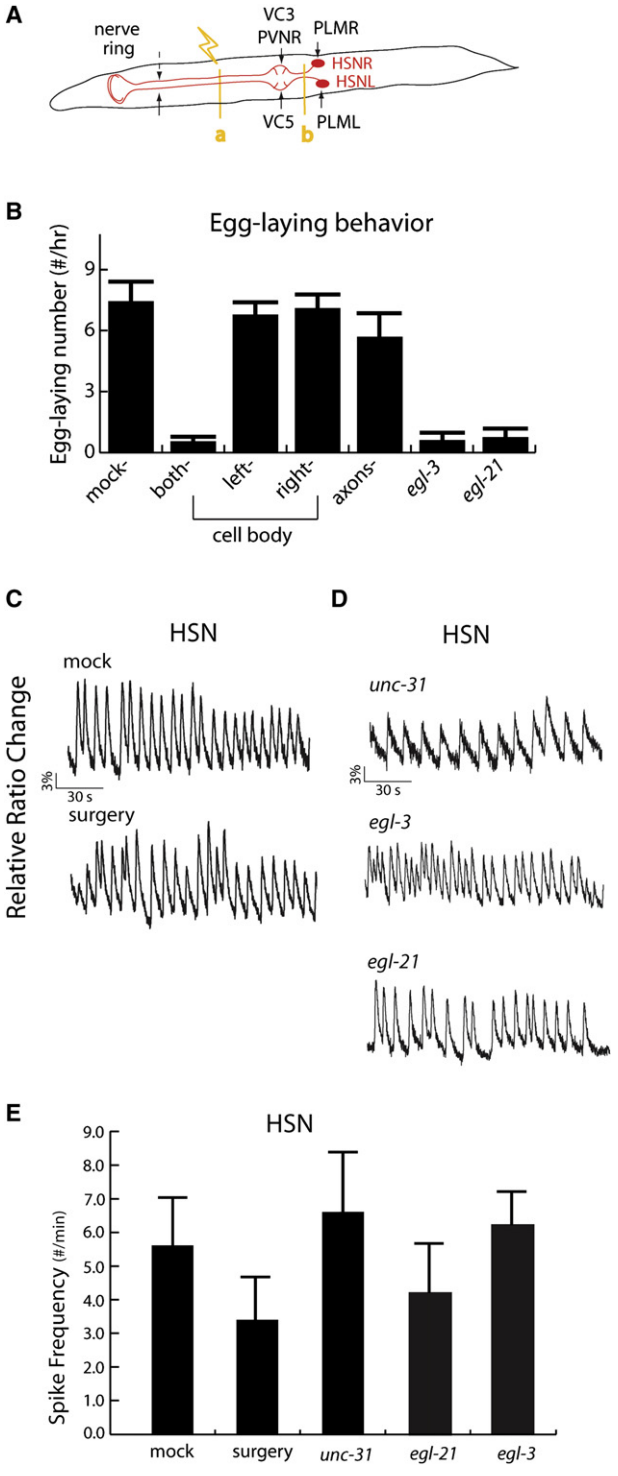

Figure 7. Independence of HSN Activity from Synaptic and Neuropeptide Inputs

(A) An illustration of synaptic inputs on the HSN cell bodies and axons, as described by White et al. [12]. The targets for femtosecond laser axotomy (a and b) are indicated by the yellow lines.

(B) Egg-laying behavior of laser-operated worms. Elimination of HSN inputs from the nerve ring (cutting at position a) does not cause a detectable egg-laying defect, consistent with the hypothesis that HSN is autonomously active. HSN doubly ablated animals and *egl-3* and *egl-21* mutants laid significantly fewer eggs than did mock-ablated animals ($p < 0.05$; Mann-Whitney rank test).

(C) Calcium transients in the HSN cell bodies of axotomized and mock-ablated animals. Axons were severed near the cell body (position b), and emission ratios from cameleon expressed in the HSN cell body were measured under low-osmolarity conditions.

(D) Calcium transients in the HSN cell bodies of *unc-31(e928)*, *egl-3(ok979)*, and *egl-21(n476)* mutant worms. Animals were imaged under permissive, low-osmolarity conditions. Although significant variability was observed in all types, mutant neurons showed oscillation frequencies comparable to or sometimes higher than WT neurons.

