***C. elegans* enteric motor neurons fire synchronized action potentials underlying the defecation motor program**

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Abstract

The *C. elegans* nervous system was thought to be strictly analog, constituted solely by graded neurons. We recently discovered neuronal action potentials in the sensory neuron AWA; however, the extent to which the *C. elegans* nervous system relies on analog or digital neural signaling and coding is unclear. Here we report that the enteric motor neurons AVL and DVB fire all-or-none calcium-mediated action potentials that play essential roles in the rhythmic defecation behavior in *C. elegans*. Both AVL and DVB synchronously fire giant action potentials to faithfully execute all-or-none expulsion following the intestinal pacemaker. AVL fires unusual compound action potentials with each positive calcium-mediated spike followed by a potassium-mediated negative spike. The depolarizing calcium spikes in AVL are mediated by a CaV2 calcium channel UNC-2, while the negative potassium spikes are mediated by a repolarization-activated potassium channel EXP-2. Whole-body behavior tracking and simultaneous neural imaging in free-moving animals suggest that action potentials initiated in AVL in the head propagate along its axon to the tail and activate DVB through the INX-1 gap junction. Synchronized action potential spikes between AVL and DVB, as well as the negative spike and long-lasting afterhyperpolarization in AVL, play an important function in executing expulsion behavior. This work provides the first evidence that in addition to sensory coding, *C. elegans* motor neurons also use digital coding scheme to perform specific functions including long-distance communication and

temporal synchronization, suggesting further, unforeseen electrophysiological diversity remains to be discovered in the *C. elegans* nervous system.

Introduction

The ancient enteric nervous system plays a role not only in digestion, but in the broader regulation of physiology and behavior across the animal kingdom. For example, studies of the crustacean stomatogastric nervous system have revealed numerous insights into the cellular and circuit mechanisms that generate rhythmic motor patterns (Marder and Bucher, 2007). The intestine and a simple enteric nervous system underlying the *C. elegans* defecation have been a powerful model system to study gut-brain communication and ultradian rhythms. This rhythmic defecation behavior, called the defecation motor program (DMP), consists of a series of stereotyped motor sequences activated once every ~45 s (Branicky and Hekimi, 2006). At the behavioral level, the three distinct, sequential motor steps are referred to as pBoc (posterior Body muscle contraction), aBoc (anterior Body muscle contraction), and Exp (Expulsion, or enteric muscle contraction) (Thomas, 1990). At the anatomical level, these motor steps are driven by a simple gut-brain circuit consisting of the intestine, two enteric motor neurons called AVL and DVB (McIntire et al., 1993), and downstream contractile apparatus including body-wall muscles and enteric muscles specialized in expulsion (Thomas, 1990). Extensive studies using genetic screening, calcium imaging, and behavioral analysis have collectively brought forth the following model: propagating calcium waves in the intestine, mediated by inositol triphosphate (IP3) receptors, generate intrinsic rhythmicity as the pacemaker of the system (Dal Santo et al., 1999; Peters et al., 2007; Teramoto and Iwasaki, 2006); periodic calcium-mediated release of protons from the posterior intestine directly activates the contraction of posterior body-wall muscles (Beg et al., 2008); the intestinal rhythmicity is relayed to the enteric neurons via calcium-mediated release of a neuropeptide-like protein NLP-40 from the intestine, activating the AEX-2/G protein-coupled receptor (GPCR) on AVL and DVB (Mahoney et al., 2008; Wang et al., 2013); activated AVL and DVB, synchronized by electrical synapses INX-1 (Choi et al., 2021), release the neurotransmitter GABA at the neuromuscular junctions (NMJs) to activate downstream enteric muscles through excitatory GABA receptors EXP-1 (Beg and Jorgensen, 2003); coordinated contraction and relaxation of enteric muscle groups finally lead to discrete expulsion events, expelling the gut contents (Thomas, 1990). Despite much progress on the understanding of calcium oscillations, rhythm regulation, and peptidergic signaling made over a span of 30 years by studying the *C. elegans* defecation cycle (Branicky and Hekimi 2006), the biophysics and activity dynamics of enteric neurons

and their contribution to the time-keeping mechanism of the behavioral rhythm is not well understood.

The rhythmicity, synchronization, and long-distance communication featured in the *C. elegans* defecation behavior suggest digital signaling that is best served by spiking neurons. Indeed, at least three lines of evidence from previous studies indicate that AVL and DVB may possess all-or-none properties. First, calcium transients observed in AVL and DVB preceding expulsion are discrete with uniform amplitude and kinetics (Choi et al., 2021; Wang et al., 2013; Wang and Sieburth, 2013). Second, genetic manipulations of the defecation circuit almost always affect AVL and DVB activities in an all-or-none fashion, i.e., they may eliminate or restore neuronal activity or in some cases affect the timing, but not the magnitude, of the calcium transients (Choi et al., 2021; Wang et al., 2013; Wang and Sieburth, 2013). Third, injection of neuropeptide NLP-40 into the tail region evoked DVB calcium transients that are indistinguishable from spontaneously occurring ones (Wang et al., 2013). Because it is unlikely that exogenous injection would deliver the precise amount of neuropeptides in the correct time scale as the endogenously released counterpart, these results strongly suggest that DVB is gated by an all-or-none thresholding mechanism.

Action potentials digitalize continuous inputs into precisely timed spikes and are crucial for neural coding and signal propagation in most known nervous systems. The *C. elegans* nervous system was thought to be an exception with only graded signaling until the recent discovery of the first neuronal action potentials in an olfactory neuron, AWA (Liu et al., 2018). Thus, the *C. elegans* nervous system employs a hybrid system for information processing, but it is not clear to what extent the digital and analog coding schemes are utilized across different neural cell types or neural functions. The all-or-none property and temporal precision inherently associated with the action potential excel at synchronizing spatially distributed activity, relaying information across distances without decay, and setting time windows for neural oscillation and plasticity. Here we directly examine the intrinsic electrophysiological properties of AVL and DVB and identify both neurons as spiking motor neurons that fire all-or-none action potentials in *C. elegans*. Through recordings from animals with loss- or gain-of-function mutations in various ion channels, we define the ionic basis and the molecular mechanisms underlying these action potentials. Simultaneous imaging of both neurons and the intestine in free-moving animals suggest that the action potential in AVL not only functions in long-distance signal propagation but its specialized features also set timing for neural synchronization that is important for expulsion behavior. These newly discovered spiking neurons reveal unanticipated heterogeneity in the *C. elegans* nervous system and call for future in-depth investigations of the significance of its biophysical diversity in neural computation and animal behavior.

Results

Enteric neurons AVL and DVB fire action potentials with distinct kinetics

The *C. elegans* enteric nervous system consists of two GABAergic motor neurons, AVL in the lateral ganglion and DVB in the dorso-rectal ganglion. Cell-killing experiments indicate that they drive the expulsion step of the defecation cycle (Choi et al., 2021; McIntire et al., 1993). Since expulsion mediated by synchronized action of multiple enteric muscles is an episodic, all-or-none behavior, we suspected that AVL and DVB may possess specialized electrophysiological properties best suited for this task. Thus, we recorded from cell bodies of AVL and DVB in dissected worms with GFP expressed in all GABA neurons using whole-cell patch clamp to determine their intrinsic electrophysiological properties and underlying ion channel kinetics. Under current-clamp recording configuration, all-or-none action potentials were evoked in both AVL and DVB by applying series of current-injection stimuli (Fig 1A and B). Several motor neurons have been electrophysiologically characterized including VA and VB (Liu et al., 2017; Liu et al., 2014), VD and DD (unpublished data) neurons but none were found to fire action potentials. Thus, AVL and DVB are the first two spiking motor neurons identified in *C. elegans* to date. As comparisons, neither RIS, another GABAergic neuron located in close proximity to AVL in the head region, nor DVC, another neuron located right next to DVB in the tail region, fired action potentials under any current-injection stimuli we tested (unpublished data). These results suggest that action potential firing is not a general property for GABAergic neurons, nor dictated by the neuron's physical location in the body, but most likely a specialized feature for the two enteric neurons with specific functions in the defecation behavior.

The kinetics of action potentials in AVL and DVB were distinct from each other, as well as from those in AWA (Liu et al., 2018). In particular, under suprathreshold stimuli, DVB membrane potential depolarized gradually at first, then evolved into an abrupt upstroke overshooting 20mV after reaching a threshold of about -20mV (Fig 1B). The peak of the upstroke was followed by a fast downstroke and a modest afterhyperpolarization (AHP), forming a broad spike of nearly 400 ms in half-maximum width (Fig 1B and C). DVB usually fired single or double spikes with similar threshold, amplitude, spike width and overall shape, the defining features of all-or-none action potentials. Similarly, AVL also fired broad action potential spikes but with smaller peak amplitude (Fig 1B and C). More strikingly, however, the AHP following each positive spike in AVL evolved into a much more prominent and regenerative negative spike in the hyperpolarization direction (Fig 1A and C).

The action potentials in AVL and DVB are likely mediated by voltage-gated calcium currents, since the *C. elegans* genome lacks genes encoding voltage-gated sodium channels (Bargmann, 1998). In this case, the large and stereotyped calcium-mediated action

Figure 1. Enteric neurons AVL and DVB fire action potentials with distinct kinetics.

