Neuroscience

Pharyngeal Mechanosensory Neurons Control Food Swallow in *Drosophila melanogaster*

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Abstract

As the early step of food ingestion, the swallow is under rigorous sensorimotor control. Nevertheless, the mechanisms underlying swallow control at a molecular and circuitry level remain largely unknown. Here, we find that mutation of the mechanotransduction channel genes *nompC*, *Tmc*, or *piezo* impairs the regular pumping rhythm of the cibarium during feeding of the fruit fly *Drosophila melanogaster*. A group of multi-dendritic mechanosensory neurons, which co-express the three channels, wrap the cibarium and are crucial for coordinating the filling and emptying of the cibarium. Inhibition of them causes difficulty in food emptying in the cibarium, while their activation leads to difficulty in cibarium filling. Synaptic and functional connections are detected between the pharyngeal mechanosensory neurons and the motor circuit that controls swallow. This study elucidates the role of mechanosensation in swallow, and provides insights for a better understanding of the neural basis of food swallow.

eLife assessment

This **valuable** study investigates the role of mechanosensory feedback during swallowing in adult Drosophila. The authors provide **convincing** evidence that three mechanotransduction channel genes are required for ingestion rhythms and localize the role of these genes to a specific subpopulation of pharyngeal mechanosensory neurons. However, there is **incomplete** evidence to support the conclusions that these sensory neurons are necessary for swallowing, respond to stretch during swallowing, and connect to the motor neurons that control swallowing. This work may be of interest to neuroscientists interested in motor control of feeding behavior.

Introduction

Pharyngeal sensory organs serve as the final checkpoint before food ingestion, monitoring the chemical and physical properties of food[11–14]. Real-time sensory feedback generated during swallow peristalsis is vital to the process of expelling food bolus from the mouth to esophagus[4–7]. The pharyngeal sensation can also impact the internal state and appetite of animals, while

feeding status can in turn affect swallowing behavior[8,9]. In humans, tactile and pressure receptors on the tongue and palate receive sensory inputs from distributed areas (e.g., bolus texture, shape, and size) and relay information to the brain[20,21]. Chewing and ingesting tough or viscous foods can cause increased "oro-exposure time" and reduced food intake as a result[22,23].

The pharynx contains a plethora of gustatory and mechanosensory neurons, and their functions are being gradually elucidated[10–15]. Physical properties of the food offer important information regarding its texture and influence animals' inclination to eat[16,41]. Although it has been extensively studied how the food texture is sensed by peripheral sensory organs in both *Drosophila* and other animals[17–19], how the internal sensory neurons monitor the physical quality of food to regulate ingestion remains elusive.

Feeding behaviors such as chewing and sucking require rhythmic and coordinated contraction of muscle groups, and are thought to be controlled by central pattern generators (CPGs). CPGs have been proposed to control many complex motor behaviors in different systems. They are defined as a neural assembly by their intrinsic oscillation and independence from sensory input[1,2]. In mammals, the muscles that coordinate food swallow are controlled by the CPGs in the brain stem[2,29]. However, the identity of CPGs and their downstream circuits remain poorly understood.

In *Drosophila*, the swallow is driven by food pumping induced by the suction and compression of the cibarium[3]. Pump frequency is independent of the concentration of sucrose or feeding state, but is largely regulated by food viscosity[4]. Silencing of certain groups of motor neurons could disrupt pumping and ingestion, while activation could elicit arrhythmic pumping[4]. Rhythmic fluid ingestion has also been studied in other insects[25], but the molecular and neural basis controlling ingestion is still undiscovered.

Here we identify a group of multi-dendritic mechanosensory neurons in the fly's cibarium (md-C neurons) which are essential for swallow control. Inhibition of these neurons leads to difficulty in cibarium emptying and lower ingestion efficiency, while activation of them causes higher pump frequency and sometimes difficulty in cibarium filling. md-C neurons interact with the motor neurons in the brain to control swallowing. Our work provides insights into the regulation mechanism of swallow.

Results

Mechanotransduction channel genes are required for swallowing behavior

Flies exhibit a rhythmic swallow pattern when ingesting food[3,4] (**Figure 1A**). The whole action is composed of two steps: food is sucked into the cibarium (filling) and then expelled into the foregut (emptying). The frequency is not influenced by the taste but the viscosity of the food[4], suggesting that the mechanical force exerted by food bolus passing the cibarium is essential in maintaining the swallow rhythm. We thus tested the swallowing behavior of the mutant alleles of three mechanotransduction channel genes (*nompC*, *piezo*, *Tmc*), and found that these flies exhibited a lower pump frequency compared to control groups. Disruption of the mechanoreceptor function had a profound impact on the emptying phase of swallowing (**Figure 1B-1D**, video 1), with filling and emptying taking longer and the filling/emptying time ratio decreasing significantly (**Figure 1— figure supplement 1A-C**). About 1/3 of *nompC* mutants exhibited difficulty in cibarium emptying (emptying time > 0.3s), indicating the essential role of mechanotransduction channel genes in swallowing behavior. However, the *nompC* mutant flies

did not show a significant impairment in feeding efficiency, likely due to an increased volume of each pump (**Figure 1C**). These findings suggest that mechanosensation mainly contributes to the emptying phase of swallowing.

We investigated the involvement of mechanoreceptors in regulating swallow frequency in response to food viscosity. We fed flies with a water solution containing varying concentrations of methylcellulose (MC) to increase viscosity and found that flies with mutated *Tmc* or *piezo* genes consistently exhibited incomplete emptying when fed with water solutions containing 1% MC or higher concentrations, while only about 20% nompC mutants and wild-type flies sporadically exhibited incomplete filling when fed with water solution of the same viscosity (**Figure 1—figure supplement 1E-H**, video 4). These findings suggest that all three mechanoreceptors are necessary for sensing the swallowing process and providing feedback to the downstream motor circuit to regulate pump strength, while for foods with certain viscosity, *Tmc* or *piezo* mutants pump might be unable to support complete cibarium emptying. We propose that *nompC* plays a role in initiation, while *Tmc* and *piezo* control the driving strength of swallowing.

Moreover, double mutation of *Tmc* and *nompC* led to a more severe emptying difficulty, with pump frequency and ingestion rate both decreasing significantly (**Figure 1E**) and food tends to stay in the pharyngeal area for a longer time (Video 1). As a result, a higher pump volume was detected, although the overall intake efficiency was decreased due to the incomplete swallow process (**Figure 1E**). In contrast, the double mutant of *Tmc* and *piezo* showed a pump frequency of about 5Hz, similar to flies of the mutant of either gene (**Figure 1**—figure supplement 1D).

Based on our findings that *Tmc*, *nompC* and *piezo* are important for swallowing, we reasoned that neurons co-expressing these genes may play an essential role in swallow control. So, we explored the expression pattern of each gene, or the intersection between each two of the three genes in the brain and in the cibarium, and identified a set of multi-dendritic sensory neurons in the cibarium (**Figure 2—figure supplement 1**, **Figure 2F**). In the brain, SEZ was commonly labelled by either driver, while *Tmc-GAL4* and *nompC-QF* intersection shows a clear projection pattern (**Figure 2F**). And by expressing nuclear-localized RFP, RedStinger, we revealed that somata of these multi-dendritic neurons situated in the cibarium but not in the brain (**Figure 2G**). These multi-dendritic neurons in the cibarium may sense the swallowing process, and project to SEZ to interact with the neural circuits that control ingestion.

md-C neurons are essential for swallow control

To explore the role of these pharyngeal multi-dendritic neurons, we expressed TNT (tetanus toxin)[38] in *Tmc* positive neurons to block the synaptic transmission, and found that flies' swallowing became arrhythmic and the pump frequency decreased significantly, with about 48.1% (n=27) flies displayed difficulty in cibarium emptying (Video 2; **Figure 2A, 2D**).

Tmc was also reported to participate in food texture sensation and proprioceptive control[19,35–37], to restrict the manipulation to a more specific cell population, we used the FLP-out system to express Kir2.1 in neurons expressing both TMC and NOMPC. The intersection exclusively labels two pairs of neurons around the cibarium (**Figure 2F**) and they were named as md-C (cibarium multi-dendritic) neurons for brevity. We found that flies with md-C neurons inhibited displayed severe dysphagia (difficulty in swallowing), during which flies could hardly ingest water from the cibarium into the foregut. In some cases, water in the cibarium seemed to be pumped along the esophagus. However, no water was visible in the flies' crop, indicating that water in the cibarium was not successfully emptied (Video 2). As a result, flies' intake volume decreased dramatically (**Figure 2C**), indicating that md-C neurons are necessary for rhythmic pump and food ingestion.

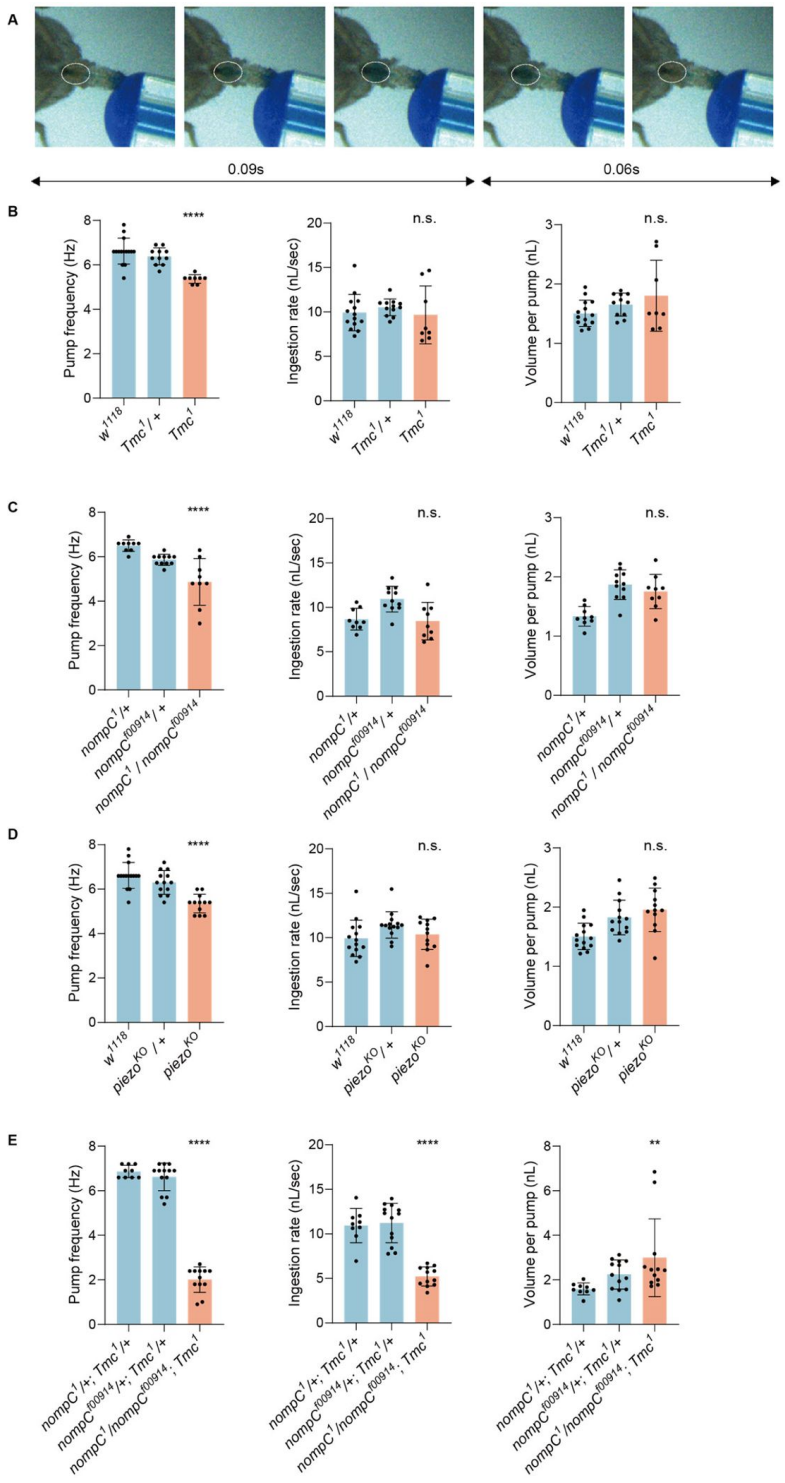

Figure 1.

Mechanoreceptor genes are essential for swallow control of *Drosophila*. (A) Swallow patterns of liquid food. Filling and emptying process constitutes a cycle. (B-D) Swallowing behavior of *Tmc*, *nompC* and *piezo* mutant flies. Pump frequency represents the swallowing speed, ingestion rate indicates the efficiency of food intake, while volume per pump shows how much a fly ingests with one pump. N = 8 to 15 in each group. (E) Double mutans of *Tmc* and *nompC* show more severe dysphagia. n = 9 to 13 in each group. In all analyses, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used, and statistical differences were represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data were represented as means ± SEM.