(E) Average spike frequencies in the HSN for axotomized and mutant animals.

correlated in activity with the VCs and are necessary for VC activation. Thus, the HSNs appear to activate the egg-laying muscles both directly and indirectly, through the VCs.

Calcium spikes in the motor neurons and (perhaps surprisingly) the vulval muscles do not necessarily correlate with egg-laying events. It is not clear from vulval-muscle calcium imaging what distinguishes transients that lead to egg laying from those that do not. Interestingly, interfering with acetylcholine neurotransmission appears to block egg laying but does not completely suppress vulval-muscle calcium transients. This, along with the correlation between motor-neuron spikes and egg laying, is consistent with the possibility that egg-laying events require cholinergic neurotransmission from the motor neurons. Previous behavioral analyses have suggested, likewise, that ACh is involved in inducing individual egg-laying events during periods of active egg laying [16]. Potentially, cholinergic excitation might be necessary for coordination of simultaneous excitation of all eight vulval muscles, which may be required for a successful egg-laying event.

Interestingly, HSNs appear to exhibit spontaneous activity that does not require extrinsic neuronal input. This suggests that the control of egg laying is accomplished by modulation of the intrinsic activity of the HSNs. In fact, we observed that touch stimuli inhibit egg laying by transiently suppressing the spontaneous activity of the HSN neurons. This may represent a general mechanism for regulating egg laying in *C. elegans*. For example, EGL-47 encodes a G-protein-coupled receptor that negatively regulates egg laying through control of HSN activity [44]. Although the EGL-47 ligand is not known, it is thought to be a neuropeptide that might act either synaptically or humorally to control egg laying in response to sensory or homeostatic cues. In principle, other peptide or monoamine neuromodulators [24, 42, 45] might also exert their effects on egg laying by regulating the activity of the HSNs.

Neuropeptides and monoamine neuromodulators may also act directly on the vulval muscles. Consistent with this possibility, *egl-3* and *egl-21* mutants, although strongly egg-laying defective, exhibited normal patterns of HSN activity. This suggests that one or more neuropeptides may be required for potentiation of the HSNs' and the VCs' ability to evoke egg-laying events. The mode of regulation that we have observed in the egg-laying circuit, in which hormones and neuromodulators modify the activity of an autonomously active motor circuit, is conceptually and mechanistically similar to the control of involuntary motor programs by the autonomic nervous system in mammals. Thus, egg laying in *C. elegans* may serve as a useful model for understanding how neural circuits of this type respond to environmental context and behavioral state.

Our results also provide information about the role of the VC motor neurons in the egg-laying circuit. We found that calcium transients in the VCs were dependent on the HSNs and were temporally correlated with HSN calcium transients. These findings lead us to infer that the HSNs excite the VCs.

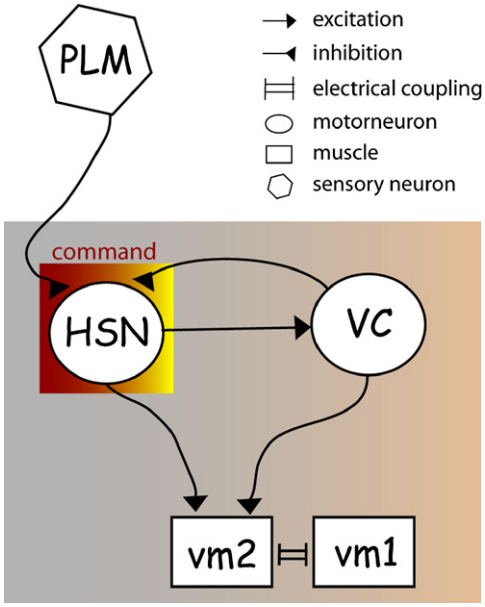

Figure 8. Functional Connectivity in the Egg-Laying Motor Circuit

Shown are the excitatory and inhibitory connections inferred from these studies. Both the HSNs and VCs excite the vulval muscles, and the HSNs excite the VCs, generating a feed-forward circuit exciting the vulval muscles. The VCs also inhibit the HSNs, creating a negative feedback loop between the HSNs and the VCs. The PLMs inhibit the HSNs to mediate touch inhibition of egg laying.

