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# Serotonin and $G_o$ Modulate Functional States of Neurons and Muscles Controlling *C. elegans* Egg-Laying Behavior

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## Summary

**From nematodes to humans, animals employ neuromodulators like serotonin to regulate behavioral patterns and states. In the nematode *C. elegans*, serotonin has been shown to act in a modulatory fashion to increase the rate and alter the temporal pattern of egg laying [1–4]. Though many candidate effectors and regulators of serotonin have been identified in genetic studies [5–16], their effects on specific neurons and muscles in the egg-laying circuitry have been difficult to determine. Using the genetically encoded $Ca^{2+}$ indicator cameleon, we found that serotonin acts directly on the vulval muscles to increase the frequency of $Ca^{2+}$ transients. In contrast, we found that the spontaneous activity of the egg-laying motorneurons was silenced by serotonin. Mutations in G protein $\alpha$ subunit genes altered the responses of both vulval muscles and egg-laying neurons to serotonin; specifically, mutations in the $G_q\alpha$ homolog *egl-30* blocked serotonin stimulation of vulval muscle $Ca^{2+}$ transients, while mutations in the $G_o\alpha$ homolog *goa-1* prevented the silencing of motorneuron activity by serotonin. These data indicate that serotonin stimulates egg laying by directly modulating the functional state of the vulval muscles. In addition, serotonin inhibits the activity of the motorneurons that release it, providing a feedback regulatory effect that may contribute to serotonin adaptation.**

## Results and Discussion

In *C. elegans*, egg laying occurs through contraction of eight electrically coupled vulval muscles, which are innervated by the two serotonergic HSN and six cholinergic VC motorneurons. Despite the extensive genetic analyses of egg-laying behavior, the activity patterns of individual muscles and neurons in the egg-laying circuit (and the relationship of cell physiology to egg-laying phenotypes) have been difficult to examine. To address these questions, we used the $Ca^{2+}$-sensitive protein cameleon to optically image the activity of muscles and neurons in intact behaving animals. Cameleon is a GFP-based $Ca^{2+}$ indicator that uses fluorescence resonance energy transfer (FRET) to measure intracellular $Ca^{2+}$ transients that accompany depolarization in excitable cells [17, 18]. Since cameleon is genetically encoded, it can be specifically targeted to individual muscle and neuronal cells to allow noninvasive imaging of depolarization-induced $Ca^{2+}$ transients during normal behavior [19].

To monitor the activity of individual vulval muscles in vivo, we obtained a transgenic line (gift from Timothy Yu, Bargmann lab) that expresses cameleon under the control of the *myo-3* promoter [20]. Animals were immobilized on agarose pads, and $Ca^{2+}$ transients were monitored as described [19]. A typical focal plane of image capture allowed the simultaneous monitoring of two vulval muscles (Figure 1). Though full egg-laying events were rare under our recording conditions, we observed many small vulval muscle contractions, and during these events, the YFP intensity/CFP intensity ratio transiently increased. This ratio change was typically accompanied by an increase in YFP emission intensity and a reciprocal decrease in CFP intensity, consistent with the expected increase in FRET when cameleon binds $Ca^{2+}$. Thus, cameleon could be used to reliably detect vulval muscle $Ca^{2+}$ transients in intact behaving animals.

Using cameleon, we recorded simultaneously from pairs of vm1 or vm2 muscle cells to investigate their patterns of activity in vivo. We found no significant differences between the frequency of $Ca^{2+}$ events in the vm1 and vm2 muscles (Table 1). The activities of individual vulval muscle cells, imaged simultaneously in individual animals, showed significant temporal correlation. For example, 23.2% of events in a pair of simultaneously imaged muscles were correlated within a window of 100 ms, significantly higher than would be expected by chance (Figure 1D). Baseline vulval muscle $Ca^{2+}$ transients were sporadic and temporally clustered (Figure 2). Periods of high activity typically lasted 3–4 s and contained multiple, small $Ca^{2+}$ events; inactive periods lasted an average of 30 s and occasionally persisted over 1–2 min in duration.

We next investigated whether the egg-laying motorneurons were required for vulval muscle $Ca^{2+}$ transients. To address this question, we ablated all six VC neurons in an *egl-1*(*n986*) mutant background (in which the HSN neurons undergo inappropriate programmed cell death) and then imaged vulval muscle $Ca^{2+}$. We observed that in the absence of the HSNs and VCs, the vulval muscles still exhibited $Ca^{2+}$ transients, although there was a downward trend in frequency (Figure 2, Table 1). Thus, spontaneous vulval muscle activity persists in the absence of neuronal input, consistent with behavioral predictions that the egg-laying motorneurons play a modulatory role in stimulation of the vulval muscles.