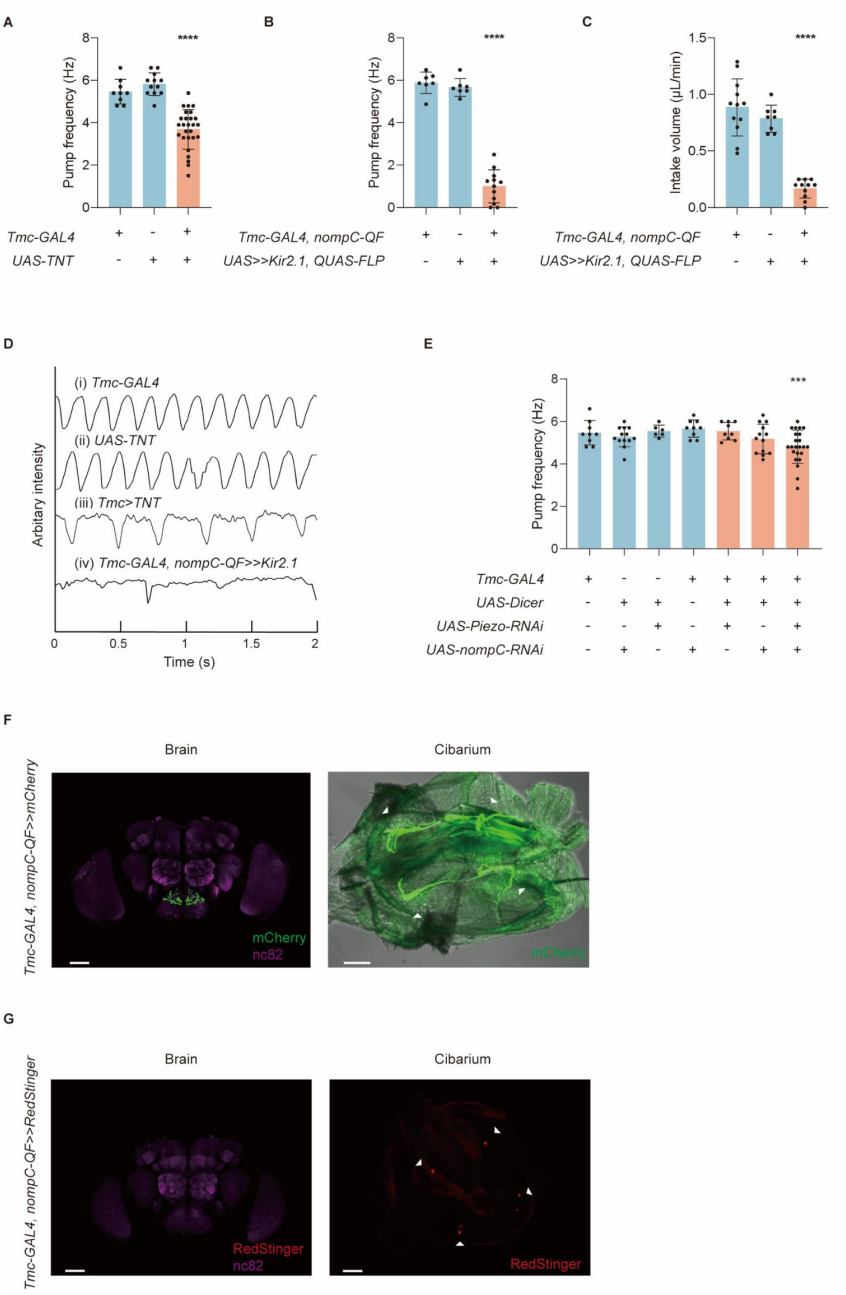

Figure 2.

Mechanosensory neurons and mechanoreceptors are essential to swallow. (A) Blocking synaptic transduction of *Tmc* positive neurons leads to a lower pump frequency, and the flies display difficulty in cibarium emptying (emptying time >0.3s). n = 9 to 27 in each group. (B-C) Inhibiting *Tmc-GAL4* and *nompC-QF* double-labeled neurons by Kir2.1 results in a lower pump frequency and intake volume per minute. n = 7 to 12 in each group. (D) Arbitrary intensity of cibarium when flies of different genotype swallow. The ordinate value was graphed using the opposite value of the original arbitrary intensity in the cibarium, so the declining line represents emptying process (the same for **figure 3B**). (E) Pump frequency after knocking down the expression level of the mechanoreceptor genes. n = 6 to 23 in each group. (F) Projection pattern of md-C neurons in the brain and cibarium. About two pairs of multi-dendritic neurons are situated along the pharynx. Genotype: *Tmc-GAL4; UAS-FRT-mCherry / nompC-QF; QUAS-FLP*. Scale bar = 50 µm. (G) Somata of md-C neurons situated in the cibarium but not in the cibarium. Genotype: $X^{UAS\text{- }RedStinger}$; *Tmc-GAL4, UAS-FRT-GAL80-STOP-FRT/ +; nompC-QF, QUAS-FLP/+*. Scale bar = 50 µm. In all analyses, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used, and statistical differences were represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data were represented as means ± SEM.

We performed a double RNAi experiment for *nompC* and *Piezo* using *Tmc-GAL4* as a driver as the double mutant of these genes is lethal. Knocking down the expression level of *nompC* and *Piezo* resulted in a significantly lower pump frequency, similar to the frequency observed in flies with knockdown of either *nompC* or *Piezo* (**Figure 2E**). These results suggest that the channels may function complementarily.

While inhibiting md-C neurons caused difficulty in cibarium emptying, activating them optogenetically resulted in difficulty in cibarium filling. We activated md-C neurons with CsChrimson[39] or ReaChR[40]. In some cases, no dye could be seen in the cibarium although flies strongly extended their proboscis against water (**Figure 3A**, video 3). This phenomenon could last for seconds but after several trials, flies can usually recover. In other cases, flies with md-C neurons activated show incomplete filling, indicated by decreased cibarium expansion, which was usually not observed in control groups (**Figure 3B**). Additionally, when flies consistently pump at a normal range (observed dye boundary in the cibarium is wider than 90% width of mouthpart), their pump frequency increased significantly (**Figure 3C**). Flies with md-C neurons activated showed a reduced volume per pump (**Figure 3C**), consistent with the small range of pump caused by incomplete filling. We thus speculated that the activation of md-C neurons could accelerate the emptying process but inhibit the filling process, which is opposite to their inhibition.

md-L neurons in the labellum[19] could also be labelled by the intersection of *Tmc-GAL4* and *nompC-QF* (**Figure 3—figure supplement 1A**). To test whether the activation of md-C neurons alone was sufficient for the inhibition of cibarium filling, we cut the flies' labellum to ablate md-L neurons. We found that 36 hours after labellum ablation, the signals of md-L neurons could no longer be observed in the mouth (**Figure 3—figure supplement 1A**), indicating that the axons of md-L likely decomposed after 36 hours, rendering them ineffective in influencing swallowing. Besides, we found that after labellum ablation for 36 hours, light-stimulation of md-C neurons still triggered filling difficulty despite the absence of md-L neurons, while these flies could pump at a normal range in the dark (**Figure 3—figure supplement 1B-C**, video 5). We thus reasoned that md-C neurons in the cibarium but not md-L neurons were responsible for mechanical sensation during swallowing. We stained the muscles in the cibarium with phalloidin and labeled md-C neurons with GFP and found that both md-C neurons and muscles were in close proximity around cibarium (**Figure 4A**), suggesting that md-C neurons could be activated by muscle stretch during the expansion of cibarium.

Next, we wondered whether md-C neurons were activated by the action of swallowing. By expressing GCaMP6m in md-C neurons and cutting a small window in the head capsule, we could detect the fluorescent change of the md-C neurons' projections in the brain when flies were swallowing sugar food. An increase of fluorescence intensity in the SEZ was observed when flies were ingesting food (**Figure 4J-K**). As food ingestion delivered both gustatory and mechanosensory information, we asked whether md-C neurons were sensitive to taste input. The whole fly head was put into saline and a small window was cut[11]. When sucrose was added to the saline, no signal changes were detected (**Figure 4K**), suggesting that md-C neurons responded to mechanical force but not gustatory cues during food ingestion.

md-C neurons form synapses with motor neurons

So far, we have shown that md-C neurons regulate swallowing probably by sensing the expansion of the cibarium. But how do they coordinate with the downstream neural circuits to control the swallow? Two groups of motor neurons, MN11 and MN12, have been reported to control the muscles that execute the swallow process[4,6]. We thus asked whether md-C neurons relay information of the cibarium volume to the motor neurons to control the swallow. We first tested their potential synaptic connection with GRASP (GFP reconstitution across synaptic partners)[27,28], expressing one part of GFP in md-C neurons and the other part in MN12 or MN11. Signals could be detected in the SEZ where the neural projections of the two group neurons

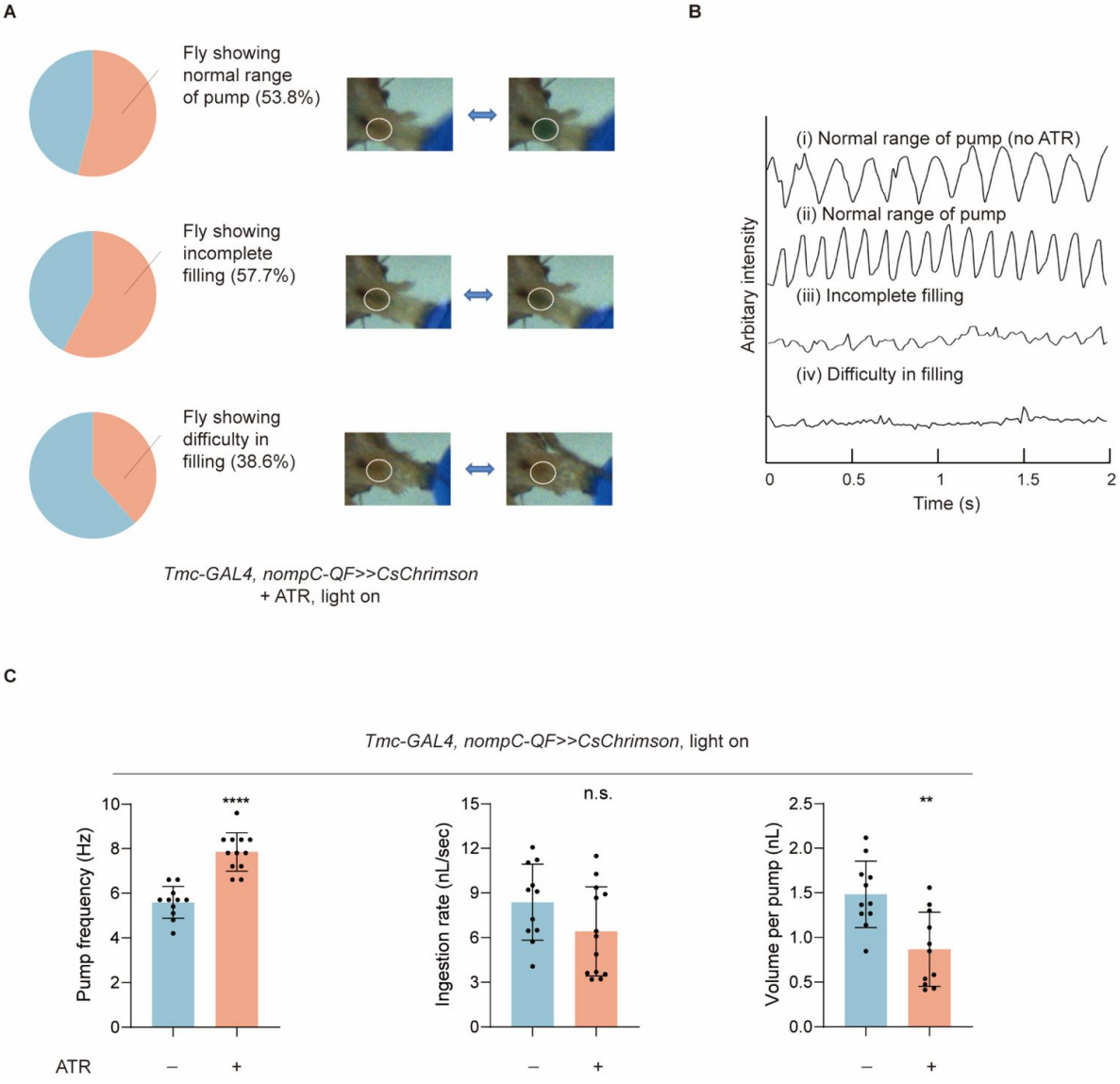

Figure 3

Activation of md-C neurons led to dysphagia. (A) Optogenetic activation of md-C neurons with CsChrimson during food intake could induce accelerated swallowing, incomplete filling (the expansion range of cibarium decreased to about half the maximum) and difficulty in filling. Distinct states driven by CsChrimson light stimulation of md-C neurons were displayed, with the proportions of flies exhibiting each state. n=27. (B) Arbitrary intensity of fly cibarium when md-C neurons were stimulated as (A) or not optogenetically stimulated (no ATR). (C) Swallowing behavior of flies with or without md-C neurons stimulated. n = 11 to 13 in each group. Pump frequency was calculated only when fly consistently pumped at a normal range (observed dye boundary in the cibarium is wider than 90% width of mouthpart). ATR, all trans retinal. In all analyses, two-tailed unpaired t-tests were used, and statistical differences were represented as follows: **P < 0.01, and ****P < 0.0001. Data were represented as means ± SEM.

