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Source: Zoological Science, 33(3) : 311-319

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs150170

ZOOLOGICAL SCIENCE **33**: 311–319 (2016)

Multiple Neuropeptide-Coding Genes Involved in Planarian Pharynx Extension

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Planarian feeding behavior involves three steps: moving toward food, extending the pharynx from their planarian's ventral side after arriving at the food, and ingesting the food through the pharynx. Although pharynx extension is a remarkable behavior, it remains unknown what neuronal cell types are involved in its regulation. To identify neurons involved in regulating pharynx extension, we quantitatively analyzed pharynx extension and sought to identify these neurons by RNA interference (RNAi) and in situ hybridization. This assay, when performed using planarians with amputation of various body parts, clearly showed that the head portion is indispensable for inducing pharynx extension. We thus tested the effects of knockdown of brain neurons such as serotonergic, GABAergic, and dopaminergic neurons by RNAi, but did not observe any effects on pharynx extension behavior. However, animals with RNAi of the *Prohormone Convertase 2* (*PC2*, a neuropeptide processing enzyme) gene did not perform the pharynx extension behavior, suggesting the possible involvement of neuropeptide(s in the regulation of pharynx extension. We screened 24 neuropeptide-coding genes, analyzed their functions by RNAi using the pharynx extension assay system, and identified at least five neuropeptide genes involved in pharynx extension. These was expressed in different cells or neurons, and some of them were expressed in the brain, suggesting complex regulation of planarian feeding behavior by the nervous system.

Key words: planarian, feeding behavior, pharynx extension, neurotransmitter, neuropeptide, RNAi

INTRODUCTION

Observation of the feeding behavior of the freshwater carnivorous planarian *Dugesia japonica* revealed that this behavior can be divided into three major processes: moving toward the food source (chemotaxis, Fig. 1A), extension of the pharynx (pharynx extension, Fig. 1B), and ingestion of food through the extended pharynx (ingestion, Fig. 1C) (Pearl, 1903; Inoue et al., 2015). Some amino acids induce chemotaxis in planarian (Coward and Johannes, 1968; Ash et al., 1973; Miyamoto and Shimozawa, 1985). However, although the structure and regeneration of the pharynx have been studied extensively (Kobayashi et al., 1998, 1999; Adler et al., 2014), planarian feeding behavior has not been analyzed.

The planarian central nervous system (CNS) has been extensively studied in recent years and its regeneration process is also well characterized (Cebrià et al., 2002; Agata et al., 2008; Umesono et al., 2011). The planarian CNS is composed of two morphologically distinct structures: an inverted U-shaped bi-lobed brain with nine lateral branches on each outer side, and a pair of two longitudinal ventral nerve cords (VNCs) (Supplementary Figure S1; Agata et al., 1998). Combinations of planarian behavior assay systems and RNAi experiments have identified neurons involved in particular behaviors, such as movement, phototaxis and thermotaxis (Inoue et al., 2004; Takano et al., 2007; Nishimura et al., 2008b; Inoue et al., 2014). A negative phototactic behavior assay demonstrated that two neuropeptide-coding genes, *1020HH* and *eye53*, are essential for the recovery of negative phototactic behaviors during regeneration (Inoue et al., 2004). It has also been shown that GABAergic neurons, which may connect with visual center neurons in the brain, are indispensable for negative phototaxis in planarians (Nishimura et al., 2008b; Akiyama et al., unpublished data). A thermosensing behavior assay system revealed that brain

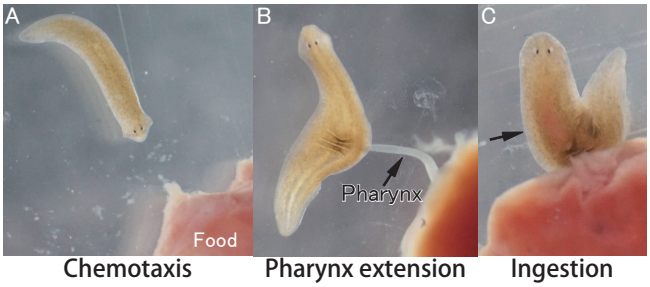

Fig. 1. Planarian feeding behavior consists of three steps. **(A)** Chemotaxis: Planarians move to food (chicken liver). **(B)** Pharynx extension: Planarians extend their pharynx from the ventral side of the body after arriving at food. Pharynx is indicated by a black arrow. **(C)** Ingestion: Planarians ingest food via the pharynx. The color of the intestinal duct changes to red (the color of the food, black arrow).