Behavioral data implicated serotonin, a neuromodulator released from the HSN egg-laying motorneurons, in the control of egg-laying behavior [2–4]. When we treated animals with exogenous 5HT, we observed a significant increase in the frequency of $Ca^{2+}$ events from a baseline of 5.63 $min^{-1}$ to a rate of 35.01 $min^{-1}$ ($p < 0.001$, Kolmogorov-Smirnov test). Serotonin treatment also led to a dramatic change in the distribution of events, as the clustered pattern of transients in untreated worms gave way to a more continuous train of $Ca^{2+}$ events (approxi-

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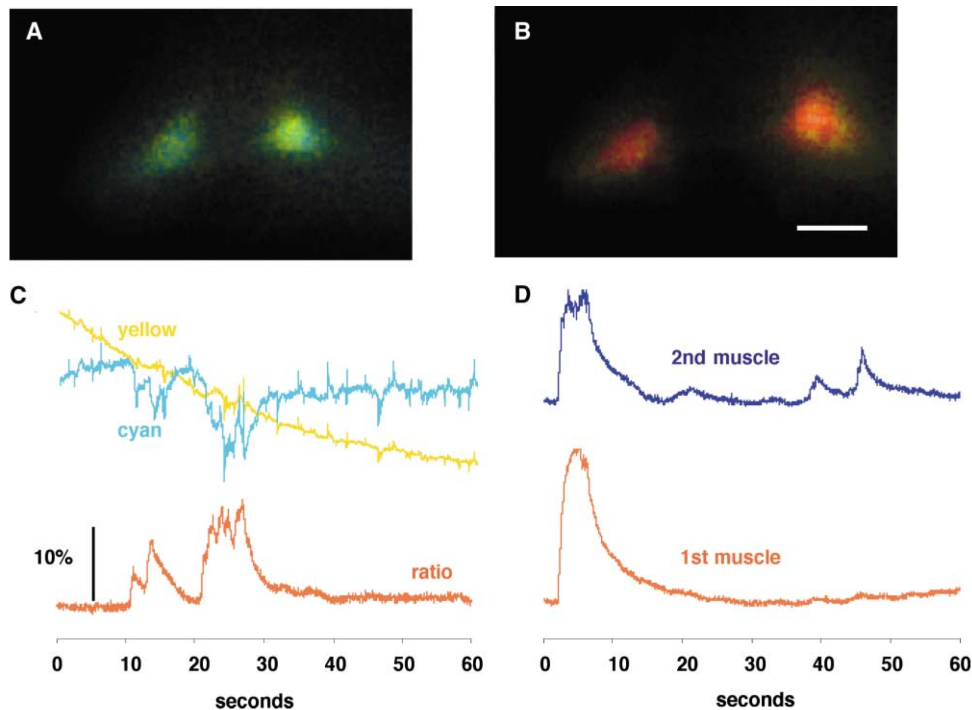


Figure 1. $Ca^{2+}$ Imaging of *C. elegans* Vulval Muscle with Cameleon

(A) Pseudocolor ratio image of vulval muscles at rest. Red indicates high ratio, while blue indicates low ratio.

(B) The same vulval muscles during contraction. Note the red pseudocolor shift. (A) to (B) spans an approximately 35% change in Y/C ratio. Scale bar is 10 μm.

(C) Sample 1 min ratio trace (red) from a separate pair of vulval muscles, with N2 [no drug], corrected for photobleaching. Component yellow and cyan intensity curves (prior to photobleaching correction) are also shown. Note reciprocal deflections of yellow and cyan. Vertical scale bar represents 10% Y/C ratio increase.

