The pharyngeal nervous system orchestrates feeding behavior in planarians

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Planarians exhibit traits of cephalization but are unique among bilaterians in that they ingest food by means of goal-directed movements of a trunk-positioned pharynx, following protrusion of the pharynx out of the body, raising the question of how planarians control such complex set of body movements for achieving robust feeding. Here, we use the freshwater planarian Dugesia japonica to show that an isolated pharynx amputated from the planarian body self-directedly executes its entire sequence of feeding functions: food sensing, approach, decisions about ingestion, and intake. Gene-specific silencing experiments by RNA interference demonstrated that the pharyngeal nervous system (PhNS) is required not only for feeding functions of the pharynx itself but also for food-localization movements of individual animals, presumably via communication with the brain. These findings reveal an unexpected central role of the PhNS in the linkage between unique morphological phenotypes and feeding behavior in planarians.

INTRODUCTION

Bilaterians comprise a huge number of species that are diverse in morphology and behavior, enabling their survival and reproduction in particular environments. The freshwater planarian Dugesia japonica exhibits traits of cephalization, having a brain and sensory organs such as eyes for photosensation and auricles for chemosensation at the anterior end of the body (1–3). By contrast, its feeding organs (mouth and pharynx) are positioned in the middle portion of the body at a considerable distance from the anterior head (4). Elucidating how planarians achieve robust feeding would be a substantial advance toward identifying neuronal mechanisms underlying a linkage between unique morphological phenotypes and behavior in a given species.

Feeding behavior of the planarian is composed of a complex set of body movements (5): (i) localization movements of individual animals toward a food target (e.g., liver homogenate), (ii) protrusion of the pharynx out of the mouth in response to the target food, and (iii) active intake of the target food from the distal opening of the pharynx into the gut (Fig. 1, A and B). Recent progress in applying a unique technique of RNA interference (RNAi), regeneration-dependent conditional gene knockdown (termed Readyknock), has provided evidence that the brain plays a central role in the integration and processing of sensory information to execute a number of planarian’s behaviors, including food-localization movements (6–8). However, the brain’s role in the regulation of goal-directed protrusive movements of the pharynx in response to chemosensory signals still remains unknown (5). Notably, the planarian pharynx is composed mainly of muscle cells (9), and it undergoes flexible movements (elongation, shortening, and bending in any direction) during feeding and thus can be proposed to be an example of a muscular hydrostat, like cephalopod tentacles and the elephant trunk (10–12). The robust detection and intake of appropriate food by the trunk-positioned pharynx during food-localization movements encouraged us to speculate that the pharyngeal nervous system (PhNS) might have greater functional capacity than we previously thought (13).

In this study, we used the planarian D. japonica to show that the PhNS acts as a functional module to establish a linkage between unique morphological phenotypes and feeding behavior, with important implications for our understanding of how bilaterians increase morphological and behavioral diversity during evolution.

RESULTS

The feeding motor program of the pharynx is generated by the pharynx itself

We recorded the behavior of individual pharynxes isolated from the D. japonica body for 45 s under different conditions of the food within a target object. In the absence of liver homogenate within a target, isolated pharynxes underwent their distal-end-first movements by crawling but never reached the target, resulting in no intake of it (15 of 15; Fig. 1, C and F to I, and movie S1). When liver homogenate was present within a target, isolated pharynxes moved toward the target and successfully took it in with their distal opening with high reproducibility (13 of 15; Fig. 1, D and F to I, and movie S2). Thereafter, rhythmic activity of the pharyngeal pump pushed the ingested matter out of the proximal end of the pharynx (Fig. 1D and movie S2). Quantitative analysis revealed that the planarian pharynx exhibits its own default activity of moving with a constant velocity regardless of whether there is food or not (Fig. 1H). It is noteworthy that the isolated pharynx of the planarian Dugesia ryukyuensis could also take in food successfully, but that of Dugesia tigrina could not because of its poor goal-directed movements toward the food, under the same assay conditions (fig. S1), indicating differences of behavioral traits even among Dugesia species.

Next, we examined whether the isolated pharynx can make appropriate food choices. Turmeric, which is well known as the main spice in curry, has been used as an insect repellent for “kimonos” (traditional Japanese gowns) in Japan. When we fed individual planarians a mixture of liver homogenate and turmeric (62.5 μg/μl as a
standard turmeric concentration), they exhibited strong feeding aversion and never took the mixture in (30 of 30; fig. S2). Isolated pharynxes also exhibited strong feeding aversion against unsuitable food that contained liver homogenate and a full dose or half dose of turmeric (Fig. 1, E and I, and movie S3), providing evidence that they sensed the liver homogenate. These observations indicate that the pharynx can, by itself,
sense different chemical stimuli at the same time and execute a binary decision regarding whether to feed or not.