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Supplemental material for this article is available online.
doi:10.2108/zs150170

serotonergic neurons connected to transient receptor potential (TRP)-positive thermosensing neurons are required for planarian thermotaxis (Inoue et al., 2014). Dopaminergic neurons and serotonergic neurons are likewise essential for regulating normal movement behaviors (Nishimura et al., 2007a; Currie and Pearson, 2013). A combination of RNAi and behavior assays would thus appear to be useful for investigating genes and neural circuits regulating planarian behaviors.

Our group previously reported that brain activity is required for the chemotactic behavior in planarian (Inoue et al., 2015), but although pharynx extension is one of the most fundamental planarian behaviors, the mechanisms underlying this behavior are not well elucidated at the cellular and molecular levels. In the present study, we sough to to identify neurons involved in regulating pharynx extension through a quantitative pharynx extension assay and RNAi experiments.

MATERIALS AND METHODS

Animals

A clonal asexual strain (SSP) of *Dugesia japonica* was used for all experiments (Asami et al., 2002). Planarians were bred in water containing 0.05 g/L artificial seawater (Instant Ocean, VA, USA) at 24°C. They were fed chicken liver once a week. Six- to 8-mm-long planarians that had been starved for 7–9 days were used for all experiments. For regeneration analysis, planarians were amputated on wet filter paper on ice. All planarians were maintained and manipulated according to a protocol approved by the Animal Care and Use Committee of Kyoto University.

Behavior assay

Liver extract was prepared as previously reported (Inoue et al., 2015). For the novel pharynx extension behavior assay devised here, planarians were put into a small linear chamber (2 mm wide × 0.5 mm deep) filled with their breeding water (Supplementary Figure S2A), and then 5 μL of 10-fold diluted chicken liver extract was placed in front of them (Supplementary Figure S2A). To visualize the pharynx extension, planarian behavior was recorded using a video camera from underneath the chamber by using a mirror, as illustrated in Supplementary Figure S2A. Usually, 12 planarians were used in one series of experiments, and the number of planarians extending their pharynx was counted after video recording. We conducted 3–6 repetitions of each experiment to obtain statistically reliable results. The number of individuals that extended their pharynx was counted (number of degrees of freedom = 1). The results were shown as the average pharynx extension rate in the total experiments, and the standard error of the mean (s.e.m.) among experiments.

For the primary feeding behavior assay, 10 planarians were put at the edge of a 90-mm-diameter Petri dish, and a slice of chicken liver was placed in the center of the dish (Supplementary Figure S2B). At 20 minutes, the planarians which had arrived at the food and started to ingest it were counted.

For evaluation of the planarians' locomotor activity, the distance that planarians moved during a test period (5 min) was recorded while they moved randomly in the chamber (10 × 60 mm), and the distance moved was measured with behavior analysis software (SMART) (Panlab, Spain).

RNA interference

Double-stranded RNA (dsRNA) synthesis and RNAi treatment were performed according to Shibata et al. (2012) and Rouhana et al. (2013). For the pharynx-extension assay, planarians were fed dsRNA-containing food twice and also injected with dsRNA dissolved in H_2O to fill their intestinal ducts every three days using a microinjector (Drummond Scientific Nanoject injector, Broomall, PA, USA). Nine days after amputation, planarians were used for behavior assay and real-time RT-PCR. For negative controls, planarians were injected with dsRNA coding for *green fluorescent protein* (*GFP*), a gene that is not found in planarians. cDNA sequences for the following genes were obtained from the DDBJ/EMBL/GenBank databases: *TH*, *tyrosine hydroxylase*, a gene encoding an enzyme limiting dopamine synthesis, AB266095 (Nishimura et al., 2006); *TBH*, *tyramine β-hydroxylase*, a gene encoding an enzyme limiting serotonin synthesis, AB362394 (Nishimura et al., 2008a); *TPH*, *tryptophan hydroxylase*, a gene encoding an enzyme limiting octopamine synthesis, AB288367 (Nishimura et al., 2007b); *ChAT*, *choline acetyltransferase*, a gene encoding an enzyme limiting acetylcholine synthesis, AB536929 (Nishimura et al., 2010); *GAD*, *glutamic acid decarboxylase*, a gene encoding an enzyme limiting GABA synthesis, AB332029 (Nishimura et al., 2008b); *glutaminase*, a gene encoding an enzyme limiting glutamic acid synthesis, BAG16389 (Higuchi et al., 2008); *synaptotagmin* (*syt*), BAA85622 (Tazaki et al., 1999); and *Prohormone convertase 2* (*PC2*), AK388877 (Agata et al., 1998). To induce effective gene knockdown, we used planarians after head amputation (Sánchez Alvarado and Newmark, 1999; Takano et al., 2007).