A - B. Representative current-clamp recording traces from AVL and DVB. Top: current-injection stimulation steps (5 s long, at 1 pA increments). Left: membrane potential dynamics under color-matched stimulation steps. Right: representative single recording traces with action potentials fired. The scale bar is for both AVL and DVB traces. **C.** Statistical measurements of resting membrane potential (AVL: -45.7 ± 1.1 mV; DVB: -49.0 ± 3.6 mV), threshold (AVL: -23.3 ± 0.8 mV; DVB: -23.4 ± 1.6 mV), peak amplitude (AVL: 9.8 ± 0.8 mV; DVB: 26.3 ± 2.6 mV), afterhyperpolarization (AHP) (AVL: $-64.8.3 \pm 1.2$ mV; DVB: -38.5 ± 2.5 mV), and half-maximum spike width (AVL: 394.2 ± 40.0 ms; DVB: 358.8 ± 59.5 ms) for AVL ($n = 17$) and DVB action potentials ($n = 5$) in wild-type animals. Error bar is SEM. **D - E.** Representative calcium imaging traces from the cell bodies of AVL and DVB expressing GCaMP6f. Left: 300 s continuous recording traces. Right: isolated single calcium spikes from the left traces to demonstrate the difference in calcium signal decay.

potential spikes should be detectable using fluorescent calcium indicators expressed in AVL and DVB. Indeed, in immobilized worms expressing GCaMP6f in GABAergic neurons including AVL and DVB, spontaneous and periodic calcium spikes were detected in the cell body of both neurons (Fig 1D and E). The period of the rhythmic calcium spikes in both

neurons are in line with the ~45 sec cycle time of the defecation motor program, consistent with previous studies showing periodic calcium transients in AVL and DVB using different calcium indicators, imaging methods and genetic backgrounds (Choi et al., 2021; Wang et al., 2013; Wang and Sieburth, 2013). Despite the apparent decay of the peak amplitudes over time due to GCaMP bleaching, the uniform shape and discrete nature of these calcium spikes suggest that they resulted from all-or-none, calcium-mediated action potentials. Presumably due to the presence of the negative spikes terminating calcium signals in AVL, individual calcium spikes in AVL had a shorter spike width than those of DVB (Fig 1D and E). These results suggest that in addition to the sensory neuron AWA, some motor neurons in the *C. elegans* enteric nervous system also fire calcium-mediated action potentials.

The AVL action potential is mediated by CaV2 voltage-gated calcium channel UNC-2

To gain insight into the unusual compound action potential in AVL with an upward spike followed by a downward or negative spike, we performed ion substitution and mutant analysis to determine its ionic origin and molecular mechanism. AVL action potentials recorded from wild-type animals in standard recording solutions containing 2mM $[Ca^{2+}]_o$ had highly stereotyped waveforms under suprathreshold stimuli of various amplitudes and duration (Fig 2A). When Ca^{2+} was removed from the extracellular solution, the depolarizing spikes in AVL were abolished, consistent with the hypothesis that the depolarizing spikes in AVL are calcium-mediated action potentials (Fig 2B). Interestingly, the negative spikes were still detected following membrane repolarization, suggesting that they were generated by a calcium-independent mechanism, which was later determined to be regenerative potassium efflux (see below).

To identify the calcium channels underlying the calcium-mediated positive spike in AVL, we recorded from animals with mutations in the alpha subunit of each predicted voltage-gated calcium channel (VGCC) family in *C. elegans* (Hobert, 2013). In a reduction-of-function mutant of *egl-19*, which encodes a CaV1 L-type VGCC, complete action potentials (with a positive spike followed by a negative spike) were recorded from AVL, although in about half of the cases negative spikes were not reliably evoked (Fig 2C). In the loss-of-function (*lf*) mutant of CaV2 P/Q-type VGCC encoded by *unc-2*, complete action potentials with negative spikes are rarely evoked (in 1 out of 8 recordings) (Fig 2D). Moreover, the shape of the positive phase of the AVL spikes were aberrant in *unc-2* mutant compared to the wild type with shallower upstroke (Fig 2D) and smaller amplitude (Fig 2G). Conversely, in a gain-of-function (*gf*) mutant of *unc-2*, complete action potentials were reliably evoked in all recordings, while the duration of AVL action potentials became more variable and significantly longer in average (Fig 2E and G). These data suggest that UNC-2

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Figure 2. AVL action potential is mediated by CaV2 voltage-gated calcium channel UNC-2.

A. Representative action potential traces recorded from wild-type AVL. Left: overlay of isolated single action potential spikes recorded from different animals aligned by the steepest slope of the upstroke. Right: the same AVL fires nearly identical action potentials evoked by stimulation steps with various length. **B.** Positive (depolarizing) action potential spikes in AVL were abolished in calcium-free extracellular solutions but negative spike was still detected following membrane potential repolarization (orange trace). Left: current-injection stimulation steps (5 s long 1 pA increment). **C-F.** Representative current-clamp recording traces from AVL in different calcium channel mutants. Left: Representative recording traces under a series of current injections steps shown in B. Right: overlay of single action potential spikes from different mutants (colored traces) aligned to the wild-type traces (same as in A but in gray). **D** and **F** right inserts: zoomed-in view of the depolarization phase showing shallower upstroke in *unc-2(lf)* (**D**) and *egl-19;unc-2* (**F**). In particular, action potentials are relatively normal in *egl-19* (**C**), compromised in *unc-2(lf)* (**D**), enhanced in *unc-2(gf)*, and completely absent in *egl-19;unc-2* (**F**). **G.** Statistical comparison of action potential measurements between different genotypes. The averaged peak amplitude and spike width in *unc-2(lf)* are significantly smaller than those of WT ($p < 0.00005$ and $p < 0.05$, respectively); the averaged spike width in *unc-2(gf)* is significantly larger than that of the WT ($p < 0.05$). Resting membrane potential (V_{rest}): WT: -45.7 ± 1.1 mV; *egl-19*: -42.1 ± 2.3 mV; *unc-2(lf)*: -46.4 ± 2.5 mV; *unc-2(gf)*: -38.2 ± 13.4 mV. Peak amplitude: WT: 9.8 ± 0.8 mV; *egl-19*: 10.2 ± 1.2 mV; *unc-2(lf)*: -0.6 ± 1.9 mV; *unc-2(gf)*: 7.4 ± 2.2 mV. Threshold: WT: -23.3 ± 0.8 mV; *egl-19*: -20.8 ± 1.4 mV; *unc-2(lf)*: -22.8 ± 2.0 mV; *unc-2(gf)*: -28.1 ± 1.5 mV. Spike width: WT: 394.2 ± 40.0 ms; *egl-19*: 387.9 ± 67.6 ms; *unc-2(lf)*: 234.3 ± 48.4 ms; *unc-2(gf)*: 677.7 ± 147.8 ms. The number of animals recorded per genotype: WT: $n = 17$; *egl-19*: $n = 8$; *unc-2(lf)*: $n = 8$; *unc-2(gf)*: $n = 8$. Error bar is SEM. Statistics used was two-sample t-test.

plays a primary role in mediating the positive action potential in AVL, possibly with minor contributions from EGL-19. Consistent with this model, in *egl-19;unc-2* double mutant animals, complete action potentials were never evoked in AVL by any stimuli (Fig 2F).

To further examine if other VGCCs play significant roles in generating AVL action potentials, we recorded from mutant animals lacking *cca-1*, which encodes a CaV3 T-type VGCC (Frokjaer-Jensen et al., 2006), double mutant of *egl-19;cca-1*, as well as double mutants of *nca-1;nca-2*, which encode the homologues of the calcium-permeable NALCN channel (Jospin et al., 2007). We found that AVL action potentials recorded in these mutants were largely unchanged compared to those in the wild type (Fig S1A and B). However, the frequency of action potentials in *cca-1* and *nca-1;nca-2* mutants appear to be increased (Fig S1A and B). This casual observation, if holds true, might be explained by a decrease of background cytosol calcium levels, which likely inhibits action potential firing by calcium-dependent UNC-2 channel inactivation.

The AVL negative spike is mediated by a repolarization-activated potassium channel EXP-2

To determine the underlying ionic currents that generate the negative spike in AVL, we recorded whole-cell currents in AVL under voltage-clamp configuration. The most prominent whole-cell currents in wild-type AVL were transient outward currents with fast activating and inactivating kinetics evoked by depolarizing voltage-clamp steps (Fig 3A). More interestingly, however, was that some small, albeit substantial, outward currents were detected after the cessation of voltage-clamp steps (Fig 3A, WT insert). Analysis of the current-voltage (I-V) relationship of these small currents indicates that they are voltage-dependent and are activated when the membrane potential is repolarized from above -20 mV back to the holding potential of -60 mV (Fig 3B). These outward repolarization-activated currents could theoretically generate regenerative spikes in the negative direction by forming a positive-feedback loop (Fig 3C). The absence of AVL negative spikes in *unc-2(lf)* and *egl-19;unc-2* mutants supports this model, as the generation of negative spikes required strong prior depolarization (Fig 2D and F).

exp-2 encodes the only known repolarization-activated potassium channel in *C. elegans* (Davis et al., 1999; Fleischhauer et al., 2000; Park and Sesti, 2007; Shtonda and Avery, 2005). To determine if EXP-2 underlies the repolarization-activated currents in AVL, we recorded from AVL in *exp-2(lf)* mutant and found that these currents were completely eliminated (Fig 3A and B). Conversely, in a heterozygous *exp-2(gf)* mutant (homozygous *exp-2(gf)* is lethal), the IV curve of these repolarization-activated currents was left-shifted so that EXP-2(gf)-mediated currents were activated at lower membrane potentials (Fig 3A and

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Figure 3. AVL negative spike is mediated by a repolarization-activated K channel EXP-2

