An Hourglass Circuit Motif Transforms a Motor Program

via Subcellularly Localized Calcium Signaling in Muscle

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Abstract

Neural control of muscle function is fundamental to animal behavior. In many cases, specific muscles can generate multiple distinct behaviors. Nonetheless, individual muscle cells are generally regarded as the smallest units of motor control. Here we report that muscle cells can alter their behavioral output by contracting subcellularly. We previously discovered that noxious tastes reverse the flow of material through the *C. elegans* pharynx, a neuromuscular pump, resulting in spitting. We now show that spitting is driven by the contraction of subcellular regions of individual muscle cells. Localized calcium signals accompany these contractions. Spitting is controlled by an “hourglass” circuit motif: parallel neural pathways converge onto a single motor neuron that differentially controls multiple muscles and the critical subcellular muscle compartment. In short, we find that subcellular muscle units enable modulatory motor control and propose that subcellular muscle contraction is a fundamental mechanism by which neurons can reshape motor function.

Introduction

How animal nervous systems differentially control muscle function to generate the variety of flexible, context-appropriate behaviors necessary for survival and reproduction is a fundamental problem in neuroscience. In many cases, distinct behaviors must be performed using the same muscle or set of muscles. For example, the muscles of the human jaw and tongue control both feeding and speech, while the *Drosophila* wing muscles produce both flight and song (O’Sullivan *et al.*, 2018). Neurons can elicit such different outcomes by altering the amplitudes and relative timing of muscle motions (Briggman and Kristan, 2008; Marder and Calabrese, 1996) or, in principle, by evoking entirely distinct modes of muscle function, with distinct spatial contraction parameters.

We are analyzing the neuromuscular control of behavior using the *C. elegans* feeding organ, the pharynx, as a model neuromuscular system. The pharynx (Figure 1A) is a neuromuscular pump that contracts rhythmically (“pumps”) to generate the suction that ingests bacterial food (Avery and You, 2012). The pharynx contains 14 classes of neurons (20 neurons in total), 8 classes of muscles (20 muscles in total), 4 glands, and 16 structural cells, and the connectome of these cells has been completely described (Albertson and Thomson, 1976; Cook *et al.*, 2019).

Behavioral functions are known for 9 of the 14 pharyngeal neuron classes (15 of the 20 total neurons). The two M2 neurons, single M4, and two MC neurons promote pumping and/or ingestion (Avery and Horvitz, 1987, 1989; Trojanowski *et al.*, 2014); the two M3 and single I5 neurons regulate the timing of muscle relaxation (Avery, 1993); the

two I2 neurons inhibit pumping (Bhatla and Horvitz, 2015); the two I1 neurons promote or inhibit pumping dependent on context (Dent *et al.*, 2000; Trojanowski *et al.*, 2014; Bhatla *et al.*, 2015); the two NSM neurons regulate locomotion and feeding in response to food or food-related odors (Sawin *et al.*, 2000; Li *et al.*, 2012; Flavell *et al.*, 2013; Rhoades *et al.*, 2019); and, as described in more detail below, the single M1 neuron controls spitting behavior (Bhatla *et al.*, 2015).

We previously found that short-wavelength violet or ultraviolet (UV) light inhibits *C. elegans* feeding behavior and that this modulation likely is mediated by light-generated reactive oxygen species (ROS) and thus reflects a response to noxious taste stimuli (Bhatla and Horvitz, 2015). Consistent with this hypothesis, the same genes and neurons required for light-induced feeding inhibition are also required for the inhibition of feeding by the ROS hydrogen peroxide (H₂O₂) (Bhatla and Horvitz, 2015). Because we and others have found that light and ROS act similarly to elicit behavioral responses (Hill and Schaefer, 2009; Kim *et al.*, 2013b; Kim and Johnson, 2014; Guntur *et al.*, 2015; Du *et al.*, 2016; Arenas *et al.*, 2017; Birkholz and Beane, 2017; Guntur *et al.*, 2017), and because of the high precision with which light can be controlled as an experimental stimulus, we are using light as a tool to analyze how the neuromusculature of the pharynx produces behavioral responses to noxious taste stimuli such as H₂O₂.

We found that upon exposure to short-wavelength light, pharyngeal pumping rapidly stops (“acute inhibition”) and then transiently resumes (“burst pumping”; Figure 1B). After the removal of light, the pumping rate is depressed and slowly recovers

(“recovery”) to the 4-5 Hz of normal feeding over about a minute (Bhatla and Horvitz, 2015). Our previous studies implicated four classes of pharyngeal neurons in the pumping response to light (Bhatla and Horvitz, 2015; Bhatla *et al.*, 2015). Light inhibits pumping by altering the activity of the I1, I2, and MC neurons. Subsequently, the M1 motor neuron promotes the burst pumping phase, during which the flow of material through the anterior pharynx is reversed, producing a spitting behavior that ejects ingested material from the pharynx.

We now report that light transforms feeding pumps into spitting pumps -- i.e. reverses the direction in which material is driven by pharyngeal pumping -- by inducing sustained contractions of subcellular regions of individual muscle cells, the three pm3 pharyngeal muscle cells. Spatially restricted calcium signals are localized to the regions of pharyngeal muscle undergoing contraction and are controlled by the M1 neuron. M1 sits at the waist of an “hourglass” circuit motif, in which multiple upstream sensory neurons converge on M1, which in turn makes divergent outputs onto multiple muscle classes that control distinct subcomponents of the spitting reflex. Weak activation of M1 via a subset of upstream neurons can evoke a subset of M1’s motor outputs, producing an attenuated variant of spitting and showing how graded neuronal activation can produce context-appropriate variations of a core behavior. Together, our results provide an example of how muscle function can be modulated at a subcellular level to produce flexible, contextual behavior and identifies subcellularly localized calcium as a mechanistic basis of such modulation.

Results

Worms Spit as a Consequence of the Sustained Contraction of Anterior

Pharyngeal Muscles

We previously defined burst pumping as the transient increase in pumping rate that follows acute light-induced pumping inhibition (Figure 1B) (Bhatla and Horvitz, 2015). Because 365 nm (UV) light evokes burst pumping most robustly (Bhatla and Horvitz, 2015), we used this wavelength in the experiments described below. We further characterized the burst pumping phase and observed that there are three alterations to pharyngeal function that occur during burst pumping: (a) pumping rate increases; (b) a valve that closes to capture food during feeding instead remains open, causing pharyngeal pumps to conclude in the spitting of pharyngeal contents; and (c) a filter that otherwise restricts particle influx opens, facilitating the rinsing of the pharyngeal lumen.

In this work, we first confirmed that pumping rate increases during burst pumping, as reported previously (Bhatla and Horvitz, 2015). We scored pumping in the burst pumping phase in real time by eye, using the assay validated by Bhatla and Horvitz (2015) and based on the rate of contraction of the grinder (“grinder pumps”), a readily-observed cuticular structure in the terminal bulb (Figure 1A and 1B).

We next confirmed and extended the previous observation that worms spit during the burst pumping phase (Bhatla *et al.*, 2015). We distinguished feeding and spitting pumps by recording high-speed videos of worms feeding on a mixture of *E. coli*

bacterial food and comparably-sized 1 μm polystyrene beads (Fang-Yen *et al.*, 2009; Bhatla *et al.*, 2015). We designated pumps that trapped ingested material in the anterior pharynx as feeding pumps (Video 1) and pumps that failed to trap material as spitting pumps (Video 2; Figure 1C). We previously found that violet light (436 nm) induces spitting (Bhatla *et al.*, 2015) and replicated this result using UV light (365 nm) (Figure 1C-1E). Consistent with the hypothesis that the response to light reflects a response to light-generated ROS, the odor of H_2O_2 also induced spitting (Figure 1—figure supplement 1A).

We then identified the biomechanical mechanism by which particle flow is reversed to produce spitting. In feeding, contraction of the pharyngeal muscles opens the lumen of the pharynx, generating suction and drawing food and fluid inwards. Two cuticular structures project from pharyngeal muscle into the lumen of the anterior pharynx and buccal cavity: the metastomal flaps and the pharyngeal valve (Figure 1A) (Albertson and Thomson, 1976). The metastomal flaps were previously shown to limit the size and flow of particles that enter the pharyngeal lumen from the buccal cavity (Fang-Yen *et al.*, 2009). For this reason, we will refer to the metastomal flaps as the “metastomal filter.” The pharyngeal valve is located immediately posterior to the filter and prevents the escape of ingested particles from the pharyngeal lumen to the buccal cavity: prior to the onset of pharyngeal muscle relaxation, the pharyngeal valve closes, sealing the anterior end of the lumen to retain bacteria while fluid is released via channels that border the lumen (Fang-Yen *et al.*, 2009). Given the importance of the

pharyngeal valve in feeding, we hypothesized that spitting involves a modulation of the motions of this structure. We recorded videos of animals feeding and spitting under a coverslip such that the anterior pharyngeal muscles and lumen were clearly visualized. As reported previously (Fang-Yen *et al.*, 2009), feeding pumps involved the closure of the pharyngeal valve, thus trapping food (Figure 1F; Video 3). By contrast, spitting pumps were characterized by a strikingly different motion: rather than relaxing and closing at each pump's conclusion, the anterior tip of the pharynx remained contracted - i.e., open -- throughout the pumping cycle (Figure 1F; Video 4). This sustained contraction prevented the pharyngeal valve from closing and capturing food at the end of the pump, such that closure of the lumen expelled the pharyngeal contents. These open-valve pumping motions occurred specifically during the burst pumping phase, confirming that they are specific to spitting (Figure 1—figure supplement 1B and 1C).

Finally, a third aspect of pharyngeal behavior was altered by light in the burst pumping phase. As reported previously (Fang-Yen *et al.*, 2009), the metastomal filter was typically partially closed during feeding, such that material accumulated in the buccal cavity and filtered only gradually into the pharynx (Figure 1C and 1G; Video 1). We observed that during spitting, however, material moved freely from the buccal cavity into the pharynx (Video 2), indicating that the metastomal filter opened during spitting (Figure 1C, 1G). Thus, in combination with the opening of the pharyngeal valve, this opening of the metastomal filter produced a rinsing effect, in which large amounts of material were drawn into and then expelled from the pharynx.

Although the metastomal filter was typically partially closed during feeding, we noticed that the filter was open during an average of up to 25% of feeding pumps (Figure 1G, Video 5). Because opening the filter significantly increased the amount of material ingested during these pumps, we designated such pumps as “gulps”.

In summary, we found that the spitting reflex is characterized by three changes to pharyngeal behavior: (a) the pumping rate increases; (b) the pharyngeal valve remains open instead of closing, producing spitting; and (c) the metastomal filter opens, increasing the volume of material rinsed in and out with each pump. These changes transform the pharyngeal motor program from one that promotes feeding into one that promotes spitting from and rinsing of the procorpus (Figure 1A and 1H).

Subcellularly Localized Muscle Contractions and Calcium Increases Open the Pharyngeal Valve to Produce Spitting

We next asked which muscles control the light-induced spitting reflex. The anterior pharynx contains three pharyngeal muscle classes: pm1, pm2, and pm3 (Figure 2A). pm1 is a single hexanucleate cell, while the pm2 and pm3 classes consist of three binucleate cells each. The pm1 and pm2 muscles, small cells of unknown function, form a ring around the anterior opening of the pharynx. The metastomal filter protrudes from this ring into the buccal cavity (Figure 2A) such that contraction of the radially arranged myofilaments of the pm1 and/or pm2 muscles would be expected to pull the filter open

and thus expand the aperture of the anterior pharynx. The pm3 muscles make up the remainder of the musculature of the procorpus, and the pharyngeal valve protrudes from their anterior ends. Because the filter and valve are held open in spitting, we hypothesized that spitting involves the sustained contraction of some or all of pm1, pm2, and/or the anterior end of pm3.

