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The serotonin circuit that coordinates germline proliferation and egg laying with other reproductive functions in *Caenorhabditis elegans*

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Behaviour and physiology are altered in reproducing animals, but neuronal circuits that regulate these changes remain largely unknown. Insights into mechanisms that regulate and possibly coordinate reproduction-related traits could be gleaned from the study of sex pheromones that can improve the reproductive success of potential mating partners. In *Caenorhabditis elegans*, the prominent male pheromone, *ascr#10*, modifies reproductive behaviour and several aspects of reproductive physiology in hermaphrodite recipients, including improving oocyte quality. Here we show that a circuit that contains serotonin-producing and serotonin-uptaking neurons plays a key role in mediating effects of *ascr#10* on germline development and egg laying behaviour. We also demonstrate that increased serotonin signalling promotes proliferation of germline progenitors in adult hermaphrodites. Our results establish a role for serotonin in maintaining germline quality and highlight a simple neuronal circuit that acts as a linchpin that couples food intake, mating behaviour, reproductive output, and germline renewal and provisioning.

1. Introduction

Males and females of the same species employ a rich repertoire of signals to improve reproductive success, including sex pheromones that can modulate behaviour and reproductive physiology of potential mates [1]. The case of the most abundant male-biased ascaroside pheromone in *Caenorhabditis elegans*, *ascr#10* [2], is instructive. This small molecule alters several behaviours. Hermaphrodites exposed to physiological concentrations of *ascr#10* reduce exploratory movement [3]. A shift from global to local exploration is generally associated with increased exploitation of local resources [4]. *ascr#10* also increases mating receptivity in hermaphrodites and promotes egg laying in already reproducing animals [3].

In addition to these behavioural changes, *ascr#10* alters hermaphrodite reproductive physiology. It improves sperm guidance [5] and affects several aspects of development of the oogenic germline. Exposure to *ascr#10* increases mitotic proliferation of germline precursor cells (GPCs) [6]. In *C. elegans* hermaphrodites, GPCs proliferate and, following the irreversible switch from spermatogenesis to oogenesis, differentiate into oocytes; oocyte production can continue well into adulthood as long as sperm is available for fertilization [7]. The increased GPC proliferation in the presence of *ascr#10* has no fewer than two consequences. First, it increases stores of GPCs as the worms age [8]. Second, it improves quality of the oogenic germline. The improvement is manifested in a more youthful oocyte morphology, decreased rates of chromosomal nondisjunction, and lower embryonic lethality both in the wild-type and mutant genetic backgrounds [6]. A likely mechanism responsible for the improved oocyte quality on *ascr#10* is that the majority of the extra-generated GPCs undergo physiological cell death, and the salvaged components (nutrients, metabolites, organelles, etc.) are used