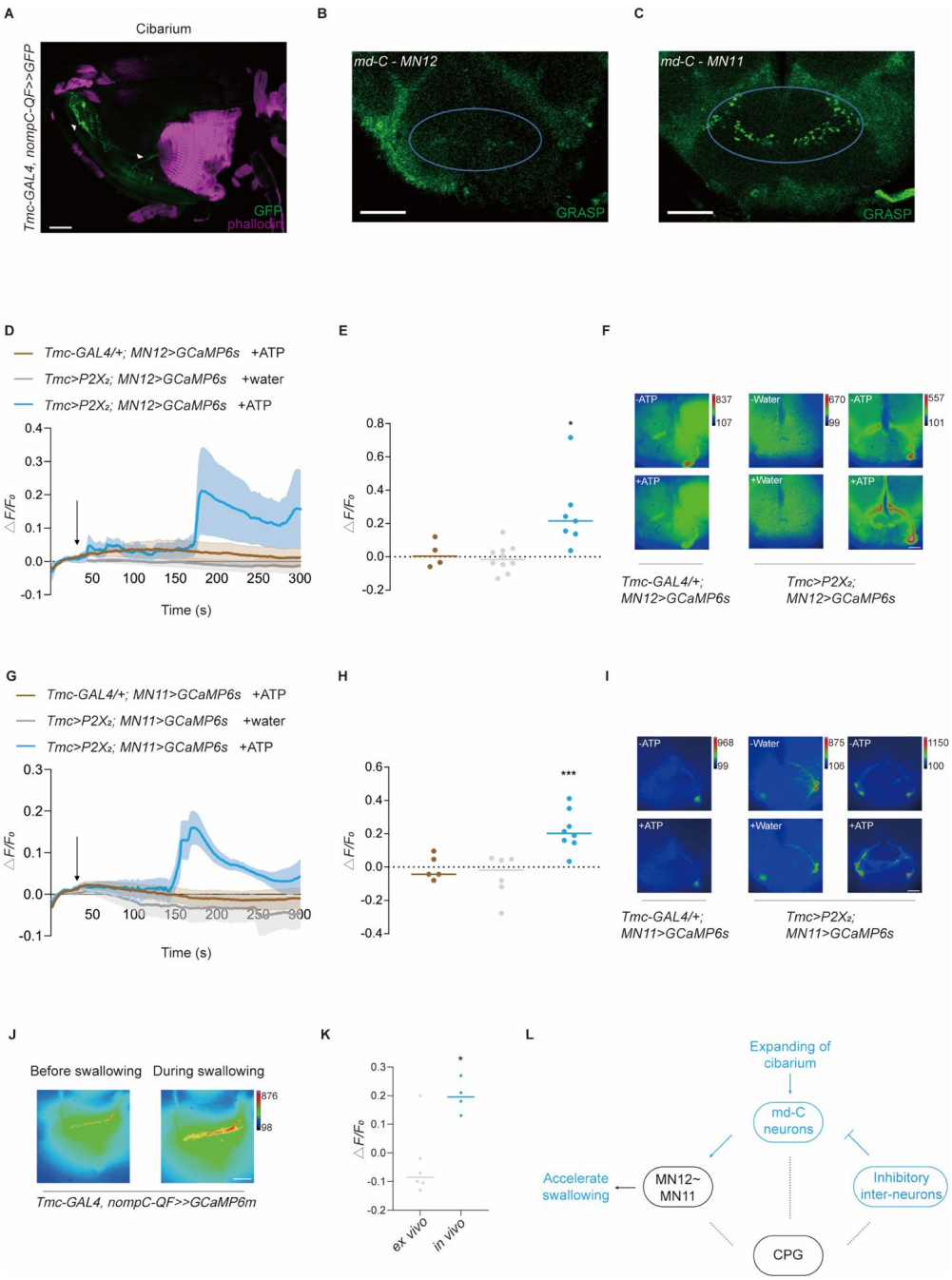

Figure 4

Interaction between motor neurons and md-C neurons revealed working pattern of swallow. (A) md-C neurons labelled by GFP antibody (green) and muscles labelled by phalloidin (magenta) showed that they are in close proximity around cibarium. Genotype: *Tmc-GAL4/UAS-FRT-mCD8-GFP; nompC-QF/QUAS-FLP*. Scale bar = 50 μm. (B-C) GRASP signals between md-C and MNs could be observed in the SEZ area. Genotype: *Tmc-GAL4, UAS-FRT-GAL80-STOP-FRT/UAS-nSyb-spGFP1-10, lexAop-CD4-spGFP11; nompC-QF, QUAS-FLP/MN-LexA*. Scale bar = 50 μm. (D-F and G-I) Activation of *Tmc*$^{+}$ neurons via $P2X_2$ increased motor neuron activity. Fluorescence changes ($\triangle F/F_0$) of GCaMP6s in motor neurons indicates calcium level changes. Water or ATP solution was added when it came to 30 seconds after record begins, as black arrow indicates. n = 4 to 10, $^{*}p < 0.05$, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used, error bars indicate mean ± SEM. Scale bar = 50 μm. (J) Significant signal changes could be detected of the md-C neurons' termini at fly's SEZ area after feeding fly with 0.1M sucrose solution when GCaMP6m is expressed in md-C neurons. Scale bar = 50 μm. (K) are traces of fluorescence changes. n = 4 to 6, $^{*}p < 0.05$, two-tailed unpaired t-test was used, error bars indicate mean ± SEM. (L) Working model for md-C-MN-CPG controlling swallow of *Drosophila*.

overlapped (**Figure 4B-C**), suggesting that md-C neurons may directly synapse onto the motor control circuit in the SEZ. By expressing $P2X_2$[30,31], an ATP receptor in *Tmc*$^+$ neurons, we also found that calcium transients could be observed in MN12 and MN11 when activating md-C neurons, while the control group show no significant differences (**Figure 4D-I**). Considering possible synaptic connections between md-C and motor neurons, we suppose activation of md-C triggered by food bolus, might directly stimulate motor neurons to accelerate swallowing. These results suggest that motor neurons associated with swallowing are activated by sensory input in the cibarium to coordinate food ingestion during swallowing.

Discussion

Here we find that a group of mechanosensory neurons in the pharynx respond to the mechanical force generated by the expansion of the cibarium during food ingestion. These neurons may form synaptic and functional connections with motor neurons in the brain, and constitute an essential part of the neural circuit that controls the swallow.

Multiple channels function in the same neurons

The mutants of either *nompC*, *Tmc,* or *piezo* showed defects in the swallow rhythm. However, we found that *nompC* seemed to play a more critical role as its mutation caused difficulty in emptying, while double mutation of *Tmc* and *piezo* did not. We speculated that *nompC* might be involved in swallowing liquid foods, as we used water in most experiments. While the md-C neurons also expressed both TMC and Piezo, we think these three channels could play different roles depending on physical properties of foods. The information generated by these three channels might be integrated in md-C neurons and helps the flies to distinguish the quality of food and regulate swallow.

Interaction between md-C neurons and the feeding control circuit

It was demonstrated that motor neuron 12 controlled muscle 12 to fill cibarium with food, while motor neuron 11 controlled muscle 11 to expel the foods into the foregut[4]. In accordance with this model, we have found that the activation of md-C neurons could activate MN11, and over-stimulation of md-C neurons could cause difficulty in filling, incomplete filling and/or higher pump frequency. On the other hand, silencing md-C neurons blocks the emptying process because expanding of cibarium fails to activate MN11 which controls emptying, leading to difficulty in swallow. Additionally, when pharyngeal mechanosensation was impaired by the mutation of the mechanotransduction channel genes, MNs may not obtain enough activation to drive muscle 11 contraction to completely empty the foods in cibarium, resulting in a slower pump rhythm, especially when foods are of high viscosity.

However, the circuits that control the swallow could be more complex than the circuit proposed above. It has been reported that the ingestion of foods is completed in a sequential activity of MNs where most parts of the proboscis are engaged[6]. Besides the bottom-up interaction between md-C neurons and MN12/11 demonstrated in this study, a top-down feedback mechanism may also exist. For example, we have found that knocking down $GABA_A$-R in md-C neurons leads to a lower pump frequency (**Figure 4**—figure supplement 1 A), suggesting that md-C neurons receive inhibitory signals during swallowing. Although it is unclear which neurons inhibit md-C neurons, we believe this is likely through a presynaptic mechanism on the axon termini of md-C neurons. Moreover, activating md-C induced calcium transients in MN12 and MN11(**Figure 4D-I**), indicating that motor neurons were stimulated by activation of md-C neurons. It is believed that sequential contraction of muscles would cause fluid bolus move through the proboscis[5], we thus hypothesize that md-C neurons might sequentially stimulate motor neurons during swallowing. Also, it is possible that sequential activation generated by MNs and md-C might crosstalk with

CPGs, which together form a steady pump frequency, depending on the mechanical quality of foods (**Figure 4L**). Another possibility is that the activation of md-C neurons acts as a switch, altering the oscillation pattern of the swallowing central pattern generator (CPG) from a resting state to a task state.

Central pattern generators (CPGs) in the control of swallow rhythm

It's widely accepted that most rhythmic behaviors of animals are controlled by the CPGs in the brain[25, 26]. For example, brain stem CPGs control the patterns of suck, lick, mastication, swallow, etc.[25,29,33,34]. Two lines of evidence supports the notion that mechanical feedback from the cibarium during ingestion regulates the activity of CPGs or their downstream circuits that execute the pattern generated by CPGs. 1, High viscosity of food caused a reduction of pumping frequency while the rhythm remained essentially regular, indicating that the pattern was modulated rather than blocked. 2, Manipulation of md-C neurons could interfere with the highly rhythmic pumping. Although the CPGs that control swallow in *Drosophila* brain are still elusive, our data provides an entry point to elucidate the CPGs circuits and the neural circuits associated with them. As their activity can be modulated by md-C neurons, they can be potentially identified by searching for the downstream of md-C neurons.

Materials and methods

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wei Zhang, wei_zhang@mail.tsinghua.edu.cn.

Materials availability

This study did not generate new unique reagents. All key resources are listed in Key resources table. Further information and requests for resources and reagents should be directed to the lead contact.

Experimental model and subject details

Animals

Fly were acquired from other labs or BDSC (Bloomington *Drosophila* Stock Center). Flies were reared on standard medium at 25°C unless otherwise noted. w^{1118} was used as wild-type control. See KRT for the full list of fly strains.

Generation of transgenic flies

The *Tmc-GAL4*^{DBD}, *Piezo-GAL4*^{AD}, *MN12-lexA*, *MN11-LexA* line was constructed with the HACK system[32] from line *Tmc-GAL4*(BDSC:66557), *Piezo-GAL4*(BDSC:59266), *MN12-GAL4* and *MN11-GAL4*(donated from Kristin Scott lab). Phack plasmid was injected into nos-Cas9 flies with standard embryo injection procedures. Injected flies were crossed with double balancer flies, and the transformants were picked up with w^{+} and RFP^{+} makers. The *Tmc-GAL4,UAS(FRT.stop)CsChrimson-mVenus/CyO*, *nompC-QF,QUAS-FLP/TM6B* and *Tmc-GAL4,UAS-FRT-GAL80-STOP-FRT/CyO* lines were generated by recombination and confirmed by confocal imaging.

Method details

Feeding assay

All flies were kept in a 24-26°C incubator under 12-12 hr light: dark cycle and about 40-60% humidity. Mated female flies were collected at eclosion and aged for 3-10 days. For neuronal silencing experiments, flies bearing Kir2.1 or TNT were kept for 7-10 days before the feeding assay. For rdl knockdown experiment in md-C neurons, 14-16 days-old flies were used. One day prior to the behavioral assays, flies were transferred to empty vials for 24 hours for water deprivation (12h for flies with *nompC* and *Tmc* mutated or flies with Kir2.1 expressed in md-C neurons).