We sought to determine the roles of these muscles in spitting. Because ablation of the pm3s results in lethality (Leon Avery, personal communication, March 2020), we laser-killed the pm1 and pm2 muscles singly and in combination to determine what, if any, function they have in spitting. Laser ablation of pm1, the pm2s, or both classes together did not eliminate burst pumping or spitting (Figure 2B-2E), indicating that, unexpectedly, the pm3s can suffice to open the pharyngeal valve and produce spitting. In other words, the contraction of a subcellular region of the pm3 muscles must be sufficient to open the valve. Specifically, the anterior regions of the pm3s underwent sustained contraction while the rest of the muscles rhythmically contracted and relaxed in animals in which the pm1 and pm2 muscles were ablated.

We were surprised to see the uncoupling of apparently separable subdomains -- the anterior end and the rest of the muscle cell -- within each of the pm3 muscle cells. We then examined the physiological mechanism that produces these sustained and spatially restricted muscle contractions in the pm3s. Given the central role of intracellular calcium in initiating muscle contraction (Kuo and Ehrlich, 2015), we expressed the calcium reporter GCaMP3 (Tian *et al.*, 2009) in pharyngeal muscle and

imaged calcium changes in the pharynxes of immobilized animals (Kim *et al.*, 2013a). Immobilization inhibits pumping, and light stimulated pumping by immobilized animals just as it does to produce burst pumping by free-moving animals. Light-stimulated pumps were both dependent on the M1 neuron, which we previously showed to be necessary for spitting (Bhatla *et al.*, 2015), and resulted in the expulsion of previously ingested mineral oil, as is the case for spitting by free-moving animals (Figure 2—figure supplement 1A and data not shown).

When we stimulated pumps with light, we detected calcium increases across the procorpus and the anterior and terminal bulbs, indicating that functional GCaMP was present in these regions (Video 6, Figure 2F-2H). Interestingly, while calcium in most pharyngeal regions rose and fell over the course of a pump, we observed sustained calcium signals at the tip of the pharynx; these signals persisted continuously throughout the pump cycle (Figure 2G). Additionally, during some trials this anterior region responded with a sustained rise in calcium even when the rest of the pharynx did not respond (Figure 2I). Consistent with previous reports (Kerr *et al.*, 2000; Shimozone *et al.*, 2004), we did not observe spatially restricted calcium signals in the anterior pharynx during feeding pumps stimulated with serotonin, indicating that these localized calcium transients are specific to spitting (data not shown). The location and temporal dynamics of the sustained calcium signals coincided precisely with the sustained muscle contractions that we observed during spitting behavior, strongly suggesting that they are part of the physiological mechanism driving subcellular contraction.

While the pm1 and pm2 muscles were not necessary for spitting, their ablation altered other aspects of pharyngeal function. First, pm2-ablated animals were partially defective in opening the metastomal filter, such that the filter was often closed during spitting (Video 7, Figure 2J). Second, consistent with a previous observation (L. Avery and C. Fang-Yen, personal communication, March 2020), ablation of pm1 eliminated the metastomal filter (Figure 2—figure supplement 1B and 1C; data not shown). For this reason, we were unable to determine whether pm1 is also involved in opening the filter. However, because pm2-ablated animals were not completely defective in opening the filter (Figure 2J) and pm1 is the only other muscle positioned to perform this function, we think it likely that pm1 contributes to the opening of the filter as well.

Taken together, and consistent with the anatomy of the anterior pharynx, our results indicate that the pm1, pm2, and pm3 muscles play specialized roles in controlling pharyngeal particle flow: the pm2 and likely the pm1 muscles actively open the metastomal filter, increasing the aperture size of the anterior lumen and thus facilitating particle influx, while a subcellular anterior compartment of the pm3s opens the pharyngeal valve via subcellular calcium signaling, thus producing spitting.

Pharyngeally-Expressed Gustatory Receptor Orthologs *lite-1* and *gur-3* Play Major and Minor Roles, Respectively, in the Light-Induced Spitting Reflex

Previously described *C. elegans* locomotory and pharyngeal responses to light depend on the gustatory receptor orthologs *lite-1* and/or *gur-3*, which are expressed in a number of pharyngeal and extra-pharyngeal neurons (Edwards *et al.*, 2008; Liu *et al.*, 2010; Bhatla and Horvitz, 2015). We asked if gustatory receptor orthologs similarly control the light-induced spitting reflex. Burst pumping rate was wild-type in animals carrying null alleles of *gur-3* (Bhatla and Horvitz, 2015) or its paralogs *egl-47/gur-1*, *gur-4*, and *gur-5* (Figure 3A and Figure 3—figure supplement 1A-1D). *lite-1* null mutants (Edwards *et al.*, 2008) could not be tested directly, because they exhibit significant defects in light-induced pumping inhibition (Bhatla and Horvitz, 2015) (Figure 3B and 3C).

To examine the role of *lite-1* in burst pumping, we tested *lite-1* mutants in an *eat-2* mutant background. *eat-2* mutants are severely defective in feeding pumping (Raizen *et al.*, 1995) but not in burst pumping (Figure 3D). Thus, the burst pumping rate rises above the slow *eat-2* feeding pumping rate and can be scored even in animals defective in light-induced inhibition of feeding. We found that *lite-1* but not *gur-3* was required for burst pumping in the *eat-2* background, a defect that was rescued by a wild-type copy of *lite-1* (Figure 3D-3G). Thus, light-induced stimulation of pumping requires *lite-1* but not *gur-3*.

These experiments established a role for *lite-1* in controlling burst pumping. To determine directly whether *lite-1* and/or *gur-3* are required for spitting -- i.e., the opening of the pharyngeal valve -- we assayed light-induced spitting in *lite-1* and *gur-3* single mutants and *lite-1 gur-3* double mutants. Both *gur-3* and *lite-1* mutants exhibited less

spitting than controls, and *lite-1* mutants also displayed a dramatic reduction in peak spitting rate compared to wild-type animals (Figure 3H-3J and 3N). More strikingly, *lite-1 gur-3* double mutants did not spit in response to light (Figure 3K). Rescue transgenes expressing wild-type copies of either *lite-1* (Bhatla and Horvitz, 2015) or *gur-3* (this study) restored light-induced spitting in *lite-1 gur-3* double mutants (Figure 3L-3N). *lite-1* mutants were additionally defective in opening the metastomal filter (Figure 3O). Although *gur-3* mutants also displayed qualitatively reduced opening of the filter, this reduction was not statistically significant (Figure 3O). Together, these results indicate that both *lite-1* and *gur-3* function in light-induced spitting and opening of the filter, with *lite-1* likely playing a greater role.

Next we assayed the pharyngeal muscle calcium response to light in *lite-1* and *gur-3* mutants. *gur-3* mutants resembled wild-type animals (Figure 3P), while *lite-1* mutants were almost completely defective in the pharyngeal muscle calcium response to light, except for a very weak, fast response (Figure 3Q). This fast response depended on *gur-3*, as the response of *lite-1 gur-3* double mutants was completely defective (Figure 3R). Thus, consistent with our behavioral observations, *lite-1* and *gur-3* function redundantly in light-induced calcium increases in pharyngeal muscle, with *lite-1* playing a major role and *gur-3* playing a minor role.

Taken together, these results indicate that *lite-1* and *gur-3* both contribute to light-induced spitting. *lite-1* is necessary for the full response -- i.e., a robust increase in pharyngeal muscle calcium, pumping rate increase, and opening of the pharyngeal

valve and metastomal filter -- while *gur-3* is sufficient in the absence of *lite-1* to produce an attenuated response (weak activation of pharyngeal muscle, partial opening of the valve, and opening of the filter).

The M1 Pharyngeal Neuron Innervates the Pharyngeal Valve and Controls Light-Induced Spitting

The M1 pharyngeal neuron is the only major source of innervation of the pm1, pm2, and pm3 muscles at the anterior tip of the pharynx (Albertson and Thomson, 1976), in the same region as the metastomal filter, the pharyngeal valve, and the subcellular muscle contractions we observed during spitting (Figure 4A), suggesting that M1 is the motor neuron that controls spitting. We previously found that M1 is required for spitting induced by violet light (Bhatla *et al.*, 2015). We first confirmed that M1 is also required for spitting induced by UV light, which, as noted above, we used throughout this study to induce spitting. We laser-killed M1 and also generated an M1 genetic-ablation strain in which the mammalian caspase ICE (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Zheng *et al.*, 1999) is expressed under an M1-specific promoter. Both laser and genetic ablation of M1 eliminated UV light-induced burst pumping and spitting (Figure 4B-4E and Figure 4—figure supplement 1A-C), and genetic ablation of M1 eliminated burst pumping in *eat-2* mutants (Figure 4—figure supplement 1D). Laser ablation of M1 also eliminated the spatially restricted calcium signals in anterior pharyngeal muscles (Figure

4F), consistent with their being induced by M1. We could not directly determine whether ablation of M1 altered the function of the metastomal filter, because M1-ablated animals did not spit and rarely pumped in the presence of light (but see below). We conclude that the M1 neuron is required for UV light-induced burst pumping, spitting, and muscle calcium physiology.

We observed that the pumping rate of M1-ablated animals recovered significantly more quickly than did controls after stimulation with light and that these pumps, in contrast to typical pumps in which the procorpus and grinder contract together, were characterized by contractions of the grinder but not the procorpus (Figure 4—figure supplement 1E-1H). These observations suggest that in addition to controlling pumping and spitting in the burst pumping phase, M1 also inhibits the grinder during the recovery phase.

Given that light-induced spitting requires M1, we asked whether M1 is acutely activated by light. We generated transgenic animals expressing the calcium reporter GCaMP6s (Chen *et al.*, 2013) in M1 and measured light-induced calcium changes. As indicated by robust GCaMP6s fluorescence increases, light increased M1 somatic calcium levels, consistent with an acute function for M1 in spitting (Figure 4G-4I, Video 8).

We next asked if *lite-1* and *gur-3* control M1's calcium response to light. *lite-1* is expressed in M1 (Bhatla and Horvitz, 2015; Taylor *et al.*, 2019). The *gur-3* expression pattern was first determined using extrachromosomal arrays expressing GFP under the

gur-3 promoter (Bhatla and Horvitz, 2015) and subsequently examined using single-cell transcriptomic analysis of the *C. elegans* nervous system (Taylor *et al.*, 2019). Although neither method detected *gur-3* expression in M1, when we integrated the previously-generated extrachromosomal arrays into the genome we noted that, in addition to being expressed in the I2 and I4 neurons as reported previously (Bhatla and Horvitz, 2015), the stably integrated *gur-3_{promoter}::GFP* transgene was also expressed in M1 and another pharyngeal neuron, MI (Figure 3—figure supplement 1E and 1F), consistent with a function for both *lite-1* and *gur-3* in M1. As was the case with pharyngeal muscle calcium increases, *lite-1* and *gur-3* were required for light-induced calcium increases in the M1 soma (Figure 4J-4L, with *lite-1* and *gur-3* respectively playing major and minor roles, indicating that the differential requirement for *lite-1* and *gur-3* in spitting occurs at or upstream of M1's activation.

We then asked if M1 might be activated directly by light, as suggested by its expression of *lite-1* and *gur-3*, or whether it instead is activated indirectly by input from other light-sensitive neurons via synaptic, humoral (i.e., dense core vesicle), or gap junction inputs. Synaptic and humoral dense-core vesicle signaling respectively require *unc-13*, which encodes the *C. elegans* homolog of UNC13A (Maruyama and Brenner, 1991; Richmond *et al.*, 1999, 2001), and *unc-31*, which encodes the *C. elegans* homolog of CADPS/CAPS (Berwin *et al.*, 1998), so we examined light-induced M1 calcium responses in these mutants. In both *unc-13(s69)* reduction-of-function mutants (Richmond *et al.*, 1999) -- null mutations are lethal (Kohn *et al.*, 2000) -- and *unc-*

31(*u280*) presumptive null mutants (Speese *et al.*, 2007), M1's calcium response was reduced in magnitude but still significant (Figure 4M-4P). These results indicate that M1 likely can function as a cellular photoreceptor but do not preclude the possibility that M1's activation occurs through residual *unc-13* function, gap junctions, or a pathway in which *unc-13* and *unc-31* function redundantly.

