Gut-cholinergic Motoneuron Communication Regulates Lipid Metabolism in Caenorhabditis Elegans

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Abstract: Recent studies reveal the gut-brain axis, with its bidirectional signaling function, plays a crucial role in host physiological regulation. Through the gut-to-brain signaling, the nervous system can influence lipid metabolism and gut physiological function via brain-to-gut signaling. However, the mechanism of how the bidirectional signaling pathway of the gut-brain axis affects the host remains elusive. In this study, the author found that the lipid metabolism pathway of Caenorhabditis elegans (C. elegans) is regulated by the bidirectional communication of cholinergic motoneurons. The ASIC-1 protein, a homolog of mammalian acid-sensitive ion channel ASICs, impacts C. elegans lipid metabolism through expression in cholinergic motoneurons. Calcium imaging experiments revealed that C. elegans cholinergic motoneurons possess H+ sensitivity, which is dependent on their ASIC-1 expression. It has been reported that various Na^+-H^+ exchangers (NHXs) expressed by C. elegans intestinal epithelial cells can release H^+ to act on ASIC-1 in cholinergic motoneurons. NHX-6, expressed by C. elegans intestinal epithelium, may regulate C. elegans lipid levels by releasing H^+ to activate ASIC-1 expressed by cholinergic motoneurons. By using RNAi technology to knock down the genes that regulate cholinergic neurotransmitter signals in cholinergic motoneurons, the author found that the NHX-6-ASIC-1 signaling regulates body fat levels through cholinergic signaling. In vivo fluorescence imaging of C. elegans confirmed that the NHX-6-ASIC-1 signaling pathway reduces the expression level of the key lipid catabolic gene *atgl-1* and increases the expression levels of the key synthetic metabolic genes *dgat-2*, *fat-5*, and *fat-7*. In summary, the results of the above studies indicate that the intestinal epithelial cell Na^+-H^+ exchanger NHX-6 releases H^+, activating the acid-sensitive ion channel ASIC-1, which in turn regulates the activity of cholinergic motoneurons and the release of the neurotransmitter acetylcholine. This initiates a conserved intestinal signaling pathway that promotes lipid synthesis metabolism, ultimately affecting the body fat levels of the C. elegans.

***Keywords*:** C. elegans, gut-cholinergic motoneuron communication, lipid metabolism, acid-sensitive ion channel, Na^+-H^+ exchanger

1. Introduction

The nervous system and the gut both play key roles in lipid metabolism [1, 2]. Through bidirectional gut-brain communication, the central nervous system also regulates body fat metabolism and gut physiological function [3, 4]. In mammals, brain-to-gut signaling is primarily generated by vagal motoneurons (efferent neurons) [3]. For example, the vagus nerve can release the neurotransmitter acetylcholine to activate G-protein coupled muscarinic receptors in the gut [5].

H^+ functions as an intercellular neurotransmitter and can mediate cell-to-cell communication [6]. In 1986, Garnier et al. found that H^+ could activate gut vagal sensory neurons [7]. However, it was unclear at the time whether and how intestinal epithelial cells could release H^+ to the vagus nerve. In 2002, Ishii et al., studying the acid protection mechanism of the duodenum, speculated based on pharmacological experiment results that intestinal epithelial cells might release H^+ through NHE1, a member of the Na^+-H^+ exchanger (NHEs) family, expressed on the basolateral membrane of intestinal epithelial cells [8]. Correspondingly, vagal sensory neurons express various H^+ sensitive ion channels, such as acid-sensitive ion channels (ASICs), transient receptor potential vanilloid receptor (TRPV), and two-pore potassium channels (K_{2p}) [9]. Among these, the ASICs family is conserved across species and has H^+ signal transduction function [6, 9, 10]. Weifang Rong et al. found that treating isolated cultured mouse small intestine vagal sensory neurons ex vivo with a non-specific ASICs antagonist significantly reduced their H^+ response current, suggesting that the gut vagus nerve may utilize ASICs to sense H^+ [11]. These studies strongly suggest that H^+ might mediate gut-brain communication through the NHEs-ASICs signaling pathway.

C. elegans serve as an ideal model for studying the fundamental mechanisms and functions of gut-neuron communication in vivo. The C. elegans gut consists solely of 20 single-type intestinal epithelial cells, which bear morphological similarities to mammalian intestinal epithelial cells[12]. The nervous system is remarkably simple, containing just 302 neurons, with

clear anatomical locations and a fully mapped neural connectome[13]. They possess most of the neurotransmitters found in mammals, along with homologous neurotransmitter receptors, transport proteins, and ion channels[14]. Their experimental cycles are short, genetic manipulation is straightforward. There are many available mutant strains, and techniques for analysing animal behaviour are well-developed and reliable. The gut is the C. elegans's primary metabolic organ, and its signalling pathways for regulating lipid metabolism are conserved in mammals[15, 16]. Similar to mammals, there is also bidirectional gut-neuron communication between the C. elegans's nervous system and its gut[17-20]. Therefore, utilising C. elegans allows for the elucidation of the intricate mechanisms of gut-neuron communication at the single-cell level, as well as revealing the impact of gut-neuron communication on animal physiological functions at the organismal level.

