Pharyngeal Mechanosensory Neurons Control Food Swallow in *Drosophila melanogaster*

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Highlights

nompC$^+$, *piezo*$^+$ and *Tmc*$^+$ neurons in the pharynx sense mechanical force during swallowing
Pharyngeal mechanoreceptor neurons regulate swallow rhythm
Dysfunction of pharyngeal mechanosensory neurons causes dysphagia
Pharyngeal mechanosensory neurons connect with brain motor neurons to coordinate swallow pattern

Abstract

As the first 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.

Keywords

Swallow; mechanosensation; feeding; *Drosophila*

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], particularly regarding the significance of pharyngeal mechanosensation in the process of food ingestion. Physical properties of the food offer important information regarding its texture and influence animals' inclination to eat[16,41]. Although it has been exclusively 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 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]. Silence 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 uncovered.

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 S1A-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 mutant animals 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 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 S1E-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 due to weakened muscle motion. 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 S1D).

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 mutil-dendritic sensory neurons in the cibarium (Figure S2, Figure 2F). In the brain, SEZ was commonly labelled by either driver, while *Tmc-GAL4-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, 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.

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*. 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 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 S3B-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 GCaMP6s 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 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. 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 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 synaptic connections between md-C and motor neurons, we suppose activation of md-C triggered by food bolus, could 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 form synaptic and functional connections with motor neurons in the brain, and may 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 receive 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 S3A), 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. Besides, 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).

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

231 CPGs that control swallow in *Drosophila* brain are still elusive, our data provides an entry point to
232 elucidate the CPGs circuits and the neural circuits associated with them. As their activity can be
233 modulated by md-C neurons, they can be potentially identified by searching for the downstream of
234 md-C neurons.
235
236
237 **Materials and methods**
238 **KEY RESOURCES TABLE**
239

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Rabbit Alexa 488	Invitrogen	Cat#A11008; RRID: AB_143165
Goat anti-Rabbit Alexa 555	Invitrogen	Cat#A21428; RRID: AB_2535849
Goat anti-Mouse Alexa 488	Invitrogen	Cat#A11001; RRID: AB_2534069
Goat anti-Mouse Alexa 647	Invitrogen	Cat#A21235; RRID: AB_2535804
Mouse monoclonal anti-Brp	Developmental Studies Hybridoma Bank	Cat# nc82; RRID: AB_2314866
Rabbit polyclonal anti-GFP	Invitrogen	Cat#A11122; RRID: AB_221569
Rabbit polyclonal anti-RFP	Rockland	Cat#600-401-379S; RRID: AB_11182807
Mouse polyclonal anti-GFP	Sigma-Aldrich	Cat# G6539; RRID: AB_259941
Chemicals, peptides, and recombinant proteins		
4% PFA	Dingguo Biotech	Cat#AR-0211;CAS:30525-89-4
Brilliant Blue	Shi-tou	Cat#GB 7655.1
Methylcellulose	Sigma	M7140-100G
All trans retinal	Sigma	R2500-500MG
phalloidin	AAT bioquest	Cat#23127
Adenosine-5	aladdin	Lot#I2118087

Experimental models: Organisms/strains		
w^{1118}	Bloomington Drosophila Stock Center	5905
Tmc-GAL4	Bloomington Drosophila Stock Center	66557
Sp/Cyo; UAS-FRT-STOP-FRT-mCherry	Bloomington Drosophila Stock Center	53743
QUAS-FLP	Bloomington Drosophila Stock Center	30127
UAS-FRT-GAL80-STOP-FRT	Bloomington Drosophila Stock Center	38880
UAS-FRT-STOP-Kir2.1-FRT	Bloomington Drosophila Stock Center	67686
UAS(FRT.mCherry)ReaChR	Bloomington Drosophila Stock Center	53743
UAS-TNT	Bloomington Drosophila Stock Center	28997
LexAop-GCaMP6s	Bloomington Drosophila Stock Center	64413
Tmc^1	Bloomington Drosophila Stock Center	66556
w[*]; P{w[+Mc] = UAS-nSyb-spGFP1-10}2, P{w[+Mc] = lexAop-CD4-spGFP11}2/CyO	Bloomington Drosophila Stock Center	64314
UAS-RedStinger	Bloomington Drosophila Stock Center	8547
Piezo-GAL4	Bloomington Drosophila Stock Center	59266
UAS-Piezo-RNAi	Vienna Drosophila RNAi Center	105132
UAS>stop>GCaMP6s	Laboratory of Chuan Zhou, Institute of Zoology, Chinese Academy of Sciences	N/A
Sp/CyO; nompC-QF	Laboratory of Yuh Nung Jan, UCSF	N/A
nompC1/CyO	Laboratory of Yuh Nung Jan, UCSF	N/A
$nompC^{f00914}$/CyO	Laboratory of Yuh Nung Jan, UCSF	N/A
$piezo^{KO}$	Laboratory of Yuh Nung Jan, UCSF	N/A
UAS-nompC-RNAi; UAS-Dicer2	Laboratory of Yuh Nung Jan, UCSF	N/A