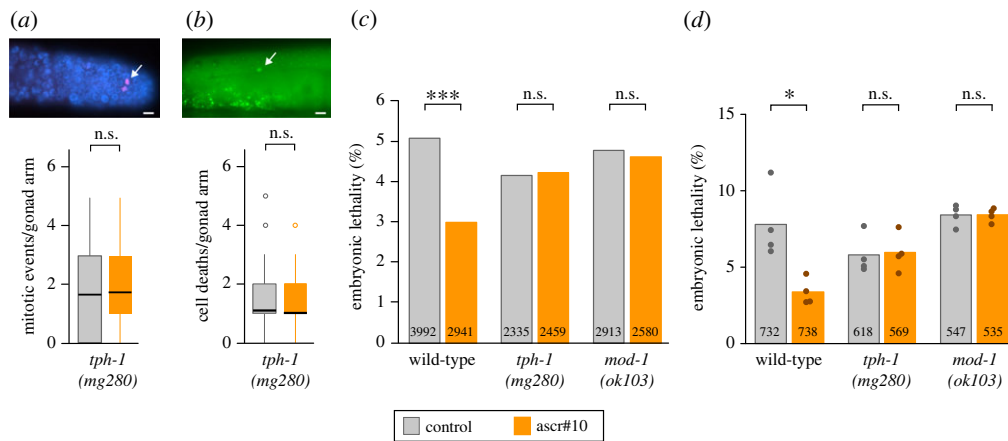


Figure 1. Serotonin signalling is required for the beneficial effects of *ascr#10* on the oogenic germline. (a) Unlike wild-type N2 hermaphrodites [6], *tph-1* hermaphrodites show no increase of germline precursor divisions in the presence of *ascr#10*. Germline proliferation was quantified as ‘mitotic events’ detected using phospho-Histone 3 (pH3) staining (magenta puncta indicated by an arrow in the inset) during day 2 of adulthood. Germline stained with DAPI, shown in blue. (b) Unlike wild-type N2 hermaphrodites [6], *tph-1* hermaphrodites show no increase of germline cell death in the presence of *ascr#10*. Quantified using SYTO12 staining (indicated with an arrow in the inset) during day 3 of adulthood. In (a,b), black bars denote means. In (a,b), scale bars in the insets are 10 μm . (c) Percentage of unhatched embryos on versus off *ascr#10* in the progeny of N2, *tph-1* and *mod-1* self-sperm depleted hermaphrodites mated on day 5 of adulthood to young males. (d) Percentage of unhatched embryos on versus off *ascr#10* in the self-progeny of N2, *tph-1* and *mod-1* hermaphrodites during days 4 and 5 of adulthood. In (c,d), total numbers of tested embryos are indicated inside relevant bars. In (d), dots represent percentage of embryonic lethality in independent experiments. Asterisks indicate levels of statistical significance (* for $p < 0.05$, *** for $p < 0.001$). Kolmogorov–Smirnov test in (a,b), binomial test in (c,d). See electronic supplementary material, table S1 for primary data and details of statistical analyses. (Online version in colour.)

to improve the quality of the surviving oocytes [6]. That an external signal can elicit these effects demonstrates that the nervous system can regulate germline quality, but the specific circuits are not known.

Some effects of *ascr#10* are known to require the function of a specific serotonin circuit that contains NSM and HSN neurons that signal via the *mod-1* receptor [3,9], the same circuit that regulates exploratory behaviour [10]. We therefore tested whether the same serotonin signalling regulates quality of the oogenic germline.

2. Results and discussion

(a) The serotonin circuit that is required for *ascr#10* improvement of oocyte quality

There are five classes of serotonergic neurons in *C. elegans* hermaphrodites; three of them (NSM, ADF and HSN) express a serotonin biosynthetic enzyme TPH-1 and can therefore produce serotonin, whereas two (AIM and RIH) can only uptake serotonin synthesized elsewhere [11]. Of the six annotated high-affinity and two low-affinity serotonin receptors, only one, MOD-1, is required for the known effects of *ascr#10* [3,9,12]. Exposure to *ascr#10* leads to increased serotonin signalling from two classes of producing neurons, NSM and HSN, that acts via the MOD-1 receptor [3,12] expressed in 40 classes of neurons [13]. We sought to test whether the NSM/HSN/MOD-1 neuronal circuit also mediates the beneficial effects of *ascr#10* on the oogenic germline.

Exposure to *ascr#10* (see Methods for details) increased GPC proliferation in wild-type N2 hermaphrodites [6], but no increase was seen in *tph-1* (figure 1a) or *mod-1* (electronic supplementary material, figure S1a) mutants. In the presence of *ascr#10*, incidence of physiological cell death in the germline increased only if GPC proliferation was increased [6]. Consistent with this results, *tph-1* mutants showed no increase of

germline cell death, likely because they were unable to increase germline proliferation in the presence of *ascr#10* (figure 1b). The ultimate effect of *ascr#10* on oocyte quality is increased probability of successful embryonic development [6]. Exposure to *ascr#10* nearly halved embryonic lethality in broods of wild-type hermaphrodites that were mated just after exhausting their self-sperm supply (figure 1c). The same was the case in self-broods of older wild-type hermaphrodites (figure 1d). Because improvements occurred regardless of the source of sperm, these findings further support the idea that *ascr#10* improves oocyte quality [6]. Loss-of-function alleles of *tph-1* or *mod-1* precluded this quality improvement by the pheromone (figure 1c,d). We concluded, that pheromone effects on the oogenic germline require serotonin signalling, specifically acting via the MOD-1 receptor.