(D) Sample ratio traces from two vulval muscles in a simultaneously imaged pair. In this instance, 35.3% of the $Ca^{2+}$ events were temporally correlated (using a defined correlation window of 100 ms). (D) is drawn to scale with (C).

mately 0.5–2 Hz) (Figure 2). Intermuscle correlation of activity increased from a fraction of 0.232 (baseline) to 0.547 under 1.3 mM 5HT, and 31 of 35 muscle/trace pairs exhibited a level of correlation achieving a significance level $p < 0.05$ (overall $p = 1.99 \times 10^{-36}$, Fisher's exact test). Thus, exogenous serotonin appeared to

Table 1. Muscle $Ca^{2+}$ Events/Min

| | Intact Nervous System (Events/Min) | | HSN(−), VC 1-6(−) (Events/Min) | |
|---|---|---|---|---|
| | No Drug | 1.3 mM 5HT | No Drug | 1.3 mM 5HT |
| N2 | **5.63** ± 0.76 (109) | [a,e]**35.01** ± 3.32 (73) | **1.08** ± 0.53 (12) | [b,e]**33.53** ± 4.22 (28) |
| N2 (longer recordings) | **6.54** ± 1.43 (21) | | | |
| vm1 only | **6.82** ± 2.05 (12) | | | |
| vm2 only | **6.16** ± 2.03 (9) | | | |
| *egl-19*(*n582*) | [a,e]**0.35** ± 0.22 (26) | **0.71** ± 0.49 (17) | | |
| *egl-30*(*n686*) | [a,e]**0.80** ± 0.60 (25) | **9.44** ± 2.29 (41) | **9.20** ± 7.24 (10) | **14.31** ± 4.66 (16) |
| *goa-1*(*n1134*) | [a,e]**20.10** ± 3.48 (30) | **26.80** ± 3.93 (20) | [b,d]**10.69** ± 2.058 (26) | [c,e]**27.04** ± 2.79 (28) |
| *goa-1*(*n1134*) *egl-30*(*n686*) | **3.44** ± 0.92 (39) | **9.71** ± 2.13 (45) | | |
| *gpa-7*(*pk610*) | **10.57** ± 3.07 (28) | [c,e]**29.65** ± 3.11 (37) | | |
| *gpa-14*(*pk347*) | **4.05** ± 1.17 (37) | [c,e]**30.17** ± 4.45 (36) | | |
| *unc-68*(*r1158*) | **9.55** ± 2.27 (40) | [c,e]**40.52** ± 6.19 (25) | | |

Listed in bold are mean event frequencies (events/min), ± SEM, (n) = number of animals/recordings used in the calculation of mean frequency of events. One of two recordings in a pair was selected at random for data analysis unless one of the pair was inactive or compromised by artifact or excessive noise—in which case, that recording was discarded and its partner utilized. Bonferroni correction was employed in cases where there were multiple comparisons.

[a]Significant Kolmogorov-Smirnov score versus N2 no drug.

[b]Significant Kolmogorov-Smirnov score versus N2 ablated, no drug.

[c]Significant Kolmogorov-Smirnov score versus same strain/ablation status, no drug.

[d]$p < 0.01$.

[e]$p < 0.001$.