UAS-GFP	Laboratory of Yuh Nung Jan, UCSF	N/A
UAS-FRT-STOP-FRT-GFP	Laboratory of Yuh Nung Jan, UCSF	N/A
UAS-rdl-RNAi	Laboratory of Xin Liang, THU	N/A
UAS-P2X_2	Laboratory of Yufeng Pan, School of Life Science and Technology, Southeast University	N/A
UAS(FRT.stop)CsChrimson-mVenus	Laboratory of Yufeng Pan, School of Life Science and Technology, Southeast University	N/A
MN12-GAL4	Laboratory of Kristin Scott, UCB	N/A
MN11-GAL4	Laboratory of Kristin Scott, UCB	N/A
MN12-LexA	This paper	N/A
MN11-LexA	This paper	N/A
Tmc-GAL4, UAS(FRT.stop)CsChrimson-mVenus/CyO	This paper	N/A
nompC-QF, QUAS-Flp/TM6B	This paper	N/A
Tmc-GAL4, UAS-FRT-GAL80-STOP-FRT/CyO	This paper	N/A
Piezo-GAL4^{AD}	This paper	N/A
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
GraphPad Prim8	GraphPad software	https://www.graphpad.com/

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.

247
248 **Materials availability**
249 This study did not generate new unique reagents. All key resources are listed in Key resources table.
250 Further information and requests for resources and reagents should be directed to the lead contact.
251
252 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
253 **Animals**
254 Fly were acquired from other labs or BDSC (Bloomington *Drosophila* Stock Center). Flies were
255 reared on standard medium at 25°C unless otherwise noted. W^{1118} was used as wild-type control.
256 See KRT for the full list of fly strains.
257
258 **Generation of transgenic flies**
259 The *Piezo-GAL4*^{AD}, MN12-lexA, *MN11-LexA* line was constructed with the HACK system[32] from
260 line *Piezo-GAL4*(BDSC:59266), *MN12-GAL4* and *MN11-GAL4*(donated from Kristin Scott lab).
261 Phack-GAL4^{AD} plasmid was injected into nos-Cas9 flies with standard embryo injection
262 procedures. Injected flies were crossed with double balancer flies, and the transformants were
263 picked up with w^+ and RFP$^+$ makers. The *Tmc-GAL4, UAS(FRT.stop)CsChrimson-mVenus/CyO*,
264 *nompC-QF, QUAS-FLP/TM6B* and *Tmc-GAL4, UAS-FRT-GAL80-STOP-FRT/CyO* lines were
265 generated by recombination and confirmed by confocal imaging.
266
267 **METHOD DETAILS**
268 **Feeding assay**
269 All flies were kept in a 24-26°C incubator under 12-12 hr light: dark cycle and about 40-60%
270 humidity. Mated female flies were collected at eclosion and aged for 3-10 days. For neuronal
271 silencing experiments, flies bearing Kir2.1 or TNT were kept for 7-10 days before the feeding assay.
272 For rdl knockdown experiment in md-C neurons, 14-16 days-old flies were used. One day prior to
273 the behavioral assays, flies were transferred to empty vials for 24 hours for water deprivation (12h
274 for flies with *nompC* and *Tmc* mutated or flies with Kir2.1 expressed in md-C neurons).
275 Before experiments, flies were anesthetized with CO_2 and mounted onto a glass slide with nail
276 polish. They were then allowed to recover in a humid chamber for 2 hours. All behavioral
277 experiments were carried out at room temperature and 40%–60% humidity. Individual flies were
278 fed with a pipette filled with water, sugar or methylcellulose water solutions. Consumption of single
279 flies was calculated by measurable glass capillary. Ingestion rate was calculated by consumption
280 divided by ingestion time, feeding lasted for 4-7 seconds except the experiment where Kir2.1 was
281 used lasted for one minute. For illustration, 0.25 ng/Ml brilliant blue food dye (Shi-tou, GB 7655.1)
282 was added to the solution. 60 fps camera was adopted to record the feeding behavior and flies with
283 total feeding duration of more than 2 seconds were analyzed.
284 For optogenetics experiments, female flies expressing CsChrimson or ReaChR were raised on food
285 containing 10 Mm all-trans retinal at 25°C and 40-60% humidity in a light-proof vial. Experiments
286 were performed with 7 to 10-day-old mated female flies in a dark room. One day before the test,
287 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. For *in vitro* experiments, 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). 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 GCaMP6s in md-C before manipulation. While for GCaMP6s expressed in MNs, F_0 was the average fluorescence of 30 images in MNs before adding 20Ml water or 20Ml water solution of 250Mm 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.