(b) Increased serotonin signalling promotes germline proliferation

Serotonin signalling is increased following exposure to *ascr#10* [3,12] and is required for *ascr#10* effects on the oogenic germline (figure 1). Can increased serotonin signalling alone, without exogenous male pheromone, affect the hermaphrodite germline as does *ascr#10*? Loss of function mutations in the serotonin transporter gene *mod-5* reduce serotonin uptake and therefore increase the amount of serotonin available at synapses effectively increasing serotonin signalling [14]. We first examined several strains for the number of GPCs, a measure that is a convenient proxy for elevated germline proliferation [6]. The number of GPCs in the otherwise untreated hermaphrodites carrying *mod-5(lf)* mutations was significantly higher than in the wild-type (average approx. equal to 61 [15]) or in any of the 31 other mutant strains we tested (figure 2a), suggesting that higher levels of serotonin signalling lead to the increase in the number of GPCs. The *mod-5* gene is expressed in four classes of serotonin neurons including producing (NSM and ADF) and uptaking-only (AIM and RIH)

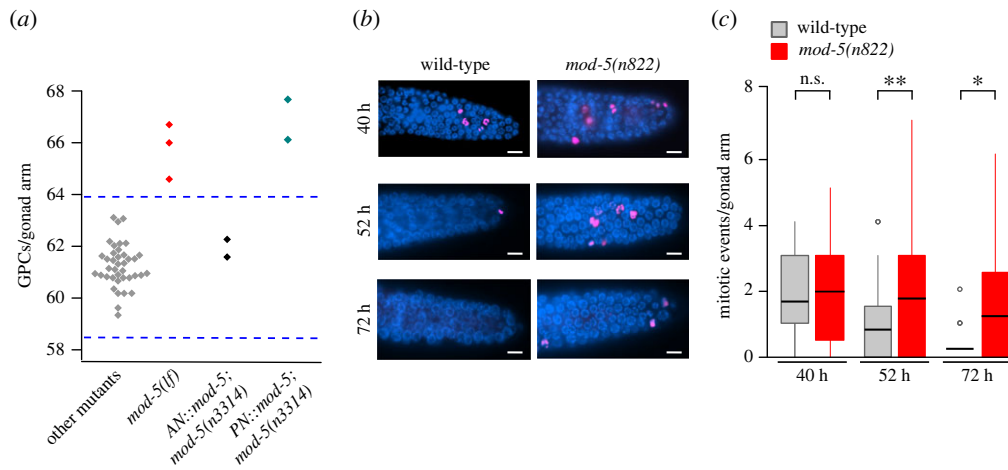


Figure 2. Increased serotonin signalling increases the number of germline precursors. (a) GPC counts from 39 experiments using 31 mutant strains tested in [3] and shown in the electronic supplementary material, table S2. Three experiments involving *mod-5* hermaphrodites—two experiments using *mod-5(n822)*, averages = 66.7 and 66, and one experiment using *mod-5(n3314)*, average = 64.6. Two experiments in which in *mod-5(n3314)* mutants MOD-5 function was restored in neurons including the absorbing AIM and RIH (*AN::*mod-5**), and two experiments in which in *mod-5(n3314)* mutants MOD-5 function was restored in neurons including the producing NSM and ADF (*PN::*mod-5**). Each diamond represents the mean value from one experiment. Dashed lines delimit three standard deviations above and below the mean of all strains except *mod-5*. See electronic supplementary material, figure S1b for more detail. (b) Representative images of pH3 staining (magenta) of gonads (DAPI stain in blue) in wild-type N2 and *mod-5* hermaphrodites aged to mid-L4 (40 h), pre-reproductive adult (52 h) and day 2 of adulthood (72 h). Scale bars are 20 μm. (c) Quantification of cell divisions (pH3 staining) in the Progenitor Zone in the germlines of N2 and *mod-5* hermaphrodites. In none of the experiments in this figure, were hermaphrodites treated with *ascr#10*. Asterisks indicate levels of statistical significance (* for $p < 0.05$, ** for $p < 0.01$; Kolmogorov–Smirnov test). See electronic supplementary material, table S1 for primary data and details of statistical analyses. (Online version in colour.)

cells [11,13]. Whereas expressing MOD-5 in the producing NSM and ADF neurons did not rescue the *mod-5* defect, expression in a set of cells that included AIMs and RIH, but no serotonin-producing neurons, restored the wild-type GPC numbers (figure 2a).

Proliferation of the germline precursor cells is reduced in adult *C. elegans* hermaphrodites compared to late larvae [16]. The untreated *mod-5(lf)* adult hermaphrodites showed increased germline proliferation (figure 2b,c) that was comparable to that of wild-type worms exposed to *ascr#10* [6].