The PhNS is required for feeding behavior of the pharynx

Next, we examined the role of the PhNS in the regulation of feeding behavior of the isolated pharynx. The PhNS displays a polarized organization along the proximal-distal (PD) axis, and a major nerve ring is positioned close to the distal end of the pharynx used for food ingestion (fig. S3), and its anatomical features are conserved among other planarian species (14-17). Chemosensory function has been ascribed to the tip of the pharynx (14, 17, 18). When an isolated pharynx was bisected along the PD axis, the distal half fragments (that contain the nerve ring) could reproduce not only the entire feeding response to liver homogenate (5 of 5; fig. S4) but also the turmeric-induced feeding aversion (5 of 5). By contrast, the proximal half fragments (that do not contain the nerve ring) never showed such food-directed movements (5 of 5; fig. S4).

Next, we performed Readyknock of D. japonica synaptotagmin (Djsyt), the gene encoding a membrane protein that is localized in synaptic vesicles and acts to trigger neurotransmitter release (19), in the PhNS. Regeneration-dependent replacement of the whole pharynx after Djsyt double-stranded RNA (dsRNA) treatment of animals caused severe reduction of the expression level of DjSYT protein throughout the pharynx and resulted in failure of movements of the isolated pharynx away from the start point, probably due to strong disturbance of the pharyngeal motor function (figs. S5 and S6). By contrast, regeneration-dependent replacement of the distal half of the pharynx caused prominent reduction of the expression level of DjSYT protein in that region, but not in the already-existing proximal half (Fig. 2, A to C, and fig. S5). This contrasting effect is due to

![Fig. 2. Behavioral traits of dsRNA-treated pharynxes with respect to food.](image-url)

(A to C) Regeneration-dependent conditional gene knockdown (Readyknock) of Djsyt in the distal half of the pharynx. (B) Control pharynx that was stained with anti-planarian cytochrome b561 (CYT b561) (magenta) and anti-planarian synaptotagmin (SYT) (green) antibodies to visualize its axonal networks at 11 days of regeneration. (C) Djsyt(RNAi) pharynx that was stained with anti–CYT b561 and anti-SYT antibodies at 11 days of regeneration. A pair of arrowheads indicates a major nerve ring. (D) Behavioral traits of gene knockdown pharynxes for the food-containing target shaded in gray. Each colored line indicates the trajectory of a distinct isolated pharynx during 45 s. In all cases, regeneration-dependent replacement of the distal half of the pharynx was done in the dsRNA-treated animals. (E) Food-directed movements of the isolated pharynxes. Distance between the distal end of isolated pharynx and the target was measured. Bars show the mean ± SE values of the isolated pharynxes. (F) Distance traveled by the distal end of isolated pharynxes during 30 s (Dunnett’s test). (G) Success rates of target intake by isolated pharynxes. (F and G) Control pharynxes, n = 28; Djsyt(RNAi) pharynxes, n = 19; DjChAT(RNAi) pharynxes, n = 19; DjTBH(RNAi) pharynxes, n = 20. Distal is to the left. D, distal; P, proximal; scale bar, 500 µm. Photo credit: Mai Miyamoto, University of Hyogo.
the fact that RNAi in newly regenerated neurons from undifferentiated stem cells (which do not have DjSYT protein) is more effective than RNAi in the already-existing terminally differentiated neurons (which have abundant DjSYT protein that has a long half-life in vivo) (8). Notably, these knockdown isolated pharynxes showed normal locomotive activity (Fig. 2F and fig. S6) but failed to find the food, resulting in a lack of intake of it (Fig. 2, D, E, and G, and fig. S6). Together, these findings provide evidence that synaptic transmission via certain neurotransmitters in the distal half of the PhNS plays an instructive role in the regulation of feeding functions of the pharynx.