To determine whether acute activation of M1 is sufficient to produce spitting, we expressed channelrhodopsin 2 (ChR2) (Nagel *et al.*, 2003; Boyden *et al.*, 2005) in M1. Because the light used to activate ChR2 also activates the endogenous pharyngeal response to light, we performed these experiments on *lite-1 gur-3* mutant animals. While no *lite-1 gur-3* mutant animals grown in the absence of the essential ChR2 cofactor all-*trans* retinal (ATR) showed spitting behavior in response to stimulation with 470 nm blue light, 25% of animals grown with ATR did so when illuminated (Figure 4Q and 4R). Thus, optogenetic activation of M1 can produce spitting behavior, although not as efficiently as UV light does in the wild-type background.

Next, we asked what neurotransmitter or neurotransmitters M1 uses to activate pharyngeal muscle. M1 expresses the cholinergic markers *unc-17* and *cha-1* (Franks *et al.*, 2009; Taylor *et al.*, 2019), and consistent with a cholinergic function for M1 we found that animals with loss-of-function mutations in genes required for signaling by acetylcholine (ACh) but not by other neurotransmitters were severely defective in burst pumping, as were *ric-1* mutants, which are defective in nicotinic ACh receptor maturation (Halevi *et al.*, 2002) (Figure 4—figure supplement 1I-1R).

We assayed the burst pumping behavior of 36 strains, each carrying mutations in one of 36 of the 37 known *C. elegans* ACh receptors (null mutation in the 37th receptor, *acr-9*, results in lethality) (The *C. elegans* Deletion Mutant Consortium, 2012). While several strains showed modest defects, none was strikingly defective, suggesting that multiple acetylcholine receptors function redundantly downstream of M1 (Figure 4—figure supplement 2A-2P').

Together these observations confirmed and extended our previous finding that M1 is necessary for spitting, additionally showing that it controls spatially-restricted muscle calcium signals, that it is acutely activated by light, and that its activation can suffice for spitting.

The *gur-3*-Expressing I2 and I4 Neurons Can Weakly Activate M1 to Open the Metastomal Filter and Pharyngeal Valve Without Increasing Pumping Rate

The M1 neuron's expression of *lite-1* and *gur-3* suggests that M1 is a likely site of action of *lite-1* and *gur-3* in the spitting response to light. However, *unc-13* and *unc-31* mutants were significantly reduced in M1 activation (Figure 4M-4P), consistent with the possibility that other *lite-1* and/or *gur-3* neurons provide stimulatory inputs to M1.

To identify additional light-sensing neurons upstream of M1, we first focused on pharyngeal neurons known to express *gur-3*. The pharyngeal I2 neurons express *gur-3* and control light-induced pumping inhibition (Bhatla and Horvitz, 2015). Since the I2s

form electrical and chemical synapses with the M1 neuron (Albertson and Thomson, 1976), we tested the hypothesis that they provide functional inputs to M1 for the induction of spitting by expressing a wild-type copy of *gur-3* under an I2-specific promoter (Bhatla and Horvitz, 2015) in *lite-1 gur-3* double mutants. I2-specific rescue of *gur-3* restored robust light-induced spitting, opening of the metastomal filter, and weak M1 calcium increases to *lite-1 gur-3* double mutants, indicating that light can activate M1 via *gur-3* function in the I2 neurons (Figure 5A-D). Laser ablation of M1 suppressed this *gur-3*-dependent spitting and opening of the metastomal filter (Figure 5E-5G), showing that the I2 neurons act via (or possibly in parallel to) M1 to induce spits and open the filter.

Since the I2 neurons can strongly stimulate spitting, we next asked if they could increase pumping rates in slow-pumping *eat-2* mutants. Light did not increase the pumping rate of *eat-2; lite-1 gur-3; I2_{promoter}::gur-3(+)* animals (Figure 5H). Thus, while the I2 neurons can activate M1 and thereby open the pharyngeal valve and metastomal filter, they are unable to activate M1 sufficiently strongly to drive pumping rate increases. This finding is consistent with our calcium imaging results indicating the I2 neurons can activate M1 only weakly (Figure 5D) and with our observation that *lite-1*, but not *gur-3*, is required for burst pumping (Figure 3D-3G).

Because prior work showed that the I2s inhibit pumping in response to light (Bhatla and Horvitz, 2015), we were initially surprised to see that *lite-1 gur-3; I2_{promoter}::gur-3(+)* animals inhibited pumping only weakly (Figure 5E). Interestingly, after

we ablated M1 in these animals, we observed robust light-induced pumping inhibition (Figure 5E, 5F, and 5I). This finding suggests that the I2 neurons' stimulatory effect on M1 outweighs their inhibitory effect on pharyngeal muscle. Initially, it seemed paradoxical that the I2s would function in this manner. However, when we compared the rate of feeding pumps (i.e., pumps that capture food) in M1- and mock-ablated *lite-1 gur-3; I2_{promoter}::gur-3* animals, it became clear that the I2s' activation of M1 actually serves to reduce the overall feeding rate, because activating M1 eliminates feeding pumps by turning them into spits (Figure 5J). Thus, rather than simply acting as nonspecific inhibitors of the rates of both feeding and spitting pumps, the I2s specifically function to reduce food intake via two mechanisms: direct inhibition of feeding by hyperpolarizing pharyngeal muscles (Bhatla and Horvitz, 2015; Bhatla *et al.*, 2015) and indirect conversion of feeding pumps into spits by activating M1 (Figure 5K).

We used an I2-specific genetic-ablation transgene (Bhatla and Horvitz, 2015) to kill the I2 neurons in wild-type and *lite-1* genetic backgrounds. Spitting persisted after ablation of the I2 neurons in both cases (Figure 5L and 5M), confirming that the I2 neurons are not necessary for spitting. Thus, *gur-3*-expressing neurons other than the I2 neurons can promote spitting.

gur-3 is also expressed in the pharyngeal I4 neuron (Figure 6A), which is of unknown function. I4 is activated by light (Bhatla and Horvitz, 2015) and synapses onto M1 (Albertson and Thomson, 1976). To determine if I4 contributes to M1's *gur-3*-dependent activation by light, we laser-killed the I2s and I4 of *lite-1* mutants singly and

in combination and assayed spitting behavior. While *lite-1* animals in which either the I2s or I4 were ablated alone exhibited spitting behavior in response to light, I4 ablation significantly reduced the spitting of *lite-1* I2-ablated animals such that I2/I4 double-ablated animals exhibited significantly delayed spitting (Figure 6B-6F), suggesting that the I2 and I4 neurons are required redundantly for rapid light-induced spitting. Consistent with the I2s and I4 acting together to promote activation of M1, we detected small light-induced M1 calcium increases in mock-, I2-, and I4-ablated *lite-1* animals but not in I2/I4 double-ablated *lite-1* animals (Figure 6G). I2-, I4-, and I2/I4-ablated animals seemed to open the metastomal filter slightly less often than did mock-ablated controls, but this small difference was not statistically significant (Figure 6H). We noticed that *lite-1*; I4-ablated animals (Figure 6D) appeared to inhibit pumping more strongly than did *lite-1*; mock-ablated animals (Figure 6B) and that this inhibition depended on the I2s (Figure 6C). Thus, it is possible that I4 regulates the I2s' inhibition of pumping. Similarly, the inhibition of *lite-1* I4-ablated animals (Figure 6D) was also much stronger than that of *lite-1 gur-3; I2_{promoter}::gur-3(+)* animals (Figure 5B and 5E), suggesting that *gur-3* functions in neurons other than the I2s and I4 to regulate the balance between excitation and inhibition of pumping. Finally, these results are also consistent with *gur-3*'s functioning in another upstream neuron that acts via I4, rather than in I4 itself. In short, our ablation data suggest but do not prove that I4 can promote spitting.

To verify that the spitting we observed in these experiments was M1-dependent, we killed M1 in combination with the I2 and I4 neurons of *lite-1* mutants. As expected,

ablation of M1 eliminated spitting in all conditions (Figure 6I-6M). Consistent with our observation that I2 inhibits pumping in M1-ablated animals (Figure 5F), we found that M1-, and M1/I4-ablated animals inhibited pumping in response to light and that triple-ablated animals lacking M1, I2, and I4 were defective in pumping inhibition (Figure 6J, 6L-6N). Some M1/I2-ablated animals inhibited pumping in response to light, suggesting that the I4 neuron might inhibit pumping in a manner similar to the I2 neurons, but this effect was not statistically significant (Figure 6K and 6N).

Because ablating the I2 and I4 neurons together failed to completely eliminate spitting, *gur-3* likely functions in one or more additional neurons. In addition to being expressed in M1 and MI, *gur-3* is also expressed in the extra-pharyngeal AVD neurons. Thus, these cells or others might be additional sites of residual *gur-3* function. In short, at least the I2 and I4 neurons are required redundantly to inhibit pumping and promote *gur-3*- and M1-dependent spitting.

Discussion

To understand how the nervous system adapts the output of a single set of muscles to a changing environment, we analyzed the spitting reflex of the *C. elegans* pharynx. This reflex comprises three parallel behavioral components: an increase in pumping rate, the opening of the pharyngeal valve, and the opening of the metastomal filter. Opening the pharyngeal valve permits the expulsion of ingested material, while opening the metastomal filter allows the worm to flush in and out large amounts of fluid and particles

not previously in the pharynx, apparently rinsing its mouth in response to a bad taste (Figure 7A). The pm2 and likely the pm1 pharyngeal muscles open the metastomal filter, while a subcellular compartment of the pm3 muscles opens the pharyngeal valve, transforming feeding motions into spitting motions. The pm3s make up the bulk of the anterior pharynx, which pumps rhythmically during spitting. Opening of the pharyngeal valve by the pm3s requires that a subcellular compartment at the anterior end of the pm3s undergoes a sustained uncoupling from the rest of the cell and stay contracted. Strikingly, the remaining posterior portion of each pm3 muscle cell continues to pump, ejecting material. The subcellular contraction of the pm3s is accompanied by localized increases in intracellular muscle calcium at the site of the pharyngeal valve. Spitting behavior, opening of the metastomal filter, and muscle calcium physiology are controlled by the M1 neuron, which directly innervates the pharyngeal muscles that control the filter and valve. The gustatory-receptor orthologs *lite-1* and *gur-3* function redundantly in the control of light-induced spitting: *lite-1* plays a major role and suffices for nearly wild-type responses to light, while *gur-3* plays a smaller role and is sufficient to partially open the valve and filter but not to increase pumping rate. *gur-3* functions in the I2 pharyngeal neurons and likely also in the I4 neuron to evoke this attenuated spitting response via the M1 neuron.

Subcellular muscle units driven by neuronally regulated subcellularly localized calcium signaling modulate motor behavior

We identified a likely physiological effector for the subcellular muscle contraction of the pm3s: calcium transients in pharyngeal muscle that colocalize with the specific muscle region that undergoes sustained contraction during spitting and from which the pharyngeal valve protrudes. We propose that these calcium transients are the drivers of the subcellular muscle contraction of the pm3s.

Subcellularly localized calcium signaling in muscle has been observed in other systems. Subcellular muscle calcium transients known as “calcium sparks” result from calcium influx via individual ryanodine receptors (Cheng *et al.*, 1993). These localized calcium increases can trigger calcium-induced calcium release from the sarcoplasmic reticulum, resulting in a wave of calcium release that drives excitation-contraction coupling (Cheng *et al.*, 1993; Kuo and Ehrlich, 2015). Calcium sparks also allow local increases in intracellular calcium to drive nearby signaling events without elevating global calcium levels (Nelson *et al.*, 1995; Navedo *et al.*, 2005; Nieves-Cintrón *et al.*, 2008).