(c) The NSM/HSN/MOD-1 circuit regulates egg laying in response to *ascr#10*

Caenorhabditis elegans hermaphrodites continuously produce embryos as long as oocytes and sperm remain available. If the rate of egg laying is lower than the rate of fertilization, embryos accumulate in the uterus (figure 3a). Serotonin signal from HSN neurons stimulates egg laying [18], while the loss of *tph-1* reduces egg laying and thus increases the number of embryos retained in the uterus [19]. Exposure to *ascr#10* increases serotonin signalling from NSM and HSN neurons and stimulates egg laying [3]. We tested the role of the NSM/HSN/MOD-1 circuit in the pheromone effect on egg laying. The loss of the TPH-1 function in all neurons expressing this enzyme or specifically in NSM or HSN neurons completely negated the stimulating effects of *ascr#10* on egg laying (figure 3b). Loss-of-function *mod-1* mutants retained about the same number of embryos as the wild-type, as reported previously [20], but were unable to increase egg laying on *ascr#10*.

(d) A model for coordinated effects of serotonin on *Caenorhabditis elegans* reproduction

Our results implicate NSM, HSN and AIM/RIH (i.e. the majority of serotonin neurons) in mediating reproductive

functions in *C. elegans* hermaphrodites (figure 3c). Increased signalling from the serotonin-producing NSM and HSN promotes several reproductive traits, whereas AIM/RIH limit this signalling by removing serotonin. Downstream of the serotonin balance established by the NSM/HSN versus AIM/RIH antagonism is the MOD-1 receptor that is expressed in 40 classes of neurons. We infer that further downstream signalling from a currently unknown subset of these neurons modulates, via currently unknown but presumably neurotransmitter and/or neuropeptide signals, several reproductive traits described here. Determining which neurons and signals are involved in these processes promises to advance understanding of how the nervous system controls reproduction and reveal the extent to which reproductive processes are coordinately regulated.

The roles of NSM and HSN neurons in reproduction are particularly interesting. Serotonin release from HSN neurons stimulates egg laying [18]. Here we showed that increased serotonin signalling (due to the loss of serotonin uptake via MOD-5) increases germline proliferation. This suggests that the signal that promotes egg laying episodes also promotes production of germline precursors, presumably to replenish oocyte stores depleted by offspring production. The pharyngeal NSM are enteric sensory neurons that detect food ingestion and release serotonin as a signal [21]. Therefore, increased serotonin signalling from NSM likely promotes processes associated with greater food ingestion. Consistent with this idea, *ascr#10* reduces exploration in a manner that depends on serotonin signalling from NSM [3,12].

These functions of NSM and HSN neurons suggest a model for the role of serotonin in orchestrating multiple reproduction-related events. On the one hand, increased serotonin signalling on *ascr#10* reduces exploration leading to a focus on consumption of local resources and greater mating receptivity [3]. On the other hand, increased serotonin signalling increases egg laying and proliferation of germline precursors.