Before experiments, flies were anesthetized with CO_2 and mounted onto a glass slide with nail polish. They were then allowed to recover in a humid chamber for 2 hours. All behavioral experiments were carried out at room temperature and 40%–60% humidity. Individual flies were fed with a pipette filled with water, sugar or methylcellulose water solutions. Consumption of single flies was calculated by measurable glass capillary. Ingestion rate was calculated by consumption divided by ingestion time, feeding lasted for 4-7 seconds except the experiment where Kir2.1 was used lasted for one minute. For illustration, 0.25 ng/mL brilliant blue food dye (Shi-tou, GB 7655.1) was added to the solution. 60 fps camera was adopted to record the feeding behavior and flies with total feeding duration of more than 2 seconds were analyzed.

For optogenetics experiments, female flies expressing CsChrimson or ReaChR were raised on food containing 10 mM all-trans retinal at 25°C and 40-60% humidity in a light-proof vial. Experiments were performed with 7 to 10-day-old mated female flies in a dark room. One day before the test, flies were transferred into empty light-proof vials for water deprivation. Before and during feeding, flies were stimulated by 1.3 mW/cm^2 590 nm light.

Immunostaining and microscopy

The brains and cibaria of 3-10-day-old female flies were dissected in PBST dissection buffer containing 0.015% Triton X-100 in 1x PBS, followed by fixation in 4% PFA solution for 30 minutes on a shaker at room temperature. Samples were then washed four times for 20 minutes each with wash buffer (0.3% Triton in 1x PBS). The tissues were transferred to block buffer (1x heat-inactivated normal goat serum with 0.3% Triton in 1x PBS), and incubated at room temperature for 30 minutes. Primary antibodies were added to the samples and incubated overnight at 4°C. The primary antibodies used included Rabbit-RFP (Rockland 39707, diluted 1:500), Rabbit-GFP (Invitrogen A11122, diluted 1:500), Mouse-nc82 (Hybridoma Band DSHB, Brunchpilot, diluted 1:500), and Mouse-GFP (Sigma-Aldrich G6539, diluted 1:200). Samples were washed four times for 20 minutes each, and then incubated with secondary antibodies on the following day. The secondary antibodies were used at a 1:200 dilution and were all from Invitrogen: Alexa Fluor 555 anti-Rabbit (A-21428), Alexa Fluor 488 anti-Mouse (A11001), and Alexa Fluor 647 anti-Mouse (A-21235). After incubation for 5 hours at room temperature or overnight at 4°C, tissues were washed four times for 20 minutes each. The brains and cibaria were attached to a slide for imaging. Confocal imaging was performed using an Olympus FV1000 microscope with a 20X air lens.

Functional imaging

Calcium imaging was carried out with an Olympus BX51WI microscope with 40X water immersion objective, Andor Zyla camera and Uniblitz shutter. To perform calcium imaging in md-C neurons during swallowing, flies were food-deprived for 12 hours and heads were fixed in the place using nail polish, the antennae and the cuticle above the SEZ were carefully removed and the exposed brain was bathed in AHL buffer. Brains were dissected in a recording chamber containing saline (NaCl 140 mM, KCl 2 mM, $MgCl_2$ 4.5 mM, $CaCl_2$ 1.5 mM, HEPES-NaOH 5 mM, PH 7.1), and 1.5 mM

$CaCl_2$ was added to the saline before use. ROIs and fluorescence changes were selected with ImageJ as peak $\Delta F/F_0 = (F_{peak} - F_0)/F_0$, where F_0 was the average fluorescence of 10 images when expressing GCaMP6m in md-C neurons before manipulation. Ex vivo control was performed as previously described[11].

While for GCaMP6s expressed in MNs, we immerse the fly in the imaging buffer. Following dissection and identification of the subesophageal zone (SEZ) area under fluorescent microscopy, we introduce 20mL water or 20mL water solution of 250mM ATP slowly into the liquid level, positioned at a distance from the brain, to minimize any potential interference with the procedure. Importantly, we did not administer ATP directly to the living fly. F_0 was the average fluorescence of 30 images in MNs before adding water or ATP to dishes containing 2mL AHL buffer.

Quantification and statistical analysis

Statistical analysis was performed with the GraphPad Prism 8 software. All error bars represent ± SEM. Two tailed unpaired t-test and two-tailed Mann-Whitney nonparametric test were used to evaluate the significance between two datasets. For all analyses, statistical notations are as follows: *, $p < 0.05$. **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$. Number of dots per bar indicates the number of tested flies (N) in each experiment. No sample size estimation and inclusion and exclusion of any data or subjects were conducted in this study.

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Author contributions

J.Q., T.Y., K.L. and T.L. performed the experiments. J.Q., T.Y. analyzed the data. W.Z. wrote the manuscript. All authors discussed and commented on the manuscript.

Declaration of interests

The authors declare no competing interests.

Appendix 1

Appendix 1—Key resources table

genetic reagent (*D. melanogaster*)	LexAop-GCaMP6s	Bloomington Drosophila Stock Center	BDSC:64413	
genetic reagent (*D. melanogaster*)	*Tmc*^{1}	Bloomington Drosophila Stock Center	BDSC:66556	
genetic reagent (*D. melanogaster*)	w[*]; P{w[+Mc] = UAS-nSyb-spGFP1-10}2, P{w[+Mc] = lexAop-CD4-spGFP11}2/CyO	Bloomington Drosophila Stock Center	BDSC:64314	
genetic reagent (*D. melanogaster*)	UAS-RedStinger	Bloomington Drosophila Stock Center	BDSC:8547	
genetic reagent (*D. melanogaster*)	Piezo-GAL4	Bloomington Drosophila Stock Center	BDSC:59266	
genetic reagent (*D. melanogaster*)	UAS(FRT.mCherry)ReaChR	Bloomington Drosophila Stock Center	BDSC:53743	
genetic reagent (*D. melanogaster*)	UAS-Piezo-RNAi	Vienna Drosophila RNAi Center	VDRC:105132	

genetic reagent (*D. melanogaster*)	UAS-FRT-STOP-FRT-GCaMP6m	other		From Laboratory of Chuan Zhou, Institute of Zoology, Chinese Academy of Sciences
genetic reagent (*D. melanogaster*)	nompC-QF	Yang et al., 2021		From Laboratory of Yuh Nung Jan, UCSF
genetic reagent (*D. melanogaster*)	$nompC^{1}$/CyO	Yang et al., 2021		From Laboratory of Yuh Nung Jan, UCSF
genetic reagent (*D. melanogaster*)	$nompC^{f00914}$/CyO	Yang et al., 2021		From Laboratory of Yuh Nung Jan, UCSF
genetic reagent (*D. melanogaster*)	$piezo^{KO}$	Zhang et al., 2013		From Laboratory of Yuh Nung Jan, UCSF
genetic reagent (*D. melanogaster*)	UAS-nompC-RNAi; UAS-Dicer2	Yang et al., 2021		From Laboratory of Yuh Nung Jan, UCSF
genetic reagent (*D. melanogaster*)	UAS-rdl-RNAi	Yang et al., 2021		From Laboratory of Xin Liang, THU

genetic reagent (*D. melanogaster*)	UAS-P2X$_2$	Yang et al., 2021		From Laboratory of Yufeng Pan, School of Life Science and Technology, Southeast University
genetic reagent (*D. melanogaster*)	UAS(FRT.stop) CsChrimson-mVenus	Wu et al., 2019		From Laboratory of Yufeng Pan, School of Life Science and Technology, Southeast University
genetic reagent (*D. melanogaster*)	MN12-GAL4	Manzo et al., 2012		From Laboratory of Kristin Scott, UCB
genetic reagent (*D. melanogaster*)	MN11-GAL4	Manzo et al., 2012		From Laboratory of Kristin Scott, UCB
genetic reagent (*D. melanogaster*)	MN12-LexA	This paper		Hacked from MN12-GAL4
genetic reagent (*D. melanogaster*)	MN11-LexA	This paper		Hacked from MN11-GAL4
genetic reagent (*D. melan*	Tmc-GAL4, UAS(FRT.stop) CsChrimson-mVenus/CyO	This paper		Recombined from BDSC:66557 and UAS(FRT.stop

ogaster)				)CsChrimson-mVenus
genetic reagent (*D. melanogaster*)	nompC-QF, QUAS-Flp/TM6B	This paper		Recombined from BDSC:30127 and nompC-QF
genetic reagent (*D. melanogaster*)	Tmc-GAL4, UAS-FRT-GAL80-STOP-FRT/CyO	This paper		Recombined from BDSC:66557& 38880
genetic reagent (*D. melanogaster*)	Piezo-GAL4^{AD}	This paper		Hacked from BDSC:59266
genetic reagent (*D. melanogaster*)	Tmc-GAL4^{DBD}	This paper		Hacked from BDSC:66557
antibody	Goat anti-Rabbit Alexa 488	Invitrogen	Cat#A11008; RRID: AB_143165	1:200
antibody	Goat anti-Rabbit Alexa 555	Invitrogen	Cat#A21428; RRID: AB_2535849	1:200
antibody	Goat anti-Mouse Alexa 488	Invitrogen	Cat#A11001; RRID: AB_2534069	1:200

antibody	Goat anti-Mouse Alexa 647	Invitrogen	Cat#A21235; RRID: AB_2535804	1:200
antibody	Mouse monoclonal anti-Brp	Developmental Studies Hybridoma Bank	Cat# nc82; RRID: AB_2314866	1:500
antibody	Rabbit polyclonal anti-GFP	Invitrogen	Cat#A11122; RRID: AB_221569	1:500
antibody	Rabbit polyclonal anti-RFP	Rockland	Cat#600-401-379S; RRID: AB_11182807	1:500
antibody	Mouse polyclonal anti-GFP	Sigma-Aldrich	Cat#G6539; RRID: AB_259941	1:200
chemical compound, drug	4% PFA	Dingguo Biotech	Cat#AR-0211;CAS:30525-89-4	
chemical compound, drug	Brilliant Blue	Shi-tou	Cat#GB 7655.1	
chemical compound, drug	Methylcellulose	Sigma-Aldrich	M7140-100G	

chemical compound, drug	All trans retinal	Sigma-Aldrich	R2500-500MG	
chemical compound, drug	Phalloidin	AAT bioquest	Cat#23127	
chemical compound, drug	Adenosine-5	aladdin	Lot#I2118087	
software, algorithm	ImageJ and Fiji	NIH; Schindelin et al., 2012	https://imagej.nih.gov/ij/ http://fiji.sc/	
software, algorithm	GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/scientific-software/prism/	
software, algorithm	Adobe Illustrator	Adobe Systems	https://www.adobe.com/products/illustrator.html	

Video 1. Feeding behavior of wild-type flies and mechanoreceptor mutant flies.

Video 2. Flies with md-C neurons inhibited displayed difficulty in cibarium emptying.

Video 3. Optogenetic activation of md-C neurons caused accelerated swallowing, incomplete cibarium filling and difficulty in filling.

Video 4. *Tmc*[1] and *piezo*^{KO} flies displayed incomplete emptying when fed with 1% MC water.

Video 5. Optogenetic activation of md-C neurons could still trigger dysphagia in flies without labellum.