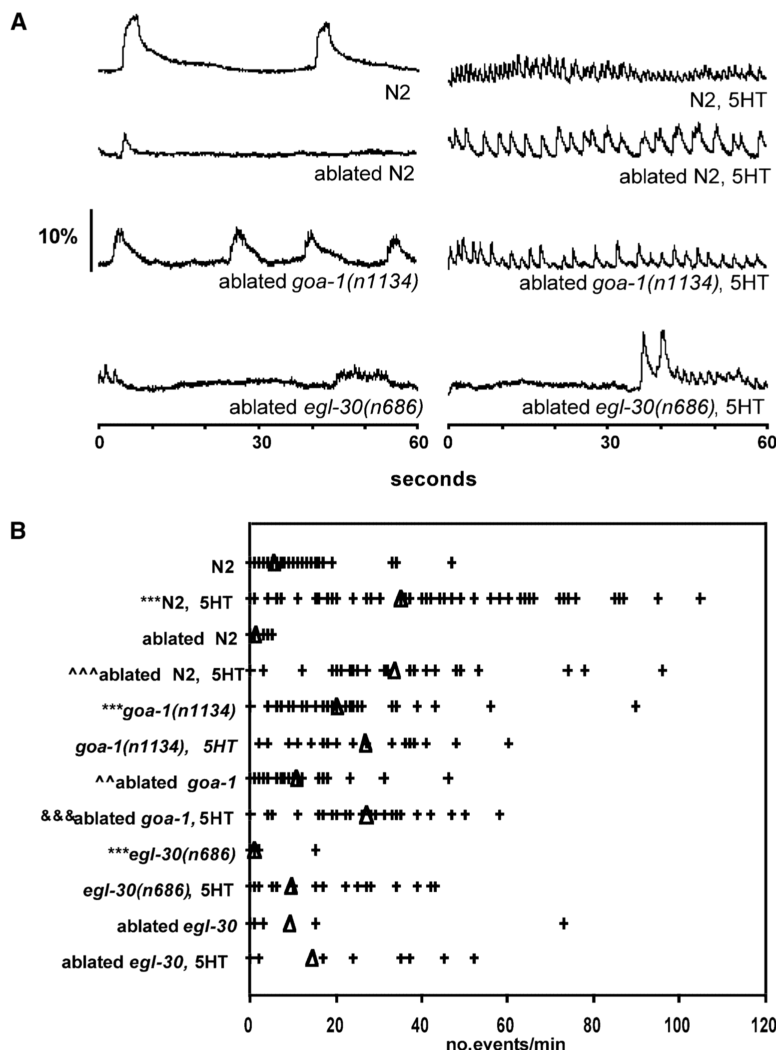


**Figure 2. Effects of Serotonin and G Proteins on Vulval Muscle $Ca^{2+}$ Transients**

(A) Sample vulval muscle ratio traces and selected distributions of event frequency. Shown are sample 1 min ratio traces for wild-type (N2) and neuronally ablated ($HSN^-$, $VC1\text{-}6^-$) wild-type, *goa-1*(*n1134*), and *egl-30*(*n686*) in the presence of 1.3 mM serotonin.
(B) Scatter-plot distributions of vulval muscle events/min. Means are indicated by triangles, and individual data-points (each representing a one-minute trace) by crosses. Asterisk indicates significant Kolmogorov-Smirnov score versus N2 [no drug]. Ampersand symbol indicates significant Kolmogorov-Smirnov score versus same strain/ablation status [no drug]. Caret symbol indicates significant Kolmogorov-Smirnov score versus N2 ablated [no drug]. One symbol denotes $p < 0.05$; two symbols, $p < 0.01$; and three symbols, $p < 0.001$.

modulate the functional state of the vulval muscles, switching them from a pattern of sporadic $Ca^{2+}$ activity to a pattern of continual $Ca^{2+}$ activity.

In principle, serotonin could exert its effects directly on the vulval muscles, or it could act indirectly by altering the activity of the egg-laying motorneurons. To resolve this issue, we ablated the egg-laying motorneurons and assayed the effect of serotonin on vulval muscle $Ca^{2+}$ transients. We found that ablated animals exhibited a continuous train of $Ca^{2+}$ transients on serotonin essentially identical to that exhibited by unablated wild-type animals (Figure 2, Table 1). Thus, the ability of serotonin to increase the frequency of $Ca^{2+}$ events was not markedly affected by the absence of the egg-laying motorneurons, indicating that serotonin directly stimulates the activity of the vulval muscles.

To investigate the molecular requirements for serotonergic modulation of the vulval muscles, we tested the effects of mutations in genes known to affect egg-laying behavioral responses on serotonin's ability to induce changes in vulval muscle $Ca^{2+}$ dynamics. Previous behavioral studies indicated that reduction-of-function mutations in the L type $Ca^{2+}$ channel gene *egl-19* strongly interfere with the ability of serotonin to stimulate egg laying [2, 3, 21]. When we imaged $Ca^{2+}$ transients in one such allele, *egl-19*(*n582*), we observed very little activity (Table 1), and in the presence of serotonin, we observed no significant increase in the frequency of $Ca^{2+}$ transients. In contrast, null mutants of ryanodine receptor *unc-68* [22] showed a normal pattern of $Ca^{2+}$ transients in the absence of drug and responded as robustly as wild-type animals to exogenous serotonin. This suggests a key role for the EGL-19 voltage-gated $Ca^{2+}$ channel in the generation of serotonin-evoked $Ca^{2+}$ transients in the vulval muscles.