A. Representative whole-cell currents recorded from AVL in wild-type (left panel), *exp-2(lf)* (middle panel), and *exp-2(gf)* mutant animals (right panel). Top: voltage-clamp steps used: holding potential is -60mV, each step is 500 ms long in 10mV increment. Insets: zoomed-in view of currents from 0 to ~70 ms after returning to holding from serial voltage steps. **B.** Current-voltage (IV) relationships of normalized repolarization-activated currents for WT (black square), *exp-2(lf)* (red triangle), and *exp-2(gf)* (blue circle). WT and *exp-2(gf)* IV curves were fitted with Boltzmann function (color-matching curves). **C.** Schematic of action potential generation in AVL. The negative potassium spike is intrinsically coupled to the preceding positive calcium spike via a positive feedback loop mediated by repolarization-activated potassium channel EXP-2. **D-E.** Representative action potential traces recorded from *exp-2(lf)* and *exp-2(gf)* AVL. Right panels: overlay of isolated single action potential spikes from *exp-2(lf)* (purple traces, note the absence of negative spikes) and *exp-2(gf)* (orange traces, note the narrow spike width) aligned to the wild-type traces (gray). **F.** Statistical comparison of action potential measurements between different genotypes. The averaged spike width in *exp-2(gf)* is significantly narrower than that of the WT ($p < 0.005$). WT measurements are the same as in Fig 1C and 2G. Resting membrane potential (V_{rest}): *exp-2(lf)*: -43.1 ± 1.1 mV; *exp-2(gf)*: -49.0 ± 2.8 mV. Peak amplitude: *exp-2(lf)*: 9.1 ± 0.4 mV; *exp-2(gf)*: 9.4 ± 2.4 mV. Threshold: *exp-2(lf)*: -24.8 ± 0.8 mV; *exp-2(gf)*: -20.2 ± 1.4 mV. Spike width: *exp-2(lf)*: 334.7 ± 34.5 ms; *exp-2(gf)*: 144.9 ± 12.8 ms. The number of animals recorded per genotype: *exp-2(lf)*: $n = 7$; *exp-2(gf)*: $n = 5$. Error bar is SEM. Statistics was two-sample t-test.

B). Consistent with the prediction that these potassium currents underlie the negative spike in AVL, action potentials recorded in *exp-2(lf)* under current-clamp completely lacked negative spikes while the positive calcium spikes were intact compared to wild-type AVL (Fig 3D). In contrast, action potentials recorded in *exp-2(gf)* AVL became significantly narrower in spike width, likely resulting from early onset of the negative spike (Fig 3E and F).

To examine if the transient depolarization-induced currents in AVL that are intact in the *exp-2(lf)* mutant (Fig 3A) also affect action potential generation, we further identified its ionic origin and tested its potential effect on action potential dynamics. The fast activating and inactivating kinetics of these transient currents are reminiscent of the SHL-1 currents isolated from AWA (Liu et al., 2018) or reconstituted in *Xenopus* oocytes (Fawcett et al., 2006). Indeed, in *shl-1(lf)* mutant, these transient currents were eliminated under voltage clamp while the EXP-2-mediated currents remained intact (Fig S2A). Under current clamp, action potentials including both positive and negative spikes were not affected in *shl-1* (Fig S2C). Consistent with this result, in *exp-2;shl-1* double mutant, in which practically all outward whole-cell currents were eliminated under voltage clamp, the evoked action potential dynamics under current clamp were indistinguishable from that of *exp-2*, i.e. normal positive spikes without negative spikes (Fig S2C). These data suggest that SHL-1 does not play a significant role in generating action potentials in AVL.

Modeling of the AVL action potential

To ask whether the EXP-2 current is sufficient to generate the negative spikes, we developed a Hodgkin-Huxley type model of the AVL action potential. (See Methods for the detailed model descriptions.) Our AVL model incorporated the various inferred calcium and potassium channels: UNC-2, EGL-19, CCA-1, SHL-1, EXP-2, NCA, and EGL-36 (Kv3) (a voltage-gated potassium channel). The model reproduced many of the qualitative features of the AVL membrane potential dynamics. The voltage-clamp simulations show a prominent transient outward current similar to the experimental data. The small voltage-dependent outward currents after the termination of the voltage clamp step seen in wild-type AVL were also qualitatively reproduced by the model (Fig 4A and B). Under current-clamp simulation, there is an excellent agreement in the shape of AVL action potential between the model and the experimental data (Fig 4C and 3C).

In order to illustrate the role of individual ion channels during an action potential, we further examined the dynamics of each channel in the model (Fig S3A displays the temporal evolution of the normalized conductance of each modeled channel in wild-type AVL). The stimulating current was set to 10 pA to reliably evoke an action potential. After the current injection, the CCA-1 inward calcium current was activated first followed by the activation of

Figure 4. Modeling of AVL action potentials

A. Simulation of voltage-clamp of AVL in the wild type (left panel) and the *exp-2(lf)* mutant (right panel). Top: voltage-clamp steps used in numerical simulation: holding potential is -60mV, each step is 500 ms long in 10mV increment. Inserts: zoomed-in view of currents from 0 to ~60 ms after returning to holding potential from serial voltage-clamp steps. **B.** Current-voltage (IV) relationships of normalized simulated repolarization-activated currents for WT (black squares), *exp-2(lf)* (red triangles), and *exp-2(gf)* (blue circles). WT and *exp-2(gf)* IV curves were fitted with Boltzmann functions (color-matching curves). **C.** Representative action potential modeling simulations for wild-type AVL. Top: current-injection steps used in numerical simulation: each step is 5 s long in 2 pA increment. **D-E.** Representative action potential simulations for *exp-2(lf)* and *exp-2(gf)* mutants under the same series of current-injection steps to wild-type AVL.

UNC-2 and EGL-19 calcium currents (Fig S3A). Meanwhile, SHL-1 generated a transient outward potassium current to counter the calcium currents and to limit the peak voltage of the positive spike. These currents contributed to the depolarization phase of the AVL action potential. During the voltage plateau phase of the model, EGL-19 generated a slowly inactivating current, as previously observed experimentally in *C. elegans* pharynx muscles (Shtonda and Avery, 2005). The UNC-2 calcium currents and the EGL-36 outward potassium current also contributed to maintenance of the plateau phase (Fig S3). Finally, when the AVL membrane potential repolarized across a threshold, the EXP-2 channel activated, rapidly, thus generating an outward current to evoke the negative spike.

To further elucidate the role of EXP-2, we modeled *exp-2(gf)* and *exp-2(lf)* by modifying the parameters used in the AVL model (see Methods for detailed description). Consistent with the experimental data (Fig 3A-E), the I-V curve of the repolarization-activated currents in the *exp-2(gf)* simulation was left-shifted (Fig 4B) and the currents in *exp-2(lf)* simulation were almost eliminated (Fig 4B and C). The current-clamp simulations showed AP shapes in *exp-2(gf)* and *exp-2(lf)* that matched the electrophysiological data: a

narrower spike width in *exp-2(gf)*, and the elimination of the negative spike in *exp-2(lf)*. These results support our model that the repolarization-activated channel EXP-2 generates the potassium-mediated negative action potential in AVL.

In addition, we also modeled *shl-1* mutant and *exp-2;shl-1* double mutant to examine the role of SHL-1 potassium channel. The simulation results agree with the experimental data in Fig S2C. The current clamp simulation of *shl-1* mutant showed a similar AP shape to wild-type AVL (Fig S3D); and the simulation of *exp-2;shl-1* double mutant showed a similar AP shape to *exp-2(lf)*, with the absence of the negative spike (Fig S3E). The combined experimental and modeling results suggest that SHL-1 can generate a transient inward potassium current to counteract the calcium currents in the upstroke phase, but does not play a significant role in other phases.

AVL and DVB synchronously fire propagating action potentials

What is the physiological and functional significance of enteric neurons firing action potentials in defecation behavior? What is the timing of action potentials in AVL and DVB relative to each other and to the intestinal calcium clock? How does spike timing affect the downstream expulsion behavior? To address these questions, we expressed GCaMP6f in AVL and DVB as well as in the intestine and recorded calcium dynamics in the enteric system in freely-moving animals. We used a closed-loop live tracking microscope (Li et al., 2021) to monitor the worm's body-length to register body muscle contraction and expulsion events and correlated these motor steps to simultaneously recorded calcium dynamics in the gut-neuron circuit. Since AVL and DVB are positioned roughly at the same z-axis level, cell bodies from both neurons can be imaged in the same focal plane. In wild-type animals, rhythmic intestinal calcium waves, calcium spikes in AVL and DVB indicative of action potentials, and body muscle contraction steps were reliably recorded (Fig 5, S4 and Movie 1). The peak of posterior body muscle contractions preceded the peak of anterior body muscle contractions by ~2.5 s and the start of the intestinal calcium wave by ~1 s (Fig S4C); the averaged cycle length was ~45 s with small variability (Fig S4C and D). Synchronized calcium spikes in AVL and DVB coincided with anterior body muscle contractions and expulsions but lagged about 3 sec behind the intestinal calcium wave (Fig 5C-E). These measurements are consistent with the existing model that the posterior body muscle contraction is directly activated by calcium-mediated proton release from the intestine (Beg et al., 2008) and that AVL and DVB drive anterior body muscle contraction and expulsion steps downstream of the intestinal clock (Branicky and Hekimi, 2006; Choi et al., 2021; McIntire et al., 1993; Peters et al., 2007; Teramoto and Iwasaki, 2006; Thomas, 1990; Wang et al., 2013). Most interestingly, under ideal imaging conditions, we detected synchronous

Figure 5. AVL and DVB synchronously fire propagating action potentials

A-B. Representative images showing synchronous GCaMP fluorescence in AVL and DVB. Time 0 is an arbitrary frame before neural activation. AVL soma (left panel, arrowhead), AVL axon (left and middle panels, arrow), preanal NMJs (right panel, arrow), DVB soma (right panel, arrowhead). This imaging strain also labels DVC (marked by a red * in the right panel) which is irrelevant to this study. **C.** Representative (normalized) calcium activities in AVL (blue) and DVB (red) soma. (All activities normalized with maximum set to 1.) Vertical line and markers: The timing of expulsion (green vertical line), the onset (asterisk), and the peak (circle) of AVL and DVB spikes. **D.** Corresponding worm body length as a function of time. Blue curve is frame-by-frame and black is a smoothed version of the blue curve. (Smoothing used local regression to a second order polynomial.) Red and blue asterisk/circle: same as in **C**. **E.** Corresponding (normalized) intestinal calcium activities along the worm body axis (A: Anterior, P: Posterior). Curves (from left to right): the timing of the onset (white), the half-peak (black) and the peak (white) of calcium activities along the worm body axis. Vertical lines in **D-E** (from left to right): the earliest onset of intestinal calcium activity, the peak of pBoc, the onset of AVL, the peak of DVB, and the peak of aBoc.

calcium spikes in the entire AVL axon together with calcium spikes in AVL and DVB cell bodies (Fig 5A and B), consistent with a previous study showing AVL to be isotonicly activated (Choi et al., 2021). These observations suggest that action potentials propagate along the AVL axon which likely underlies the synchronous firing of AVL and DVB.