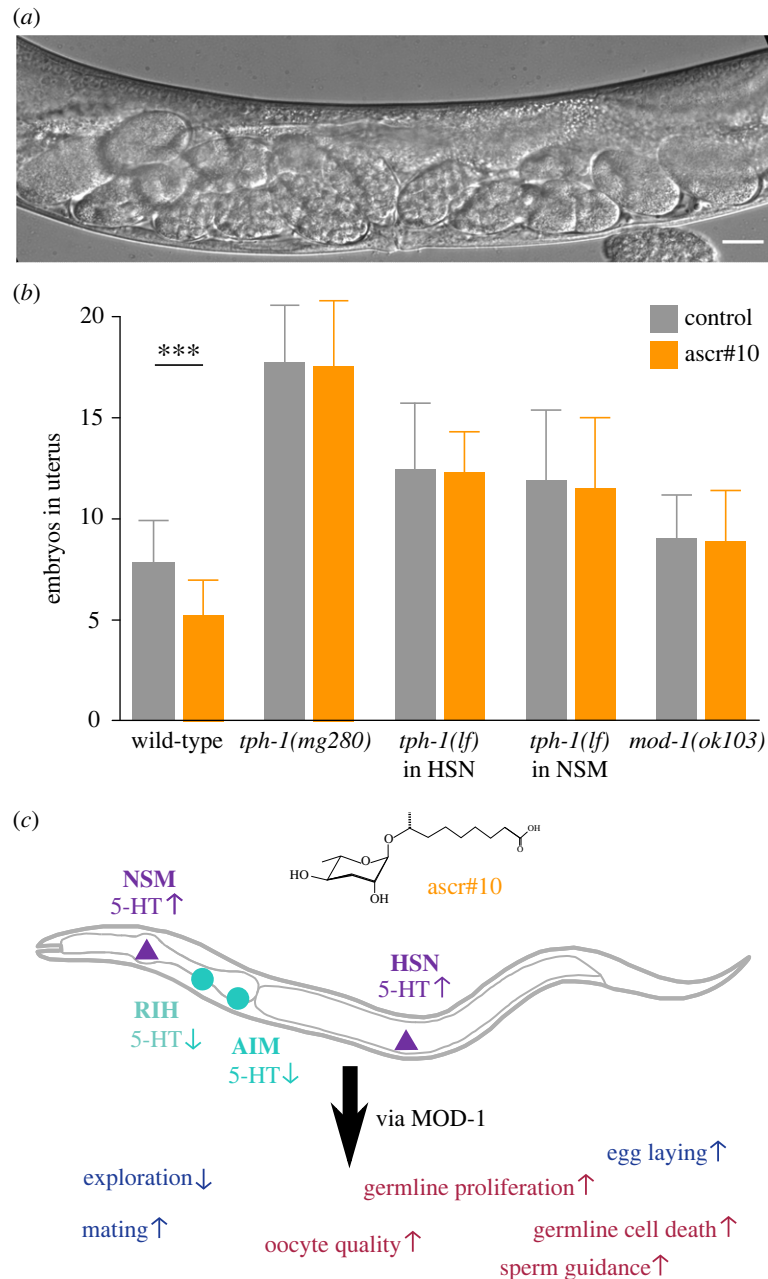


Figure 3. The serotonin circuit that coordinately regulates multiple reproductive functions in *C. elegans*. (a) An image of embryos accumulated in the uterus; *tph-1(mg280)* is shown. Scale bar is 20 μm . (b) The comparison of the number of embryos accumulated in the uterus on versus off *ascr#10*. Asterisks indicate statistical significance (***) for $p < 0.001$; Kolmogorov–Smirnov test). See electronic supplementary material, table S1 for primary data and details of statistical analyses. (c) A hypothesis regarding the role of serotonin in promoting reproductive behaviour and physiology. Increased serotonin (5-HT) signalling from *tph-1*-expressing neurons NSM and HSN (purple) affects a number of reproductive behaviours (blue) and germline physiology (red) via the MOD-1 receptor. Up and down arrows next to traits indicate whether they are up- or downregulated by increased serotonin signalling. RIH and AIM neurons reduce serotonin signalling. The male pheromone *ascr#10* stimulates serotonin signalling from NSM and HSN. The way in which *ascr#10* improves sperm guidance in the hermaphrodite reproductive tract [5] is not discussed in detail here, but sperm guidance is positively regulated by serotonin signalling [17]. (Online version in colour.)

The physiological cell death of the majority of extra germline precursors liberates nutrients, organelles, etc. that are used to improve the quality of surviving oocytes. In this way, the circuit that consists of the serotonin-producing NSM and HSN neurons, the MOD-5 transporter in AIM/RIH neurons, and the MOD-1 receptor coordinates resource consumption required for greater reproductive output, with reproduction-promoting behaviours, and matches quality and quantity of produced oocytes to demands imposed by germline expenditure and the presence of potential mates. As such, this circuit may serve as an important regulator of the trade-off between somatic maintenance and germline investment. As discussed elsewhere [6], *ascr#10* promotes greater resource allocation to

progeny production at the expense of the soma—although this pheromone improves the quality of the oogenic germline [6], it shortens the lifespan [8,22]. It is not currently clear whether male signalling to hermaphrodites via *ascr#10* reflects sexual conflict or cooperation.

(e) Is serotonin a conserved regulator of reproduction?

Evidence indicates that serotonin regulates reproduction-related traits in different species in ways that may be conserved. For example, serotonin signalling reduces exploratory movement in *C. elegans* [10], *D. melanogaster* [23] and mice [24]. In *D. melanogaster*, serotonin is involved in regulating

a dietary switch that occurs in females following mating and manages the balance of nutrients, a role that may be conserved in other animals [25]. In *Drosophila* females, mating causes changes in the levels and distribution of serotonin in the termini of neurons that innervate reproductive organs [26]. In mosquitoes, serotonin promotes ovarian development [27]. In mammals, serotonin exerts complex effects on the germline [28] and on reproduction-related behaviours [29]. Some, of these effects may be shared with other vertebrates [30]. Future comparative work will benefit from standardizing experimental paradigms across species. We also suggest that precise definition of the circuits (sources of signal, receptors and sites of action) involved in regulating specific behaviours and physiological processes, as we reported here, will be particularly important for inferring conserved roles of serotonin in regulating reproductive functions in animals.