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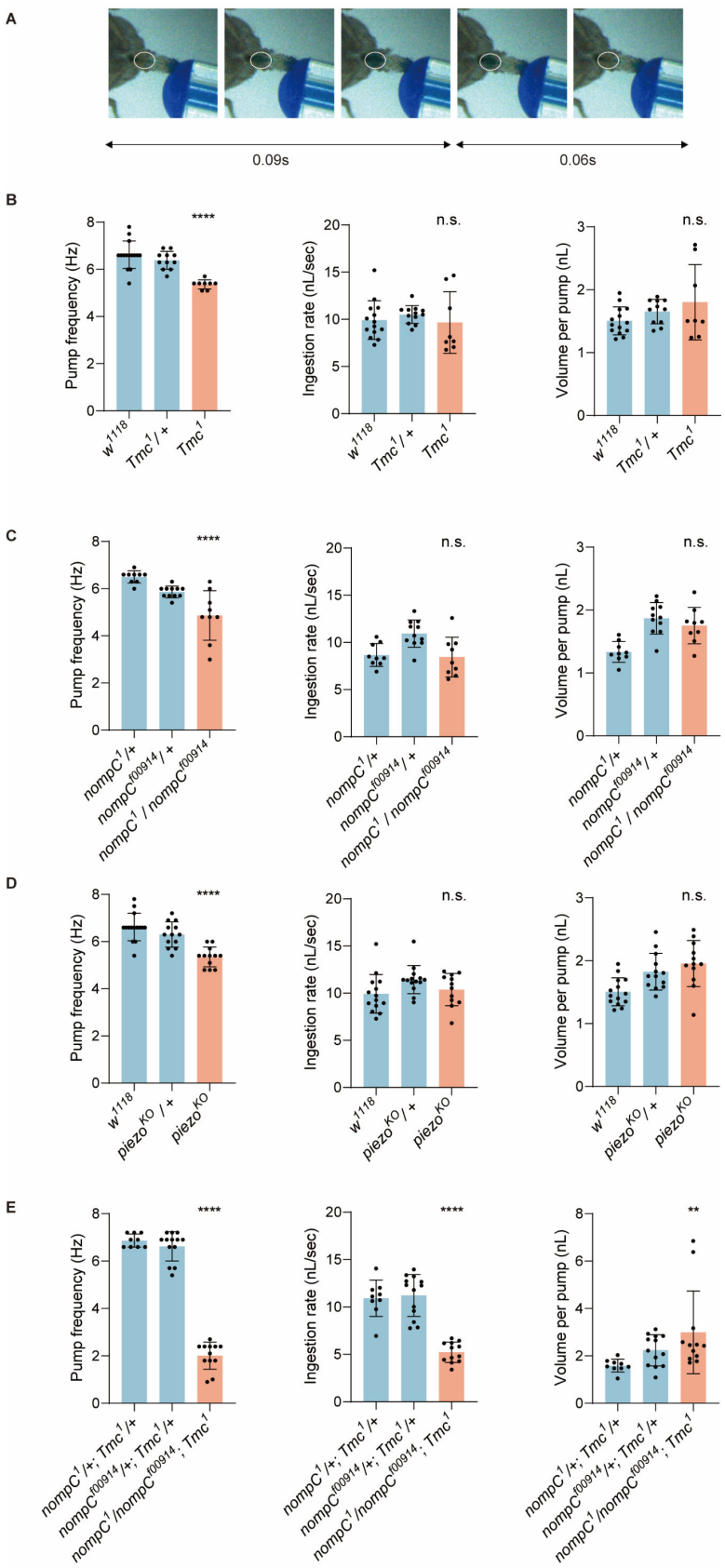

A
0.09s
0.06s
B
Pump frequency (Hz)
Ingestion rate (nL/sec)
Volume per pump (nL)
****
n.s.
n.s.
w1118
Tmc1/+
Tmc1
C
nompC1/+
nompCf00914/+
nompC1/nompCf00914
D
piezoKO/+
piezoKO
E
nompC1/+; Tmc1/+
nompCf00914/+; Tmc1/+
nompC1/nompCf00914; Tmc1
**