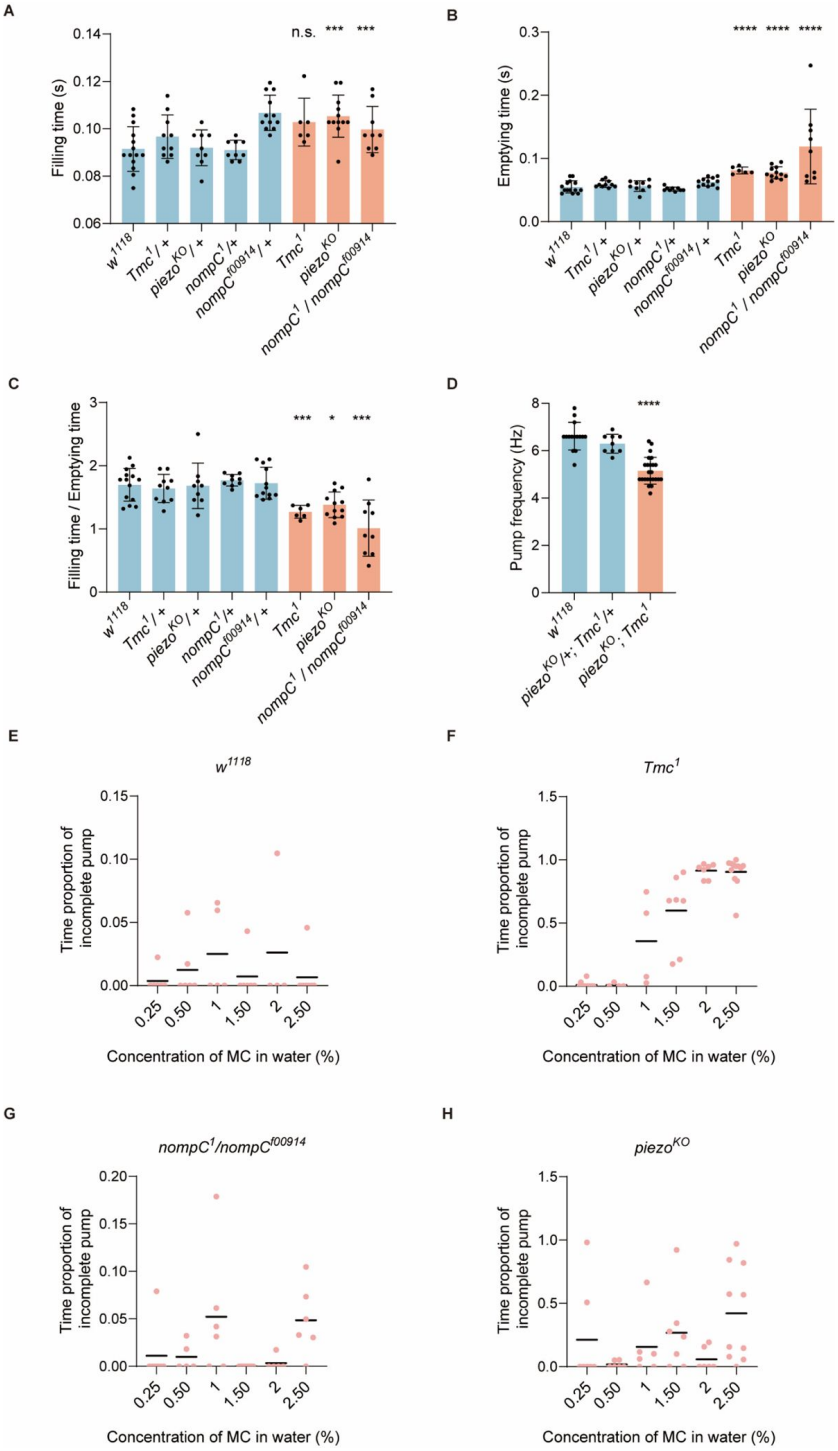

Figure 1—figure supplement 1

Mutation of mechanoreceptors leads to a lower pump frequency caused by longer emptying time. (A-B) Filling time and emptying of the cibarium in flies of different genotypes. n = 6 to 14. (C) Ratio of filling time dividing emptying time indicates the proportion of two processes in one cycle. n = 6 to 14. (D) *Tmc* and *piezo* double mutation showed a similar phenotype with single mutation for either gene in swallowing behavior. n = 9 to 32. (E-H) Proportion of time with incomplete emptying during swallowing when flies were fed with methylcellulose solution. n = 4 to 11. Data were represented as means. In all analyses, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons except nonparametric test in D was used, and statistical differences were represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data were represented as means ± SEM

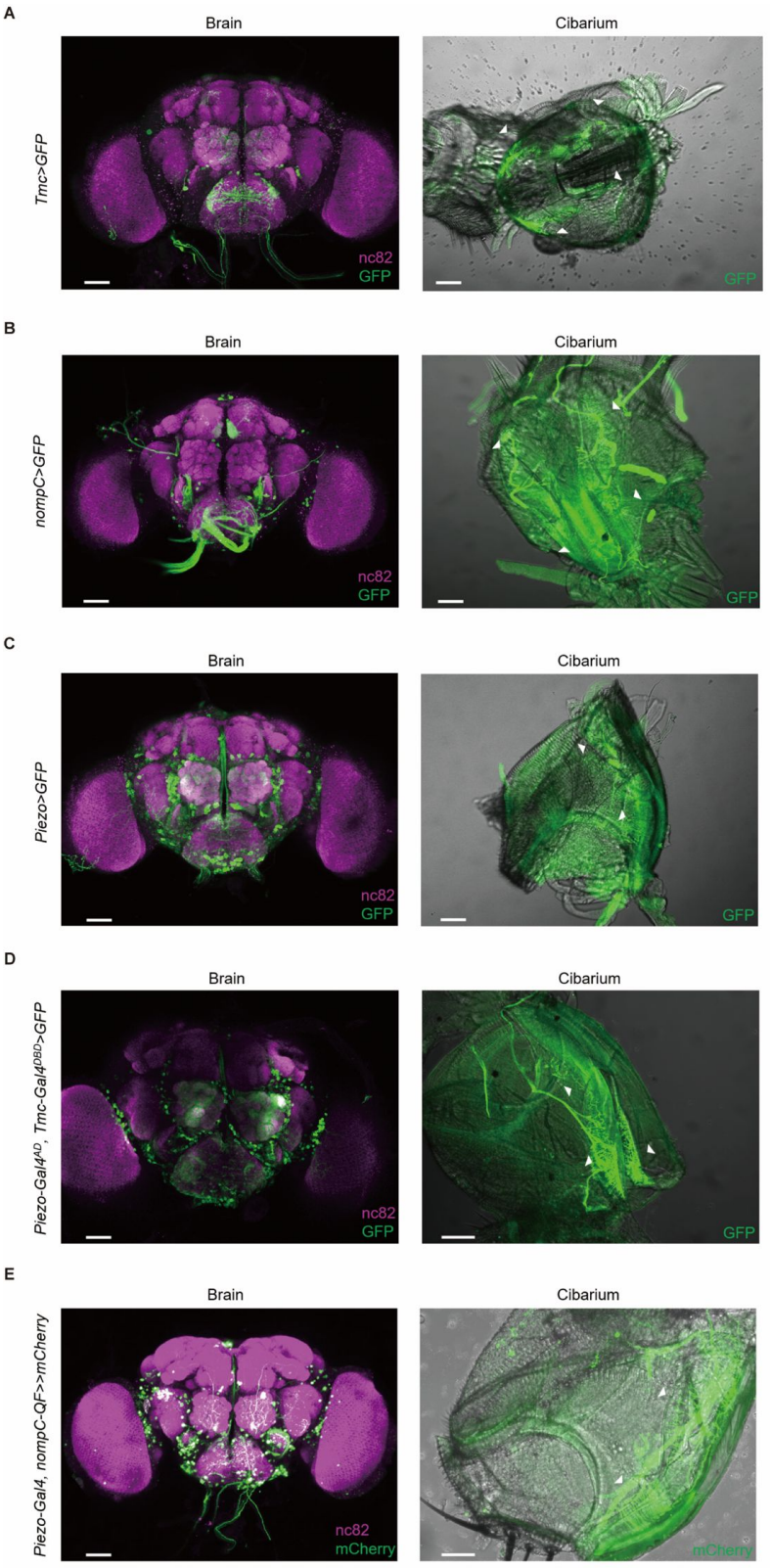

Figure 2—figure supplement 1

Expression pattern of *Tmc*, *nompC*, *piezo*, *Tmc* & *piezo* and *nompC* & *piezo* in the brain and cibarium. Scale bar= 50 μm.

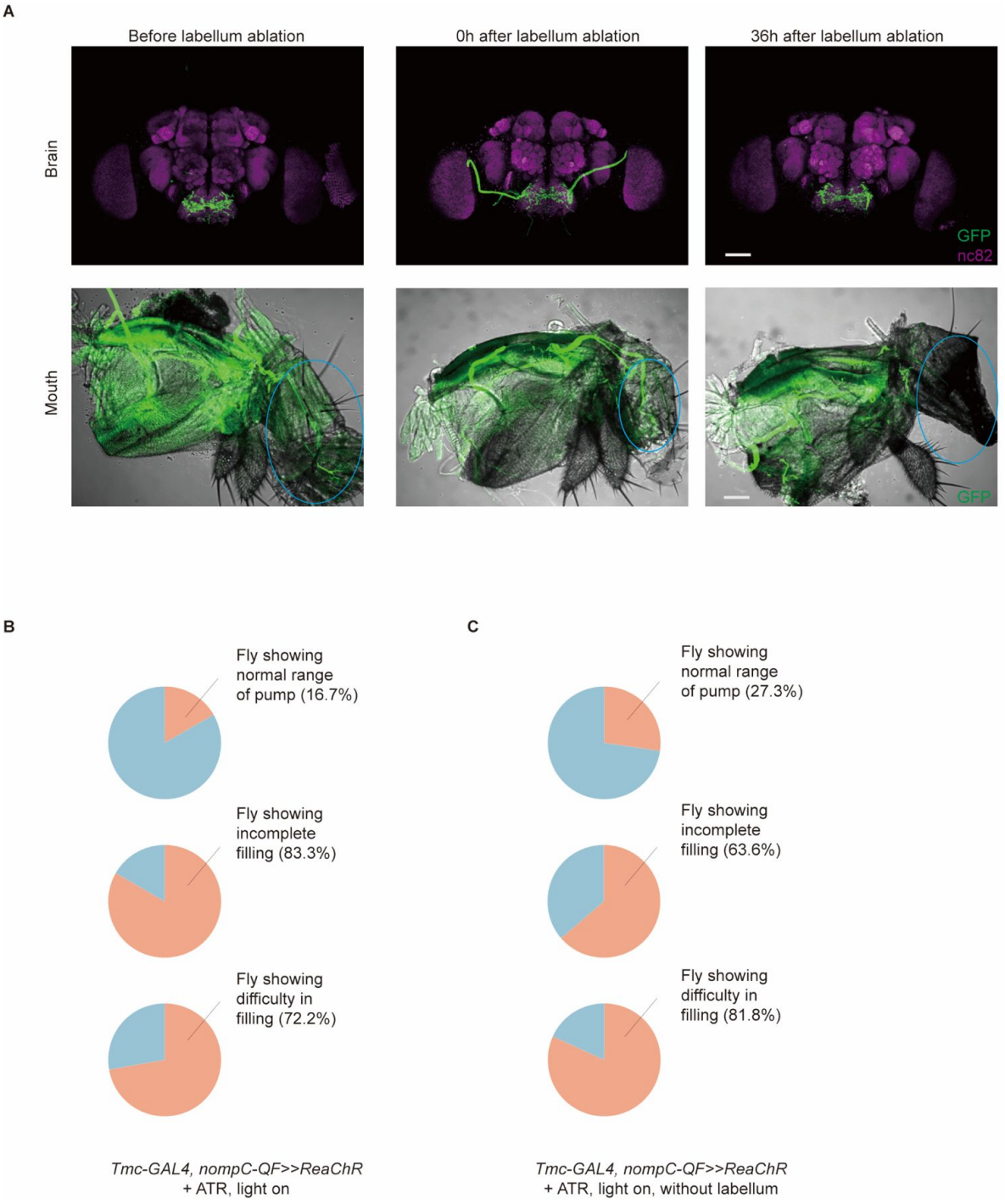

Figure 3—figure supplement 1

md-C neurons, but not md-L neurons, are essential for sensorimotor control of swallow rhythm. (A) In the brain, the projecting pattern of *Tmc-GAL4 & nompC-QF>>GFP* exhibited no significant changes post labellum ablation. Following the ablation of the labellum for 36 hours, the GFP signals of md-L in the mouth were scarcely observable when GFP expression was driven by *Tmc-GAL4 & nompC-QF*. (B) Flies expressing ReaChR in md-C neurons display dysphagia when stimulated by light. n=18. (C) Flies with labellum ablated for 36 hours still showed response to light stimulation of md-C, indicating that md-L neurons are not necessary for control of swallow rhythm. n=11.

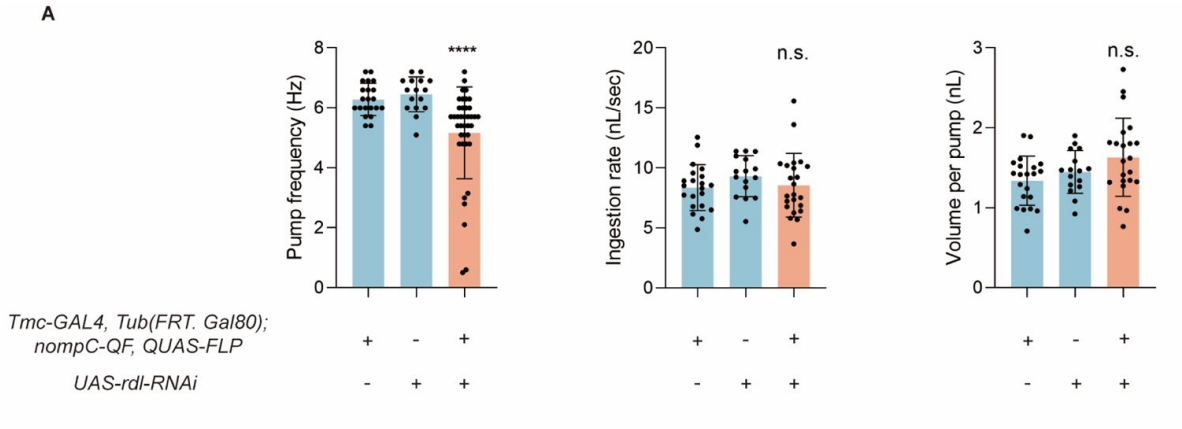

Figure 4—figure supplement 1

Regulation of md-C neurons requires the inhibitory inter-neurons. (A) Knocking down the expression of *$GABA_A$-R* in md-C neurons caused a lower pump frequency. n = 16 to 25, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used in B and C while nonparametric test was used in A, and statistical differences were represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data were represented as means ± SEM.