3. Materials and methods

The following stains were used: wild-type N2 (CGC), MT15434 *tph-1(mg280)* (CGC), MT9668 *mod-1(ok103)* (CGC), AN::*mod-5 mbr-1p::mod-5;mod-5(n3314)* (Sze lab), PN::*mod-5 tph-1p::mod-5;mod-5(n3314)* (Sze lab), MT8944 *mod-5(n822)* (Koelle lab), MT9772 *mod-5(n3314)* (CGC), CX13576 *tph-1(mg280) II; kySi56 IV; kyEx4107[egl-6::nCre]* (aka *tph-1(lf)* in HSN (Bargmann lab) and CX13572 *tph-1(mg280) II; kySi56 IV; kyEx4057[ceh-2::nCre]* (aka *tph-1(lf)* in NSM) (Bargmann lab). Standard, previously published methods were used [3,6,9,12,15]. All experimental treatments were processed in parallel with matched controls. Worms were synchronized by hypochlorite treatment and overnight incubation in M9, after which the synchronized L1 larvae were placed (in small populations, 10 or 30 worms, depending on the experiment) on agar plates seeded with *E. coli* OP50. Pre-reproductive adults (48 h post-release from L1 arrest) were transferred to OP50-seeded plates that were either control or conditioned with synthetic *ascr#10* (gift of F. C. Schroeder). This protocol ensured that hermaphrodites were exposed to pheromone after the switch from spermatogenesis to oogenesis and during their pheromone-sensitive age [6]. For assessing embryonic lethality following mating, day 5 hermaphrodites (these have exhausted self-sperm) were singled and mated to one young male for 2 h. Numbers of live and dead progeny were counted for 3 days thereafter. For assessing embryonic lethality in self-broods, progeny produced during the last day of self-fertility (end of day 4 through day 5 of adulthood) was considered. More detailed protocol can be found in [6]. For quantifying germline proliferation, gonads were dissected and stained with Anti-Histone H3 (phospho S10) antibodies following a modified

protocol of [31], as described in [6], and only prophase nuclei were counted. For counting cell death events, we used SYTO12 (Invitrogen) and the protocol by [32] as detailed in [6]. For GPC counts, on day 5 of adulthood, hermaphrodites were stained with DAPI (4',6-diamidino-2-phenylindole) as described [8] and the germline precursor cells were counted. The boundary between the Progenitor Zone and the more proximal Transition Zone is defined by the appearance of crescent-shaped nuclei that have progressed to leptotene/zygotene stages of meiotic prophase [33,34]. The AN::*mod-5* (absorbing neurons) and PN::*mod-5* (producing neurons) strains (gift of J. Y. Sze) were reported in [11]. In these *mod-5* mutant strains, the expression of the wild-type MOD-5 was restored under control of heterologous promoters. The expression of the AN::*mod-5* was directed by a *mbr-1* promoter. The expression of the *mbr-1* gene can be detected in 28 classes of neurons [13], but the only overlap with the pattern of *mod-5* expression is in AIM and RIH neurons. The expression of the PN::*mod-5* was directed by the BCD region of the *tph-1* promoter [35]. The overlap between the expression pattern of this promoter and the *mod-5* gene is in two neurons: NSM and ADF. To quantify the number of embryos in the uterus, 72 h adult hermaphrodites were transferred to either control or *ascr#10* plates for 1 h. After that time, the worms were placed individually in domed PCR caps containing 20 μ l of hypochlorite solution and allowed to dissolve. After the hermaphrodite body disintegrated, the number of fertilized embryos was counted. Some strains were also tested at 96 h post-release from L1 arrest. The strains in which the *tph-1* was deleted in either NSM or in HSN neurons were reported in [10].

Data accessibility. The data are provided in the electronic supplementary material [36].

Authors' contributions. E.Z.A.: conceptualization, data curation, formal analysis, investigation, methodology and writing—review and editing; S.D.: data curation and investigation; I.R.: conceptualization, data curation, formal analysis, funding acquisition, investigation, project administration, supervision, visualization and writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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