Similar to the situation with mammalian cortical neurons, some C. elegans neurons also possess multifunctionality[21]. For example, cholinergic motoneurons in the C. elegans's ventral nerve cord may function as sensory neurons[22]. Interestingly, two research groups led by Jorgenson and Nehrke respectively reported in 2008 that C. elegans intestinal epithelial cells can use the mammalian NHEs homologous protein NHX-7 to release H^+ into the body cavity, causing rhythmic oscillations of H^+ signals in the body cavity[23, 24]. Due to the anatomical structure of C. elegans, the gut may use H^+ to regulate cells exposed to the body cavity[14]. In the anatomical position, it can act on cholinergic motoneurons in the ventral nerve cord of C. elegans. ASIC-1 protein belongs to DEG/ENaC/ASIC family and is highly conserved among different species. The C. elegans ASIC-1 protein is a homologous protein of mammalian acid-sensing ion channel ASICs [25]. The author used C. elegans as a model animal to investigate the function and mechanism of action of ASIC-1 expressed by cholinergic motoneurons in the ventral nerve cord and the novel mechanism of gut-cholinergic motoneuron communication. The effect of gut-cholinergic motoneuron communication on host physiological function needs further study.

2. Materials and Methods

2.1 Strains and Transgenic C. elegans

Worm strains were cultured on NGM plates with Escherichia coli OP50 at 20°C. The author used *Pmyo-2::mStrawberry* (ng/μL) as a co-injection marker. Transgenic worm strains were generated using microinjection.

2.2 Oil Red O Staining

Oil Red O stock solution was prepared at 5 mg/mL, and 60% Oil Red O staining solution was prepared freshly for each use. For each experiment, 400-500 worms of each genotype were needed. Synchronized worms were obtained using bleach to lyse worm bodies and hatch eggs. On the first day of adulthood, worms were washed 2-3 times with PBS. They were then fixed in 1% paraformaldehyde (PFA) fixative for 1 hour and subjected to three freeze-thaw cycles. Dehydration was done in 60% isopropanol (PBS) solution for 15 minutes. The 60% Oil Red O staining solution was prepared right before use, shaken well, allowed to stand for 10 minutes, and then filtered through a 0.22 μm filter. Staining was performed at 20°C for 18 hours. The exposure value was adjusted and kept constant at 10x magnification.

2.3 Pharyngeal Pumping Count Assay

Well-fed L4 worms were cultured overnight at 20°C, and on the first day of adulthood, the number of pharyngeal contractions was counted under a high-power Zeiss microscope over 10 seconds. Each count included 15-20 worms and was repeated at least three times.

2.4 Measurement and Analysis of worms Movement Speed

The room temperature was maintained at 20°C. The Track-A-Worm system was used to automatically track the movement trajectory of individual worms. The movement parameters were analysed using the Track-A-Worm system software.

2.5 Live C. elegans Fluorescence Imaging

The GFP fluorescence intensity of control and specific mutant worms was captured using a Nikon imaging microscope system. Thirty worms were imaged, in three groups of ten. Worms were anaesthetised on agar plates with 10mM sodium azide. The same magnification and exposure time were used for imaging the fluorescence of the control and treatment groups. The resulting fluorescence intensity statistics were processed using ImageJ.

2.6 In Vivo worms Dissection and Neuronal Stimulation Techniques

Experiments were performed at 20°C. Worms were immobilised with Vetbond Tissue Adhesive (3M), restricting them to a fixed area while allowing their head and tail to move freely. The incision length for in vivo dissection was controlled to be between 200-300μm. The dissected worms were placed in a recording trough filled with 1ml of electrophysiological

extracellular fluid. Using an Eppendorf FemtoJet 4i microinjector, solutions of varying pH values were delivered to the target neurons through a ~3MΩ glass electrode using a pressure of 2psi for 3 seconds. The composition of the electrophysiological extracellular solution (in mM) was: 140 NaCl, 5 KCl, 5 $CaCl_2$, 5 $MgCl_2$, 11 dextrose, and 5 HEPES (pH 7.2)

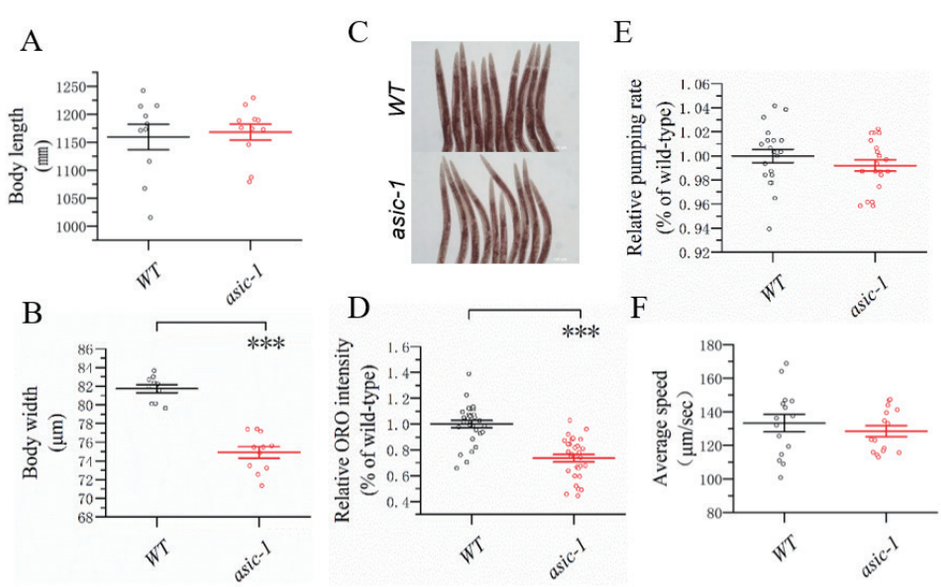
2.7 Calcium Imaging Experiment

In vivo calcium imaging technique was used. Experiments were performed at 20°C. Worms were immobilised on a coverslip coated with silicone resin using Vetbond Tissue Adhesive (3M) and placed in a recording trough filled with electrophysiological extracellular fluid. An Eppendorf FemtoJet 4i microinjector was used to deliver a series of pH solutions to the anterior section of the ventral nerve cord at a pressure of 0.3psi for 30 seconds. Neuronal calcium signal changes were recorded for 3 minutes at 1 frame per second using a Nikon TS2R inverted fluorescence microscope with a 60x objective lens connected to a CMOS camera (Hamamatsu, C11440-22CU). Fluorescence intensity changes were analysed using NIS-Elements imaging software (version 4.51).