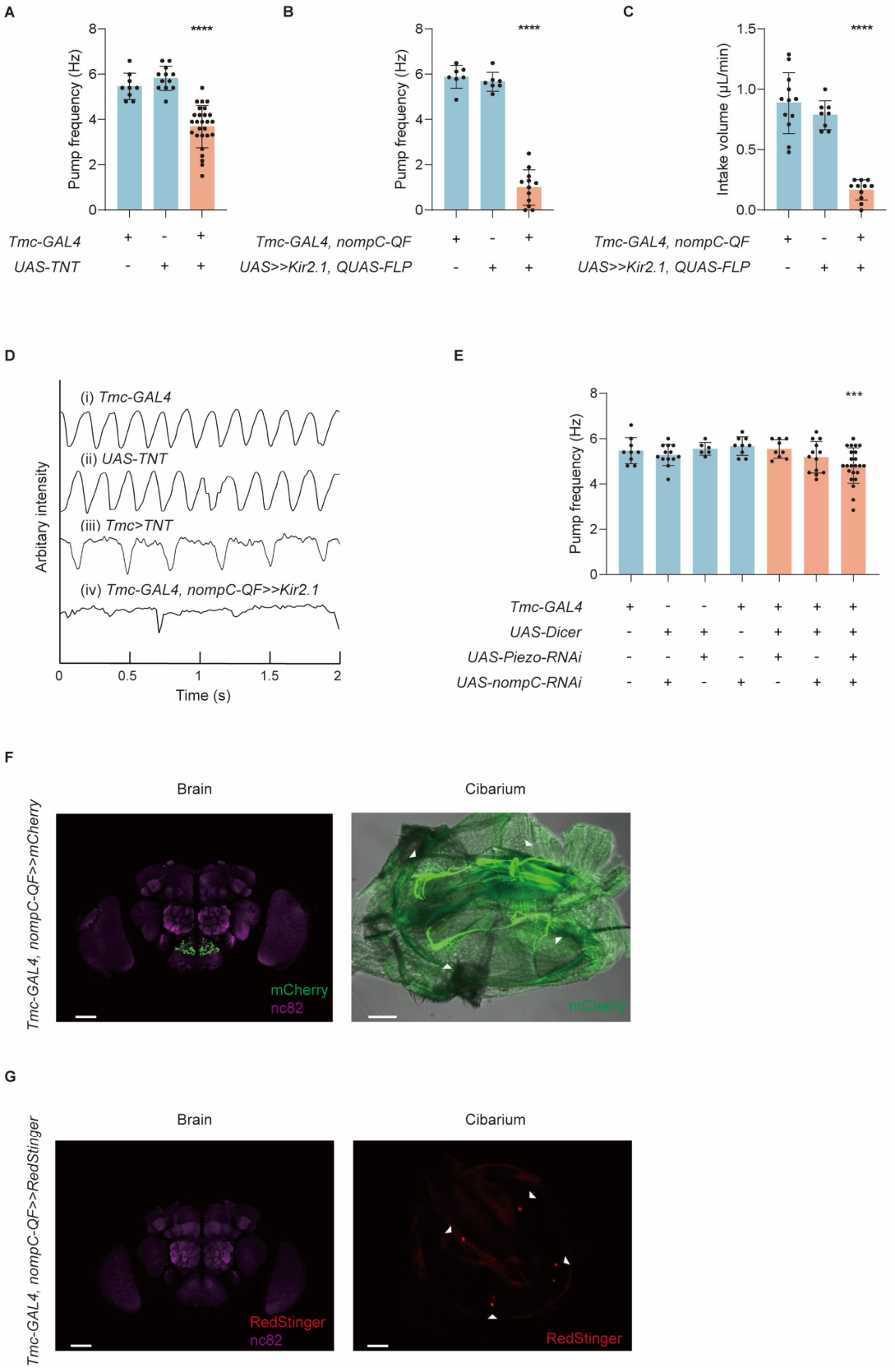
Figure 1. Mechanoreceptor genes are essential for swallow control of *Drosophila*.

(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 server 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.


A
Pump frequency (Hz)
****
Tmc-GAL4 + - +
UAS-TNT - + +
B
Pump frequency (Hz)
****
Tmc-GAL4, nompC-QF + - +
UAS>>Kir2.1, QUAS-FLP - + +
C
Intake volume (μL/min)
****
Tmc-GAL4, nompC-QF + - +
UAS>>Kir2.1, QUAS-FLP - + +
D
Arbitary intensity
(i) Tmc-GAL4
(ii) UAS-TNT
(iii) Tmc>TNT
(iv) Tmc-GAL4, nompC-QF>>Kir2.1
Time (s)
E
Pump frequency (Hz)
***
Tmc-GAL4 + - - + + + +
UAS-Dicer - + + - + + +
UAS-Piezo-RNAi - - + - + - +
UAS-nompC-RNAi - + - + - + +
F
Brain
Cibarium
Tmc-GAL4, nompC-QF>>mCherry
mCherry
nc82
mCherry
G
Brain
Cibarium
Tmc-GAL4, nompC-QF>>RedStinger
RedStinger
nc82
RedStinger

451

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, 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 mutil-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.