Cloning of genes coding for neuropeptide precursors of *D. japonica*

Partial or full-length sequences of neuropeptide genes, putative orthologs of neuropeptide genes in other planarian species (Collins et al., 2010), were obtained from our EST database (Nishimura et al., 2012) and transcriptome database (Kashima et al., unpublished) of *D. japonica*. To clone these genes, we performed PCR using specific primers (Supplementary Table S1) corresponding to the planarian sequences of interest using cDNA derived from intact planarians (Yazawa et al., 2009). These PCR fragments were cloned using pCR II plasmid (Invitrogen, CA, USA).

In situ hybridization

Digoxigenin-labeled antisense RNA probes were prepared and whole-mount in situ hybridization was performed as described previously (Agata et al., 1998; Tasaki et al., 2011).

To clearly detect signals in the pharynx, the pharynx was amputated from the planarian body on ice, and then fixed and hybridized with the probe of interest. The in situ hybridization of the isolated pharynx was performed using a modification of the protocol for whole-mount in situ hybridization as follows: fixation for 10 min, no decoloring or hydration, treatment with 5 μg/mL proteinase K for 10 min, re-fixation for 10 min and hybridization with probes for 16 hours. Photographs were taken using a Leica M205FA microscope (Leica Microsystems, Germany).

For observation of transverse sections, after detection of in situ hybridization signals of whole-mount planarians or of the isolated pharynx, the samples were fixed with 1% glutaraldehyde in PBS for one hour and subsequently cut transversely.

To distinguish the dorsal- and ventral-side in transverse sections, whole-mount planarians were incubated with 10 μg/ml Hoechst 33342 (Thermo Fisher Scientific) in PBS and then fixed with 1% glutaraldehyde in PBS for one hour, and subsequently these planarian samples were cut transversely. The in situ hybridization signals of the sections were detected using an Olympus BX62 microscope. In these transverse sections, the ventral nerve cords (VNCs) did not show any Hoechst 33342 staining signals because the VNCs consisted only of axons.

Semi-quantitative RT-PCR analysis

Reverse transcription was carried out using total RNA from six planarians at nine days post-amputation using a Quantitect Reverse Transcription kit (Qiagen, Netherlands). Semi-quantitative-PCR

(qPCR) was performed as reported previously (Yazawa et al., 2009) using the primers listed in Supplementary Table S2. The expression level of genes in *GFP*(*RNAi*) planarians was taken as = 1. Measurements were performed four times for technical replicates and three times for biological replicates and were normalized by the expression level of *DjEF-1*, D49924 (Mineta et al., 2003).

Statistical analysis

The statistical significance of differences between pharynx extension rate test results was determined by the chi-square test; p values greater than 0.05 were taken as not significant (NS) when examining the total value obtained from several experiments. The statistical significance of differences was evaluated by comparison with the value in intact animals in amputation assays, and by comparison of the value in *GFP*(*RNAi*) planarians with the value in other RNAi planarians in other assays.

The statistical significance of differences between untreated planarians and RNAi planarians in the distance-moved and qPCR assays was determined by the Steel test after F-tests for the equality of variances: p values greater than 0.05 were taken as not significant (NS).

Data deposition

Neuropeptide cDNA sequences were deposited in DDBJ/EMBL/GenBank databases with the accession numbers listed in Table 4.