Additionally, VC calcium transients are temporally correlated with egg-laying events, even in the absence of the HSNs; thus, the VCs appear to play a redundant role in exciting the vulval muscles. These data help explain the apparently paradoxical findings that VC ablation can confer either an egg-laying-defective or an egg-laying-constitutive phenotype, depending on the experimental context. Our results indicating that the VCs negatively regulate egg laying by inhibiting HSN activity are consistent with previous genetic results indicating the HSNs as a target of negative regulation by the VCs [15]. Likewise, the apparent redundancy of the VCs' stimulatory effect on the vulval muscles is consistent with the earlier observation that VC ablation leads to slower egg laying most clearly in animals lacking functional HSNs [16].

The functional architecture of the egg-laying circuit (Figure 8) has intriguing parallels in *C. elegans*, as well as in other organisms. A recent analysis [46] found that the connectivity pattern seen in the egg-laying motor circuit, in which two reciprocally connected cells direct synaptic output to the same target, was significantly overrepresented in the *C. elegans* nervous system. It is thought that such overrepresented motifs may represent functional units, analogous to circuit elements in a computer, that perform key computational functions [47]. In the case of the egg-laying circuit, this circuit combines the properties of a negative feedback loop (the HSNs excite the VCs, which in turn feedback- inhibit the HSNs) and feed-forward stimulation (the HSNs excite the vulval muscles, both directly and indirectly, through the VCs). Interestingly, the aforementioned connectivity pattern was recently shown to also be overrepresented in the mammalian cerebral cortex [48]. Thus, studies of the *C. elegans* egg-laying circuit may provide fundamental insights into the properties of microcircuits that underlie the workings of much more complicated brains.

Experimental Procedures

General Methods and Strains

All nematodes were grown at 20.5°C ± 0.5°C on standard Nematode Growth Medium (NGM) seeded with *E. coli* strain OP50 as the food source, except the *cha-1(y226)* temperature-sensitive mutant, which was maintained at 15°C. Nematodes were assayed 24 hr after the late fourth larval (L4) stage at 20.5°C ± 0.5°C, except *cha-1(y226)*, which was assayed 30 hr after later L4 stage. Integrated transgenic arrays *ljIs25[myo-3::YC2.0]* and *ljIs19[cat-1::iYC2.1]* were generated by gamma irradiation (5000 rad) of existing transgenic lines (*kyEx302* and *ljEx65*, respectively), followed by seven and six generations of backcrossing, respectively. Neither line showed obvious differences, compared to the WT N2 strain, in egg-laying behavior. Mutant nematode strains, *egl-1(n986)*, *lin-39 (n709ts)*, *tph-1(mg280)*, *and cha-1(y226)*, were crossed to *ljIs25* for generation of cameleon lines for Ca^{2+}-imaging experiments. For surgery experiments, we used the transgenic strain SK4013, a WT N2 background strain with the integrated transgene *zdIs13[tph-1::gfp]* [31, 49], obtained from Scott Clark.

Ca^{2+}-Imaging Experiments

Combined fluorescent and visible microscopy was used to view both the Ca^{2+} signal and egg-laying events simultaneously. The microscope equipment was largely as described previously [10]: in brief, a Zeiss Axioskop 2 upright microscope equipped with a Hamamatsu Orca ER CCD camera, a Hamamatsu W-View emission image splitter, and a Uniblitz Shutter (Vincent Associates). Filter/dichroic pairs were: excitation, 420/40; excitation dichroic 455; cyan fluorescent protein (CFP) emission, 480/30; emission dichroic 505; yellow fluorescent protein (YFP) emission, 535/30 (Chroma). A minimal level of fluorescent light was used for reduction of photobleaching and phototoxicity; for our microscope, this involved using 20%–60% of the maximal power to the lamp, with a neutral density (ND) filter of 2.0. Weak visible light, barely revealing egg-laying events, was used to minimize interference with the cameleon fluorescent signal (as shown in Figure S1). Fluorescent images were acquired and saved with MetaVue 4.6 (Universal Imaging) at a frequency of either 33hz or 10 Hz, for muscle and neuron imaging, respectively (binning 4×4), with a 63X Zeiss Achroplan water-immersion objective used. Photobleaching was corrected by fitting of the baseline YFP/CFP ratio to a single exponential decay; bleedthrough was corrected with the use of an RCFP value of 0.6 [50].