Despite these examples of localized calcium in muscle in physiological settings, how subcellular muscle calcium signaling contributes to behavior is poorly understood. The *C. elegans* pm5 pharyngeal muscle cells function to swallow food from the anterior pharynx to the terminal bulb via a peristaltic wave of subcellular contraction (Avery and Horvitz, 1987), and subcellular calcium signals in the pm5s likely underlie swallowing behavior (Shimozono *et al.*, 2004; Kozlova *et al.*, 2019). These transients have been proposed to resemble calcium waves in cardiac muscle (Kozlova *et al.*, 2019), a

phenomenon driven by the propagation of calcium-induced calcium release from internal stores (Cheng *et al.*, 1993). Subcellular signals have also been observed in enteric muscles, which can contract peristaltically, but the functional significance of these signals is unknown (Yamazawa and Iino, 2002). Our results identify a different and novel role for subcellular muscle contraction and calcium signaling: sustained, neuronally induced contraction of a stable, dedicated subcompartment of muscle, thus modulating function and transforming one behavioral output into another. While calcium sparks and *C. elegans* peristalsis behavior occur on a timescale of tens of milliseconds, the contractions and calcium signals that we observed can last at least 10 seconds. This timescale is more similar to that of calcium sparklets produced by L-type voltage-gated calcium channels (Navedo *et al.*, 2005). We propose that subcellular muscle contraction via subcellular calcium signaling is a mechanism by which the nervous system can modulate muscle function to alter motor outputs and so generate distinct behaviors, and further suggest that *C. elegans* spitting behavior offers a system for the analysis of the molecular bases of subcellular calcium signaling in muscle using a highly genetically tractable organism.

Independent control of the pharyngeal valve and metastomal filter generates four distinct pumping behaviors

We observed that, in addition to opening the pharyngeal valve, spitting worms open the metastomal filter and that this opening depends in part on the pm2 muscles. This finding

suggests that in addition to passively filtering incoming material as reported previously (Fang-Yen *et al.*, 2009), the metastomal filter can be actively opened to allow unrestricted entry of material into the pharynx. In the context of spitting pumps, opening the filter permits new material to be rinsed in and out, while closing the filter allows egestion without rinsing. We observed that the filter sometimes also opens during feeding, producing “gulps” with greatly increased intake. Thus, the independent control of the metastomal filter and pharyngeal valve produces four distinct pumping behavior variants -- feeding, gulping, spitting with rinsing, and spitting without rinsing. Given that M1 is the major source of innervation of the pm1 and pm2 muscles, from which the metastomal filter protrudes (Albertson and Thomson, 1976), we think it likely that M1 can function in the production of gulps by opening the metastomal filter without producing an accompanying contraction in the regions of pm3 that open the pharyngeal valve. Such a behavior might allow enhanced consumption of certain preferred food or, because it draws large amounts of material into the mouth, might allow the worm to more efficiently sample the environment while foraging. The possibility that a single neuron might control opposite behaviors (i.e., spitting and gulping) via the differential activation of multiple downstream muscles and subcellular muscle compartments is intriguing.

The M1 neuron drives spitting from the waist of an hourglass circuit motif

Our circuit studies show that the motor neuron M1 is a key regulator of spitting. We speculate that in addition to controlling the muscles that produce spitting, M1 functions as a central integrator of noxious taste information in the pharyngeal nervous system. Several lines of evidence support this hypothesis. A recent analysis of the pharyngeal connectome found that M1 exhibits a prominent and highly connected position in the pharyngeal network (Cook *et al.*, 2019), suggesting that M1 plays an important role in network function. Second, M1 is particularly well-positioned to integrate noxious taste stimuli, as it receives direct inputs from each of the other five *lite-1*- and/or *gur-3*-expressing pharyngeal neurons (i.e., the paired I2 and single I4, M4, M5, and MI neurons) (Albertson and Thomson, 1976; Bhatla and Horvitz, 2015). Consistent with this reported connectivity, we found that two of these neuron classes, the I2s and I4, contribute to M1's activation by light. Third, it is likely that M1 is itself light- and ROS-sensitive, as it expresses both *lite-1* (Bhatla and Horvitz, 2015) and *gur-3* (this study) and we show here that it can respond robustly to light even when synaptic and humoral inputs are substantially reduced or eliminated, respectively. For these reasons, we propose that M1 sits at the narrow waist of an “hourglass” circuit motif (Figure 7B). In this motif, multiple upstream parallel pathways converge on M1, which in turn makes divergent outputs onto the multiple downstream muscle groups that control the discrete motor outputs of the spitting reflex. Such an “hourglass” structure was recently described in a theoretical analysis of the larger *C. elegans* non-pharyngeal nervous

system (Sabrin *et al.*, 2020). We propose that the neuromuscular circuit we describe here constitutes a miniature variation of an “hourglass” circuit organization.

The magnitude of M1 activation by the I2 and I4 neurons evokes a distinct subset of hourglass motor outputs

We explored circuitry upstream of the M1 neuron and identified the I2 and I4 neurons as likely sites of *gur-3* function in light-induced spitting. The I2s inhibit pumping in response to light, and I4 was previously shown to be activated by light (Bhatla and Horvitz, 2015), but no function for I4 had previously been reported. Because I4 expresses *gur-3* and controls responses to light, we speculate that I4 is also a cellular receptor for light and ROS. Interestingly, although the pharyngeal connectome and our data suggest that M1 acts as a bottleneck through which signals must pass to evoke spitting, the I2/I4 circuit can selectively produce a subset of M1-dependent behaviors by weakly activating M1. Weak *gur-3*-dependent stimulation of M1 is sufficient to open the pharyngeal valve and metastomal filter, but not to increase pumping rates. Because UV light robustly activates the I2s (Bhatla and Horvitz, 2015), the fact that the I2s activate M1 only weakly suggests that their input to M1 is similarly weak. The weakness of this connection might be a design feature of the pharyngeal nervous system, allowing strong activation of the I2 neurons to be converted into weak activation of the M1 neuron, thereby producing an attenuated variation of spitting. We speculate that I4 might function in a similar way, and that the I2s and I4 serve as modules to trigger attenuated spitting, while input from other

cells and/or endogenous *lite-1* function in M1 produce spitting in a more robust manner, enabling the pharyngeal nervous system to generate graded variations of the spitting reflex and thus fine-tune its output to the environment. One problem that must be solved by an hourglass circuit structure with one or a few cells in the bottleneck is how to differentially evoke downstream outputs when all upstream inputs must be compressed together at the hourglass waist. We suggest that the M1 spitting circuit illustrates a simple solution to this problem: graded activation of M1 in the hourglass waist activates progressively larger subsets of downstream outputs. Outputs of such a circuit can thus be tuned by adjusting the magnitude of upstream inputs, allowing upstream neurons to input additively if their connections to the bottleneck are equally weighted or to evoke a subset of downstream outputs if they only weakly activate the bottleneck (as is the case with the I2 neurons).

Previous studies showed that the I2s inhibit pumping and that M1 is required for spitting (Bhatla and Horvitz, 2015; Bhatla *et al.*, 2015). We found that in addition the I2s can promote pumps (spitting pumps) and that M1 can inhibit pumping (it does so in the recovery phase). Thus, each of these neuron classes can function in superficially opposite ways in different contexts. Although it might seem counterproductive for a given neuron to both promote and inhibit pumping, both of these outputs share the effect of reducing food intake, since spitting pumps eject previously ingested food. We suggest that these differentially timed inhibitory and excitatory neural inputs to pharyngeal muscle allow the pharynx to generate a sequence of actions, first inhibiting

pumping and/or stimulating spitting to eject noxious material, then increasing pumping rate and rinsing in the burst pumping phase if noxious stimulation continues, and finally inhibiting all pumping, including spitting, as the animal flees the site of the noxious encounter. Additionally, because spitting with an opened metastomal filter results in rinsing new material into the pharynx, it might be maladaptive if spitting persists when the animal is in a large region of noxious material; in this case, circuits promoting inhibition on a longer timescale than spitting would serve to limit additional exposure to noxious material.

More generally, we propose that subcellular muscle contraction mediated by subcellularly localized muscle calcium signaling is a fundamental mechanism by which nervous systems can reshape motor outputs and suggest that hourglass motifs might function broadly in neuronal circuit organization for the production of complex adaptive behavior in response to changing environments.

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627 and advice.

628

629 **Competing interests:** The authors declare that no competing interests exist.

630 **Materials and methods**

631 ***C. elegans* strains and transgenes (listed in order of appearance):**

632 N2 (wild type)

633 CB1072 *unc-29(e1072)*

634 MT26279 *lin-15(n765ts); nls507 [inx-4_{promoter}::GFP; lin-15(+)]*

635 MT23338 *nls686 [gpa-16_{promoter}::gcamp3::unc-54 3' UTR; lin-15(+)] III; lin-15AB(n765ts)*

636 MT21783 *gur-3(ok2245)*

637 KG1180 *lite-1(ce314)*

638 MT21793 *lite-1(ce314) gur-3(ok2245)*

639 DA1113 *eat-2(ad1113)*

640 MT25982 *eat-2(ad1113); gur-3(ok2245)*

641 MT22899 *eat-2(ad1113); lite-1(ce314)*

642 MT25983 *eat-2(ad1113); lite-1(ce314) gur-3(ok2245)*

643 MT25999 *eat-2(ad1113); lite-1(ce314) gur-3(ok2245); nls687 [lite-1_{promoter}::gfp::lite-1; lin-*
644 *15(+)]*

645 MT25809 *lite-1(ce314) gur-3(ok2245) lin-15(n765ts); nEx2281 [lite-1_{promoter}::lite-1::gfp;*
646 *lin-15(+)]*

- 647 MT25810 *lite-1(ce314) gur-3(ok2245) lin-15(n765ts); nEx2157 [gur-3 gDNA; lin-15(+)]*
- 648 MT23415 *nls686 III; gur-3(ok2245) lin-15(n765ts)*
- 649 MT23417 *nls686 III; lite-1(ce314) lin-15(n765ts)*
- 650 MT23370 *nls686 III; lite-1(ce314) gur-3(ok2245) lin-15(n765ts)*
- 651 MT25631 *lin-15(n765ts); nls864 [glr-2_{promoter}::gfp::unc-54 3' UTR; lin-15(+)]*
- 652 MT25732 *lin-15(n765ts); nls864; nEx2905 [lury-1_{promoter}::ice::sl2::mcherry::unc-54 3'*
- 653 *UTR; ttx-3_{promoter}::mcherry]*
- 654 MT23192 *lin-15(n765ts); nls678 [glr-2_{promoter}::gcamp6s::unc-54 3' UTR; lin-15(+)]*
- 655 MT23250 *gur-3(ok2245) lin-15(n765ts); nls678*
- 656 MT23230 *lite-1(ce314) lin-15(n765ts); nls678*
- 657 MT23369 *lite-1(ce314) gur-3(ok2245) lin-15(n765ts); nls678*
- 658 MT23343 *unc-13(s69); lin-15(n765ts); nls678*
- 659 MT23410 *unc-31(u280); lin-15(n765ts); nls678*
- 660 MT25468 *lite-1(ce314) gur-3(ok2245) lin-15(n765ts); nEx2815 [lury-*
- 661 *1_{promoter}::chr2::sl2::mcherry::unc-54 3' UTR]*
- 662 MT23606 *lite-1(ce314) gur-3(ok2245) lin-15AB(n765ts); nEx2144 [flp-*
- 663 *15_{promoter}::mcherry::gur-3, lin-15(+)]*