Whole-mount in situ hybridization (WISH) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) for genes encoding various rate-limiting enzymes of the biosynthesis of specific neurotransmitters revealed that at least four types of monoaminergic neurons—cholinergic (20), octopaminergic (21), dopaminergic (22), and serotonergic (23) ones—are highly concentrated at the distal end of the pharynx (fig. S3). We found that defective cholinergic signaling following RNAi of the gene *D. japonica choline acetyltransferase* (*DjChAT*) or defective octopaminergic signaling following RNAi of the gene *D. japonica tyramine β-hydroxylase* (*DjTBH*) in the PhNS resulted in failure of food-directed movements of the knockdown isolated pharynxes (Fig. 2, D to G). By contrast, defective dopaminergic signaling following RNAi of the gene *D. japonica tyrosine hydroxylase* (*DjTH*) or defective serotonergic signaling following RNAi of the gene *D. japonica tryptophan hydroxylase* (*DjTPH*) in the PhNS allowed these knockdown isolated pharynxes to complete their entire feeding functions equivalent to those of egfp dsRNA-treated isolated pharynxes as a control, resulting in successful food intake (5 of 5). A food aversion assay revealed that both of *DjTH* knockdown and *DjTPH* knockdown isolated pharynxes underwent chemotactic movements toward turmeric-containing unsuitable food, as we expected (Fig. 3, A to E), but exhibited a significant defect of their feeding aversion. They took in the unsuitable food more actively than the control isolated pharynxes (Fig. 3, B, C, and F).

**The pharyngeal octopaminergic signaling is required for feeding behavior at the individual animal level**

Last, we examined the role of the pharyngeal octopaminergic signaling in the regulation of feeding behavior at the individual animal level by performing Readyknock of *DjTBH*. Intact animals that had received *DjTBH* dsRNA or egfp dsRNA as a control were amputated into three body fragments (head, trunk containing a pharynx, and tail) and allowed to regenerate for 10 days, which was a sufficient period for the recovery of feeding functions under normal conditions.
Anti-DjTBH antibody staining revealed that DjTBH was mainly expressed in two organs, the brain and pharynx, in control regenerates (Fig. 4A and fig. S8). In DjTBH dsRNA-treated animals, regenerates from head fragments showed selective silencing of DjTBH in the pharynx while leaving that in the brain normal, whereas regenerates from trunk fragments showed selective silencing of DjTBH in the brain while leaving that in the pharynx normal, and regenerates from tail fragments resulted in silencing of DjTBH in both the brain and the pharynx (Fig. 4A). In control animals, we confirmed that all regenerates from the three body fragments recovered all steps of their normal motor functions during feeding: localization movements of individual animals toward liver homogenate, pharynx protrusion, and successful food intake (Fig. 4, B to E). DjTBH(RNAi) trunk fragments also showed feeding behaviors equivalent to those of the control trunk fragments, suggesting that octopaminergic signaling in the brain is dispensable for feeding behavior (Fig. 4, B to E). In contrast, DjTBH(RNAi) head fragments showed severe defects in their localization movements toward the food (Fig. 4, B and C), resulting in failure to ingest food (Fig. 4, D and E). We also found that DjTBH(RNAi) tail fragments showed the same defects as those observed in DjTBH(RNAi) head fragments (Fig. 4, B to E). Quantitative analyses demonstrated that there were no statistically significant differences in the feeding behaviors of control and DjTBH(RNAi) planarians.

Fig. 4. Pharyngeal octopaminergic signaling is required for feeding behavior at the individual animal level. (A) Octopaminergic neurons (magenta) in the brain and pharynx of regenerated individuals treated with Readyknock of DjTBH. Anterior is to the top. Cyan, nucleus; scale bars, 20 μm. (B) Heatmap view of averaged odor-localization behavior of individually assayed control and DjTBH(RNAi) planarians (n = 20). Open circle, target area; S, start area; scale bar, 500 μm. (C) Time spent by the assayed control and DjTBH(RNAi) planarians in the target area (Wilcoxon test, ***, P < 0.005; *, P < 0.05). (D) Food intake in control and DjTBH(RNAi) planarians. Food incorporated into the gut was visualized by red color. Anterior is to the left. Scale bars, 200 μm. (E) Feeding index of individually assayed control and DjTBH(RNAi) planarians (n = 20). Defective pharyngeal octopaminergic signaling resulted in the failure of food ingestion at the individual animal level (Wilcoxon test, ***, P < 0.005; ***, P < 0.01). Photo credit: Takeshi Inoue, Gakusyuin University.
differences between DjTBH(RNAi) head and tail fragments (Fig. 4, C and E), indicating that loss of octopaminergic signaling in the brain does not make any contribution to the behavioral defects in DjTBH(RNAi) tail fragments. We conclude that the pharyngeal octopaminergic signaling is required for food-localization movements of individual animals. This unexpected finding suggests that the planarian pharynx may communicate with the brain for orchestrating feeding behavior at the individual animal level.