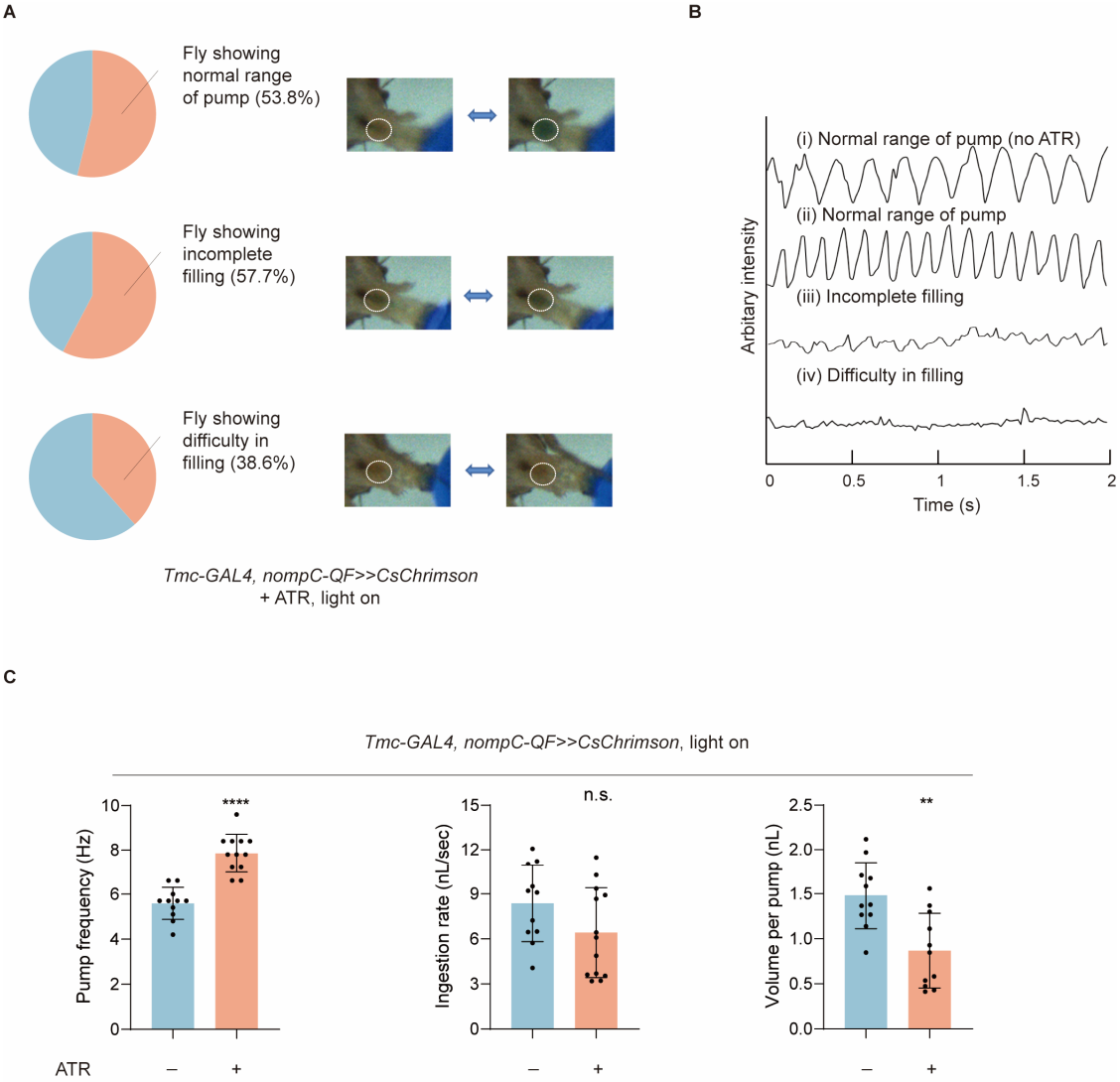


Figure 3 Activation of md-C neurons led to dysphagia.

(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. n=27.

(B) Arbitrary intensity of cibarium when CsChrimson was stimulated.

(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.