664 MT23545 *lite-1(ce314) gur-3(ok2245) lin-15AB(n765ts); nls678 ; nEx2144*

665 MT26001 *eat-2(ad1113); lite-1(ce314) gur-3(ok2245) nls791 X [integrated transgene*

666 *derived from nEx2144: flp-15_{promoter}::mcherry::gur-3, lin-15(+)]*

667 MT21421 *nls569 [flp-15_{promoter}::csp-1b; ges-1_{promoter}::gfp]*

668 MT21791 *lite-1(ce314) nls569*

669

670 **Supplemental strains:**

671 MT2249 *egl-47(n1082dm)*

672 RB850 *egl-47(ok677)*

673 RB845 *gur-4(ok672)*

674 FX06169 *gur-5(tm6169)*

675 MT24110 *lin-15(n765ts); nls780 [gur-3_{promoter}::gfp; lin-15(+)]*

676 MT25804 *oxls322 [myo-2_{promoter}::mcherry::h2b; myo-3_{promoter}::mcherry::h2b] II ; nls310*

677 *[nlp-13_{promoter}::gfp] V; nEx2905*

678 MT25950 *eat-2(ad1113); nls865 [integrated transgene derived from nEx2905: lury-*

679 *1_{promoter}::ice::sl2::mcherry::unc-54 3' UTR; ttx-3_{promoter}::mcherry]*

680 CB933 *unc-17(e245)*

- 681 PR1152 *cha-1(p1152)*
- 682 RM509 *ric-3(md158)*
- 683 CB156 *unc-25(e156) dnj-17(ju1162)*
- 684 MT6308 *eat-4(ky5)*
- 685 CB1112 *cat-2(e1112)*
- 686 MT10661 *tdc-1(n3420)*
- 687 MT14984 *tph-1(n4622)*
- 688 MT15434 *tph-1(mg280)*
- 689 MT22280 *ser-4(ok512); mod-1(ok103); ser-1(ok345) ser-7(tm1325)*
- 690 CB211 *lev-1(e211)*
- 691 MT6637 *acr-2(n2595 n2420)*
- 692 RB1659 *acr-3(ok2049)*
- 693 TU1803 *deg-3(u662) des-2(u695)*
- 694 NC293 *acr-5(ok180)*
- 695 FX03723 *acr-6(tm3723)*
- 696 FX863 *acr-7(tm863)*
- 697 RB1195 *acr-8(ok1240)*

698 FX02515 *acr-10(tm2515)*

699 VC40816 *acr-11(gk827759)*

700 VC188 *acr-12(ok367)*

701 ZZ15 *lev-8/acr-13(x15)*

702 FX05819 *acr-14(tm5819)*

703 RB1172 *acr-15(ok1214)*

704 RB918 *acr-16(ok789)*

705 VC40773 *acr-17(gk804825)*

706 VC40487 *acr-17(gk661490)*

707 VC20403 *acr-18(gk324494)*

708 VC491 *acr-19(ok967)*

709 VC20325 *acr-20(gk161737)*

710 RB1250 *acr-21(ok1314)*

711 VC20417 *acr-23(gk231233)*

712 EG8095 *acr-23(ox429)*

713 RB2119 *acr-23(ok2804)*

714 VC20738 *acr-24(gk387895)*

715 VC40292 *acr-24(gk557791)*

716 MT21980 *deg-3(u773)*

717 CB904 *unc-38(e264)*

718 MT1512 *lev-7/unc-63(x13)*

719 FX03268 *acc-1(tm3268)*

720 VC40182 *acc-2(gk501637)*

721 VC1757 *acc-2(ok2216)*

722 FX03219 *acc-2(tm3219)*

723 FX03174 *acc-3(tm3174)*

724 CX12724 *acc-4(ok2371)*

725 MT22153 *lgc-40(tm3377)*

726 VC20746 *pbo-5(gk963058)*

727 RB1379 *pbo-6(ok1564)*

728 RB896 *gar-1(ok755)*

729 RB756 *gar-2(ok520)*

730 VC670 *gar-3(gk337)*

731

Molecular biology

Transgenes were generated by standard cloning methods. DNA sequences used in the generation of transgenes were amplified with the primers indicated below.

Generated using the infusion cloning technique (Takara Bio, Mountain View, CA) and injected as plasmids:

nls864 - *pSS028* [*glr-2*_{promoter}::*gfp*::*unc-54* 3' UTR]

nEx2905 and *nls865* - *pSS042* [*lury-1*_{promoter}::*ice*::*sl2*::*mcherry*::*unc-54* 3' UTR]

nEx2815 - *pSS034* [*lury-1*_{promoter}::*chr2*::*sl2*::*mcherry*::*unc-54* 3' UTR]

*glr-2*_{promoter}: TTGGGACAAATGTGGAAACGA,

TTCGCTTTTTACAGAGTTAACTCTGC

*lury-1*_{promoter}: TTCATTAAGTAATCCGTTTAGGGCAAAT,

GATTGGATTTTCTGGAATAATCGGGT

ice: ATGGCCGACAAGGTCCTGA,

TTAATGTCCTGGGAAGAGGTAGAAACATC

Generated and injected as PCR product:

nls686 - *pcNB1* [*gpa-16*_{promoter}::*gcamp3*::*unc-54* 3' UTR]

nEx2157 – *pcNB20* [*gur-3*_{promoter}::*gur-3* gDNA::*gur-3* 3' UTR]

nls678 - [*glr-2*_{promoter}::*gcamp6s*::*unc-54* 3' UTR]

753

754 *gpa-16*_{promoter}: ACCAACCTGAACAGCGAATC,

755 AAAGGGAATTTTATGTGAATAATATCG

756 *gur-3*_{promoter}::*gur-3* gDNA::*gur-3* 3' UTR: GCCTGATGGAACACACTTCCAAC

757 GTTTACCCCGTCTCTATTTCCGCTTTG

758 *gcamp6s*: ATGGGTTCTCATCATCATCATC,

759 GCCCGTACGGCCGACTAGTAGG

760

761 **Grinder Pumping Assay**

762 Previously, we used a xenon bulb (Till Photonics Polychrome V, 150 W; Thermo Fischer
763 Scientific, Waltham, MA) to deliver 365 nm UV light through an inverted microscope
764 (Axiovert S100; Zeiss, Oberkochen, Germany) to individual worms on an agar pad
765 (Bhatla and Horvitz, 2015). Because of the low throughput of this approach, we
766 designed a device to deliver 365 nm UV light to standard Petri dishes used for culturing
767 worms and used this apparatus to assay behavioral responses to 365 nm UV light as
768 described previously for 436 nm violet light (Bhatla and Horvitz, 2015). The apparatus
769 consists of a UV-light-emitting LED (M365F1; Thorlabs, Newton, NJ) coupled by a fiber-
770 optic cable to a collimating lens. The LED system delivers 365 nm UV light in a 3-4 mm
771 spot at a power of ~1.5mW/mm² to young (1-day post-L4) hermaphrodite adults moving
772 freely on a Petri dish, producing a burst pumping response indistinguishable from that
773 obtained previously (Bhatla and Horvitz, 2015). Custom Matlab software was used to

control the LED, and the contraction rate of the grinder before, during, and after application of light was observed by eye via a dissecting microscope and manually recorded via this software (Bhatla and Horvitz, 2015). All behavioral data were visualized as described in (Bhatla and Horvitz, 2015; Bhatla *et al.*, 2015). All behavioral assays and imaging experiments were performed in the same location, at which temperature averaged 19°C, with a standard deviation of 1.2°C.

Pharyngeal transport assay

Pharyngeal transport assays were performed as described previously (Bhatla *et al.*, 2015). To visualize the direction of flow in the pharynx during each pump, worms were fed a 50% dilution of 1 μ m polystyrene beads (Polysciences, Warrington, PA) in M9 buffer. Young (1-day post-L4) hermaphrodite adults were placed with bacteria on an NGM agar pad on a coverslip and observed with a 20x objective on an inverted microscope (Axiovert S100; Zeiss). Videos were recorded at 1000 frames per sec using a high-speed video camera (Fastcam Mini; Photron, Tokyo, Japan). 365 nm UV light (0.88-1.5 mW/mm²) was presented to the worm for 10 sec (Till Photonics Polychrome V, 150 W xenon bulb; Thermo Fischer Scientific). Hydrogen peroxide was administered as described previously (Bhatla and Horvitz, 2015). Videos were viewed and manually annotated, using custom Matlab software, for specific behavioral events: ingestion (beads being retained in the procorpus after procorpus relaxation), spitting (beads flowing out of the procorpus), and pumping (movement of the grinder). In some cases,

the worm moved temporarily out of the focal plane of the microscope or field-of-view of the camera. These unscorable frames were annotated as "missing" and excluded from further analysis. The position of the metastomal filter was scored separately based on whether beads passed freely through or accumulated in the buccal cavity during feeding or spitting, and the fraction of feeding and spitting pumps with the filter open or closed was calculated for the period before and during illumination. Animals with fewer than five scorable pumps in a given 10 sec interval were censored from these analyses.

Muscle motion visualization assay

To visualize the timing of pharyngeal muscle motions during each pump, young (1-day post-L4) hermaphrodite adults were placed with bacteria on an NGM agar pad underneath a coverslip and observed with a 20x objective on an inverted microscope (Axiovert S100; Zeiss). Videos were recorded at 86 frames per sec using an EMCCD camera (iXon⁺; Andor, Belfast, United Kingdom). 365 nm UV light was presented to the worm for 10 sec (Till Photonics Polychrome V, 150 W xenon bulb; Thermo Fischer Scientific). Slow-moving *unc-29(e1072)* animals were filmed to facilitate video capture. Videos were viewed and manually annotated based on whether the anterior tip of the pharynx closed or remained open at the end of each pump.

Laser ablations

We used a pulsed nitrogen laser (MicroPoint; Andor) coupled to a compound microscope (Axioplan; Zeiss) to conduct laser microsurgery of individual pharyngeal neurons, as previously described (Fang-Yen *et al.*, 2012). Briefly, worms were immobilized using 10 mM sodium azide and mounted for viewing through a 100x oil objective. Ablations were performed on hermaphrodite larvae of stages 1 or 2 (L1 or L2), with cell nuclei identified by either Nomarski differential interference contrast optics or cell-specific GFP reporters. Specifically, M1 was identified by Nomarski optics or by the M1-expressed GFP reporter *nIs864*, which drives expression of GFP in M1 via the *glr-2* promoter (Brockie *et al.*, 2001), while pm1 was identified by GFP reporter *nIs507*, which drives expression of GFP in pm1 via the *inx-4* promoter (Altun *et al.*, 2009). The pm2 muscles, the I2 and I4 neurons, and, in some experiments, the M1 neuron were identified by Nomarski optics. Ablations were confirmed the following day by appearance under Nomarski optics and/or by the loss of cell-specific fluorescence. On day 3 or 4, adults were assayed for their response to light. Mock-ablated animals were mounted alongside ablation animals and received identical treatments (i.e., observation of nuclear morphology and/or GFP expression) at each step. Animals were allocated into mock and ablation groups randomly.

Genetic ablation

M1 was ablated genetically by overexpressing the mammalian caspase ICE fused to an *sl2::mcherry* sequence in M1 driven by the *lury-1* promoter (Ohno *et al.*, 2017). For all

animals assayed, genetic ablation of M1 was confirmed beforehand using an M1-specific GFP marker (*nls864*), which drives expression of GFP in M1 via the *glr-2* promoter (Brockie *et al.*, 2001). The *nEx2905* genetic ablation transgene also killed the M2 neurons in approximately 50% of animals assayed (Figure S4A), as confirmed using an M2-specific GFP marker (*nls310*) (Luo and Horvitz, 2017). The *sl2::mcherry* sequence used drove varying degrees of mCherry expression in pharyngeal muscle, and we found that the brightness of this mCherry expression correlated with the rate of M2-killing, such that selecting animals with dim pharyngeal mCherry expression reduced the rate of M2-killing to 1-2% (Figure S4C). Animals assayed in Figure 4D and 4E were not selected in this way. We did not determine the rate of M1 or M2 killing in *nls865* animals.

Optogenetic depolarization

Optogenetic manipulations were performed as described previously (Bhatla *et al.*, 2015). Cultivation plates were seeded with 300 μ L of OP50 *E. coli* mixed with 0.5 μ L of 100 mM all-*trans* retinal (ATR+) or ethanol alone (ATR-) and stored in the dark. Transgenic hermaphrodite worms carrying the channelrhodopsin 2 (*chr2*) coding sequence (Nagel *et al.*, 2003; Boyden *et al.*, 2005) fused to a *sl2::mcherry* sequence were cultivated on these plates in the dark from egg to adulthood. For behavioral assays, one-day-old animals were analyzed as described above ('Pharyngeal Transport Assay'), except that 470 nm light was used in the place of 365 nm UV light. *nEx2815*

[*lury-1_{promoter}::chr2::sl2::mcherry::unc-54 3' UTR*] was expressed in both M1 and the M2s driven by the *lury-1* promoter (Ohno *et al.*, 2017). To avoid confounding optogenetic activation of the M2s, we assayed only mosaic animals pre-selected for expression of mCherry in M1 but not the M2s. Animals were allocated into ATR- and ATR+ groups randomly.