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Reviewer #1 (Public Review):

Qin et al. set out to investigate the role of mechanosensory feedback during swallowing and identify neural circuits that generate ingestion rhythms. They use Drosophila melanogaster swallowing as a model system, focusing their study on the neural mechanisms that control cibarium filling and emptying in vivo. They find that pump frequency is decreased in mutants of three mechanotransduction genes (nompC, piezo, and Tmc), and conclude that mechanosensation mainly contributes to the emptying phase of swallowing. Furthermore, they find that double mutants of nompC and Tmc have more pronounced cibarium pumping defects than either single mutants or Tmc/piezo double mutants. They discovered that the expression patterns of nompC and Tmc overlap in two classes of neurons, md-C and md-L neurons. The dendrites of md-C neurons warp the cibarium and project their axons to the subesophageal zone of the brain. Silencing neurons that express both nompC and Tmc leads to severe ingestion defects, with decreased cibarium emptying. Optogenetic activation of the same population of neurons inhibited filling of the cibarium and accelerated cibarium emptying. In the brain, the axons of nompC∩Tmc cell types respond during ingestion of sugar but do not respond when the entire fly head is passively exposed to sucrose. Finally, the authors show that nompC∩Tmc cell types arborize close to the dendrites of motor neurons that are required for swallowing and that swallowing motor neurons respond to the activation of the entire Tmc-GAL4 pattern.

Strengths:
-The authors rigorously quantify ingestion behavior to convincingly demonstrate the importance of mechanosensory genes in the control of swallowing rhythms and cibarium filling and emptying
-The authors demonstrate that a small population of neurons that express both nompC and Tmc oppositely regulate cibarium emptying and filling when inhibited or activated, respectively
-They provide evidence that the action of multiple mechanotransduction genes may converge in common cell types

Weaknesses:
-A major weakness of the paper is that the authors use reagents that are expressed in both md-C and md-L but describe the results as though only md-C is manipulated
-Evidence that the defects they see in pumping can be specifically attributed to md-C is based

on severing the labellum and allowing md-L neurons to degrade.
-GRASP is known to be non-specific and prone to false positives when neurons are in close proximity but not synaptically connected. A positive GRASP signal supports but does not confirm direct synaptic connectivity between md-C/md-L axons and MN11/MN12.
-MN11/MN12 LexA lines are not included in the manuscript and their expression patterns (shared with the reviewers in the author response) do not appear to contain any motor neurons. Double labeling with previously described MN11 and MN12 motor neuron Gal4 lines is needed to support the claim that these LexA lines in fact label MN11 and MN12.
-As seen in Figure Supplement 2, the expression pattern of Tmc-GAL4 is broader than md-C alone. Therefore, the functional connectivity the authors observe between Tmc expressing neurons and MN11 and 12 cannot be traced to md-C alone
-Example traces of md-C calcium imaging during ingestion in vivo are not included, and evidence that md-C neurons respond to mechanical force is lacking
-A positive control (perhaps demonstrating that sugar sensory neurons respond to sucrose in this preparation) is needed to assess whether the lack of response to sucrose ex vivo in Figure 4K is informative
-Proximity between md-C neurons and muscles is not evidence that they sense stretch
-Reporting of posthoc tests needs to be improved throughout the manuscript, as it is not clear which comparisons are noted with asterisks in the figures.

Overall, this work convincingly shows that swallowing and swallowing rhythms are dependent on several mechanosensory genes. Qin et al. also characterize a candidate neuron, md-C, that is likely to provide mechanosensory feedback to pumping motor neurons, but the results they present here are not sufficient to assign this function to md-C alone. This work will have a positive impact on the field by demonstrating the importance of mechanosensory feedback to swallowing rhythms and providing a potential entry point for future investigation of the identity and mechanisms of swallowing central pattern generators.

https://doi.org/10.7554/eLife.88614.2.sa1

Reviewer #3 (Public Review):

Strengths:
There are many reports on the effect of chemical properties of foods on feeding in fruit flies, but only limited studies reported how physical properties of food affect feeding especially pharyngeal mechanosensory neurons. First, they found that mechanosensory mutants, including nompC, Tmc, and Piezo, showed impaired swallowing, mainly the emptying process. Next, they identified cibarium multidendritic mechanosensory neurons (md-C) are responsible for controlling swallowing by regulating motor neuron (MN) 12 and 11, which control filling and emptying, respectively.

Weaknesses:
While the involvement of md-C and mechanosensory channels in controlling swallowing is convincing, it is not yet clear which stimuli activate md-C. Can it be an expansion of cibarium or food viscosity, or both? In addition, if rhythmic and coordinated contraction of muscles 11 and 12 is essential for swallowing, how can simultaneous activation of MN 11 and 12 by md-C achieve this? Finally, previous reports showed that food viscosity mainly affects the filling rather than the emptying process, which seems different from their finding.

https://doi.org/10.7554/eLife.88614.2.sa0

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Qin et al. set out to investigate the role of mechanosensory feedback during swallowing and identify neural circuits that generate ingestion rhythms. They use Drosophila melanogaster swallowing as a model system, focusing their study on the neural mechanisms that control cibarium filling and emptying in vivo. They find that pump frequency is decreased in mutants of three mechanotransduction genes (nompC, piezo, and Tmc), and conclude that mechanosensation mainly contributes to the emptying phase of swallowing. Furthermore, they find that double mutants of nompC and Tmc have more pronounced cibarium pumping defects than either single mutants or Tmc/piezo double mutants. They discover that the expression patterns of nompC and Tmc overlap in two classes of neurons, md-C and md-L neurons. The dendrites of md-C neurons warp the cibarium and project their axons to the subesophageal zone of the brain. Silencing neurons that express both nompC and Tmc leads to severe ingestion defects, with decreased cibarium emptying. Optogenetic activation of the same population of neurons inhibited filling of the cibarium and accelerated cibarium emptying. In the brain, the axons of nompC∩Tmc cell types respond during ingestion of sugar but do not respond when the entire fly head is passively exposed to sucrose. Finally, the authors show that nompC∩Tmc cell types arborize close to the dendrites of motor neurons that are required for swallowing, and that swallowing motor neurons respond to the activation of the entire Tmc-GAL4 pattern.

Strengths:

- *The authors rigorously quantify ingestion behavior to convincingly demonstrate the importance of mechanosensory genes in the control of swallowing rhythms and cibarium filling and emptying*
- *The authors demonstrate that a small population of neurons that express both nompC and Tmc oppositely regulate cibarium emptying and filling when inhibited or activated, respectively*
- *They provide evidence that the action of multiple mechanotransduction genes may converge in common cell types*

Thank you for your insightful and detailed assessment of our work. Your constructive feedback will help to improve our manuscript.

Weaknesses:

- *A major weakness of the paper is that the authors use reagents that are expressed in both md-C and md-L but describe the results as though only md-C is manipulated-Severing the labellum will not prevent optogenetic activation of md-L from triggering neural responses downstream of md-L. Optogenetic activation is strong enough to trigger action potentials in the remaining axons. Therefore, Qin et al. do not present convincing evidence that the defects they see in pumping can be specifically attributed to md-C.*

Thank you for your comments. This is important point that we did not adequately address in the original preprint. We have obtained imaging and behavioral results that strongly suggest md-C, rather than md-L, are essential for swallowing behavior.

36 hours after the ablation of the labellum, the signals of md-L were hardly observable when GFP expression was driven by the intersection between Tmc-GAL4 & nompC-QF (see F Figure 3—figure supplement 1A). This observation indicates that the axons of md-L likely degenerated after 36 hours, and were unlikely to influence swallowing. Moreover, the projecting pattern of Tmc-GAL4 & nompC-QF>>GFP exhibited no significant changes in the brain post labellum ablation.

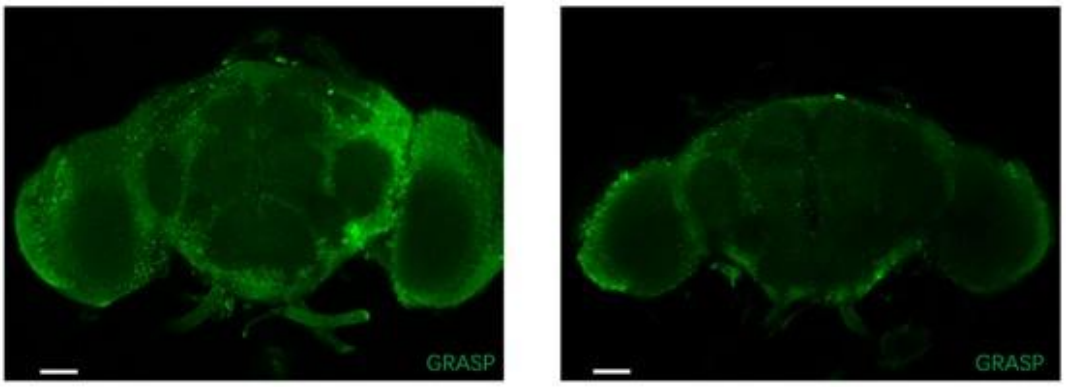
Furthermore, even after labellum ablation for 36 hours, flies exhibited responses to light stimulation (see Figure 3—figure supplement 1B-C, Video 5) when ReaChR was expressed in md-C. We thus reasoned that md-C but not md-L, plays a crucial role in the swallowing process.

> - *GRASP is known to be non-specific and prone to false positives when neurons are in close proximity but not synaptically connected. A positive GRASP signal supports but does not confirm direct synaptic connectivity between md-C/md-L axons and MN11/MN12.*

In this study, we employed the nSyb-GRASP, wherein the GRASP is expressed at the presynaptic terminals by fusion with the synaptic marker nSyb. This method demonstrates an enhanced specificity compared to the original GRASP approach.

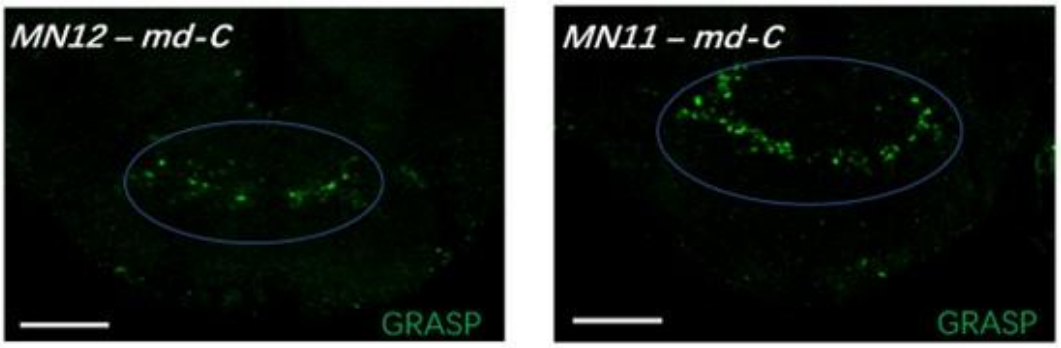
Additionally, we utilized +/ UAS-nSyb-spGFP1-10, lexAop-CD4-spGFP11 ; + / MN-LexA fruit flies as a negative control to mitigate potential false signals originating from the tool itself (Author response image 1, scale bar = 50μm). Beside the genotype Tmc-Gal4, Tub(FRT. Gal80) / UAS-nSyb-spGFP1-10, lexAop-CD4-spGFP11 ; nompC-QF, QUAS-FLP / MN-LexA fruit flies discussed in this manuscript, we also incorporated genotype Tmc-Gal4, Tub(FRT. Gal80) / lexAop-nSyb-spGFP1-10, UAS-CD4-spGFP11 ; nompC-QF, QUAS-FLP / MN-LexA fruit flies as a reverse control (Author response image 2). Unexpectedly, similar positive signals were observed, indicating that, positive signals may emerge due to close proximity between neurons even with nSyb-GRASP.

Author response image 1



It should be noted that the existence of synaptic projections from motor neurons (MN) to md-C cannot be definitively confirmed at this juncture. At present, we can only posit the potential for synaptic connections between md-C and motor neurons. A more conclusive conclusion may be attainable with the utilization of comprehensive whole-brain connectome data in future studies.