Synchronous firing of AVL and DVB underlies reliable expulsion

To further improve imaging and tracking quality and facilitate data analysis for longer recording covering multiple defecation cycles, we constructed a new imaging line with co-expression of mCherry and GCaMP in the enteric nervous system and the intestine (Fig 6A), and employed a two-color imaging procedure to simultaneously acquire both GCaMP dynamics and mCherry fluorescence signals (Movie 2). Calcium-independent mCherry fluorescence was used as reference for localizing the cell body and minimizing motion artifacts (Li et al., 2021). To objectively identify the three motor steps of the defecation cycle, we analyze the body contraction by dividing the worm body into two halves and measuring each half of the body length separately (Fig S5A). Posterior body muscle contractions are

strong, thus were clearly detected in all cycles coinciding with intestinal calcium waves (Fig 6B and S5B). From the computed body-lengths alone, sometimes the smaller anterior body muscle contraction and expulsion events were indistinguishable from noise; however, even in most of these cases, these two steps could be easily identified by eye. When both anterior body muscle contraction and expulsion events were clearly visible in the same cycle, we found that they were always initiated at roughly the same time (Fig 6B and S5B).

Using the dual-color strain and imaging setup in the wild-type genetic background, we found that calcium spikes fired in AVL almost always slightly preceded DVB spikes with an average temporal advance of 215 ± 176 ms (Fig 6G). In some cases, AVL and DVB spikes were nearly overlapping with very small delays between them, but DVB spikes were not detected before AVL spikes (Fig 6G). This result and the observation of propagating calcium spikes along the AVL axon (Fig 5B) suggest that spike signals flow from AVL to DVB. In a recent study, Choi et al. reported that AVL and DVB activities are electrically coupled by the gap junction INX-1 located at the preanal ganglia NMJs (Choi et al., 2021). Consistent with this finding, we found that calcium spikes in AVL and DVB were de-synchronized in *inx-1(lf)* mutant background (Fig 6C and S5C), and DVB spikes were only detected in 43% of cycles in *inx-1(lf)* compared to 100% in WT (Fig 6D). In cycles in which both AVL and DVB fired, the delay time between the AVL and DVB spikes was significantly increased in *inx-1* (2847 ± 2307 ms) (Fig 6G). DVB firing spikes alone without AVL firing was never observed in either WT (n=17 cycles) or *inx-1* (n=14 cycles) (Fig 6D). Taken together, these results support a model in which AVL fires action potentials first, which propagate orthodromically to the preanal ganglia and activate DVB to fire action potentials through INX-1 gap junction.

Our behavioral tracking and simultaneous recording of calcium spikes in AVL and DVB also support previous studies suggesting that both enteric neurons are semi-redundantly required for reliable expulsion (Choi et al., 2021; McIntire et al., 1993). In wild-type animals, expulsion was reliably detected in 94.7% cycles (18 out of 19 cycles) and was always associated with synchronized AVL/DVB spikes (Fig 6E). In the *inx-1* mutant, when AVL fired alone in a cycle, expulsions were only observed in 50% of cycles (n = 8) (Fig 6E), while when both AVL and DVB fired, even with longer delays, expulsions were detected in 100% of cycles (Fig 6E). However, the exact timing of expulsions in the *inx-1* mutant was more variable: expulsion coinciding with the AVL spike in 33% of cycles, and with the DVB spike in 67% of cycles (n = 6) (Fig 7C). These data confirm that reliable expulsion behavior required activation of both AVL and DVB (Choi et al., 2021; McIntire et al., 1993), and also suggest that the timing of expulsion is encoded by the synchronicity of the spike code from two spatially well-separated but electrically-coupled enteric neurons.

Figure 6. Synchronous action potential firing in AVL and DVB underlies reliable expulsion
A. Representative fluorescent and DIC images of the dual-color imaging line used for Figs 6 and 7. An anesthetized L4 worm with co-expression of mCherry and GCaMP6f in all GABA neurons and the intestine. **B-C.** Top panels: Representative calcium activities of AVL (red) and DVB soma (blue) in wild-type (**B**) and *inx-1* mutant animals (**C**). Middle panels: Corresponding traces showing simultaneous measurements of the length of the anterior (red) and posterior (blue) half of the centerline. Bottom panels: Corresponding normalized intestinal calcium activities along the worm body axis (A: Anterior, P: Posterior). Green vertical lines: Timing of expulsion. **D.** Fraction of AVL and DVB firing detected in DMP cycles. **E.** Fraction of expulsion detected under the circumstances where only AVL fires, only DVB fires, or both the neurons fire. In **D** and **E**, the values for AVL or DVB firing alone in WT and DVB firing alone in *inx-1* are zero. **F.** Fraction of DMP cycles associated with expulsion occurrence. **G.** Time delay between AVL and DVB spikes in wild-type and *inx-1* mutants. Asterisks indicate significant differences: * $P < 0.05$ in Student's t test. **H.** Top: a representative current-clamp recording trace of AVL under repetitive stimuli (each stimulus is 3-s long at 2 pA) at various intervals. Bottom: the averaged trace of prolonged current-clamp recording of AVL under a 1-s long 8 pA stimulus. Gray: SEM, $n = 7$. Red dotted lines label the resting membrane potential.

AVL action potentials are followed by long-lasting afterhyperpolarization

Our observation of asynchronous DVB spikes only in the *inx-1* mutant but not in the wild type supports a recently proposed model of INX-1 gap junction suppressing ectopic DVB spiking

during intercycles (Choi et al., 2021). To explore the electrophysiological mechanism of this hypothesis, we recorded AVL for prolonged periods of time after inducing action potentials (Fig 6H). Strikingly, we found that in addition to the negative spike, there was a long-lasting AHP following each action potential (Fig 6H). We accurately measured the length of the long-lasting AHP by inducing an action potential like waveform in AVL using a 1 sec-long stimulus (Fig 6H right), and found the averaged AHP lasted for over 40 sec, matching the duration of the intercycle. This long AHP is independent of the negative spike, as it was present in *exp-2(lf)* mutant (data not shown). Since INX-1 forms bidirectional homotypic gap junctions between AVL and DVB (Choi et al., 2021), this slow hyperpolarization might pass through the gap junction and keep DVB inactive during intercycles in wild-type animals. In the *inx-1* mutant, elimination of this inhibitory entrainment force by the removal of the gap junction could allow DVB to fire at its own pace.

The AVL negative spike inhibits excessive expulsion per defecation cycle

To determine the physiological function of the AVL negative spike during defecation, we performed live imaging and behavioral tracking in the *exp-2(lf)* mutant in which AVL fire normal calcium action potentials without negative spikes (Fig 3D). We found that *exp-2(lf)* did not affect the cycle period nor the delay between AVL and DVB spikes (Fig 7D); however, in ~30% of total cycles, an additional expulsion was observed immediately

Figure 7. The AVL negative spike inhibits excessive expulsion per defecation cycle

A. Top: Representative (normalized) calcium activities of AVL (red) and DVB soma (blue) in *exp-2(lf)* mutants. Middle: Corresponding changes of the length of the anterior (red) and posterior (blue) half of the worm body. Bottom: Corresponding normalized intestinal calcium activities along the worm body axis (A: Anterior, P: Posterior). Green vertical lines: Timing of expulsion. **B.** Fraction of DMP cycles associated with different numbers of expulsion occurrence. **C.** Fraction of expulsions per cycle associated with AVL or DVB firing or both. **D.** Time delay between AVL and DVB spike in wild-type, *exp-2(lf)* and *inx-1* mutants. Asterisks indicate significant differences: * P<0.05 in Student's t test. **E.** Time delay between two consecutive expulsions in *exp-2(lf)* mutants (n=6) and the spike width of AVL action potentials in wild-type animals (n=17).

following the normally timed expulsion (Fig 7A, B and S5D). The averaged delay between the two consecutive expulsions in *exp-2(lf)* was 430 ± 155 ms ($n=6$), which corresponds well with the averaged spike width of AVL action potential (394.2 ± 40.0 ms, $n=17$) (Fig 7E). This result suggests that negative spikes in AVL may prevent excessive expulsions by terminating calcium signals in AVL and/or DVB in a timely manner. Furthermore, in *exp-2(lf)* only ~40% of cycles were associated with a normal single expulsion, and in ~30% cycles no expulsions were detected at all (Fig 7B), suggesting that the AVL negative spike may play more important roles in regulating the enteric rhythm and coordinating motor steps during defecation behavior.