DISCUSSION

Here, we have established a behavioral assay system for the isolated pharynx in planarians and provided evidence of the sensory and behavioral independence of the pharynx from the central nervous system (CNS). Our findings showed that four distinct types of pharyngeal neurons (cholinergic, octopaminergic, dopaminergic, and serotonergic neurons) are required for executing two distinct behavioral responses (food attraction and aversion) of the pharynx itself (Fig. 5A). Our findings also suggest that the planarian pharynx may send commands to the brain for guiding food-localization movements of individual animals (Fig. 5B), which would enable appropriate positioning of the pharynx for achieving robust food intake. Previous dye tracer experiments revealed that certain pharyngeal neurons connect directly with the brain by extending their long axons (24). The pharyngeal octopaminergic neurons are one of the possible candidates for such neurons.

We have not excluded the possibility that the brain may play a role in the regulation of food-directed protrusive movements of the pharynx in planarians. This possibility is implied by the fact that decapitated D. japonica does not protrude the pharynx when it is placed near food (5). However, we propose a different idea to explain this phenomenon; namely, the brain may signal for executing the mouth opening that allows pharynx protrusion in response to food. Regarding this possibility, we also speculate that the pharynx might send commands to the brain for executing mouth opening when the pharynx senses food. We will assess these hypotheses in future experiments.

Our findings also have important implications regarding the evolution of morphological and behavioral diversity within the phylum Platyhelminthes (flatworms), which comprises species that exhibit broad variation in the position of feeding organs along the anterior-posterior axis (25). The earliest-branching clades, such as the orders Catenulida and Macrostomorpha, retain feeding organs, a mouth and nonprotrusive pharynx, at a position close to the anterior end (25, 26). We speculate that the posteriorizing of feeding organs from their ancestral anterior position in the Platyhelminthes lineage leading to planarians may impose a pressure to increase the sensory and behavioral independence of the pharynx from the sensory–CNS circuits that direct the whole animal movements toward food. The establishment of a division of labor between the PhNS and CNS for the regulation of feeding behavior may have enhanced the evolutionary fitness as it relates to dynamic changes in a flatworm body plan.

MATERIALS AND METHODS

Animals

Two clonal strains (HI and GI) of the planarian D. japonica, which are maintained in autoclaved tap water at 22° to 24°C, were used in this study (27, 28). HI (about 20 mm in body length) was used for a behavioral assay of the isolated pharynx. These planarians were starved for at least 1 week before experiments.

Behavioral assay of isolated pharynxes

The bottom surface of a disposable petri dish (3 cm in diameter) was coated with 1% Blocking reagent (Roche) in PBST [phosphate-buffered saline (PBS) containing 0.1% Triton X-100], which allows isolated pharynxes to move smoothly without being attached to the dish. Three concentric circles (with radius 1, 2, or 3 mm) were carved on the underside of the bottom surface of the dish using a compass. (i) A mixture of 20 μl of pink-colored chalk powder solution [the powder and pure water (1:3) in volume], 10 μl of pure water, and 10 μl of 2% agarose; (ii) a mixture of 20 μl of the chalk powder solution, 10 μl of beef liver homogenate, and 10 μl of 2% agarose; or (iii) a mixture of 10 μl of turmeric (Mascot) in pure water (250 μg/μl), 10 μl of pure water, 10 μl of beef liver homogenate, and 10 μl of 2% agarose was used as a target object. Each mixture (1.3 μl; a minimal pipetting amount for robust uptake and discharge by means of a low retention micropipette tip) was carefully put on the center of the concentric circles of the assay dish and kept at ~20°C for 30 min to allow the agarose to gel. An intact planarian was immobilized on ice, and its epidermal tissues were removed using forceps for easy manipulation of a pharynx. Thereafter, an intact pharynx (about 1 mm in length during muscle contractions) was surgically dissected from the planarian body and was immediately put into an assay dish containing
about 5 ml of 5/8 Holfreter’s solution at 22° to 23°C. To avoid reduction of locomotive activity of the isolated pharynx, we determined minimally essential conditions for the behavioral assay (pharynx’s travel distance and assay period). The distal end of an isolated pharynx faced a target object and was placed at a position between a 2-mm-radius and 3-mm-radius concentric circles using fine-point brushes for starting the behavioral assay. The trajectory of the movement of each isolated pharynx was recorded for 45 s using an ILCE-QX1 interchangeable lens digital camera (Sony). ImageJ was used to obtain the data essential for quantitative analyses based on snapshots of the pharynx behavior recorded every 3 s. The distal end of isolated pharynxes was tracked.