We next explored the effects of G protein-signaling mutants on vulval muscle activity and its modulation by serotonin. Of the 20 G protein $\alpha$ subunits in the *C. elegans* genome [9], five are known to be expressed in the vulval muscles: *goa-1*, *gsa-1*, *gpa-7*, *gpa-14*, and *gpa-16*. Viable loss-of-function alleles exist for *goa-1*, *gpa-7*, and *gpa-14*; we therefore assayed the effects of these mutations on vulval muscle $Ca^{2+}$ transients in the presence and absence of serotonin. Mutations in *gpa-7* and *gpa-14* had no significant effect on baseline vulval muscle activity or its enhancement by 5HT (Table 1). *goa-1*(*n1134*) worms, however, displayed a higher level of baseline activity than wild-type (20.10 versus 5.63

events/min, $p < 0.001$ by Kolmogorov-Smirnov; Figure 2, Table 1). To determine whether these effects were due to muscle-intrinsic action of GOA-1, we imaged animals in which the egg-laying motorneurons were ablated in the *goa-1*(*n1134*) mutant background. We observed that these ablated mutant animals showed elevated $Ca^{2+}$ activity compared with neuronally ablated wild-type animals (10.69 versus 1.08 events/min, $p < 0.01$ by Kolmogorov-Smirnov; Figure 2, Table 1). These data indicated that GOA-1 negatively regulates vulval muscle activity, at least in part, through cell-intrinsic action within the muscle cells.

We next investigated the effect of *goa-1* on the response of the vulval muscles to serotonin. *goa-1*(*n1134*) animals exhibited an already elevated frequency of vulval muscle $Ca^{2+}$ events in the absence of 5HT, and treatment with exogenous serotonin did not increase this vulval muscle activity further. However, the frequency of $Ca^{2+}$ events in neuron-ablated *goa-1* animals was robustly stimulated by serotonin (from 10.69 to 27.04 events/min; $p < 0.001$ by Kolmogorov-Smirnov; Figure 2, Table 1). Thus, GOA-1 apparently is not required for vulval muscle serotonin response but, rather, appears to act independently and antagonistically to serotonin to inhibit the frequency of vulval muscle $Ca^{2+}$ transients.

Another candidate G protein effector of serotonin in the egg-laying system is the *C. elegans* $G_q\alpha$ homolog, EGL-30. *egl-30* null alleles are lethal, and the strongest loss-of-function alleles profoundly affect viability, fertility, and feeding ability [7]. Thus, we assayed the effect of an *egl-30* reduction-of-function allele, *n686*, that had a strong egg-laying defect but was otherwise fertile and healthy. We observed that *egl-30*(*n686*) mutant animals showed a significant reduction in spontaneous vulval muscle $Ca^{2+}$ transients compared to wild-type (Figure 2, Table 1). In addition, *egl-30*(*n686*) mutants were markedly less responsive to the stimulatory effects of serotonin on vulval muscle $Ca^{2+}$ activity. Consistent with epistasis results reported in previous behavioral studies [11], we found that the *egl-30*(*n686*)*; goa-1*(*n1134*) double mutant was also serotonin resistant (Table 1). To assess the dependence of this serotonin resistance on the egg-laying motorneurons, we ablated the HSNs and VCs in *egl-30*(*n686*) mutant animals and imaged $Ca^{2+}$ transients in the presence and absence of serotonin. We observed (Figure 2, Table 1) no significant stimulation of vulval muscle $Ca^{2+}$ activity by serotonin in these neuronally ablated animals. Together, these data suggest that the modulation of vulval muscle $Ca^{2+}$ dynamics by serotonin is largely dependent on EGL-30.

Although our analysis of motorneuron ablated animals demonstrated a direct action of serotonin and GOA-1 on the vulval muscle, an assortment of previous behavioral studies also implied possible actions of both serotonin and $G_o$ in controlling the activity of the egg-laying motorneurons. We therefore investigated the effects of serotonin and *goa-1* on the neurons in the egg-laying motor circuit. To monitor egg-laying motorneuron activity in behaving animals, we generated a transgenic line (*cat-1::iYC2.1*) expressing cameleon in the HSN and VC4/5 neurons, and imaged spontaneous neuronal $Ca^{2+}$ transients under the same conditions used for vulval muscle imaging. Using this approach, we were able to record from both classes of motorneurons, but the HSNs gave us the most robust recordings. We observed spontaneous and aperiodic $Ca^{2+}$ transients in approximately 25% of all 1 min recordings from N2 HSNs, with an overall mean of 1.00 events/min (Figure 3).