Author response image 2



- *As seen in Figure 2—figure supplement 1, the expression pattern of Tmc-GAL4 is broader than md-C alone. Therefore, the functional connectivity the authors observe between Tmc expressing neurons and MN11 and 12 cannot be traced to md-C alone*

It is true that the expression pattern of Tmc-GAL4 is broader than that of md-C alone. Our experiments, including those flies expressing TNT in Tmc+ neurons, demonstrated difficulties in emptying (Figure 2A, 2D). Notably, we encountered challenges in finding fly stocks bearing UAS>FRT-STOP-P2X2. Consequently, we opted to utilize Tmc-GAL4 to drive UAS-P2X2 instead. We believe that the results further support our hypothesis on the role of md-C in the observed behavioral change in emptying.

Overall, this work convincingly shows that swallowing and swallowing rhythms are dependent on several mechanosensory genes. Qin et al. also characterize a candidate neuron, md-C, that is likely to provide mechanosensory feedback to pumping motor neurons, but the results they present here are not sufficient to assign this function to md-C alone. This work will have a positive impact on the field by demonstrating the importance of mechanosensory feedback to swallowing rhythms and providing a potential entry point for future investigation of the identity and mechanisms of swallowing central pattern generators.

Reviewer #2 (Public Review):

In this manuscript, the authors describe the role of cibarial mechanosensory neurons in fly ingestion. They demonstrate that pumping of the cibarium is subtly disrupted in mutants for piezo, TMC, and nomp-C. Evidence is presented that these three genes are co-expressed in a set of cibarial mechanosensory neurons named md-C. Silencing of md-C neurons results in disrupted cibarial emptying, while activation promotes faster pumping and/or difficulty filling. GRASP and chemogenetic activation of the md-C neurons is used to argue that they may be directly connected to motor neurons that control cibarial emptying.

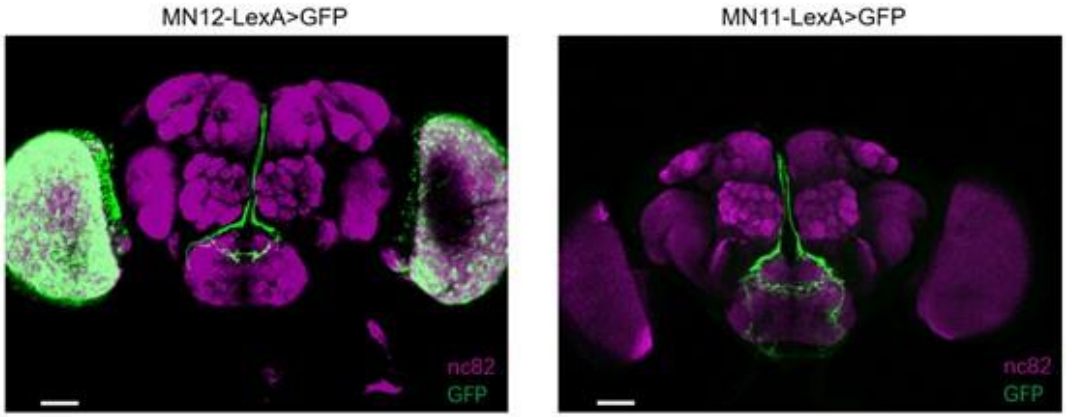
The manuscript makes several convincing and useful contributions. First, identifying the md-C neurons and demonstrating their essential role for cibarium emptying provides reagents for further studying this circuit and also demonstrates the important of mechanosensation in driving pumping rhythms in the pharynx. Second, the suggestion that these mechanosensory neurons are directly connected to motor neurons controlling pumping stands in contrast to other sensory circuits identified in fly feeding and is an interesting idea that can be more rigorously tested in the future.

At the same time, there are several shortcomings that limit the scope of the paper and the confidence in some claims. These include:

a) the MN-LexA lines used for GRASP experiments are not characterized in any other way to demonstrate specificity. These were generated for this study using Phack methods, and their expression should be shown to be specific for MN11 and MN12 in order to interpret the GRASP experiments.

Thanks for the suggestion. We have checked the expression pattern of MN-LexA, which is similar to MN-GAL4 used in previous work (Manzo et al., PNAS., 2012, PMID:22474379) . Here is the expression pattern:

Author response image 3



b) There is also insufficient detail for the P2X2 experiment to evaluate its results. Is this an in vivo or ex vivo prep? Is ATP added to the brain, or ingested? If it is ingested, how is ATP coming into contact with md-C neuron if it is not a chemosensory neuron and therefore not exposed to the contents of the cibarium?

The P2X2 experimental preparation was done ex vivo. We immersed the fly in the imaging buffer, as described in the Methods section under Functional Imaging. Following dissection and identification of the subesophageal zone (SEZ) area under fluorescent microscopy, we introduced ATP slowly into the buffer, positioned at a distance from the brain

c) In Figure 3C, the authors claim that ablating the labellum will remove the optogenetic stimulation of the md-L neuron (mechanosensory neuron of the labellum), but this manipulation would presumably leave an intact md-L axon that would still be capable of being optogenetically activated by Chrimson.

Please refer to the corresponding answers for reviewer 1 and Figure 3—figure supplement 1.

d) Average GCaMP traces are not shown for md-C during ingestion, and therefore it is impossible to gauge the dynamics of md-C neuron activation during swallowing. Seeing activation with a similar frequency to pumping would support the suggested role for these neurons, although GCaMP6s may be too slow for these purposes.

Profiling the dynamics of md-C neuron activation during swallowing is crucial for unraveling the operational model of md-C and validating our proposed hypothesis. Unfortunately, our assay faces challenges in detecting probable 6Hz fluorescent changes with GCaMP6s.

In general, we observed an increase of fluorescent signals during swallowing, but movement of alive flies during swallowing influenced the imaging recording, so we could not depict a

decent tracing for calcium imaging for md-C neurons. To enhance the robustness of our findings, patching the md-C neurons would be a more convincing approach. As illustrated in Figure 2, the somata of md-C neurons are situated in the cibarium rather than the brain. patching of the md-C neuron somata in flies during ingestion is difficult.

> *e) The negative result in Figure 4K that is meant to rule out taste stimulation of md-C is not useful without a positive control for pharyngeal taste neuron activation in this same preparation.*

We followed methods used in the previous work (Chen et al., Cell Rep., 2019, PMID:31644916), which we believe could confirm that md-C do not respond to sugars.

> *In addition to the experimental limitations described above, the manuscript could be organized in a way that is easier to read (for example, not jumping back and forth in figure order).*

Thanks for your suggestion and the manuscript has been reorganized.

> ***Reviewer #3 (Public Review):***
>
> *Swallowing is an essential daily activity for survival, and pharyngo-laryngeal sensory function is critical for safe swallowing. In Drosophila, it has been reported that the mechanical property of food (e.g. Viscosity) can modulate swallowing. However, how mechanical expansion of the pharynx or fluid content sense and control swallowing was elusive. Qin et al. showed that a group of pharyngeal mechanosensory neurons, as well as mechanosensory channels (nompC, Tmc, and Piezo), respond to these mechanical forces for regulation of swallowing in Drosophila melanogaster.*
>
> *Strengths:*
>
> *There are many reports on the effect of chemical properties of foods on feeding in fruit flies, but only limited studies reported how physical properties of food affect feeding especially pharyngeal mechanosensory neurons. First, they found that mechanosensory mutants, including nompC, Tmc, and Piezo, showed impaired swallowing, mainly the emptying process. Next, they identified cibarium multidendritic mechanosensory neurons (md-C) are responsible for controlling swallowing by regulating motor neuron (MN) 12 and 11, which control filling and emptying, respectively.*
>
> *Weaknesses:*
>
> *While the involvement of md-C and mechanosensory channels in controlling swallowing is convincing, it is not yet clear which stimuli activate md-C. Can it be an expansion of cibarium or food viscosity, or both? In addition, if rhythmic and coordinated contraction of muscles 11 and 12 is essential for swallowing, how can simultaneous activation of MN 11 and 12 by md-C achieve this? Finally, previous reports showed that food viscosity mainly affects the filling rather than the emptying process, which seems different from their finding.*

We have confirmed that swallowing sucrose water solution activated md-C neurons, while sucrose water solution alone could not (Figure 4J-K). We hypothesized that the viscosity of the food might influence this expansion process.

While we were unable to delineate the activation dynamics of md-C neurons, our proposal posits that these neurons could be activated in a single pump cycle, sequentially stimulating MN12 and MN11. Another possibility is that the activation of md-C neurons acts as a switch,

altering the oscillation pattern of the swallowing central pattern generator (CPG) from a resting state to a working state.

In the experiments with w1118 flies fed with MC (methylcellulose) water, we observed that viscosity predominantly affects the filling process rather than the emptying process, consistent with previous findings. This raises an intriguing question. Our investigation into the mutation of mechanosensitive ion channels revealed a significant impact on the emptying process. We believe this is due to the loss of mechanosensation affecting the vibration of swallowing circuits, thereby influencing both the emptying and filling processes. In contrast, viscosity appears to make it more challenging for the fly to fill the cibarium with food, primarily attributable to the inherent properties of the food itself.

> ***Reviewer #4 (Public Review):***
>
> *A combination of optogenetic behavioral experiments and functional imaging are employed to identify the role of mechanosensory neurons in food swallowing in adult Drosophila. While some of the findings are intriguing and the overall goal of mapping a sensory to motor circuit for this rhythmic movement are admirable, the data presented could be improved.*
>
> *The circuit proposed (and supported by GRASP contact data) shows these multi-dendritic neurons connecting to pharyngeal motor neurons. This is pretty direct - there is no evidence that they affect the hypothetical central pattern generator - just the execution of its rhythm. The optogenetic activation and inhibition experiments are constitutive, not patterned light, and they seem to disrupt the timing of pumping, not impose a new one. A slight slowing of the rhythm is not consistent with the proposed function.*

Motor neurons implicated in patterned motions can be considered effectors of Central Pattern Generators (CPGs)(Marder et al., Curr Biol., 2001, PMID: 11728329; Hurkey et al., Nature., 2023, PMID:37225999). Given our observation of the connection between md-C neurons and motor neurons, it is reasonable to speculate that md-C neurons influence CPGs. Compared to the patterned light (0.1s light on and 0.1s light off) used in our optogenetic experiments, we noted no significant changes in their responses to continuous light stimulation. We think that optogenetic methods may lead to overstimulation of md-C neurons, failing to accurately mimic the expansion of the cibarium during feeding.

Dysfunction in mechanosensitive ion channels or mechanosensory neurons not only disrupts the timing of pumping but also results in decreased intake efficiency (Figure 1E). The water-swallowing rhythm is generally stable in flies, and swallowing is a vital process that may involve redundant ion channels to ensure its stability.

> *The mechanosensory channel mutants nompC, piezo, and TMC have a range of defects. The role of these channels in swallowing may not be sufficiently specific to support the interpretation presented. Their other defects are not described here and their overall locomotor function is not measured. If the flies have trouble consuming sufficient food throughout their development, how healthy are they at the time of assay? The level of starvation or water deprivation can affect different properties of feeding - meal size and frequency. There is no description of how starvation state was standardized or measured in these experiments.*

Defects in mechanosensory channel mutants nompC, piezo, and TMC, have been extensively investigated (Hehlert et al., Trends Neurosci., 2021, PMID:332570000). Mutations in these channels exhibit multifaceted effects, as illustrated in our RNAi experiments (see Figure 2E). Deprivation of water and food was performed in empty fly vials. It's important to note that

the duration of starvation determines the fly's willingness to feed but not the pump frequency (Manzo et al., PNAS., 2012, PMID:22474379).

In most cases, female flies were deprived water and food in empty vials for 24 hours because after that most flies would be willing to drink water. The deprivation time is 12 hours for flies with nompC and Tmc mutated or flies with Kir2.1 expressed in md-C neurons, as some of these flies cannot survive 24h deprivation.

> *The brain is likely to move considerably during swallow, so the GCaMP signal change may be a motion artifact. Sometimes this can be calculated by comparing GCaMP signal to that of a co-expressed fluorescent protein, but there is no mention that this is done here. Therefore, the GCaMP data cannot be interpreted.*

We did not co-express a fluorescent protein with GCaMP for md-C. The head of the fly was mounted onto a glass slide, and we did not observe significant signal changes before feeding.

.>Abstract: I disagree that swallow is the first step of ingestion. The first paragraph also mentions the final checkpoint before food ingestion. Perhaps sufficient to say that swallow is a critical step of ingestion.

Indeed, it is not rigorous enough to say “first step”. This has been replaced by “early step”.