3. Experimental Results

3.1 ASIC-1 Regulates Lipid Metabolism in C. elegans

The C. elegans ASIC-1 protein is a homologous protein of the mammalian acid-sensitive ion channel ASICs and belongs to the DEG/ENaC/ASIC family[25]. Some proteins in this family make up mechanosensitive channels that sense mechanical stimuli, while others can form acid-sensitive ion channels[26]. The previous study showed that adult worms with an *asic-1* deletion mutation are significantly thinner compared to the wild type. The measured data showed that the length of the *asic-1* deletion mutant was not significantly changed compared with the wild type, but the width was significantly reduced (Figures 1A and B). Compared with the wild type, the body fat level of the *asic-1* mutants is significantly reduced (Figures 1C and D), indicating that ASIC-1 can increase the body fat level in C. elegans. The body fat level in C. elegans is related to movement, food intake, and lipid metabolism[27]. To explore the specific mechanism by which the *asic-1* mutation affects the body fat level in C. elegans, the author tested the locomotion and food intake of *asic-1* deletion mutant worms. Using the Track-a-worm automatic tracking and recording analysis system[28] to compare the movement speed of the wild type and *asic-1* mutants, the author found that the average movement speed of the *asic-1* mutant worms was not significantly different from the wild type (Figure 1E). By counting the rate of pharyngeal pumping, the author found that there was no significant change in the *asic-1* mutants compared to the wild type (Figure 1F), indicating that the *asic-1* mutation does not affect the food intake of C. elegans. Therefore, ASIC-1 may increase the body fat level in C. elegans by regulating lipid metabolism.



Note: (A) Compared with the wild-type worms (WT), the *asic-1* deletion mutation does not affect the body length of the worms. (B) Compared with WT, the width of the *asic-1* deletion mutant is significantly reduced (n = 10). ***p<0.001; (C) Representative images of Oil Red O staining of WT and *asic-1* deletion mutants (Day 1 adults), scale bar 100μm; (D) Compared with WT, the lipid content of *asic-1* mutants is significantly reduced. Data are expressed as relative values ±SEM (n = 30). ***p<0.001. Compared with WT, there is no difference in the pharyngeal pumping rate (E) and average speed (F) of the *asic-1* mutants. ±SEM (n = 20). Unpaired t-test was used.

Figure 1. ASIC-1 regulates lipid metabolism in C. elegans

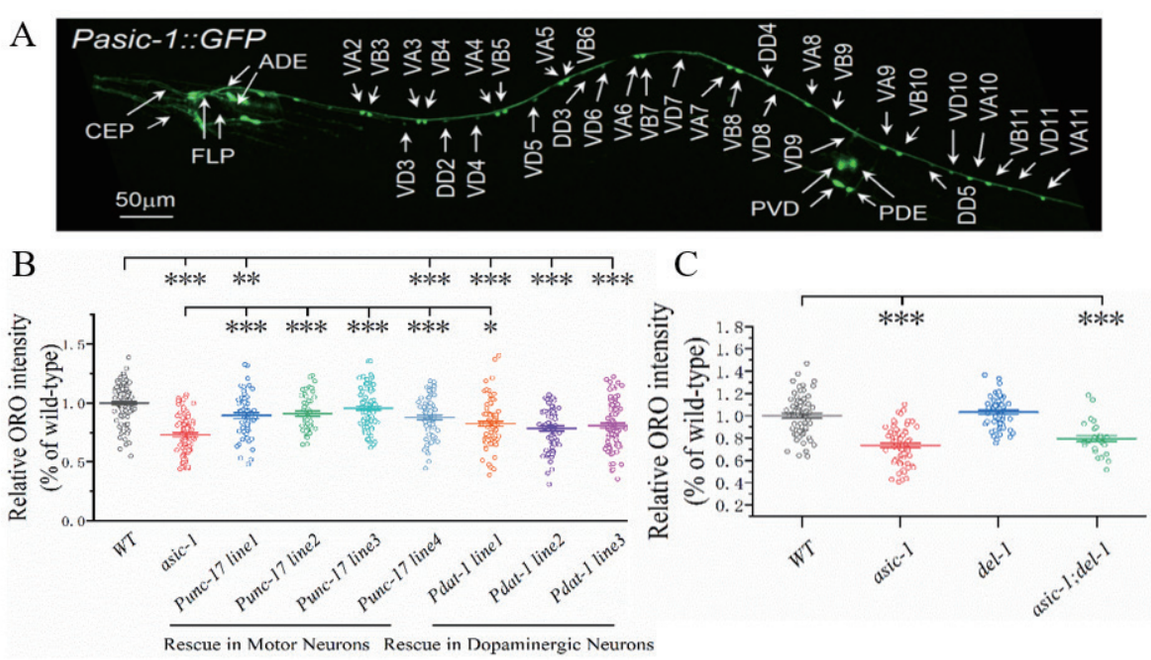
3.2 ASIC-1 Mainly Regulates Body Fat Levels in Cholinergic motoneurons

To identify the cells in which ASIC-1 exerts its regulatory role, the author constructed an *asic-1* promoter transcription fusion plasmid (*Pasic-1::GFP*, the "*P*" before the gene stands for promoter) and used this plasmid to construct transgenic worms expressing *Pasic-1::GFP* through microinjection. Through fluorescence imaging, the author found that *ASIC-1* is mainly expressed in the ventral cord cholinergic motoneurons and dopaminergic neurons (Figure 2A).