Immunohistochemistry of whole-mount individual planarians
Planarians were treated with 1% HNO3, 50 mM MgCl2 solution for 5 min at room temperature to remove mucus and fixed in 4% PFA, 5% methanol, 50% PBS solution for 30 min at room temperature. Fixed animals were bleached in 5% H2O2, methanol solution under light overnight. After rehybridization, animals were treated with proteinase K (5 μg/ml) and subsequently postfixed with 4% PFA, 5% methanol, 50% PBS solution for 30 min at room temperature. Planarians were stained using rabbit anti-planarian TBH antibody (1:2000 dilution) (21). For the secondary antibody, Alexa Fluor 594–labeled anti-rabbit IgG (H+L) antibody (Thermo Fisher Scientific) was used. Cell nuclei were labeled with Hoechst 33342. Fluorescence was detected using an FV10i confocal laser scanning microscope (Olympus; a 60×/1.34–numerical aperture oil immersion objective lens). Images were processed using FV10-ASW (Olympus). All images were obtained using the same photography conditions to allow direct comparison between experimental animals and controls.

Reverse transcription and qRT-PCR
Ten to 15 pharynxes were divided into halves along the PD axis, and total RNA was isolated from each half using Isogen-LS (Nippon Gene). First-strand complementary DNA was synthesized using the extracted RNA as template and a PrimeScript RT Reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa). qRT-PCR was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). Quantitative analysis of the amount of each gene product was carried out as previously described (32) using Thermal Cycler Dice Real Time System II (TaKaRa). Measured amounts were normalized by the expression level of a constitutively transcribed housekeeping gene, Djgapdh. The mean of three biological replicate qRT-PCR assays was reported. Oligonucleotide primer sequences used for the assays are as follows: Djgapdh, 5′-ACCACCAACTGTGTAGCTCCTCTTA-3′ (forward) and 5′-GATGGTCATCAAGCAGCTTTGCA-3′ (reverse); DjMHC-A, 5′-CTCTGGAAAGAGCTGATCAAGCTGAACAA-3′ (forward) and 5′-TGGATTTACAGTATGTCACCAGGTGCA-3′ (reverse); DjSyt, 5′-GGCATCTGGTGGACATGTG-3′ (forward) and 5′-TTCCGCGATTCTTGAGG-3′ (reverse); DjChAT, 5′-AGCGATCTGATCCTGATTG-3′ (forward) and 5′-TGTCCTCCTGTTTGGCCT-3′ (reverse); DjTBH, 5′-GGTTTCTGATTATTTTAACCTTCCG-3′ (forward) and 5′-TGTGTTTCAATTTTTAACCTTCC-3′ (reverse); DjTHTH, 5′-CAAATCCTTACATGATATACAGG-3′ (forward) and 5′-CCATTTGCTGTTTTAAAATATCTTCC-3′ (reverse); DjTTPH, 5′-CTCCTATGCAGCTGTTGAGGAAATTCTT-3′ (forward) and 5′-AAATCCTCCAGCAGCTGTTTAAATATCTTTCC-3′ (reverse).

Food odor localization assays of individual planarians
Behavioral experiments of individual planarians were performed in a dark room with only red light, the wavelength of which was not sensed by planarians (35). For the assay, the field was divided into quadrants, and a target food was put into the center of a quadrant of the dish. A mixture of 10 μl of pink-colored chalk powder solution, 25 μl of chicken liver homogenate, and 5 μl of 2% agarose was used as target food. A planarian was put into the center of the diagonal quadrant of the quadrant where the target food was placed. Planarian behavior was recorded for 180 s using an ILCE-7 camera (Sony) fixed above the assay field. Trajectories were tracked once per second, and
Feeding index
For quantification of the intake of food, planarians were allowed to feed freely on target food for 30 min and then were put on ice for observation. Pink-colored chalk was detected using a Texas Red fluorescence filter, and the planarian’s shape was detected by bright-field microscopy. Fluorescence was quantified using the ImageJ program, and fluorescence intensity was expressed as the food intake after binarization with a certain threshold. Food index was calculated using Eq. 1

\[
\text{Feeding index} = \frac{IF}{BA}
\]  

(1)

where IF is the area of the fluorescence and BA is the area of the body margin of an individual.

Statistical analysis
Statistical analysis was carried out using one-way analysis of variance (ANOVA), two-tailed Student’s t test, Dunnett’s multiple comparisons test, Wilcoxon test, or Fisher’s exact test where applicable. A value of \( P < 0.05 \) was considered statistically significant in all tests.

SUPPLEMENTARY MATERIALS
Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/15/eaa0882/DC1

View/request a protocol for this paper from Bio-protocol.

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