We next assessed the effects of exogenous serotonin and *goa-1* on motorneuron activity. In the presence of exogenous serotonin (1.3 mM), we unexpectedly observed a complete silencing of the HSNs: out of 29 1 min wild-type HSN recordings, none showed any detectable activity. This difference was highly significant ($p = 0.0069$ compared with [no drug], Fisher's exact test before Bonferroni correction), and suggested that in addition to stimulating the vulval muscles, serotonin acts as an inhibitory modulator of the motorneurons from which it is released. Mutations in *goa-1* did not significantly change the baseline activity of the HSNs in the absence of serotonin—out of 27 HSN recordings made from *goa-1*(*n1134*), 33% exhibited activity, with an overall mean of 1.85 events/min (sample trace in Figure 3B). However, the HSNs from *goa-1*(*n1134*) appeared strongly resistant to serotonin-induced silencing of neuronal activity, as 28% ($n = 25$) of 1 min traces on 5HT showed activity ($p = 0.0027$ compared to wild-type on serotonin, Fisher's exact test before Bonferroni correction), averaging 0.88 events/min. In contrast, HSNs from *egl-30*(*n686*) mutant animals were effectively silenced by serotonin. Thus, GOA-1 appears to play two distinct roles in the egg-laying circuitry: in the vulval muscles it functions antagonistically to serotonin to inhibit muscle activity, while in the HSNs it mediates serotonin silencing of neuronal activity.

Taken together, our imaging results provide a straightforward cellular basis for the effects of serotonin on egg-laying behavior. Our results demonstrate that the stimulatory effect of serotonin on egg laying occurs at least in part through direct modulation of vulval muscle $Ca^{2+}$ dynamics. In is interesting to note that the increase in the frequency of vulval muscle activity caused by serotonin did not require neuronal input from the HSNs or VCs, in contrast to our expectations based on behavioral studies [3]. Thus, the effect of serotonin on the behavioral pattern of egg laying may involve additional complexities beyond its effects on the vulval muscles. Serotonin also affected the activity of the HSN motorneurons, except in this case, the action of serotonin was inhibitory. Since the HSNs are themselves serotonergic, this inhibition could serve as a feedback mechanism by which serotonin could terminate its own release from the HSNs and thus maintain a clustered pattern of egg laying. It is known that long-term exposure to high concentrations of serotonin inhibits egg laying [23]. Our results here suggest that this long-term adaptation to serotonin could result from prolonged inhibition of HSN activity combined with a desensitization of serotonin's stimulatory effect on the vulval muscles.

Our results likewise confirm and extend a number of hypotheses concerning the $G_o/G_q$ signaling network in *C. elegans*, which was previously implicated in the modulation of egg laying by serotonin [5–7]. Both GOA-1 and EGL-30 were shown to affect vulval muscle activity and serotonin response in the absence of the egg-laying motorneurons, indicating an important functional role