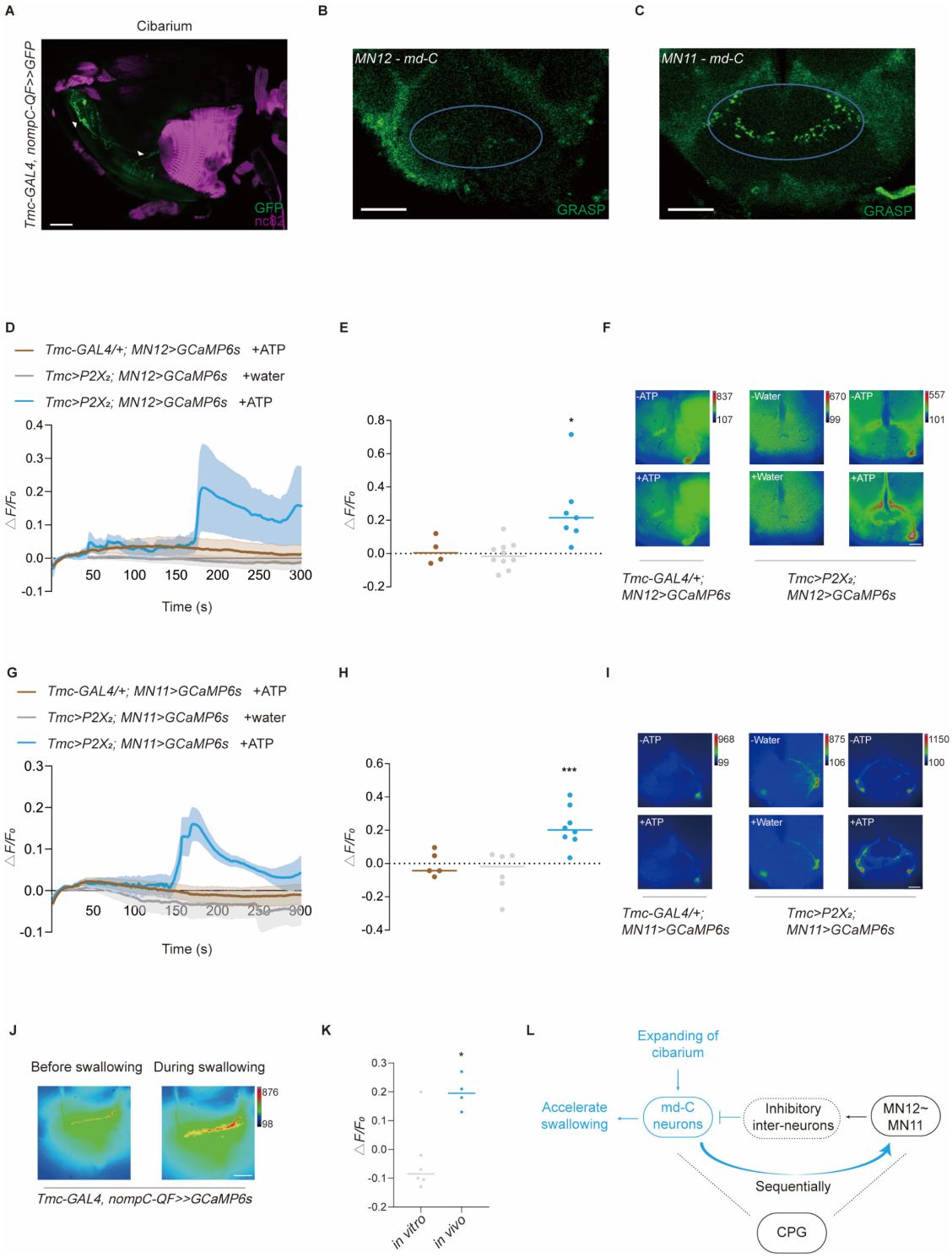


Figure 4 Interaction between motor neurons and md-C neurons revealed working pattern of swallow.

(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 after adding either ATP solution or water. 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 0.5M sucrose solution when GCaMP6s 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*.