Discussion

Here we identify two new spiking neurons, AVL and DVB, in the *C. elegans* enteric nervous system, with distinct membrane potential dynamics and putative functions. DVB fires giant calcium-mediated action potentials while AVL fires unusual compound action potentials with each giant calcium spike followed by a potassium-mediated negative spike. Electrophysiological recording and biophysical modeling suggest that a CaV2 type voltage-gated calcium channel UNC-2 and a repolarization-activated potassium channel EXP-2 underlie the molecular machinery generating the time-locked compound action potential in AVL. Using simultaneous neuron-gut imaging and behavioral tracking in freely-moving animals, we found that AVL and DVB synchronized action potential firing through electrical coupling by INX-1 gap junction, and that the AVL negative spike prevented excessive expulsions in a given defecation cycle. Our study provides new insight into temporal coding, spike propagation and signal integration within the *C. elegans* enteric neural circuit underlying motor pattern generation and behavioral rhythm.

The AVL negative spike

The negative spikes recorded in AVL resemble those observed in the pharyngeal muscles of *C. elegans* and other nematodes (Byerly and Masuda, 1979; Davis et al., 1999) as well as in lobster muscles (Reuben et al., 1961). It is worth noting that all these excitable cells firing negative spikes are elements of apparatuses that produce biological rhythms, suggesting that the negative spike in AVL may be involved in generating and/or modulating the enteric rhythm underlying the defecation motor program. Our electrophysiological recording results suggest that the AVL negative spike is generated by EXP-2-mediated repolarization-activated potassium currents. From our model voltage dynamics, the AVL negative spike appears to be an upside-down version of the Hodgkin-Huxley action potential (Fig 3C), with the activation and inactivation of EXP-2 analogous to the activation of Na and the activation

of K in the Hodgkin-Huxley. More specifically, in wild-type AVL, the repolarization of preceding calcium-mediated action potential activates EXP-2, allowing potassium efflux which induces further repolarization and more EXP-2 activation, forming a positive feedback loop that results in a regenerative negative spike. Thus, the positive calcium spike and negative potassium spike are intrinsically coupled, producing a biophysical mechanism to maintain and regulate the duration of calcium influx during each action potential firing.

To our knowledge, AVL is the only known neuron that fires this characteristic negative spike mediated by a repolarization-activated potassium channel in *C. elegans* or other animals. In addition to AVL, *exp-2* is also expressed in pharyngeal muscles and a few other neurons in *C. elegans* (Davis et al., 1999). Presumably through a similar mechanism, EXP-2 in pharyngeal muscles also functions in regulating the rhythmic pharyngeal action potentials and the pumping rate (Davis et al., 1999). It may be worth looking into other *exp-2* expressing neurons to investigate their potential roles in generating or regulating rhythmic behaviors in *C. elegans*.

The ion channel composition that generates action potentials in DVB was not electrophysiologically examined here; however, previous calcium imaging studies suggest that EGL-19 mediates calcium transient in DVB with possible minor contribution from additional calcium channels (Choi et al., 2021; Wang and Sieburth, 2013). Furthermore, our whole-cell currents recorded in wild-type DVB show very little inward or outward currents under a broad range of voltage-clamp steps (Fig S2B). This result suggests that the repolarization of DVB action potential does not rely on potassium conductances but rather mainly on voltage-gated calcium channel inactivation.

Our observation of frequent double-expulsion events in the *exp-2(lf)* mutant suggests a plausible physiological function of the AVL negative spike. Excessive expulsions at the wrong time not only waste energy but may also cause more severe detrimental problems to the defecation cycle, e.g., ~30% cycles in *exp-2(lf)* lack expulsion steps (Fig 7B). This consequence could be induced by disrupting the normal temporal rhythm through unknown retrograde feedback signals from enteric muscles to the enteric nervous system. Interestingly, in the *inx-1* mutant where DVB action potentials are out of alignment, double expulsions are never observed. This suggests that most likely the AVL negative spike functions in limiting calcium signals from AVL itself rather than from DVB through INX-1. Future experiments with *exp-2(lf);inx-1* double mutant would draw more conclusive pictures.

Timing of anterior body muscle contraction and expulsion

Almost all ethograms in the literature label the expulsion step behind the anterior body muscle contraction step by one time-unit (usually 1 sec) (Liu and Thomas, 1994; Teramoto

and Iwasaki, 2006; Thomas, 1990), sometimes with graphical representation of one full second in between the two steps (Kwan et al., 2008). Using our customized whole-animal imaging and behavioral tracking setup, we detect nearly concurrent anterior body muscle contraction and expulsion events in freely-moving wild-type animals (Fig 6B and S5B), which may seem inconsistent with the literature regarding the timing of these two steps. However, manually scoring anterior body muscle contraction for plotting ethograms is known to be not wholly reliable due to the locomotory movements of the head region (Thomas, 1990). The whole-worm tracking of the defecation cycle in the current study is, to the best of our knowledge, the first unbiased quantitative measurement of the timing of these two motor steps in freely-moving animals. The near concurrence of anterior body muscle contraction and expulsion is consistent with their common control by intestinal neuropeptide signaling (Mahoney et al., 2008; Thomas, 1990) and the instantaneous action potential propagation along the AVL axon from the head to tail (Fig 5B).

Neural regulation of the defecation behavior

The identification of action potentials in AVL and DVB highlights a previously underestimated role played by the enteric nervous system in regulating the defecation cycle. In retrospect, at least three features of the defecation motor program demand the all-or-none property and temporal precision that are best supported by spiking neurons. First, the expulsion step requires three muscle groups including intestinal muscle, sphincter muscle, and anal depressor muscle to contract and relax in synchrony to execute a successful expulsion. The broad calcium-mediated action potentials synchronously fired by AVL and DVB ensure an ample dose of GABA release to orchestrate postsynaptic muscle activities. Second, the defecation motor program requires temporal and spatial coordination of multiple organs from head to tail including the intestine, anterior and posterior body-wall muscles, two spatially separated enteric neurons, and multiple muscle groups for executing expulsion. The intestinal calcium wave that lasts several seconds without well-defined temporal and spatial initiation and termination time points generates the crude pacemaker signal but is unlikely to provide precision control and coordination across all the downstream players. Rather, near-instantaneous action potential spikes could digitalize intestinal calcium waves and propagate the spike code from AVL to DVB to precisely synchronize the apparatus between the head and tail during each cycle. Third, presumably all behavioral rhythms and the underlying clocks and pattern generators last the entire lifetime of an animal. This likely requires robust error-correcting and fail-safe mechanisms to maintain stability by preventing accumulation of temporal fluctuations or even system breakdowns. Rhythmic firing of action potentials, the presence of negative spikes and prolonged AHPs in many rhythm-generating systems strongly suggest a universal rhythm regulation scheme.

Our work suggests that spiking neurons AVL and DVB with distinct membrane dynamics may play differential roles in the defecation motor program. Specifically, DVB may function mainly as a workhorse to facilitate driving downstream muscle contractions, while AVL may act as a neuronal timekeeper functioning at multiple timescales: At the ultrafast, millisecond time scale, AVL instantaneously relays gut pacemaker signals through propagating action potential along its axon from head to tail to activate DVB across gap junctions; at the fast timescale on the order of seconds, AVL negative spikes function to timely terminate calcium signals and therefore suppress excessive expulsion; at the slow behavioral time scale of the defecation cycle, the long-lasting AHP in AVL suppresses ectopic DVB firing during intercycles by negative entrainment through gap junctions. All in all, this study supports a model in which spiking enteric neurons provide additional timing information at both fast and slow scales to fine-tune and coordinate the defecation motor steps initiated by the intestinal calcium clock.

INX-1 gap junctions mediate coordinated action potential firing in AVL and DVB

Recently, a study by Choi et al. demonstrated that the INX-1 gap junction between AVL and DVB functions in bidirectional electrical coupling of this neuron pair underlying expulsion behavior (Choi et al., 2021). Our independent study using different imaging and behavioral tracking setup of animals with different genetic backgrounds supports the prior conclusion and further provides a plausible electrophysiological mechanism. Both studies detected near synchronous calcium spikes in AVL and DVB in wild-type animals (Fig 5C and 6B and (Choi et al., 2021)). The instantaneous calcium spike propagation along the AVL axon (Fig 5B and (Choi et al., 2021)) and the slight temporal advance of the AVL spike (Fig 7D) suggest that NLP-40 release from the anterior intestine induces AVL action potentials first at the cell body or at the axon and that AVL propagates the action potential along the axon to preanal NMJs and evokes action potentials in DVB through INX-1 gap junctions. Furthermore, both studies detected ectopic DVB firing in the *inx-1* mutant at a slower rate and mis-aligned with respect to the rest of the steps (averaged DVB firing cycle was 84.6 s in this study, $n = 3$, Fig 6C and (Choi et al., 2021)), and expulsions that occur in the absence of INX-1 gap junctions (Fig 7B and C) or AVL activation (Choi et al., 2021). These observations suggest that NLP-40 diffused across the pseudocoelom can activate DVB and induce expulsion, at least in the absence of AVL. In wild-type animals, such out-of-phase DVB activation is suppressed by INX-1 (Choi et al., 2021), which can be explained by our observation of the long-lasting AHP in AVL at the time scale of the intercycle (Fig 6H): prolonged AVL AHP hyperpolarizes DVB through INX-1 gap junctions during intercycles and prevent out-of-phase action potential firing in DVB induced by slow-diffusing NLP-40. Taken together, we propose the following scenario: AVL bidirectionally entrains the enteric nervous system through INX-1 gap

junctions, allowing the gut pacemaker signals encoded in the AVL action potentials to be propagated across the whole-body length to DVB; Eventually the integrated spike code from AVL and DVB determine the probability of an all-or-none behavior output – the expulsion.