Calcium imaging

Calcium imaging was performed as described previously (Bhatla and Horvitz, 2015). Cellular calcium changes were monitored using either GCaMP3 (Tian *et al.*, 2009) expressed using the *nls686* transgene in pharyngeal muscle driven by the *gpa-16* promoter (Jansen *et al.*, 1999) or GCaMP6s (Chen *et al.*, 2013) expressed in M1 using the *nls678* transgene driven by the *glr-2* promoter (Brockie *et al.*, 2001). Adult hermaphrodite worms were immobilized using polystyrene beads on 10% agarose pads under a coverslip (Kim *et al.*, 2013a), and neurons were imaged using an inverted microscope (Axiovert S100; Zeiss) with a 40x air objective. To stimulate animals with UV light and image GCaMP fluorescence simultaneously, light was flickered between 365 nm UV and 485 nm light at a rate of 2 Hz. Behavioral spitting responses to flickered 365 nm / 485 nm light were indistinguishable from behavioral responses to 365 nm light (not shown). Videos were recorded using an EMCCD camera (iXon⁺; Andor) and analyzed using custom Matlab software as described previously (Bhatla and Horvitz, 2015; Bhatla *et al.*, 2015). The immobilized pumping assay described in Figure S2A

was performed under the same conditions described above for calcium imaging, with the exception that animals were stimulated with only 485 nm light.

Behavioral Statistics

For all measurements, each animal was scored once. Biological replicates were performed using separate populations of animals on different days and consisted of 20 animals unless indicated otherwise. Statistics for significance of differences between fractions in metastomal filter analyses were calculated as follows. Fractions were log-transformed, and any fractions with a numerator of zero were approximated to 0.1 prior to transformation. Similar results were obtained by approximating numerators of zero with 0.01. Log-transformed datasets were assessed for significance with an unpaired t-test when a single pair of conditions was compared or unpaired ordinary one-way ANOVA analysis when multiple conditions were compared. These analyses assumed that log-transformed data followed a normal distribution with similar standard deviations. Statistics for significance between average spitting rates were calculated as follows. For each animal, the total number of spits observed during stimulation with light was divided by the number of scorable frames in the light administration phase for that animal to give the average pumping rate during light administration. Significance of differences between groups was assessed by unpaired, ordinary one-way ANOVA analysis, with the assumption that the data followed a Gaussian distribution with similar standard deviations. Pumping inhibition was calculated by dividing the average pumping rate

899 during light stimulation ($10 < t < 20$) by the average pumping rate in the feeding phase
 900 ($0 < t < 10$). All statistics were calculated using Prism (GraphPad Software, San Diego,
 901 CA).

902

903 **Author contributions**

904 SRS - Conceptualization, Methodology, Investigation, Resources, Formal analysis,
 905 Validation, Writing (original draft), Writing (reviewing and editing), Visualization

906 NB - Conceptualization, Methodology, Investigation, Resources, Formal analysis,
 907 Writing (reviewing and editing)

908 ELLQ - Formal analysis, Writing (reviewing and editing)

909 HRH - Conceptualization, Writing (reviewing and editing), Supervision, Funding
 910 Acquisition

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- 1099

Summary of figures and supplemental material

1101

Main figures:

1103 **Figure 1**

1104 **Figure 2**

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1106 **Figure 4**

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1110

Supplemental material:

1112 **Figure 1—figure supplement 1**

1113 **Figure 2—figure supplement 1**

1114 **Figure 3—figure supplement 1**

1115 **Figure 4—figure supplement 1**

1116 **Figure 4—figure supplement 2**

1117 **Video 1**

1118 **Video 2**

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1122 **Video 6**

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1124 **Video 8**

Figure 1. Worms Spit as a Consequence of the Sustained Contraction of Anterior Pharyngeal Muscles

(A) Anatomy of the *C. elegans* pharynx; a, anterior; d, dorsal.

(B) Grinder pumping response to 365 nm UV light (from Bhatla and Horvitz [2015]). Left, adult wild-type *C. elegans* head showing one pump of the grinder; center, raster plot of individual animals, each grinder contraction shown by a tick; right, backward moving average of the pumping rate of the same animals. Labels indicate the “acute inhibition,” “burst,” and “recovery” phases of the response. Scale bar, 20 μ m.

(C) Representative images of worms feeding and spitting in the polystyrene bead feeding assay. Pumps were scored as “feeding” if they resulted in the retention of particles in the anterior pharynx (arrows), while pumps were scored as “spitting” if they released beads from the procorpus into the environment or if beads ingested during a procorpus contraction were not retained. In feeding pumps, the metastomal filter was typically partially closed, such that food accumulated in the buccal cavity (arrowheads and inset). Spitting pumps involved the opening of the metastomal filter such that the buccal cavity did not fill.

(D) Raster plot of representative results from polystyrene bead feeding assay. Animals carried the transgene *nls678* (*M1_{promoter}::gcam6s*). “*M1_{promoter}*” refers to the *glr-2* promoter.

(E) Quantification of average grinder pumping and spitting rates of animals shown in (D).

(F) Representative images of the pumping cycles of feeding (left column) and spitting worms (right column). When open, the lumen of the pharynx (arrowheads) is visible as a white line. The food-trapping pharyngeal valve (left arrowhead) is located at the anterior end of the lumen. In the absence of UV light (left column), the lumen and pharyngeal valve open (yellow arrowheads) and close (white arrowheads) nearly synchronously, enabling the trapping and eventual ingestion of food (Fang-yen *et al.*, 2009). By contrast, in the presence of UV (right column) light the opening of the lumen and the pharyngeal valve become uncoupled. While the lumen continues to open and close, the muscle region at the site of the pharyngeal valve undergoes sustained contraction and thus opens the valve such that the conclusion of a pump ejects material from the pharynx. Animals were *unc-29(e1072)* mutants to facilitate video capture.

(G) Quantification of frequencies at which the metastomal filter was open in feeding and spitting pumps in (D). Center bar, mean; error bars, standard deviation (SD). ****, $p < 0.0001$; t test.

(H) Diagrammatic summary of feeding and spitting behavior. Shading around traces indicates standard error of the mean (SEM). See also **Figure supplement 1**.

Figure 2. Subcellularly Localized Muscle Contractions and Calcium Increases

Open the Pharyngeal Valve to Produce Spitting

(A) Anatomy of the anterior pharynx showing pharyngeal muscles pm1, pm2, and pm3.

Double-headed arrows indicate orientation of muscle fibers.

(B-E) Mock-ablated (n = 9 animals), pm1-ablated (n = 5), pm2-ablated (n = 8), and

pm1/pm2 double-ablated animals (n = 4) are wild-type in light-induced spitting. All

animals carried transgene *nls507* (*pm1_{promoter}::gfp*) to mark pm1. “*pm1_{promoter}*” refers to

the *inx-4* promoter.

(F) Diagram of the eight classes of pharyngeal muscle (pm1-8; borders indicated by thin

solid black lines; lumen indicated with thick solid black lines). Dashed boxes indicate

regions analyzed for GCaMP3 fluorescence changes.

(G) Representative images from video of pharyngeal muscle GCaMP3 calcium imaging.

Yellow arrowhead indicates spatially restricted calcium increases. Images false-colored

based on the intensity of fluorescence changes. See also Video 6.

(H) Light induces increases in pharyngeal muscle calcium. Regions analyzed are

indicated in (F). n = 13 animals.

(I) Spatially restricted calcium increases occur even when light does not induce pumps.

Regions adjacent to the anterior tip of the pharynx do not exhibit sustained calcium

signals when the pharynx is not pumping. Regions analyzed are indicated in (F). n = 9

animals.

1183 (J) Laser ablation of the pm2 pharyngeal muscles partially suppresses the light-induced
 1184 opening of the metastomal filter. Center bar, mean; error bars, SD. ***, $p < 0.001$; t test.
 1185 $n \geq 5$ animals.
 1186 Shading around traces indicates SEM. Animals in (G-J) carried transgene *nls686*
 1187 (*pharyngeal muscle_{promoter::gcamp3}*). “*pharyngeal muscle_{promoter}*” refers to the *gpa-16*
 1188 promoter. See also **Figure supplement 1**.

Figure 3. Pharyngeally-Expressed Gustatory Receptor Orthologs *lite-1* and *gur-3* Play Major and Minor Roles, Respectively, in the Light-Induced Spitting Reflex

(A) *gur-3* is not required for light-induced burst pumping. n = 3 biological replicates (60 animals total).

(B-C) The contribution of *lite-1* to burst pumping cannot be evaluated directly because *lite-1* mutants are partially defective in pumping inhibition. n_{lite-1} = 2 biological replicates (40 animals total); $n_{lite-1 gur-3}$ = 20 animals.

(D-F) Burst pumping of *eat-2(ad1113)* mutants depends on *lite-1* but not *gur-3*. n = 3 biological replicates (60 animals total).

(G) *lite-1* rescuing transgene *nls687 (lite-1_{promoter}::gfp::lite-1)* rescues the burst pumping defect of *eat-2; lite-1 gur-3* mutants. n = 3 biological replicates (60 animals total).

(H-K) Light-induced spitting is reduced in *gur-3* and *lite-1* single mutants and eliminated in *lite-1 gur-3* double mutants. n = 10 animals except n_{gur-3} = 9.

(L-M) Expression of wild-type *lite-1* (transgene *nEx2281; lite-1_{promoter}::lite-1::gfp*) or *gur-3* (transgene *nEx2157; gur-3_{promoter}::gur-3 gDNA*) respectively restores wild-type or partial spitting to *lite-1 gur-3* double mutants. n = 10 animals.

(N) Quantification of average spitting rate during light exposure of animals from (H-M).

(O) Quantification of the frequencies at which the metastomal filter was open in feeding and spitting pumps in (H-M).

1208 (P-R) Light-induced pharyngeal muscle calcium increases depend on *lite-1* (which
 1209 accounts for 99% of the response) and *gur-3* (which makes a small contribution to the
 1210 initial activation of pharyngeal muscle; black arrows). $n \geq 20$ animals.
 1211 Shading around traces indicates SEM. All *gur-3* alleles are null allele *gur-3(ok2245)* and
 1212 all *lite-1* alleles are null allele *lite-1(ce314)*. Center bar, mean; error bars, SD. ****, $p <$
 1213 0.0001; ***, $p < 0.0005$, **, $p < 0.005$; n.s., not significant; ordinary one-way ANOVA.
 1214 See also **Figure supplement 1**.

1215

Figure 4. The Pharyngeal Neuron M1 Controls the Spitting Reflex

(A) The anatomy of M1 (red) suggests that it controls spitting directly, as it is the only neuron to make neuromuscular junctions (bolded blue segment) with the region of pharyngeal muscle that undergoes sustained contraction during spitting.

(B-C) M1 laser-ablated, but not mock-ablated, animals are completely defective in light-induced spitting and burst pumping. Animals carried GFP reporter transgene *nls864* (*M1_{promoter}::gfp*) to mark M1. “*M1_{promoter}*” refers to the *glr-2* promoter. $n \geq 12$ animals.

(D-E) M1 genetically-ablated animals, but not transgene-negative sibling controls, are completely defective in light-induced spitting and burst pumping. Ablated animals carried genetic-ablation transgene *nEx2905* (*M1_{promoter}::ice::sl2::mcherry*) and all animals carried GFP reporter transgene *nls864*. “*M1_{promoter}*” refers to the *lury-1* promoter. $n = 10$ animals.

(F) Spatially restricted calcium signals depend on M1. Region analyzed is indicated in Figure 2F. $n \geq 11$ animals. Animals carried pharyngeal muscle GCaMP3 transgene *nls686*.

(G) Representative example of light-induced GCaMP6s fluorescence increase in M1. Animals carried transgene *nls678* (*M1_{promoter}::gcamp6s*). “*M1_{promoter}*” refers to the *glr-2* promoter. The unchanging signal common to both images was caused by GCaMP6s fluorescence in additional *glr-2*-expressing neurons in the nerve ring. See also Video 8.