Accordingly, the author constructed two types of plasmids, *Punc-17Δ1::asic-1 (cDNA)::SL2::mStrawberry* and *Pdat-1::asic-1 (cDNA)::SL2::GFP*, using the cholinergic motoneuron-specific promoter Punc-17Δ1and the dopaminergic neuron-specific promoter *Pdat-1*, respectively. the author selected four strains, line1-line4: *asic-1*;[*Punc-17Δ1::asic-1(cDNA)::SL2::mStrawberry,Pmyo-2::mStrawberry*] with clear red fluorescence expression in ventral cholinergic motoneurons, and three strains each of eight pairs of transgenic C. elegans with clear green fluorescence expression in dopaminergic neurons, line1-line3: *asic-1*;[*Pdat-1::asic-1(cDNA)::SL2::GFP, Pmyo-2::mStrawberry*], for neuron-specific gene rescue experiments.

Using Oil Red O staining, with wild-type worms and *asic-1* mutant worms as control groups, the author found that the specific expression of wild-type *asic-1* cDNA in cholinergic motoneurons can significantly restore the body fat levels of *asic-1* mutant worms. However, the specific expression of wild-type *asic-1* cDNA in dopaminergic neurons had little effect on the body fat levels of *asic-1* mutant worms (Figure 2B). These results indicate that *ASIC-1* regulates lipid metabolism in C. elegans through cholinergic neurons.

The previous work showed that ASIC-1 and another DEG/ENaC/ASIC family protein DEL-1 are the main components of the mechanically sensitive channels in cholinergic motoneurons. Additionally, studies have shown that mammalian homologues of C. elegans ASIC-1, ASICs, are acid-sensitive ion channel proteins and mainly constitute postsynaptic H^+ receptors in the brain [29, 30]. To determine whether DEL-1 can regulate body fat levels in cholinergic motoneurons as a mechanically sensitive channel protein, the author found that compared with wild-type worms, the body fat levels of *del-1*(*ok150*) deletion mutant worms did not change significantly, and the body fat levels of *asic-1*;*del-1*(*ok150*) double mutants were the same as those of *asic-1* single mutants (Figure 2C). These results indicate that the mechanically sensitive channels in cholinergic neurons do not affect body fat levels, and the regulatory effect of ASIC-1 on body fat may be produced as an acid-sensing channel.



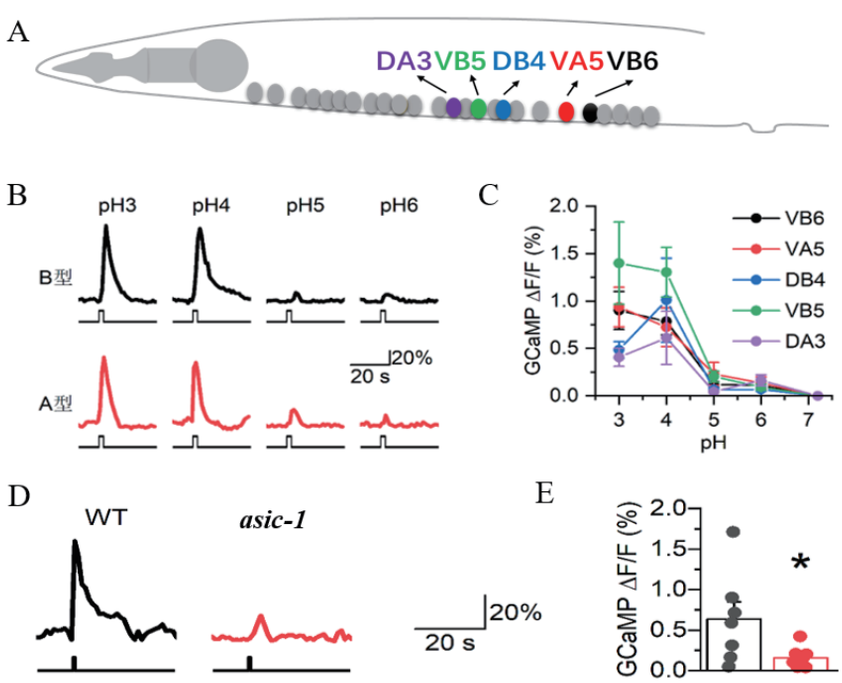
Note: (A) The *Pasic-1::GFP* integrated transgene array shows a scale of 50μm; (B) The expression of *asic-1* in cholinergic neurons specifically restored by the *Punc-17Δ1* promoter is sufficient to rescue the increase in lipid levels in *asic-1* mutants; The expression of *asic-1* in dopaminergic neurons specifically restored by the *Pdat-1* promoter does not rescue the increase in lipid levels in *asic-1* mutants. Data are shown as relative values ±SEM (n=30) *P<0.05, **P<0.01, ***P<0.001. (C) Compared with WT, the body fat content of *del-1(ok150) X* deletion mutant worms shows no difference, and *asic-1*;*del-1(ok150)X* double mutant worms show no difference in lipid levels compared with *asic-1* single mutants ±SEM (n=30) ***p<0.001. Analyzed by one-way ANOVA Tukey test.