Biophysical diversity of the *C. elegans* nervous system

To date, three spiking neurons, including a sensory neuron and two motor neurons, have been identified in *C. elegans* (Liu et al., 2018 and this study). This suggests that the *C. elegans* nervous system is likely more heterogenous than previously thought. Digital neural coding seems to play significant roles in certain computational and behavior functions in *C. elegans*, i.e., encoding sensory stimuli in AWA (Liu et al., 2018), and long-distance signal propagation and temporal coordination of AVL and DVB coupled by gap junctions. We are just beginning to appreciate the importance of the biophysical diversity and their functional roles among different neuronal cell types in the *C. elegans* nervous system. In order to understand circuit function and system-level activity dynamics, the intrinsic biophysical properties of individual neurons need to be thoroughly investigated with electrophysiological recording, optical imaging techniques, as well as theoretical modeling. Equipped with the complete connectome of this small brain, it is conceivable to develop an anatomically correct and biophysically accurate systems model in the future to gain a comprehensive biophysical and functional understanding of the *C. elegans* nervous system. Using this approach, this work provides a case study of investigating motor pattern generation and gut-brain communication - two universal biological functions across the animal phyla.

Author Contributions

Q.L. conceived the project and designed the experiments. L.T. oversaw the live-imaging experiments and modeling. Q.L. carried out electrophysiological experiments and constructed the imaging strains used in this work. J.J., Y.S., H.L. and Q.L. carried out live-imaging experiments. J.J. analyzed the behavior tracking and imaging data. Y.S. and R.Z. developed the biophysical models. Q.L. wrote the paper with input from all authors.

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Methods

C. elegans culture and strains

All animals used in this study were young adult hermaphrodites (~3-days old) maintained at room temperature (22-23°C) on nematode growth medium (NGM) plates seeded with *E. coli* OP50 bacteria as a food source (Brenner, 1974). Wild-type animals were Bristol strain N2 or PD1074. CX18004 *kyIs772* strain was made by integration (UV irradiation) of CX17967 *kyEx6380*. Extrachromosomal array *kyEx6380* was made by co-injecting N2 strain with 50ug/ul plasmid containing Punc-47mini::GCaMP6f and 5ug/ul plasmid containing Pinx-16::GCaMP6f. The unc-47 mini promotor (Punc-47mini) is 196bp upstream of ATG of the *unc-47*, amplified using the primer pair of aattaaGGCCGGCCggtctaataatccccggtctcttc and gagacgacagcgtcacatttatttcattacagGGCGCGCCaattaa. The inx-16 promotor (Pinx-16) is amplified using the primer pair of aattaaGGCCGGCCaagacaaagtgactcagt and aatttcagggtacacaaaGGCGCGCCaattaa. CX18153 *kyIs777* strain was made by integration of the extrachromosomal arrays CX18103 *kyEx6441*; *kyEx6442* which were crossed from CX18089 *kyEx6441* and CX18090 *kyEx6442*. *kyEx6441* was made by injecting PD1074 strain with 30ug/ul plasmid containing Punc-47::GCaMP6f::SL2::mCherry::let858 3'UTR; *kyEx6442* was made by co-injecting PD1074 strain with 50ug/ul plasmid containing Punc-47mini::GCaMP6f::SL2::mCherry::unc-54 3'UTR and 5ug/ul plasmid containing Pinx-16::GCaMP6f. The *unc-47* promotor was amplified using the primer pair of aattaaGGCCGGCCactaaacttctacgtcaaaaagttgacaaaac and gagacgacagcgtcacatttatttcattacagGGCGCGCCaattaa.

A complete strain list used in this study is provided in Table 1.

Electrophysiology

Electrophysiological recordings were performed as previously described (Liu et al., 2018). The gluing and dissection were performed at room temperature under an Olympus SZX16 stereomicroscope equipped with a 1X Plan Apochromat objective and widefield 10X eyepieces. Briefly, an adult animal was immobilized on a Sylgard-coated (Sylgard 184, Dow Corning) glass coverslip in a small drop of DPBS (D8537; Sigma) by applying a cyanoacrylate adhesive (Vetbond tissue adhesive; 3M) along the dorsal side of the head or tail region. A puncture in the cuticle away from the incision site was made to relieve hydrostatic pressure. A small longitudinal incision was then made using a diamond dissecting blade (Type M-DL 72029 L; EMS) along the glue line either between two pharyngeal bulbs for AVL recording or in the tail region for DVB recording. The cuticle flap was folded back and glued to the coverslip with GLUture Topical Adhesive (Abbott

Laboratories), exposing the neuron of interest. The coverslip with the dissected preparation was then placed into a custom-made open recording chamber (~1.5 ml volume) and treated with 1 mg/ml collagenase (type IV; Sigma) for ~10 s by hand pipetting. The recording chamber was subsequently perfused with the standard extracellular solution using a custom-made gravity-feed perfusion system for ~10 ml.

All electrophysiological recordings were performed with the bath at room temperature under an upright microscope (Axio Examiner; Carl Zeiss, Inc) equipped with a 40X water immersion lens and 16X eyepieces. AVL and DVB were identified by GFP, mCherry, or GCaMP markers under fluorescent illumination and their physical position. Preparations were then switched to the differential interference contrast (DIC) setting for patch-clamp. Electrodes with resistance (RE) of 20–30 MΩ were made from borosilicate glass pipettes (BF100-58-10; Sutter Instruments) using a laser pipette puller (P-2000; Sutter Instruments) and fire-polished with a microforge (MF-830; Narishige). We used a motorized micromanipulator (PatchStar Micromanipulator; Scientifica) to control the electrodes back filled with standard intracellular solution. The standard pipette solution was (all concentrations in mM): [K-gluconate 115; KCl 15; KOH 10; MgCl₂ 5; CaCl₂ 0.1; Na₂ATP 5; NaGTP 0.5; Na-cGMP 0.5; cAMP 0.5; BAPTA 1; Hepes 10; Sucrose 50], with pH adjusted with KOH to 7.2, osmolarity 320–330 mOsm. The standard extracellular solution was: [NaCl 140; NaOH 5; KCl 5; CaCl₂ 2; MgCl₂ 5; Sucrose 15; Hepes 15; Dextrose 25], with pH adjusted with NaOH to 7.3, osmolarity 330–340 mOsm. For calcium-free extracellular solution, 2mM CaCl₂ was replaced with 2mM EGTA. Chemicals were from Sigma-Aldrich. Liquid junction potentials were calculated and corrected before recording. Whole-cell current clamp and voltage clamp experiments were conducted on an EPC-10 amplifier (EPC-10 USB; Heka) using PatchMaster software (Heka). Two-component capacitive compensation was optimized at rest, and series resistance was compensated to 50%. Analog data were filtered at 2 kHz and digitized at 10 kHz. For quality control, only patch clamps with a seal resistance above 1 GΩ and uncompensated series resistance below 100 MΩ were accepted for further analysis.

Simultaneous behavior tracking and calcium imaging

We used a customized optical imaging system to track a single worm and simultaneously image its intestinal and neuronal cells (Li et al., 2021). Our closed-loop setup consisted of a high-speed CMOS camera for locomotion and behavior tracking and a two-camera neural-circuit imaging subsystem. Using a fast tracking algorithm, the behavior tracking system drove a two-axis piezo stage to realize worm-motion compensation, enabling rapid and stable cellular-resolution neural circuit imaging.

The transgene line *kyls772* and *kyls777* in wild-type and various genetic background (Table 1) were used for imaging. L3-L4 stage animals were transferred to customized NGM plates covered seeded with a thin layer of OP50 lawn. GCaMP and mCherry signals were recorded with a 20×/0.75 objective, and two sCMOS cameras (Hamamatsu, ORCA-Flash4.0 V3) at a frame rate of 24Hz.

Data analysis

Electrophysiology

Statistics, curve fitting and graphing were conducted using OriginPro 2020b (OriginLab). Action potential spikes for averaging were manually selected for the largest peak amplitude for each preparation. Measurements of resting membrane potential, threshold, peak amplitude, AHP, and spike width were made by hand with the measure function in Fitmaster (Heka). Electrophysiological recording traces shown in all figures were re-sampled at 2.5kHz

for reducing file size. Boltzmann function for IV curve fitting was $y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} + A_2$

Behavior & calcium imaging

Analysis of behavior and calcium imaging data was performed using custom MATLAB scripts. To perform worm shape analysis, the centerline of each worm was first extracted frame-by-frame using a centerline localization algorithm, and then equally divided into 49 segments (Li et al., 2021). The position of one of the VD/DD motor neurons was used as a landmark to divide the worm centerline into anterior and posterior halves. Then the length of each half was calculated separately to distinguish pBoc and aBoc. For intestinal imaging, the normal to the centerline at each point partitioned the worm intestine into equally-spaced segments. The intestinal GCaMP or mCherry fluorescence intensity from anterior to posterior was defined by the average fluorescence intensity within each segment. For neuronal imaging, the GCaMP or mCherry fluorescence intensities of AVL and DVB soma were defined as the average fluorescence intensity of a region of interest (ROI) in the soma region. The calcium activity (R) was calculated as the ratio of GCaMP to mCherry fluorescence intensities, and were normalized by its minimum (R_0) as $(R-R_0)/R_0$. The timing of neuronal firing was defined as the time when its calcium dynamic begins to rise from the baseline. The timing of expulsion was defined as the start of an obvious enteric muscles contraction that can be observed by the naked eye.