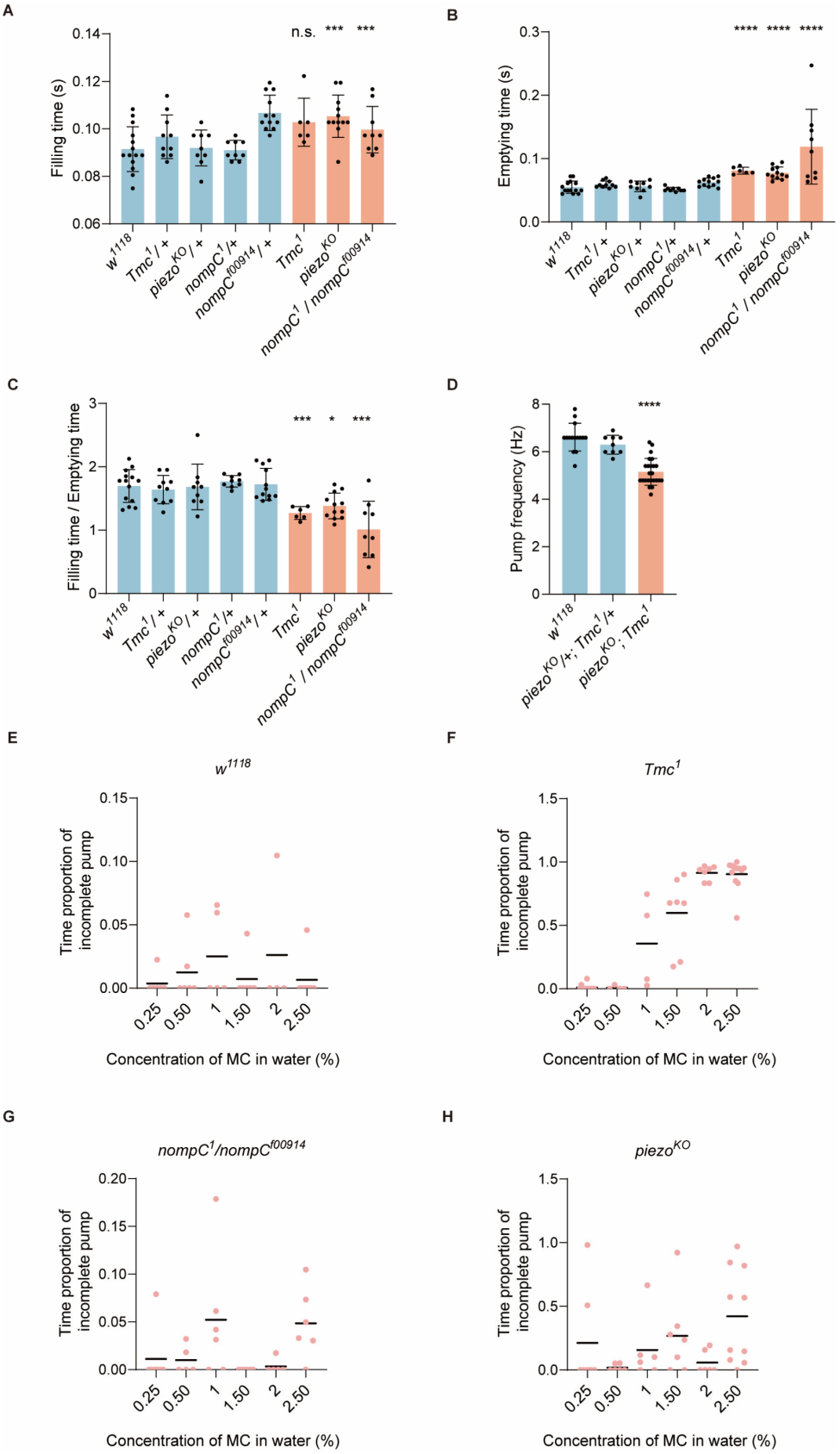

A
Filling time (s)
0.14
0.12
0.10
0.08
0.06
n.s.
***
***
w1118
Tmc1/+
piezoKO/+
nompC1/+
nompCf00914/+
Tmc1
piezoKO
nompC1/nompCf00914
B
Emptying time (s)
0.3
0.2
0.1
0.0
****
****
****
C
Filling time / Emptying time
3
2
1
0
***
*
***
D
Pump frequency (Hz)
8
6
4
2
0
****
piezoKO/+; Tmc1/+
piezoKO; Tmc1
E
w1118
Time proportion of incomplete pump
0.15
0.10
0.05
0.00
0.25
0.50
1
1.50
2
2.50
Concentration of MC in water (%)
F
Tmc1
1.5
1.0
0.5
0.0
G
nompC1/nompCf00914
0.20
0.15
0.10
0.05
0.00
H
piezoKO

Figure S1 Mutation of mechanoreceptors leads to a lower pump frequency caused by longer emptying time.

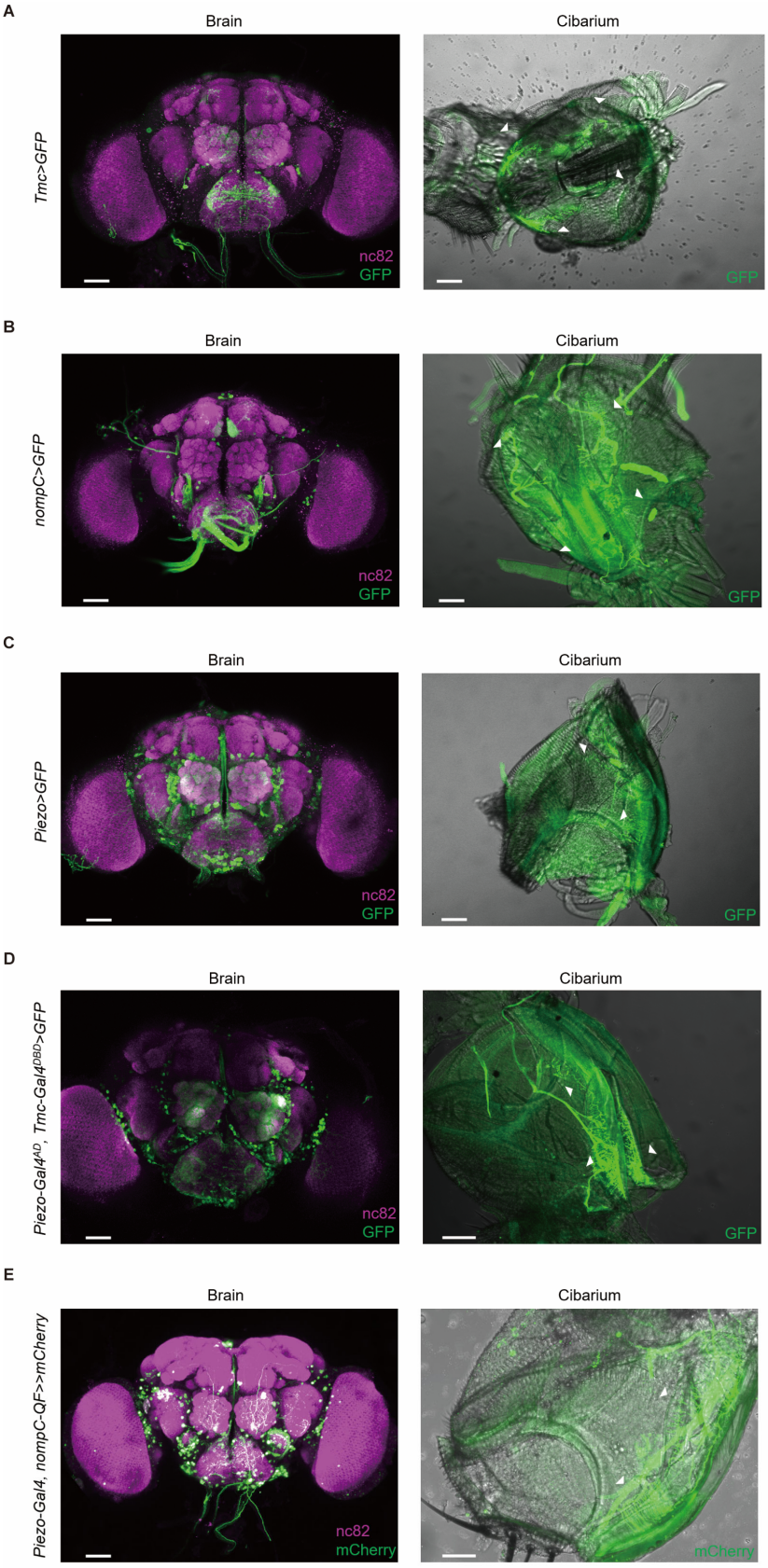
(A-B) Filling time and emptying of the cibarium in flies of different genotypes. n = 6 to 14.