(H) Heatmap of light-induced GCaMP6s fluorescence increases (F/F_0) in the M1 soma. Each row is a different animal; $n = 20$.

1237 (I) Average response of animals shown in (H).

1238 (J-L) Light-induced calcium increases in the M1 neuron depend on *lite-1* (which
1239 accounts for 99% M1's response) and *gur-3* (which makes a small but consistent
1240 contribution to M1's initial activation; black arrows). n = 3 biological replicates (more
1241 than 60 animals total).

1242 (M) Light-induced calcium increases in M1 are reduced but still significant in *unc-*
1243 *13(s69)* mutants. n = 2 biological replicates (greater than 41 animals total).

1244 (N) Quantification of M1's calcium response to light in *unc-13(s69)* mutants in (M). Error
1245 bars, SEM; *, p < 0.05; t test.

1246 (O) Light-induced calcium increases in M1 are reduced but still significant in *unc-*
1247 *31(u280)* mutants. n = 20 animals.

1248 (P) Quantification of M1's calcium response to light in *unc-31(u280)* mutants in (O).
1249 Error bars, SEM; **, p < 0.01; t test.

1250 (Q-R) A subset (~25%) of *lite-1 gur-3* animals expressing an M1-specific ChR2
1251 transgene *nEx2815 (M1_{promoter}::chr2::sl2::mcherry)* grown in the presence, but not the
1252 absence, of ATR spit in response to 470 nm light. "*M1_{promoter}*" refers to the *lury-1*
1253 promoter. n ≥ 10 animals.

1254 Shading around traces indicates SEM. All *gur-3* alleles are null allele *gur-3(ok2245)*,
1255 and all *lite-1* alleles are null allele *lite-1(ce314)*. See also **Figure supplement 1** and
1256 **Figure supplement 2**.

Figure 5. The *gur-3*-Expressing I2 Neurons Can Activate M1 to Produce an Attenuated Form of Spitting

(A-B) I2-specific expression of *gur-3* restores light-induced spitting to *lite-1 gur-3* mutants. n = 5 animals.

(C) Quantification of the frequencies at which the metastomal filter was open in feeding and spitting pumps in (A) and (B). Center bar, mean; error bars, SEM; ****, p < 0.0001; t test. n = 5 animals.

(D) I2-specific expression of *gur-3* restores the light-induced M1 calcium response of *lite-1 gur-3* mutants. n ≥ 26 animals.

(E-F) Laser ablation of M1 suppresses the I2-specific *gur-3* rescue of *lite-1 gur-3* spitting defects. n = 11 animals.

(G) Quantification of the frequencies at which the metastomal filter was open in feeding and spitting pumps in (E) and (F). Center bar, mean; error bars, SEM; ****, p < 0.0001; t test. n ≥ 8 animals.

(H) I2-specific expression of *gur-3* via transgene *nls791 (I2_{promoter}::mcherry::gur-3(+))* does not rescue the burst pumping defect of *eat-2; lite-1 gur-3* animals in the grinder pumping assay. n = 20 animals.

(I) Comparison of grinder pumping rates in (E) and (F). While mock-ablated animals from (E) do not inhibit pumping in response to light, M1-ablated animals from (F) exhibit robust light-induced inhibition, indicating that the I2s' promotion of pumping via M1 outweighs their inhibition of pumping.

1278 (J) Comparison of feeding pumping rates from (E) and (F).

1279 (K) Model for the effects of the I2 neurons on M1 and pumping. The I2 neurons reduce

1280 overall feeding by two mechanisms: direct inhibition of feeding pumps and M1-

1281 dependent promotion of spitting pumps.

1282 (L) Genetic ablation of the I2 neurons in a wild-type background does not eliminate light-

1283 induced spitting. n = 5 animals.

1284 (M) Genetic ablation of the I2 neurons of *lite-1(ce314)* mutants does not eliminate light-

1285 induced spitting. n = 6 animals.

1286 Shading around traces indicates SEM. Unless indicated otherwise, “I2-specific

1287 expression of *gur-3*” refers to transgene *nEx2144 (I2_{promoter}::mcherry::gur-3(+))*. “Genetic

1288 ablation of the I2 neurons” refers to transgene *nIs569 (I2_{promoter}::csp-1b)* (Bhatla and

1289 Horvitz, 2015). “*I2_{promoter}*” refers to the *flp-15* promoter.

Figure 6. The I4 Neuron Functions with the I2 Neurons to Promote Spitting and Inhibit Feeding

(A) Diagram indicating the cellular morphology of M1 and the *gur-3*-expressing I2 and I4 pharyngeal neurons. The I2 neurons are bilaterally symmetric; only one I2 neuron is depicted.

(B) Mock-ablated *lite-1* animals inhibit pumping and spit in response to light. n = 8 animals.

(C) I2-ablated *lite-1* animals spit in response to light but do not inhibit pumping. n = 8 animals.

(D) I4-ablated *lite-1* animals inhibit pumping and spit in response to light. n = 10 animals.

(E) I2/I4 double-ablated *lite-1* animals exhibit delayed spitting and do not inhibit pumping in response to light. n = 8 animals.

(F) Quantification of average spitting rate during light exposure of animals from (B-E).

(G) I2- and I4-ablated *lite-1* animals exhibit small M1 calcium responses; I2/I4 double-ablated animals do not have detectable M1 calcium responses. n ≥ 8 animals.

(H) Quantification of the frequencies at which the metastomal filter was open in feeding and spitting pumps in (B-E). n ≥ 4 animals.

(I) Mock-ablated *lite-1* animals inhibit pumping and spit in response to light. n = 5 animals.

1310 (J-L) M1-ablated (n = 6 animals), M1/I2-ablated (n = 6), and M1/I4-ablated (n = 7) *lite-1*
 1311 animals inhibit pumping, but do not spit, in response to light.
 1312 (M) M1/I2/I4 triple-ablated *lite-1* animals neither inhibit pumping nor spit in response to
 1313 light. n = 4 animals.
 1314 (N) Quantification of pumping inhibition in (I-M). Ablation of the I2 neurons eliminates
 1315 light-induced pumping inhibition.
 1316 Shading around traces indicates SEM. All animals were *lite-1*(ce314) null mutants and
 1317 carried transgene *nls678* (*M1_{promoter}::gcamp6s*). “*M1_{promoter}*” refers to the *glr-2* promoter.
 1318 Center bar, mean; error bars, SD. **, p < 0.005; *, p < 0.05; n.s., not significant; ordinary
 1319 one-way ANOVA.

Figure 7. Models of Spitting Behavior and Neural Circuitry

(A) Model for feeding and spitting. During feeding, the M1 neuron is inactive. Thus, the metastomal filter is partially closed, restricting particle influx, and the pharyngeal valve closes at the end of each pump, trapping food. During spitting induced by light or reactive oxygen species (ROS), M1 is active, thus inducing spitting of ingested material by opening the pharyngeal valve and facilitating rinsing of the pharynx by opening the metastomal filter.

(B) Model for neural circuitry controlling light- and ROS-induced spitting. Light or ROS stimulate the M1, I2, and I4 neurons via LITE-1 and GUR-3. The I2 and I4 neurons excite M1, which is situated in the “waist” of an “hourglass” circuit motif. In turn, M1 makes diverging outputs to multiple pharyngeal muscle classes, each driving distinct aspects of the spitting reflex: the pm1 and pm2 muscles open the metastomal filter, a subcellular compartment of the pm3 muscles opens the pharyngeal valve, and the remainder of pm3 pumps. The M1 and I2 neurons each also inhibit pumping.

Figure 1—figure supplement 1.

- (A) The odor of 9M hydrogen peroxide induces spitting. Animals are *unc-29(e1072)* mutants to facilitate video capture. n = 4 animals.
- (B) Raster plot showing occurrence of “closed” (black) and “open” (green) pumps in pharyngeal muscle motion visualization assay. n = 7 animals.
- (C) Moving averages of animals from (B), indicating that the burst pumping phase consists almost completely of “open” pumps.
- Shading around traces indicates SEM.

Figure 2—figure supplement 1.

(A) Quantification of pumps induced by 485 nm light in 10 seconds in immobilized pumping assay. Laser ablation of M1, but not other pharyngeal neurons, eliminates light-induced pumping.

(B-C) pm1-ablated animals lack the metastomal filter and thus ingest more material into the procorpus than mock-ablated controls do. Dark material in the pharyngeal lumen is a mixture of *E. coli* and 1 µm polystyrene beads. In mock-ablated animals, accumulation is visible in the pharyngeal isthmus but is minimal in the procorpus of most animals. pm1-ablated animals accumulate markedly more material in the procorpus. We also noticed qualitatively that pm1-ablated animals often spit material less efficiently than control animals, but this difference could stem from the differences in the average amount of ingested material in the pharynx in each condition. All animals carry transgene *nls507 (pm1_{promoter}::gfp)*. “pm1_{promoter}” refers to the *inx-4* promoter.

Figure 3—figure supplement 1.

(A) *egl-47/gur-1(n1082dm)* mutants were normal in burst pumping. n = 20 animals.

(B) *egl-47/gur-1(ok677)* mutants were normal in burst pumping. n = 20 animals.

(C) *gur-4(ok672)* mutants were normal in burst pumping. n = 20 animals.

(D) *gur-5(tm6169)* mutants were normal in burst pumping. n = 20 animals.

(E-F) Integrated *gur-3* transcriptional reporter transgene *nIs780 (gur-3_{promoter}::gfp)* is expressed in the MI and M1 pharyngeal neurons and an unidentified head neuron (not shown), in addition to being expressed in the I2, I4, and AVD neurons as previously reported by Bhatla and Horvitz (2015).

Shading around traces indicates SEM.

Figure 4—figure supplement 1.

(A) Overexpression of the mammalian caspase ICE in M1 via the transgene *nEx2905* (*M1_{promoter} – lury-1::ice::sl2::mCherry*) kills M1 with high efficiency and the M2 pharyngeal neurons with moderate efficiency. The presence of M1 and the M2s was determined by the expression of transgenes *nls864* (*M1_{promoter} – glr-2::gfp*) and *nls310* (*nlp-13_{promoter}::gfp*), respectively.

(B) M1 genetic-ablation transgene eliminates burst pumping in the grinder pumping assay. Ablation animals carried transgene *nEx2905* (*M1_{promoter} – lury-1::ice::sl2::mcherry*); all animals carried *nls864* (*M1_{promoter} – glr-2::gfp*). n = 40 animals.

(C) Screening animals based on the brightness of pharyngeal mCherry expression allows the selection of animals lacking the M1 neuron but not the M2 neurons. When pharyngeal muscle mCherry expression is high, animals are more likely to lack the M2s. Animals with dim pharyngeal muscle mCherry expression very rarely lack M2s; thus dim mCherry expression can be used to enrich for animals lacking only M1. Center bar, mean; error bars, SEM. n = 3 biological replicates (more than 65 animals total).

(D) Genetic ablation of M1 eliminates burst pumping in *eat-2* mutants. Ablation animals carried transgene *nls865* (*M1_{promoter} – lury-1::ice::sl2::mCherry*). n = 3 biological replicates (60 animals total).

(E) Comparison of grinder pumping rates of mock- and M1-ablated animals from Figure 4B and 4C. In addition to eliminating burst pumping and spitting, M1 ablation enhances the rate of pumping recovery after illumination. n ≥ 12 animals.

1386 (F) Laser-ablated animals from Figure 4B exhibit abnormal “uncoupled” pumps, during
 1387 which the grinder pumps while the procorpus does not. $n \geq 12$ animals.

1388 (G) Comparison of grinder pumping rates of control and M1-ablated animals from Figure
 1389 4D and 4E. In addition to eliminating burst pumping and spitting, M1 ablation enhances
 1390 the rate of pumping recovery after illumination. $n = 10$ animals.