Figure 2. ASIC-1 primarily regulates lipid levels in cholinergic motoneurons

3.3 ASIC-1 is the Major Constituent Protein of Acid-sensing Channels in Cholinergic motoneurons

To verify the role of ASIC-1 as an acid-sensing channel in the regulation of lipid metabolism in cholinergic motoneurons, the author first tested the acid sensitivity of cholinergic motoneurons using calcium imaging technology. The cholinergic motoneurons in the ventral nerve cord of the C. elegans mainly include type A (A-MNs) and type B motoneurons (B-MNs), which control the backward and forward movement of the worms, respectively. Depending on whether the motoneurons control the ventral or dorsal muscles, A-MNs and B-MNs can be further divided into ventral neurons (12 VAs and 11 VBs) and dorsal neurons (9 DAs and 7 DBs). The author selected DA3, VA5 to represent A-MNs, and VB5, VB6, DB4 to represent B-MNs. The cell bodies of these neurons are located in the front half of the worms body and can be identified by their anatomical positions (Figure 3A).

Using the techniques of in vivo dissection of worms and in vivo neuronal stimulation, the author performed pH3, pH4, pH5, and pH6 stimulation on cholinergic motoneurons. Using calcium imaging technology, the author found that acid stimulation induced a significant increase in calcium signals in cholinergic motoneurons. The acid response was very rapid, without significant delay, indicating that acid stimulation indeed activated cholinergic motoneurons (Figure 3B). Further, the author found that the acid response of cholinergic motoneurons showed a clear pH-dependent effect, i.e., the lower the pH, the stronger the response, and the response reached its maximum at pH 4 (Figure 3C). These results indicate that cholinergic motoneurons have the ability to sense H^+ and may express H^+ receptor ion channels. For convenience, the author only used pH 4 acid stimulation in subsequent experiments, and the results only show VB6 and VA5 representing A-MNs and B-MNs, respectively. Next, the author tested whether ASIC-1 mediates the acid sensing response of cholinergic motoneurons. Using calcium imaging technology, the author found that the *asic-1* deletion mutation greatly eliminated the acid response of cholinergic motoneurons (Figure 3D-E), indicating that the acid-sensitive ion channel in cholinergic motoneurons is primarily composed of ASIC-1.



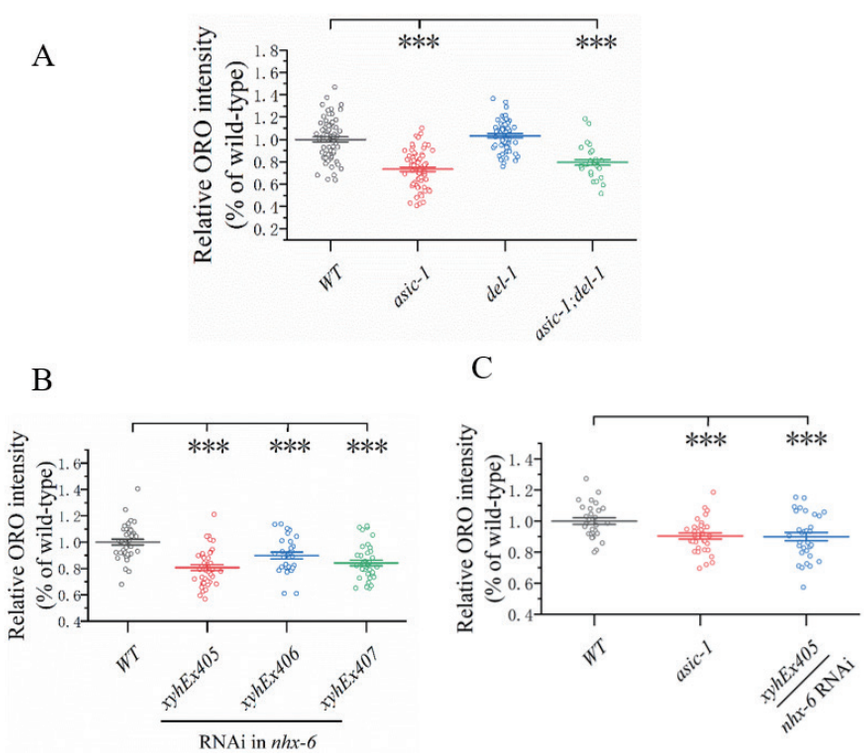
Note: (A) Anatomical position schematic of DA3, VA5, VB5, VB6, DB4. (B) Calcium imaging results show that acid stimulation elicited a significant calcium signal increase in type A (A-MNs) and type B motoneurons (B-MNs). (C) Calcium imaging results show that cholinergic motoneurons display a pH-dependent response to acid stimulation. n≥7, data are relative values ± SEM. (D-E) Calcium imaging results show that compared to WT, cholinergic motoneurons display a pH-dependent response to acid stimulation. n≥7±SEM. *p<0.05. One-way ANOVA with Tukey's post hoc test.

Figure 3. Cholinergic motoneurons possess acid-sensing ability

3.4 The Intestine Regulates Body Fat Levels Using Na^+-H^+ Exchanger

The above results indicate that cholinergic motoneurons express the H^+ receptor ion channel ASIC-1, which can be activated by H^+. The author next identified the source of H^+ that activates cholinergic motoneurons. It has been reported that