(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


A
Brain
Cibarium
Tmc>GFP
nc82
GFP
GFP
B
Brain
Cibarium
nompC>GFP
nc82
GFP
GFP
C
Brain
Cibarium
Piezo>GFP
nc82
GFP
GFP
D
Brain
Cibarium
Piezo-Gal4AD, Tmc-Gal4DBD>GFP
nc82
GFP
GFP
E
Brain
Cibarium
Piezo-Gal4, nompC-QF>>mCherry
nc82
mCherry
mCherry

523

524 **Figure Supplement 2 Expression pattern of *Tmc*, *nompC*, *piezo*, *Tmc* & *piezo* and *nompC* &**
525 ***piezo* in the brain and cibarium.** Scale bar= 50 μm.

526

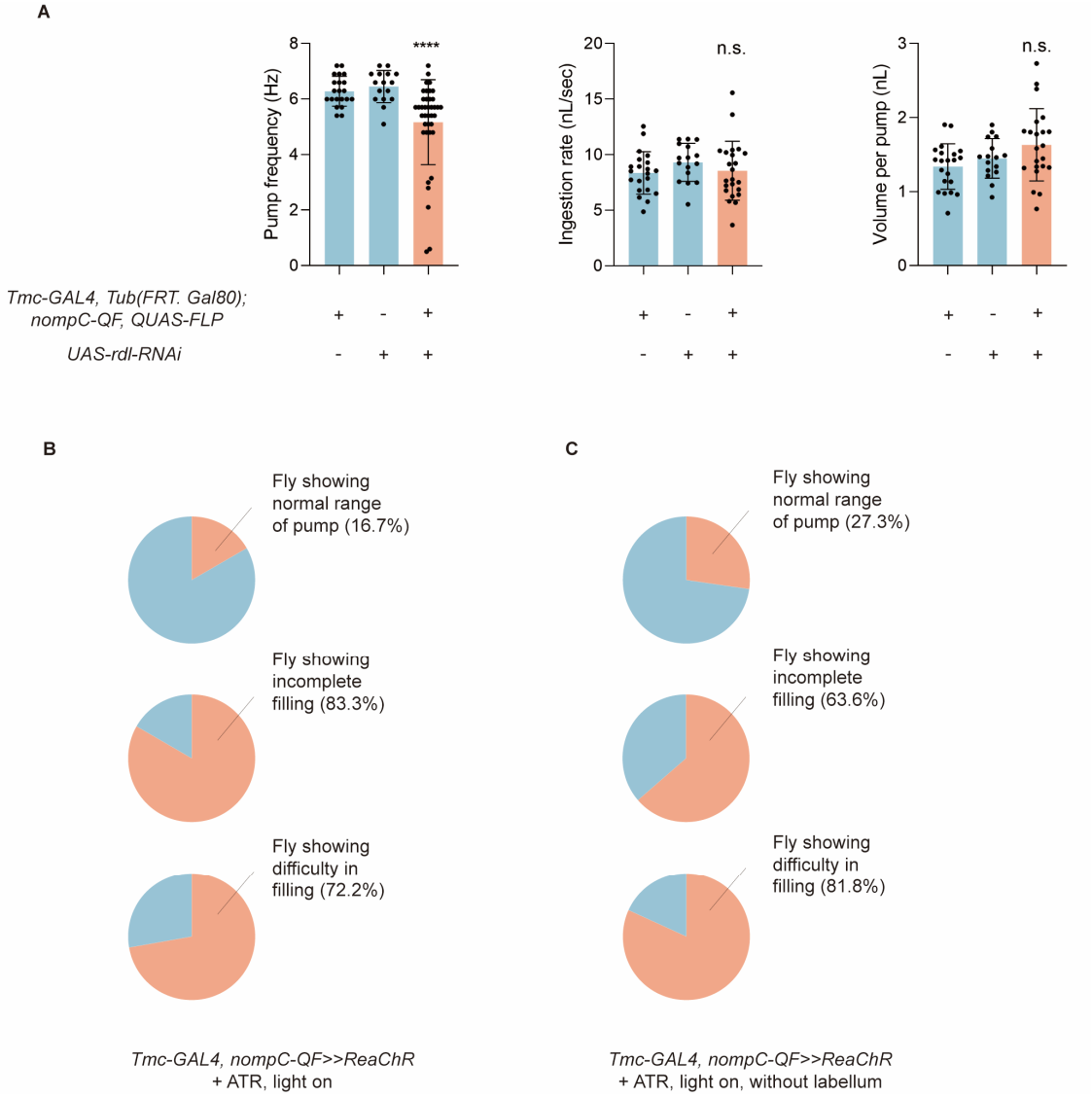


Figure Supplement 3 Regulation of md-C neurons requires the inhibitory inter-neurons.
(A) Knocking down the expression of *$GABA_A$-R* in md-C neurons caused a lower pump frequency. n = 16 to 25, one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used in B and 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.
(B) Flies expressing ReaChR in md-C neurons display dysphagia when stimulated by light. n=18.
(C) Flies with labellum ablated still showed response to light stimulation of md-C, indicating that md-L neurons are not necessary for control of swallow rhythm. n=11.

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.