1391 (H) M1 genetic-ablation animals from Figure 4E exhibit abnormal “uncoupled” pumps,
 1392 during which the grinder pumps while the procorpus does not. $n = 10$ animals.

1393 (I) *unc-17(e245)* vesicular acetylcholine transporter mutants defective in vesicular
 1394 acetylcholine transport were severely defective in both feeding and burst pumping. $n = 3$
 1395 biological replicates (60 animals total).

1396 (J) *cha-1(p1152)* choline acetyltransferase mutants defective in acetylcholine synthesis
 1397 were severely defective in both feeding and burst pumping. $n = 20$ animals.

1398 (K) *ric-3(md158)* mutants defective in nicotinic acetylcholine receptor maturation were
 1399 severely defective in both feeding and burst pumping. $n = 20$ animals.

1400 (L) *unc-25(e156)* glutamate decarboxylase mutants defective in GABA synthesis were
 1401 normal in burst pumping. $n = 20$ animals.

1402 (M) *eat-4(ky5)* vesicular glutamate transporter mutants defective in vesicular glutamate
 1403 transport were defective in acute inhibition (Bhatla and Horvitz, 2015) but normal in
 1404 burst pumping. $n = 20$ animals.

1405 (N) *cat-2(e1113)* tyrosine hydroxylase mutants defective in dopamine synthesis were
 1406 normal in burst pumping. $n = 20$ animals.

1407 (O) *tdc-1(n3420)* tyrosine decarboxylase mutants defective in tyramine and octopamine
1408 synthesis were normal in burst pumping. n = 20 animals.

1409 (P) *tph-1(n4622)* tryptophan hydroxylase mutants defective in serotonin synthesis were
1410 mildly defective in both feeding and burst pumping. n = 3 biological replicates (60
1411 animals total).

1412 (Q) *tph-1(mg280)* tryptophan hydroxylase mutants defective in serotonin synthesis were
1413 mildly defective in both feeding and burst pumping. n = 3 biological replicates (60
1414 animals total).

1415 (R) *ser-4(ok512); mod-1(ok103); ser-1(ok345) ser-7(tm1325)* quadruple serotonin
1416 receptor mutants were defective in feeding but normal in burst pumping. n = 20 animals.

1417 Shading around traces indicates SEM. “*M1_{promoter – lury-1}*” refers to the *lury-1* promoter
1418 and “*M1_{promoter – glr-2}*” refers to the *glr-2* promoter. Panels (E-H) are derived from videos
1419 of bead feeding assays, all other behavior plots were grinder pumping assays scored in
1420 real time.

Figure 4—figure supplement 2: Burst pumping responses of acetylcholine receptor mutants to UV light.

(A) *lev-1/acr-1(e211)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

(B) *acr-2(n2595 n2420)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

(C) *acr-3(ok2049)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

(D) *deg-3(u662); des-2/acr-4(u695)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

(E) *acr-5(ok180)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

(F) *acr-6(tm3723)* nicotinic acetylcholine receptor mutants were subtly defective in burst pumping. n = 3 biological replicates (60 animals total).

(G) *acr-7(tm863)* nicotinic acetylcholine receptor mutants were modestly defective in burst pumping. n = 3 biological replicates (60 animals total).

(H) *acr-8(ok1240)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 3 biological replicates (60 animals total).

(I) *acr-10(tm2515)* nicotinic acetylcholine receptor mutants were normal in burst pumping. n = 20 animals.

- 1441 (J) *acr-11(gk827759)* nicotinic acetylcholine receptor mutants were normal in burst
 1442 pumping. n = 20 animals.
- 1443 (K) *acr-12(ok367)* nicotinic acetylcholine receptor mutants were normal in burst
 1444 pumping. n = 20 animals.
- 1445 (L) *lev-8/acr-13(x15)* nicotinic acetylcholine receptor mutants were normal in burst
 1446 pumping. n = 20 animals.
- 1447 (M) *acr-14(tm5819)* nicotinic acetylcholine receptor mutants were normal in burst
 1448 pumping. n = 20 animals.
- 1449 (N) *acr-15(ok1214)* nicotinic acetylcholine receptor mutants were normal in burst
 1450 pumping. n = 20 animals.
- 1451 (O) *acr-16(ok789)* nicotinic acetylcholine receptor mutants were normal in burst
 1452 pumping. n = 20 animals.
- 1453 (P) *acr-17(gk804825)* nicotinic acetylcholine receptor mutants were defective in both
 1454 feeding and burst pumping. n = 2 biological replicates (40 animals total).
- 1455 (Q) *acr-17(gk661490)* nicotinic acetylcholine receptor mutants were normal in burst
 1456 pumping. n = 20 animals.
- 1457 (R) *acr-18(gk324494)* nicotinic acetylcholine receptor mutants were defective in feeding
 1458 but normal in burst pumping. n = 20 animals.
- 1459 (S) *acr-19(ok967)* nicotinic acetylcholine receptor mutants were normal in burst
 1460 pumping. n = 20 animals.

1461 (T) *acr-20(gk161737)* nicotinic acetylcholine receptor mutants were normal in burst
1462 pumping. n = 20 animals.

1463 (U) *acr-21(ok1314)* nicotinic acetylcholine receptor mutants were normal in burst
1464 pumping. n = 20 animals.

1465 (V) *acr-23(gk231233)* nicotinic acetylcholine receptor mutants were subtly defective in
1466 feeding and defective in burst pumping. n = 3 biological replicates (60 animals total).

1467 (W) *acr-23(ox429)* nicotinic acetylcholine receptor mutants were subtly defective in
1468 burst pumping. n = 3 biological replicates (60 animals total).

1469 (X) *acr-23(ok2804)* nicotinic acetylcholine receptor mutants were normal in burst
1470 pumping. n = 20 animals.

1471 (Y) *acr-24(gk387895)* nicotinic acetylcholine receptor mutants were defective in both
1472 feeding and burst pumping. n = 2 biological replicates (40 animals total).

1473 (Z) *acr-24(gk557791)* nicotinic acetylcholine receptor mutants were defective in burst
1474 pumping. n = 3 biological replicates (60 animals total).

1475 (A') *deg-3(u773)* nicotinic acetylcholine receptor mutants were normal in burst pumping.
1476 n = 20 animals.

1477 (B') *unc-29(e1072)* nicotinic acetylcholine receptor mutants were normal in burst
1478 pumping. n = 3 biological replicates (60 animals total).

1479 (C') *unc-38(e264)* nicotinic acetylcholine receptor mutants were normal in burst
1480 pumping. n = 20 animals.

1481 (D') *lev-7/unc-63(x13)* nicotinic acetylcholine receptor mutants were normal in burst
 1482 pumping. n = 20 animals.

1483 (E') *acc-1(tm3268)* acetylcholine-gated chloride channel mutants were normal in burst
 1484 pumping. n = 20 animals.

1485 (F') *acc-2(gk501637)* acetylcholine-gated chloride channel mutants were normal in burst
 1486 pumping. n = 20 animals.

1487 (G') *acc-2(ok2216)* acetylcholine-gated chloride channel mutants were defective in
 1488 feeding and burst pumping. n = 3 biological replicates (60 animals total).

1489 (H') *acc-2(tm3219)* acetylcholine-gated chloride channel mutants were normal in burst
 1490 pumping. n = 20 animals.

1491 (I') *acc-3(tm3174)* acetylcholine-gated chloride channel mutants were normal in burst
 1492 pumping. n = 20 animals.

1493 (J') *acc-4(ok2371)* acetylcholine-gated chloride channel mutants were normal in burst
 1494 pumping. n = 20 animals.

1495 (K') *lgc-40(tm3377)* acetylcholine-gated chloride channel mutants were normal in burst
 1496 pumping. n = 20 animals.

1497 (L') *pbo-5(gk963058)* nicotinic acetylcholine receptor family mutants were normal in
 1498 burst pumping. n = 20 animals.

1499 (M') *pbo-6(ok1564)* nicotinic acetylcholine receptor family mutants were normal in burst
 1500 pumping. n = 20 animals.

1501 (N') *gar-1(ok755)* muscarinic acetylcholine receptor mutants were subtly defective in
 1502 burst pumping. n = 3 biological replicates (60 animals total).
 1503 (O') *gar-2(ok520)* muscarinic acetylcholine receptor mutants were normal in burst
 1504 pumping. n = 20 animals.
 1505 (P') *gar-3(gk337)* muscarinic acetylcholine receptor mutants were normal in burst
 1506 pumping. n = 20 animals.

Video 1: Feeding pumps in polystyrene bead feeding assay.

High frame rate video of *C. elegans* feeding in polystyrene bead feeding assay. The small, dark particles are polystyrene beads. During typical feeding pumps, beads accumulate in the buccal cavity. Beads filter gradually from the buccal cavity into the pharyngeal lumen, where they are trapped by the closure of the pharyngeal valve at the front of the pharynx upon the conclusion of each pump. Video was recorded at 1,000 frames per second. Playback is at 3% of original speed.

Video 2: Spitting pumps in polystyrene bead feeding assay.

High frame rate video of *C. elegans* spitting in response to light in polystyrene bead feeding assay. During spitting pumps, beads no longer accumulate in the buccal cavity, but instead freely enter the pharyngeal lumen. At the end of the pump, all ingested material is expelled. Video was recorded at 1,000 frames per second. Playback is at 3% of original speed.

Video 3: Feeding pumps in muscle motion visualization assay.

Video of *C. elegans* feeding beneath a coverslip. The pharyngeal lumen is visible as a white space when it is opened by the contraction of pharyngeal muscle. In feeding, the musculature of the anterior pharynx relaxes at the end of each pump, closing the pharyngeal valve and thereby trapping ingested material. Video was recorded at 86 frames per second. Playback is at 50% of original speed.

1528

1529 **Video 4: Spitting pumps in muscle motion visualization assay.**

1530 Video of *C. elegans* beneath a coverslip spitting in response to light. In contrast to
 1531 feeding pumps, spitting pumps are characterized by the sustained contraction of the
 1532 anterior musculature of the pharynx, which remains contracted open as the rest of the
 1533 pharynx continues to contract and relax. Video was recorded at 86 frames per second.
 1534 Playback is at 50% of original speed.

1535

1536 **Video 5: “Gulping” pumps in polystyrene bead feeding assay.**

1537 High frame rate video of *C. elegans* “gulping” in polystyrene bead feeding assay.
 1538 As in spitting pumps, beads pass freely through the buccal cavity and into the
 1539 pharyngeal lumen. As in feeding pumps, this material is subsequently trapped and
 1540 retained by the pharyngeal valve, resulting in ingestion. Video was recorded at 1,000
 1541 frames per second. Playback is at 3% of original speed.

1542

1543 **Video 6: Pharyngeal muscle calcium response to light.**

1544 The pharyngeal muscle calcium response to light, as reported by GCaMP3
 1545 fluorescence. Pumps result in fluorescence increases across the entirety of the anterior
 1546 pharynx. Before and between pumps, localized and sustained calcium increases occur
 1547 at the anterior tip of the pharynx. Video was recorded at 15 frames per second and

false-colored based on the intensity of fluorescence changes (blue, low intensity; red, high intensity). Playback is in real time.

Video 7: pm2-ablated animals are defective in opening the metastomal filter during spitting behavior in polystyrene bead feeding assay.

High frame rate video of pm2-ablated *C. elegans* spitting in response to light in polystyrene bead feeding assay. In contrast to typical spitting pumps, beads accumulate in the buccal cavity, indicating that the metastomal filter is partially closed. At the end of the pump, ingested material is still expelled, indicating that the pharyngeal valve is open. Video was recorded at 1,000 frames per second. Playback is at 3% of original speed.

Video 8: The pharyngeal neuron M1 is activated by light.

The pharyngeal neuron M1 is acutely activated by light, as reported by GCaMP6s fluorescence. Video was recorded at 2 frames per second and false-colored based on the intensity of fluorescence changes (blue, low intensity; red, high intensity). Playback is at 100% of original speed.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 1—figure supplement 1

Figure 3—figure supplement 1

Figure 4—figure supplement 1

Figure 4—figure supplement 2, p. 1

Figure 4—figure supplement 2, p. 2