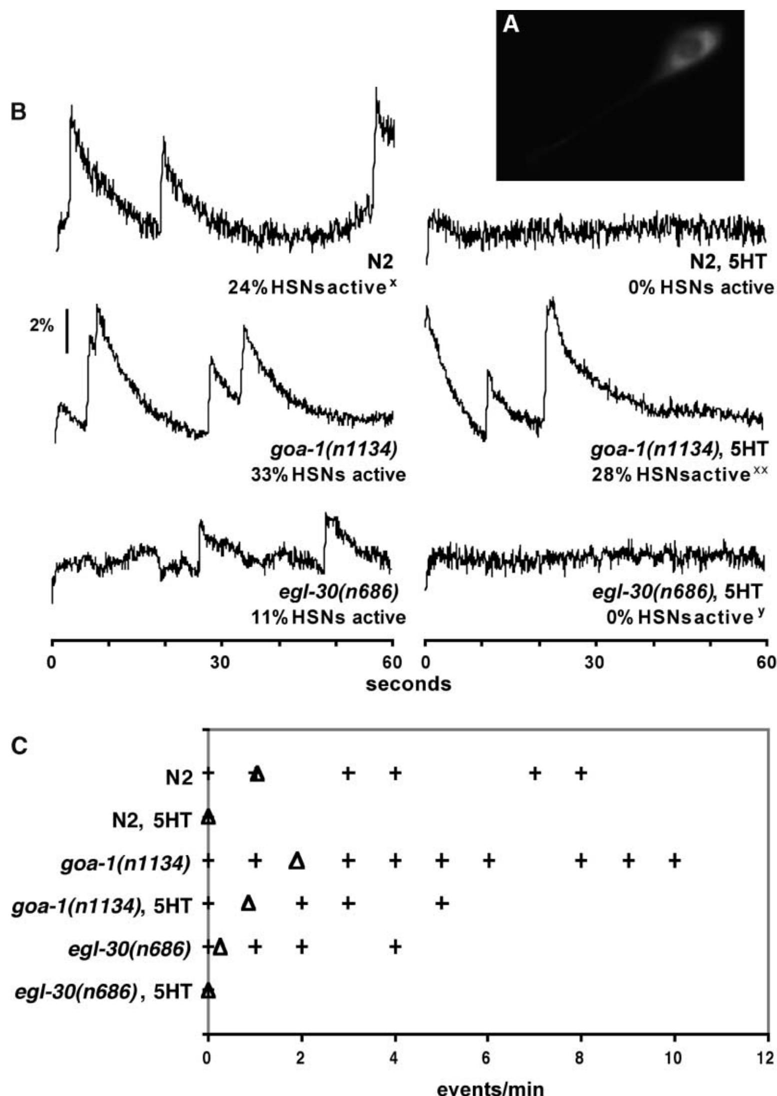


Figure 3. Silencing of HSN Neuronal Activity by Serotonin

(A) Sample field of view showing an HSN motorneuron with *cat-1::iYC2.1*. Axons were variably fluorescent. VCs 4/5 also express *cat-1::iYC2.1* but are in a different focal plane. Scale bar is 10 $\mu$m.

(B) Representative HSN traces are shown for N2, *goa-1*(*n1134*), and *egl-30*(*n686*) on/off 1.3 mM 5HT, with percent of total traces active as noted. Although N2 and *goa-1*(*n1134*) HSNs were indistinguishable in the baseline condition, only N2 HSNs were silenced on 1.3 mM 5HT. *egl-30*(*n686*) displayed a trend toward a lower percent of total traces active (relative to a second N2 control dataset), but this was not statistically significant. *egl-30(n686)*, like N2 (wild-type), was completely silenced by 5HT. $^{x}p = 0.069$ (N2 versus N2 5HT), $^{xx}p = 0.0027$ (*n1134* 5HT versus N2 5HT), $^{y}p = 0.019$ (*n686* 5HT versus *n686*), Fisher's exact test (active/inactive) before Bonferroni correction. n = 25 (N2 HSN), 27 (*n1134* HSN), 27 (*n686* HSN), 29 (N2 HSN, 5HT), 25 (*n1134* HSN, 5HT), and 71 (*n686* HSN, 5HT).

(C) Scatter-plot distributions of HSN events/min. Means are indicated by triangles, and individual data points (each representing a 1 min trace) by crosses. Because of the reduced levels of activity inherent to our neuronal recordings, statistical significance was evaluated for percent of total traces active (as noted in Figure 3B).

for these molecules within the vulval muscles themselves. Furthermore, the motorneuron-independent activity of EGL-30 was found critical for the vulval muscle response to serotonin, making EGL-30 a prime candidate for a serotonin effector in these cells. It should be noted that some serotonin sensitivity may remain in *egl-30* mutant animals; although this could be simply a consequence of the partial loss-of-function allele analyzed in our study, it is possible other G proteins may make minor contributions to serotonin signaling in the vulval muscles [9]. In the HSNs, the dependence of serotonin-evoked neuronal silencing on GOA-1 implicates $G_o$ as a serotonin effector in these neurons. This function for GOA-1 in the HSNs parallels $G_o$'s hypothesized role in mediating the silencing of ventral cord motorneurons by serotonin [10, 11, 24]. With the cellular-level assays for vulval muscle and neuronal activity described here, it should be possible to investigate the functions of other components of $G_o/G_q$ pathways [12–16] and assess genetically based hypotheses about the signaling mechanisms employed by this highly conserved and extremely intricate signal transduction network.

In addition to the vulval muscles and egg-laying motorneurons described here, a number of other excitable cells have been successfully monitored by optical imaging in intact nematodes, including the pharyngeal muscles and mechanoreceptor neurons [19, 25]. Genetically encoded $Ca^{2+}$ sensors have also been used in dissected *Drosophila*, further demonstrating their utility as in vivo neural indicators [26, 27]. Future application of these methods in other neurons should enable us to test and corroborate a wide range of cellular-level predictions about the neural basis for behavioral phenotypes.

#### Supplemental Data

Supplemental Data including imaging protocols, two supplemental figures showing correlated VC neuron activity and an example of automated peak detection, and a table showing the temporal correlation of vulva muscle activity patterns are available at http://www.current-biology.com/cgi/content/full/13/21/1910/DC1/.

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