the epithelial cells of the C. elegans intestine release H^+ into the body cavity through the Na^+-H^+ exchanger family NHX-7, causing rhythmic oscillations of the H^+ signal in the body cavity [23, 24]. Anatomically, the cholinergic motoneurons in the ventral nerve cord are excellent targets for intestinal H^+ regulation. Therefore, to test whether intestinal epithelial cells could activate cholinergic motoneurons by releasing H^+ through NHX-7. The author found that compared with wild-type worms, *nhx-7*(*ok583*) deletion mutant worms did not show a decrease in body fat levels like *asic-1* mutants, while the body fat level of *asic-1*;*nhx-7*(*ok583*) double mutants was consistent with that of *asic-1* single mutants (Figure 4A), indicating that NHX-7 does not participate in the regulation of C. elegans body fat levels. The C. elegans genome encodes 9 hypothetical Na^+-H^+ exchanger proteins, including NHX-1 to 9 [31]. Expression patterns show that NHX-7 is expressed in the basal lateral membrane of intestinal epithelial cells, but its expression is strictly limited to the rear end of the intestine. In contrast, another Na^+-H^+ exchanger protein, NHX-6, is also expressed in the basal lateral membrane of intestinal epithelial cells, but it is expressed throughout the intestine [31]. The author constructed two plasmids for RNA interference (RNAi) of intestinal nhx-6 expression using the intestine-specific promoter Pges-1, namely *Pges-1::nhx-6 RNAi_ss* and *Pges-1::nhx-6 RNAi_as*. The author obtained three lines: *xyhEx405*, *xyhEx406*, and *xyhEx407*[*Pges-1::nhx-6 RNAi, Pmyo-2::GFP*]. Through Oil Red O staining, the author found that the body fat levels of these three *nhx-6* RNAi lines were significantly reduced compared with wild-type (Figure 4B), indicating that the intestine participates in the regulation of body fat levels through NHX-6.In addition, the body fat levels of the *xyhEx405* and *asic-1* deletion mutants were significantly lower than that of wild type, and the reduction was similar (Figure 4C), suggesting that the intestine may use NHX-6 to regulate body fat levels through ASIC-1 in cholinergic motoneurons.



Note: (A) Compared with wild-type worms (WT), *nhx-7(ok583)* deletion mutation does not affect worms body fat levels, data are relative values ±SEM (n = 30) ***p<0.001. (B) Compared with WT, the body fat levels of the three *nhx-6* RNAi worms are significantly reduced, ±SEM (n = 30) ***p<0.001. (C) Compared with *asic-1* deletion mutant C. elegans, there is no difference in body fat levels of *nhx-6* RNAi, ±SEM (n = 30) ***p<0.001. One-way ANOVA with Tukey's test was used.

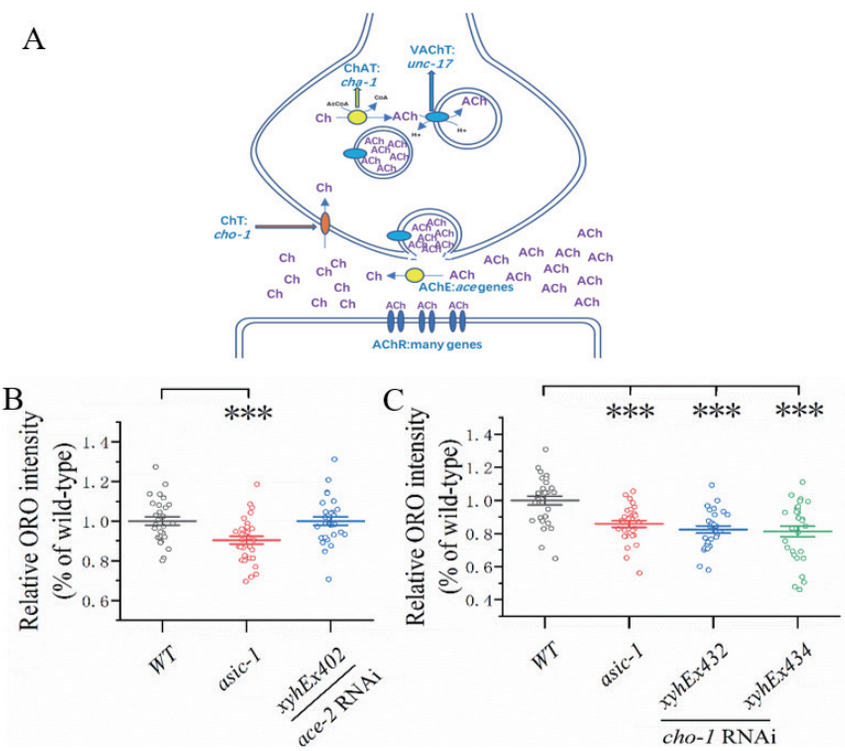
Figure 4. The Intestine Participates in the Regulation of Body Fat Levels through NHX-6

3.5 NHX-6-ASIC-1 Signaling Regulates Body Fat Levels through Cholinergic Signaling

The above experimental results suggest that C. elegans gut may use NHX-6-ASIC-1 signal transduction to affect the activity of cholinergic motoneurons to regulate body fat level. Cholinergic motoneurons primarily release the neurotransmitter acetylcholine (ACh). Acetylcholine released into the extracellular space is hydrolyzed by acetylcholinesterase (AChE) into choline, which is recycled back into neurons for the synthesis of ACh through the choline transporter (ChT: CHO-1) (Figure

5A) [32]. Therefore, a deficiency of AChE would lead to the accumulation of extracellular ACh, while a deficiency of CHO-1 would limit the synthesis rate of presynaptic ACh, leading to a decrease in ACh synthesis. To test whether NHX-6-ASIC-1 signaling regulates body fat levels via cholinergic signaling, the author selected gene mutants of AChE and ChT in the acetylcholine metabolism pathway for Oil Red O staining experiments.