Modeling of AVL action potential

To model the dynamics of the AVL membrane potential, we adopted a Hodgkin-Huxley formulation by modeling the ion channels that are participating in AVL action potentials.

From our experiments and the open access databases Wormbase and CeNGEN, we chose six voltage-gated calcium and potassium channels (UNC-2, EGL-19, CCA-1, SHL-1, EGL-36 and EXP-2) and NCA sodium leak channels to constitute the AVL model. Therefore, the dynamics of the membrane potential of AVL is described by

$$C \frac{dV}{dt} = -(I_{\text{UNC2}} + I_{\text{EGL19}} + I_{\text{CCA1}} + I_{\text{SHL1}} + I_{\text{EGL36}} + I_{\text{EXP2}} + I_{\text{NCA}} + I_{\text{L}}) + I_{\text{inj}}$$

where C is the membrane capacitance. In addition to the ionic currents mentioned above,

I_{L} and I_{inj} are the leak and the external input currents, respectively. The leak current is given

by Ohm's law:

$$I_{\text{L}} = g_{\text{L}}(V - V_{\text{L}})$$

nca-1 and *nca-2* encode the homologue of calcium-permeable NALCN channel, which can be modeled as leak channels (Nicoletti et al., 2019):

$$I_{\text{NCA}} = g_{\text{NCA}} \cdot (V - V_{\text{Na}})$$

EXP-2 is a repolarization-activated HERG (Kv11.1, KCNH2) potassium channel homolog in *C. elegans* (Fleischhauer et al., 2000; Shtonda and Avery, 2005). Because of its unusual gating characteristics, the EXP-2 gating current cannot be directly modeled within the Hodgkin-Huxley formulation (it has no existing ordinary-differential-equation model yet). Considering the similarity in kinetic properties between EXP-2 and HERG channel (Fleischhauer et al., 2000), we used a Markov chain model of HERG channel based on the kinetic scheme from the work of Mazhari et al. to model the EXP-2 channel (Mazhari et al., 2001). The Markov model can be represented by the following five-state diagram:

where C1, C2 and C3 are the close states, O is the open state, and I is an inactive state.

The forms of the various rate constants are as follows:

$$\alpha_1(V) = p_1 e^{p_2 V}, \quad \beta_1(V) = p_3 e^{-p_4 V}$$

$$K_f = p_5, \quad K_b = p_6$$

$$\alpha_2(V) = p_7 e^{p_8 V}, \quad \beta_2(V) = p_9 e^{-p_{10} V}$$

$$\alpha_i(V) = p_{11} e^{p_{12} V}, \quad \beta_i(V) = p_{13} e^{-p_{14} V}$$

$$\alpha_{i2}(V) = p_{15} e^{p_{16}V}, \quad \psi(V) = \frac{\beta_2(V) \cdot \beta_1(V) \cdot \alpha_{i2}(V)}{\alpha_2(V) \cdot \alpha_1(V)}$$

To incorporate the Markov model of the EXP-2 kinetics into the Hodgkin-Huxley formulation, we modified the parameters $p_1 \sim p_{16}$ by fitting to the experimental I-V curve of the repolarization-activated current in Fig 3A. The dynamics of the Markov model can then be described by the following ordinary differential equations (ODE):

$$\frac{dC_1}{dt} = \beta_1(V) \cdot C_2 - \alpha_1(V) \cdot C_1$$

$$\frac{dC_2}{dt} = \alpha_1(V) \cdot C_1 + K_b \cdot C_3 - [\beta_1(V) + K_f] \cdot C_2$$

$$\frac{dC_3}{dt} = K_f \cdot C_2 + \psi(V) \cdot I + \beta_2(V) \cdot O - [K_b + \alpha_{i2}(V) + \alpha_2(V)] \cdot C_3$$

$$\frac{dO}{dt} = \beta_i(V) \cdot I + \alpha_2(V) \cdot C_3 - [\beta_2(V) + \alpha_i(V)] \cdot O$$

$$I = 1 - C_1 - C_2 - C_3 - O$$

With this ODE formulation of the EXP-2 kinetics, the EXP-2 potassium current is given by

$$I_{\text{EXP2}} = g_{\text{EXP2}} \cdot O \cdot (V - V_K)$$

The calcium channel UNC-2, EGL-19, CCA-1 and the potassium channel SHL-1, EGL-36 were modeled based on Hodgkin-Huxley formulation. The UNC-2 (CaV2) calcium current is given by

$$I_{\text{UNC2}} = g_{\text{UNC2}} \cdot m_{\text{UNC2}}^2 \cdot h_{\text{UNC2}} \cdot (V - V_{\text{Ca}})$$

where g_{UNC2} is the maximum conductance and V_{Ca} is the calcium reversal potential. The dynamics of the activation and inactivation gating variables, m_{UNC2} and h_{UNC2} are described by

$$\frac{dm_{\text{UNC2}}}{dt} = \alpha_{m_{\text{UNC2}}}(V)(1 - m_{\text{UNC2}}) - \beta_{m_{\text{UNC2}}}(V)m_{\text{UNC2}}$$

$$\frac{dh_{\text{UNC2}}}{dt} = \alpha_{h_{\text{UNC2}}}(V)(1 - h_{\text{UNC2}}) - \beta_{h_{\text{UNC2}}}(V)h_{\text{UNC2}}$$

$$\alpha_{m_{\text{UNC2}}}(V) = \frac{a(V-b)}{1 - e^{-\left(\frac{V-b}{c}\right)}}, \beta_{m_{\text{UNC2}}}(V) = ae^{-\left(\frac{V-b}{c}\right)}$$

$$\alpha_{h_{\text{UNC2}}}(V) = ae^{-\left(\frac{V-b}{c}\right)}, \beta_{h_{\text{UNC2}}}(V) = \frac{a}{1 + e^{-\left(\frac{V-b}{c}\right)}}$$

where $\alpha_{m_{\text{UNC2}}}$, $\alpha_{h_{\text{UNC2}}}$, $\beta_{m_{\text{UNC2}}}$ and $\beta_{h_{\text{UNC2}}}$ are the rate constants of m_{UNC2} and h_{UNC2} , and a , b and c are parameters.

We follow the work of Nicoletti et al. to model the EGL-19, CCA-1 and SHL-1 channels (Nicoletti et al., 2019). The EGL-19 (CaV1) L-type calcium current is given by

$$I_{\text{EGL19}} = g_{\text{EGL19}} \cdot m_{\text{EGL19}} \cdot h_{\text{EGL19}} \cdot (V - V_{\text{Ca}})$$

where g_{EGL19} is the maximum conductance. The dynamics of the activation and inactivation gating variables, m_{EGL19} and h_{EGL19} , are described by

$$\frac{dm_{\text{EGL19}}}{dt} = \frac{m_{\text{EGL19},\infty}(V) - m_{\text{EGL19}}}{\tau_{m_{\text{EGL19}}}(V)}$$

$$\frac{dh_{\text{EGL19}}}{dt} = \frac{h_{\text{EGL19},\infty}(V) - h_{\text{EGL19}}}{\tau_{h_{\text{EGL19}}}(V)}$$

$$m_{\text{EGL19},\infty}(V) = \frac{1}{1 + e^{-\left(\frac{V-V_{1/2}}{k_a}\right)}} \quad h_{\text{EGL19},\infty}(V) = \left[\frac{a}{1 + e^{-\left(\frac{V-V_{1/2,1}}{k_{i,1}}\right)}} + b \right] \cdot \left[\frac{c}{1 + e^{-\left(\frac{V-V_{1/2,2}}{k_{i,2}}\right)}} + d \right]$$

$$\tau_{m_{\text{EGL19}}}(V) = \left[ae^{-\left(\frac{V-b}{c}\right)^2} \right] + \left[de^{-\left(\frac{V-e}{f}\right)^2} \right] + g$$

$$\tau_{h_{\text{EGL19}}}(V) = a \left[\frac{b}{1 + e^{-\left(\frac{V-c}{d}\right)}} + \frac{e}{1 + e^{-\left(\frac{V-f}{g}\right)}} + h \right]$$

where $m_{\text{EGL19},\infty}$ and $h_{\text{EGL19},\infty}$ are the steady-state functions of the gating variables, and

$\tau_{m_{\text{EGL19}}}$ and $\tau_{h_{\text{EGL19}}}$ are the corresponding voltage-dependent time constants.

The CCA-1 and SHL-1 channels were modeled similarly to EGL-19; their equations are listed below.