> *Introduction:*
>
> *Line 59: "Silence" should be "Silencing"*
>
> *This has been replaced.*
>
> *Results:*
>
> *Lines 91-92: I am not clear about what this means. 20% of nompC and 20% of wild-type flies exhibit incomplete filling? So nompC is not different from wild-type?*

Sorry for the mistake. Viscous foods led to incomplete emptying (not incomplete filling), as displayed in Video 4. The swallowing behavior differs between nompC mutants and wild-type flies, as illustrated in Figure 1C, Figure 1—figure supplement 1A-C and video 1&5.

When fed with 1% MC water solution (Figure 1—figure supplement 1E-H). We found that when fed with 1% MC watere solution, Tmc or piezo mutants displayed incomplete emptying, which could constitute a long time proportion of swallowing behavior; while only 20% of nompC flies and 20% of wild-type flies sporadically exhibit incomplete emptying, which is significantly different. Though the percent of flies displaying incomplete pump is similar between nompC mutant and wild-type files, you can find it quite different in video 1 and 5.

> *Line 94: Should read: “while for foods with certain viscosity, the pump of Tmc or piezo mutants might"*
>
> *What evidence is there for weakened muscle motion? The phenotypes of all three mutants is quite similar, so concluding that they have roles in initiation versus swallowing strength is not well supported -this would be better moved to the discussion since it is speculative.*

> ***Recommendations for the authors:***

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Muscles are responsible for pumping the bolus from the mouth to the crop. In the case of Tmc or piezo mutants, as evidenced by incomplete filling for viscous foods (see Video 4), we speculate that the loss of sensory stimuli leads to inadequate muscle contraction. The phenotypes observed in Tmc and piezo mutants are similar yet distinct from those of the wild-type or nompC mutant, as shown in Video 1 and 4. The phrase "due to weakened muscle motion" has been removed for clarity.

> *Line 146: If md-L neurons are also labeled by this intersection, then you are not able to know whether the axons seen in the brain are from md-L or md-C neurons. Line 148: cutting the labellum is not sufficient to ablate md-L neurons. The projections will still enter the brain and can be activated with optogenetics, even after severing the processes that reside in the labellum.*

Please refer to the responses for reviewer #1 (Public Review):” A major weakness of the paper…” and Figure 4.

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Please refer to the responses for reviewer #1 (Public Review):” A major weakness of the paper…” and Figure 4.

> *Line 162: If the fly head alone is in saline, do you know that the sucrose enters the esophagus? The more relevant question here is whether the md-C neurons respond to*

> *mechanical force. If you could artificially inflate the cibarium with air and see the md-C neurons respond that would be a more convincing result. So far you only know that these are activated during ingestion, but have not shown that they are activated specifically by filling or emptying. In addition, you are not only imaging md-C (md-L is also labeled). This caveat should be mentioned.*

We followed the methods outlined in the previous work (Chen et al., Cell Rep., 2019, PMID:31644916), which suggested that md-C neurons do not respond to sugars. While we aimed to mechanically stimulate md-C neurons, detecting signal changes during different steps of swallowing is challenging. This aspect could be further investigated in subsequent research with the application of adequate patch recording or two-photon microscopy (TPM).

> *Figure 3: It is not clear what the pie charts in Figure 3 A refer to. What are the three different rows, and what does blue versus red indicate?*

Figure 3A illustrates three distinct states driven by CsChrimson light stimulation of md-C neurons, with the proportions of flies exhibiting each state. During light activation, flies may display difficulty in filling, incomplete filling, or a normal range of pumping. The blue and red bars represent the proportions of flies showing the corresponding state, as indicated by the black line.

> *Figure 4: Where are the example traces for J? The comparison in K should be average dF/F before ingestion compared with average dF/F during ingestion. Comparing the in vitro response to sucrose to the in vivo response during ingestion is not a useful comparison.*

Please refer to the answers for reviewer #2 question d).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Suggested experiments that would address some of my concerns listed in the public review include:*
>
> *a) high resolution SEZ images of MN-LexA lines crossed to LexAop-GFP to demonstrate their specificity*
>
> *b) more detail on the P2X2 experiment. It is hard to make suggestions beyond that without first seeing the details.*
>
> *c) presenting average GCaMP traces for all calcium imaging results*
>
> *d) to rule out taste stimulation of md-C (Figure 4K) I would suggest performing more extensive calcium imaging experiments with different stimuli. For example, sugar, water, and increasing concentrations of a neutral osmolyte (e.g. PEG) to suppress the water response. I think that this is more feasible than trying to get an in vitro taste prep to be convincing.*

Please refer to the responses for public review of reviewer #2.

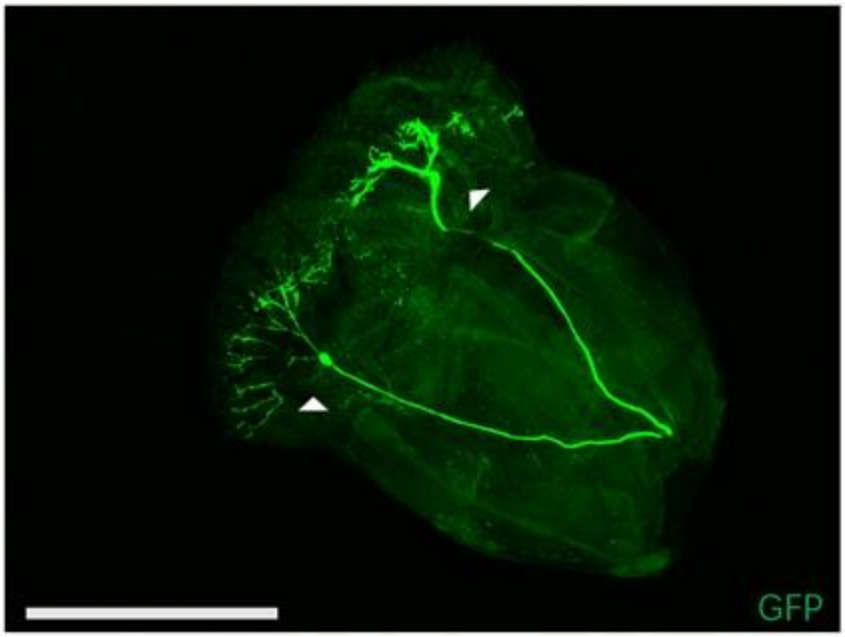
> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Below I list my suggestions as well as criticisms.*
>
> *(1) It would be excellent if the authors could demonstrate whether varying levels of food viscosity affect md-C activation.*

That is a good point, and could be studied in future work.

> *(2) It is not clear whether an intersectional approach using TMC-GAL4 and nompC-QF abolishes labelling of the labellar multidendritic neurons. If this is the case, please show labellar multidendritic neurons in TMC-GAL4 only flies and flies using the intersectional approach. Along with this question, I am concerned that labellum-removed flies could be used for feeding assay.*

Intersectional labelling using TMC-GAL4 and nompC-QF could not abolish labelling of the labellar multidendritic neurons (Author response image 4). Labellum-removed flies could be used for feeding assay (Figure 3—figure supplement 1B-C, video 5), but once LSO or cibarium of fly was damaged, swallowing behavior would be affected. Removing labellum should be very careful.

Author response image 4



> *(3) Please provide the detailed methods for GRASP and include proper control.*

Please refer to the responses for public review of reviewer #1.

> *(4) The authors hypothesized that md-C sequentially activates MN11 and 12. Is the time gap between applying ATP on md-C and activation of MN11 or MN12 different? Please refer to the responses for public review of reviewer #3. The time gap between applying ATP on md-C and activation of MN11 or MN12 didn't show significant differences, and we think the reason is that the ex vivo conditions could not completely mimic in vivo process.*
>
> *I found the manuscript includes many errors, which need to be corrected.*
>
> *(1) The reference formatting needs to be rechecked, for example, lines 37, 42, and 43.*
>
> *(2) Line 44-46: There is some misunderstanding. The role of pharyngeal mechanosensory neurons is not known compared with chemosensory neurons.*

> *(3) Line 49: Please specify which type of quality of food. Chemical or physical?*
>
> *(4) Line 80 and Figure 1B-D Authors need to put filling and emptying time data in the main figure rather than in the supplementary figure. Otherwise, please cite the relevant figures in the text(S1A-C).*
>
> *(5) Line 84-85; Is "the mutant animals" indicating only nompC? Please specify it.*
>
> *(6) Figure 1a: It is hard to determine the difference between the series of images. And also label filling and emptying under the time.*
>
> *(7) S1E-H: It is unclear what "Time proportion of incomplete pump" means. Please define it.*
>
> *(8) Please reorganize the figures to follow the order of the text, for example, figures 2 and 4*
>
> *(9) Figure 4A. There is mislabelling in Figure 4A. It is supposed to be phalloidin not nc82.*
>
> *(10) Figure 4K: It does not match the figure legend and main text.*
>
> *(11) Figure 4D and G: Please indicate ATP application time point.*

Thanks for your correction and all the points mentioned were revised.

> ***Reviewer #4 (Recommendations For The Authors):***
>
> *The figures need improvement. 1A has tiny circles showing pharynx and any differences are unclear.*
>
> *The expression pattern of some of these drivers (Supplement) seems quite broad. The tmc nompC intersection image in Figure 1F is nice but the cibarium images are hard to interpret: does this one show muscle expression? What are "brain" motor neurons? Where are the labellar multi-dendritic neurons?*
>
> *Tmc nompC intersection image show no expression in muscles. Somata of motor neurons 12 or 11 situated at SEZ area of brain, while somata of md-C neurons are in the cibarium. Image of md-L neurons was posted in response for reviewer #3 (Recommendations For The Authors):*
>
> *Why do the assays alternate between swallowing food and swallowing water?*

Thank for your suggestion, figure 1A has been zoomed-in. The Tmc nompC intersection image in Figure 2F displayed the position of md-C neurons in a ventral perspective, and muscles were not labelled. We stained muscles in cibarium by phalloidin and the image is illustrated in Figure 4A, while we didn't find overlap between md-C neurons and muscles. Image of md-L neurons were posted as Author response image 4.

In the majority of our experiments, we employed water to test swallowing behavior, while we used methylcellulose water solution to test swallowing behavior of mechanoreceptor mutants, and sucrose solution for flies with md-C neurons expressing GCaMP since they hardly drank water when their head capsules were open.

> *How starved or water-deprived were the flies?*

One day prior to the behavioral assays, flies were transferred to empty vials (without water or food) for 24 hours for water deprivation. Flies who could not survive 24h deprivation

would be deprived for 12h.

> *How exactly was the pumping frequency (shown in Fig 1B) measured? There is no description in the methods at all. If the pump frequency is scored by changes in blue food intensity (arbitrary units?), this seems very subjective and maybe image angle dependent. What was camera frame rate? Can it capture this pumping speed adequately? Given the wealth of more quantitative methods for measuring food intake (eg. CAFE, flyPAD), it seems that better data could be obtained.*
>
> *How was the total volume of the cibarium measured? What do the pie charts in Figure 3A represent?*

The pump frequency was computed as the number of pumps divided by the time scale, following the methodology outlined in Manzo et al., 2012. Swallowing curves were plotted using the inverse of the blue food intensity in the cibarium. In this representation, ascending lines signify filling, while descending lines indicate emptying (see Figure 2D, 3B). We maintain objectivity in our approach since, during the recording of swallowing behavior, the fly was fixed, and we exclusively used data for analysis when the Region of Interest (ROI) was in the cibarium. This ensures that the intensity values accurately reflect the filling and emptying processes. Furthermore, we conducted manual frame-by-frame checks of pump frequency, and the results align with those generated by the time series analyzer V3 of ImageJ.

For the assessment of total volume of ingestion, we referred the methods of CAFE, utilizing a measurable glass capillary. We then calculated the ingestion rate (nL/s) by dividing the total volume of ingestion by the feeding time.

> *The changes seem small, in spite of the claim of statistical significance.*

The observed stability in pump frequency within a given genotype underscores the significance of even seemingly small changes, which is statistically significant. We speculate that the stability in swallowing frequency suggests the existence of a redundant mechanism to ensure the robustness of the process. Disruption of one channel might potentially be partially compensated for by others, highlighting the vital nature of the swallowing mechanism.

> *How is this change in pump frequency consistent with defects in one aspect of the cycle - either ingestion (activation) or expulsion (inhibition)?*

Please refer to Figure 2, 3. Both filling and emptying process were affects, while inhibition mainly influences emptying time (Figure 1—figure supplement 1).

> *for the authors:*
>
> *Line 48: extensively*
>
> *Line 62 - undiscovered.*
>
> *Line 107, 463: multi*
>
> *Line 124: What is "dysphagia?" This is an unusual word and should be defined.*
>
> *Line 446: severe*
>
> *Line 466: in the cibarium or not?*

Thanks for your correction and all the places mentioned were revised.