The worms genome encodes three AChE genes: *ace-1*, *ace-2* and *ace-3* [32]. Among them, ACE-2 is expressed in cholinergic motoneurons in the ventral nerve cord [33]. To specifically test the role of ACE-2 in cholinergic motoneurons, the author used the specific promoter Pdel-1 to construct a transgenic worms in which ace-2 was RNA-interfered (RNAi) in cholinergic motoneurons: xyhEx402[Pdel-1::SL2::ace-2 RNAi, Pmyo-2::GFP]. The results of Oil Red O staining showed that there was no significant difference in body fat levels between RNAi worms and wild-type worms (Figure 5B), indicating that ACE-2 may not be involved in regulating body fat levels, or ACE-2 may work in conjunction with ACE-1 and ACE-3 expressed in cholinergic motoneurons, which needs to be tested further. Next, the author used the specific promoter *Pdel-1* to construct two transgenic worms in which cho-1 was RNA-interfered (RNAi) in cholinergic motoneurons: *xyhEx432* and *xyhEx433*[*Pdel-1::SL2::cho-1 RNAi, Pmyo-2::GFP*]. The results of Oil Red O staining showed that the body fat levels of *xyhEx432* and *xyhEx433* C. elegans were significantly lower than that of wild-type, and the decrease was comparable to that of *asic-1* deletion mutants (Figure 5C), indicating that CHO-1 increases body fat levels in C. elegans and preliminarily indicating that the NHX=6-ASIC-1 signaling pathway may regulate body fat levels through cholinergic signaling.



Note: (A) Schematic of the synapses of Ach-type neurons. (B) Compared with wild-type worms (WT), there is no difference in body fat levels in *ace-2 RNAi*, data are relative values ±SEM (n = 30), ***p<0.001. (C) Compared with WT, the body fat levels of the two *cho-1 RNAi* worms are significantly reduced, whereas there is no difference compared with *asic-1* mutant worms, ±SEM (n = 30) ***p<0.001. One-way ANOVA with Tukey's test was used.

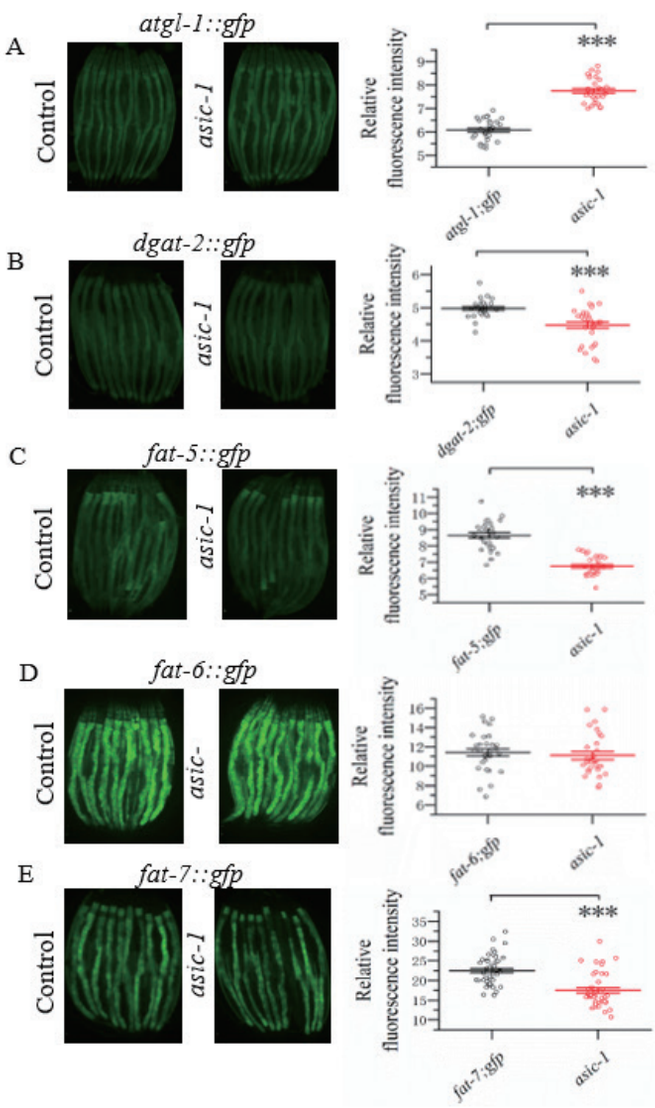
Figure 5. NHX-6-ASIC-1 Signaling Pathway May Regulate Body Fat Levels Through Cholinergic Signaling

3.6 Gut-Neuron Communication Regulates Lipid Metabolism Through NHX-6-ASIC-1-ACh Signaling

To verify whether the *asic-1* deletion mutation disrupts the regulation of worm lipid metabolism by gut-neuron communication utilizing NHX-6-ASIC-1 signaling through cholinergic signaling, the author must consider the worm's lipid metabolism because the intestine is the main organ for lipid synthesis and breakdown in C. elegans [15, 16]. Key genes involved in C. elegans lipid metabolism include: *atgl-1* (adipose triglyceride lipase) and *cpt-2* (carnitine palmitoyl transferase II) involved in breakdown metabolism, and *acly-1* (ATP-citrate lyase), *dgat-2* (diacylglycerol acyltransferase

2), *elo-2* (fatty acid elongase 2), *fasn-1* (fatty acid synthase), *fat-5/6/7* (stearoyl-CoA desaturase), and *pod-2* (acetyl-CoA carboxylase) involved in lipid synthesis [34, 35].