744 CCA-1 (CaV3) T-type calcium current:

$$745 \quad I_{\text{CCA1}} = g_{\text{CCA1}} \cdot m_{\text{CCA1}}^2 \cdot h_{\text{CCA1}} \cdot (V - V_{\text{Ca}})$$

$$746 \quad \frac{dm_{\text{CCA1}}}{dt} = \frac{m_{\text{CCA1},\infty}(V) - m_{\text{CCA1}}}{\tau_{m_{\text{CCA1}}}(V)}$$

$$747 \quad \frac{dh_{\text{CCA1}}}{dt} = \frac{h_{\text{CCA1},\infty}(V) - h_{\text{CCA1}}}{\tau_{h_{\text{CCA1}}}(V)}$$

$$748 \quad m_{\text{CCA1},\infty}(V) = \frac{1}{1 + e^{-\left(\frac{V - V_{1/2}}{k_a}\right)}}, \quad h_{\text{CCA1},\infty}(V) = \frac{1}{1 + e^{\left(\frac{V - V_{1/2}}{k_i}\right)}}$$

$$749 \quad \tau_{m_{\text{CCA1}}}(V) = \frac{a}{1 + e^{-\left(\frac{V - b}{c}\right)}} + d, \quad \tau_{h_{\text{CCA1}}}(V) = \frac{a}{1 + e^{\left(\frac{V - b}{c}\right)}} + d$$

750 SHL-1 (Kv4) potassium current:

$$751 \quad I_{\text{SHL1}} = g_{\text{SHL1}} \cdot m_{\text{SHL1}}^3 \cdot (0.7h_{\text{SHL1},f} + 0.3h_{\text{SHL1},s}) \cdot (V - V_{\text{K}})$$

$$752 \quad \frac{dm_{\text{SHL1}}}{dt} = \frac{m_{\text{SHL1},\infty}(V) - m_{\text{SHL1}}}{\tau_{m_{\text{SHL1}}}(V)}$$

$$753 \quad \frac{dh_{\text{SHL1},f}}{dt} = \frac{h_{\text{SHL1},\infty}(V) - h_{\text{SHL1},f}}{\tau_{h_{\text{SHL1},f}}(V)}$$

$$754 \quad \frac{dh_{\text{SHL1},s}}{dt} = \frac{h_{\text{SHL1},\infty}(V) - h_{\text{SHL1},s}}{\tau_{h_{\text{SHL1},s}}(V)}$$

$$755 \quad m_{\text{SHL1},\infty}(V) = \frac{1}{1 + e^{-\left(\frac{V - V_{1/2}}{k_a}\right)}}, \quad h_{\text{SHL1},\infty}(V) = \frac{1}{1 + e^{\left(\frac{V - V_{1/2}}{k_i}\right)}}$$

$$756 \quad \tau_{m_{\text{SHL1}}}(V) = \frac{a}{e^{-\left(\frac{V - b}{c}\right)} + e^{\left(\frac{V - d}{e}\right)}} + f$$

$$757 \quad \tau_{h_{\text{SHL1},f}}(V) = \frac{a}{1 + e^{\left(\frac{V - b}{c}\right)}} + d, \quad \tau_{h_{\text{SHL1},s}}(V) = \frac{a}{1 + e^{\left(\frac{V - b}{c}\right)}} + d$$

The action potential in the *exp-2;shl-1* double mutants (Fig S2C) indicated that AVL (and hence the model) required additional potassium channels that open at depolarized membrane potentials to provide outward current. We noted that *egl-36* encodes a *Shaw* subfamily (Kv3) of voltage-gated potassium channels which is expressed in AVL (Choi et al., 2021; Johnstone et al., 1997). Here we added EGL-36 into the model and with parameters based on experimental data of Johnstone et al. (1997). The EGL-36 (Kv3) potassium current activates with three components at fast, medium and slow timescales, and it is modeled by the equations below

$$I_{\text{EGL36}} = g_{\text{EGL36}} \cdot (0.33m_{\text{EGL36,f}} + 0.36m_{\text{EGL36,m}} + 0.39m_{\text{EGL36,s}}) \cdot (V - V_K)$$

$$\frac{dm_{\text{EGL36,f}}}{dt} = \frac{m_{\text{EGL36},\infty}(V) - m_{\text{EGL36,f}}}{\tau_{m_{\text{EGL36,f}}}}$$

$$\frac{dm_{\text{EGL36,m}}}{dt} = \frac{m_{\text{EGL36},\infty}(V) - m_{\text{EGL36,m}}}{\tau_{m_{\text{EGL36,m}}}}$$

$$\frac{dm_{\text{EGL36,s}}}{dt} = \frac{m_{\text{EGL36},\infty}(V) - m_{\text{EGL36,s}}}{\tau_{m_{\text{EGL36,s}}}}$$

$$m_{\text{EGL36},\infty}(V) = \frac{1}{1 + e^{-\left(\frac{V - V_{1/2}}{k_a}\right)}}$$

$$\tau_{m_{\text{EGL36,f}}} = \tau_f, \tau_{m_{\text{EGL36,m}}} = \tau_m, \tau_{m_{\text{EGL36,s}}} = \tau_s$$

During our modeling of individual ionic currents, the conductance of each channel was estimated to fit the current and voltage clamp experiment. We attempted to predict the phenotypes of four potassium channel mutants (*exp-2(gf)*, *exp-2(lf)*, *shl-1* and *exp-2;shl-1* double mutant) by modifying the parameters and fitting it to the experimental mutant data. For the *exp-2(gf)* mutant, the parameters of EXP-2 Markov model was modified by fitting to the current-voltage curve of the repolarization-activated current of *exp-2(gf)* in Fig 3B. For the loss-of-function mutants, the specific mutant channel was knocked out *in silico* by setting its conductance to zero and increasing conductance of other potassium channels.

All our simulations were written in MATLAB and the parameter values of the wildtype and mutant models can be found in Table 2.

Table 1. List of worm strains

Stain ID	Genotype
EG1285	<i>lin-15B(n765); oxIs12 [punc-47::GFP + lin-15(+)] X</i>
CX17773	<i>exp-2(sa26ad1426) V; lin-15B(n765) X; oxIs12 X</i>
CX17868	<i>+eT1 III; exp-2(sa26)/eT1[let-?n886] V; lin-15B(n765); oxIs12 X</i>
CX17774	<i>shl-1(ok1168) IV; lin-15B(n765) X; oxIs12 X</i>
CX17889	<i>shl-1(ok1168) IV; exp-2(sa26ad1426) V; lin-15B(n765) X; oxIs12 X</i>
CX17776	<i>unc-2(e55) IV; lin-15B(n765) X; oxIs12 X</i>
CX17777	<i>egl-19(n582) IV; lin-15B(n765) X; oxIs12 X</i>
CX17848	<i>oxIs608 [Punc-47::mCherry] V; cca-1(ad1650) X</i>
CX17849	<i>egl-19(n582) IV; oxIs608 V; cca-1(ad1650) X</i>
CX17890	<i>nca-2(gk5) III; nca-1(gk9) IV; lin-15B(n765) X; oxIs12 X</i>
CX17891	<i>unc-2(zf35) IV; lin-15B(n765) X; oxIs12 X</i>
CX17902	<i>egl-19(n582) IV; unc-2(e55) X; lin-15B(n765) X; oxIs12 X</i>
CX18004	<i>kyls772 [Punc-47mini::GCaMP6f @ 50ug/ul + Pinx-16::GCaMP6f @ 5ug/ul + Pceh-63::GCaMP6f @ 25ug/ul]</i>
CX18153	<i>kyls777 [Punc-47::GCaMP6f::SL2::mCherry::let858 3'UTR @ 30ug/ul + Punc-47mini::GCaMP6f::SL2::mCherry::unc-54 3'UTR @ 50ug/ul + Pinx-16::GCaMP6f @ 5ug/ul] II</i>
CX18176	<i>kyls777 II; inx-1(tm3524) X</i>
CX18316	<i>kyls777 II; exp-2(sa26ad1426) V</i>

Table 2. Model parameters

See attached excel file.

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Supplemental Figure 1

A-B. Representative action potential traces recorded from AVL in *cca-1*, *egl-19;cca-1* and *nca-1;nca-2* mutants.

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Supplemental Figure 2

A-B. Representative voltage-clamp traces recorded from AVL in *shl-1*, *exp-2;shl-1* mutant animals (A) and DVB in wild-type animals (B). **C.** Representative action potential traces recorded from AVL in *shl-1* and *exp-2;shl-1* mutants. *shl-1* AVL fires WT-like action potentials. *exp-2;shl-1* AVL fires *exp-2(lf)*-like action potentials.

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Supplemental Figure 3

A-C. Temporal evolution of the normalized conductance of each modeled calcium current and potassium current in wild-type, *exp-2(lf)* and *exp-2(gf)* AVL. Top: current-injection stimulation used. The stimulating currents are set to 10 pA, 8 pA and 12 pA for wild-type, *exp-2(lf)* and *exp-2(gf)* AVL, respectively. The normalized conductances correspond to the colored traces of the current clamp simulations of Fig. 4C, D, and E in the main text. The bottom two panels are the zoomed-in view of the panels above. **D-E.** Representative action potential simulations and the normalized conductance of each modeled current for *shl-1* mutant and *exp-2;shl-1* double mutant AVL. The temporal evolution of the normalized conductance is color matched with the corresponding current clamp simulation.

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Supplemental Figure 4

A. Definition of DMP events (start of pBoc, peak of pBoc, start of aBoc, peak of aBoc, end of aBoc) based on the change of worm body length. **B.** Changes of body length were aligned to the peak of aBoc in several cycles. Pink: average timing when intestinal calcium activities reach half-peak in each segment. Blue: when AVL is active estimated by visual inspection. **C.** Statistical comparison of DMP event intervals between wild-type individuals. Each column represents a wild-type individual. CaRise was defined as the average timing when intestinal calcium activities reach half-peak in each segment. **D.** Correlation between the intervals examined in C (Δt_1 : CaRise-pBocMax, Δt_2 : pBocMax-aBocMax, Δt_3 : CaRise-CaRise). Each point represents a wild-type individual.

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Supplemental Figure 5

A. Worm centerline was divided into anterior and posterior half, using VD/DD motor neurons as landmarks. **B-D.** Top: Representative normalized calcium activities of AVL (red) and DVB soma (blue) in wild-type (**B**), *inx-1* (**C**), and *exp-2(lf)* (**D**) mutant animals. Middle: Representative trace showing the length change of the anterior (red) and posterior (blue) half of the centerline. Bottom: Representative normalized intestinal calcium activities along the worm body axis (A: Anterior, P: Posterior). Vertical lines: Timing of expulsion.