Worms were immobilized with Nexaband S/C cyanoacrylate veterinary glue on a small agarose pad, with the buffer of choice freshly made on a microscopy slide. The worms were then quickly covered with the buffer of choice and immediately moved under the microscope for recording. For HSN and vulval-muscle imaging, the agarose pad was made in the same buffer as the bath. For VC imaging, a 2% agarose pad in M9 was used, regardless of the bath buffer, due to the fast adaptation of VCs to low-osmolarity conditions. Agarose pads were made immediately before slide preparation in order to minimize the loss of focus in long recordings (10 min), which usually is caused by swelling of the agarose pad. Worms were allowed to equilibrate for 2–4 min before the start of recording.

Acute-Osmotic-Shock Experiments

A flow chamber was created by connection of a small reservoir (RC-26GLP, Warner Instruments) to inflow buffer and vacuum lines, which allowed for continuous buffer exchange. The chamber was originally filled with 1 ml of the starting buffer and gradually changed over 1–2 min (flow rate of about 0.3–0.4 ml/min) to the new buffer after the syringe and vacuum connections were opened at the indicated time, 30 s after the beginning of the recording.

PDMS Mold for Rotating and Imaging Worms

We poured liquid unpolymerized polydimethylsiloxane (PDMS) from Sylgard 184 Silicone Elastomer Kit on top of a 12-in long-playing (LP) vinyl record to make a negative replica mold of the record channels. We polymerized the PDMS mold by baking it overnight at 50°C. By fabricating agarose pads against ridges of the PDMS mold, we formed channels on the surface of the agarose pad. L4 to young-adult worms fit within the channels, which hold them in a fixed orientation for imaging or surgery. With the observation of body marking, worms can be quickly rotated by pick to a desired orientation. At the L4 to young-adult stages, the vulva protrudes significantly from the rest of the worm's body. As the coverslip is placed on top of worms on a flat agarose pad, the protruding vulva is pushed to the side, forcing the worm into a lateral imaging orientation. The channels lower the worm into the agarose, decreasing the force of the coverslip against the vulva. When the coverslip is introduced, the worms will retain their orientation.

Femtosecond Laser Ablation of HSN Cell Bodies and Axons

Femtosecond laser ablation of the HSN neurons was performed on young-adult worms via established procedures. Young-adult worms were anesthetized with 1.5 mM sodium azide and imaged on an epi-fluorescent microscope for surgery. A regeneratively amplified Titanium:sapphire laser (λ = 800 nm) delivered a 10 kHz train of 80–90 fs pulses, which were attenuated to 2–3 nJ pulse energy and tightly focused by a 1.4 NA, 63× oil-immersion objective onto the GFP-fluorescent HSN neurons. We severed HSN axons by irradiation for less than 0.5 s, typically at a location halfway between the cell body and the nerve ring. We ablated HSN cell bodies by irradiation of the soma two or three times for 1 s each time. Usually, the disappearance of only the nuclear fluorescence was noted within the first second of irradiation. We confirmed the surgeries by fluorescent reimaging after assay.

Microscopic PLM Activation

Experiments were performed as described previously [10]. In brief, a smooth glass rod about 15 μm in diameter was mounted on a three-axis manipulator through a M-111.1DG microtranslation stage (Polytec PI) and positioned perpendicular to and barely touching the ventral side of an immobilized worm, about 5–10 μm anterior to the PLM cell body. A "buzz" stimulus, in which the probe was continuously vibrated aganist the body with 10 μm of displacement at10 Hz for 3 s, was delivered as described previously [10]. An LED light flashed once to indicate the beginning of the stimulation.

To test the statistical significance of the delay in HSN activity induced by PLM activation, we took advantage of the fact that all spike intervals from a recording of a single worm (except the one just after the stimulation) are found to follow a normal distribution, as confirmed by both the Lilliefors test and the Jarque-Bera test. Thus, a fitted normal distribution was calculated for each experiment. The time interval just after the stimulation was then compared to the fitted distribution of that single worm, and the standard *Z* score was calculated. All of the *Z* scores from the stimulating experiment and also the mock experiment were then compared with the Mann-Whitney rank test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/19/1445/DC1/.

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