Utilizing strains of C. elegans expressing these proteins fused with GFP, including ATGL-1::GFP: [*Patgl-1::atgl-1::GFP, mec-7::RFP*], DGAT-2::GFP: *unc-119(ed3);kunIs148*[*Pdgat-2::dgat-2::GFP*]}, FAT-5::GFP: *unc-119(ed3);kunIs161*[*fat-5p::fat-5::gfp*], FAT-6::GFP: *lin-15(n765)*;*waEx16*[*fat-6WG::GFP*] and FAT-7::GFP: *lin-15(n765)*;*waEx15*[*fat-7 WG::GFP*], the author constructed *asic-1* deletion mutants and their heterozygotes through hybridization. Through fluorescent imaging technology, the author found that in the *asic-1* deletion mutant, the expression level of ATGL-1 is significantly upregulated. Conversely, the expression of DGAT-2, FAT-5, and FAT-7 significantly decreased (Figure 6). *atgl-1* is a rate-limiting enzyme for triglyceride hydrolysis, which can hydrolyze lipids into free fatty acids, serving as a source of energy metabolism. *dgat-2* is a key enzyme for synthesizing triacylglycerol, which plays an important role in lipid droplet formation. *fat-5/6/7* are three D9 desaturases, which are important for producing monounsaturated fatty acids [36]. Therefore, ASIC-1 can simultaneously inhibit lipid breakdown metabolism and promote lipid synthesis metabolism, thereby increasing body fat levels. Since previous experimental results have already shown that other mutations in the NHX-6-ASIC-1-ACh signaling pathway, including *nhx-6* and *cho-1*, have similar effects on body fat levels. These results initially indicate that gut-neuron communication regulates lipid metabolism through the NHX-6-ASIC-1-ACh signaling pathway.



Note: Lipid metabolism-related genes *atgl-1*, *dgat-2*, *fat-5, fat-6*, and *fat-7* can characterize the expression in control and *asic-1* deletion mutant worms, and the effect of *asic-1* on these five lipid metabolism-related genes can be explored through imaging experiments. Adult worms in the third day period were photographed microscopically, with 30-35 worms photographed in each experiment per group. Scale ratio: 100μm. *P<0.05, **P<0.01, ***P<0.001. Analyzed by one-way ANOVA Tukey's test.

Figure 6. Imaging experiment of five lipid synthesis metabolism gene marker C. elegans

4. Discussion

In this study, the author has initially discovered the influence on C. elegans body fat level through gut-cholinergic motoneuron communication at the whole-animal level by comprehensively using Oil Red O staining, molecular genetics, calcium imaging, in vivo fluorescence imaging, and worms behavior analysis. At the cellular and neural circuit levels, the current results suggest that the Na^+-H^+ exchanger expressed in intestinal epithelial cells releases H^+, which regulates the release of acetylcholine neurotransmitter through the acid-sensitive ion channel expressed in cholinergic motoneurons. Since the intestine is the main site of lipid metabolism in C. elegans [34, 35], the NHX-6-ASIC-1-ACh signal transmission will act on the intestine, affecting lipid synthesis and decomposition metabolism, and ultimately affecting the body fat level of the C. elegans. At the molecular level, the author has identified key proteins in the gut-cholinergic motoneuron communication, including the Na^+-H^+ exchanger NHX-6 expressed in intestinal epithelial cells, the acid-sensitive ion channel protein ASIC-1 expressed in cholinergic motoneurons, and choline transporter CHO-1 that affects cholinergic signals. C. elegans can use the Na^+-H^+ exchanger NHX-6 expressed in intestinal epithelial cells to release H^+ to activate ASIC-1 to regulate host lipid metabolism through intestinal-neuron communication. NHX-6-ASIC-1-ACh signal transmission pathway regulates lipid metabolism.

By constructing specific promoter *Pdel-1* to RNA interfere (RNAi) different genes *ace-2* and *cho-1* in cholinergic motoneurons, the author initially demonstrated that the NHX-6-ASIC-1 signaling pathway may regulate body fat levels through cholinergic signaling, and identified the choline transporter CHO-1 that affects cholinergic signaling. However, whether CHO-1 works in the same pathway as ASIC-1 and whether it acts in conjunction with *ace-1, 3* still needs further verification. In addition, the downstream mechanism of intestinal regulation of lipid metabolism through cholinergic signaling remains to be further explored. Meanwhile, the mechanism of gut-cholinergic motoneuron pathway regulating the lifespan of C. elegans needs further study.

In summary, this study has discovered that intestinal epithelial cells can use the Na^+-H^+ exchanger NHX-6 to release H^+ as an intercellular transmitter, activate the H^+ receptor channel composed of ASIC-1, enhance the activity of cholinergic motoneurons, and regulate lipid metabolism in C. elegans (Figure 7). This research reveals the circuit mechanism of gut-cholinergic motoneuron communication regulating animal metabolism at the single-cell level. This suggests that the NHX-6-H^+-ASIC-1signal transmission in C. elegans may represent a conserved molecular mechanism of intestinal-neuron communication.

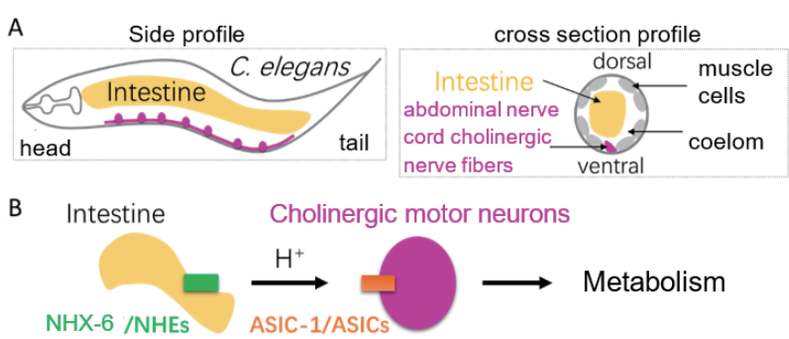


Figure 7. Pattern of gut-cholinergic motoneuron communication regulating lipid metabolism

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