RESULTS

Quantitative observation of planarian pharynx-extension

To determine the optional dilution of liver extract for use in the pharynx-extension assay, one of a range of dilutions, including undiluted extract, was put into the chamber in front of a planarian, and the number of pharynx-extending planarians was counted in three experiments and shown as the pharynx extension rate (Supplementary Table S3). Dilutions of liver extract in the range between undiluted and 1:10 diluted liver extract induced pharynx extension in more than 85% of planarians on average. However, undiluted liver extract induced not only pharynx extension but also ingestion in some cases (data not shown). We thus used 1:10 diluted liver extract in further analyses of pharynx extension.

Pharynx extension requires the head region

In order to investigate how the pharynx extension is regulated, the pharynx-extension assay was performed using planarians cut into several fragments. It had been thought that the planarian might extend its pharynx in response to liver extract using the nerve ring of the tip of the pharynx (Supplementary Figure S1; Tazaki et al., 1999; Okamoto et al., 2005). We therefore tested pharynx extension using intact, head-less (head amputated) and tail-less (tail amputated) planarians at three hours after amputation. Unexpectedly, the head-less planarians never extended their pharynx at all (Table 1), suggesting that pharynx extension requires the head, and that the pharynx by itself cannot be extended as a reflex movement. Tail-less planarians extended the pharynx normally, like intact planarians, suggesting that simple amputation of the nervous system does not affect pharynx extension.

Next, we closely observed the process of recovery of pharynx-extension ability during regeneration after head amputation. Pharynx-extension ability was gradually recovered in head-less planarians during head regeneration (Fig. 2, open circles). At day 5, half of the head-less planarians undergoing head regeneration had recovered pharynx-extension ability, and most of them had recovered this ability by seven days after amputation.

Monoaminergic and amino acidergic neurons may not be involved in pharynx extension

Previous studies identified six types of monoaminergic neurons (dopaminergic, octopaminergic, serotonergic and cholinergic) and amino acidergic neurons (GABAergic and glutamatergic) in planarian based on their expression of the gene for a rate-limiting enzyme for the respective neurotransmitter (Nishimura et al., 2007a, b, 2008a, b, 2010). To identify the subtype of brain neurons involved in pharynx extension, we analyzed the possible functions of these monoaminergic and amino acidergic neurons by combinatory analysis using the pharynx-extension assay system and RNAi. Unexpectedly, none of these six types of RNAi-treated planarians showed any statistically significant effects on pharynx extension (Table 2). There was almost no difference in the results of three independent experiments. The efficiency of RNAi was tested by qPCR (Supplementary Figure S3A). Significant reduction of the *TPH* and *ChAT* genes was not observed, so we could not exclude the possibility that *TPH* and *ChAT* are related pharynx extension.

Table 1. Pharynx extension rate of amputated planarians. **, $P < 0.005$. $t = 120$ sec; $n = 43$ or 44.

Amputated planarians	No. of planarians that extended pharynx/Number of planarians				Avg. pharynx extension rate ± s.e.m.
	Ex.1	Ex.2	Ex.3	Total	
Intact	10/12	12/12	20/20	42/44**	94.4 ± 5.5%
Head-less	0/12	0/11	0/20	0/43**	0.0 ± 0.0%
Tail-less	10/12	11/12	16/20	37/44**	85.0 ± 3.5%

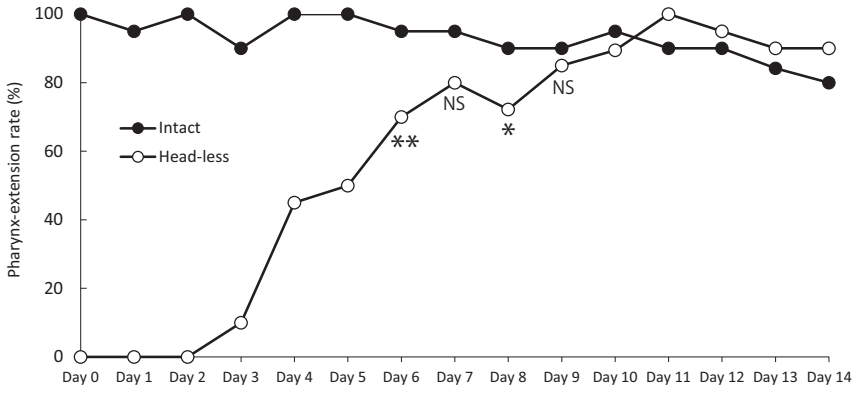

Fig. 2. Recovery of pharynx-extension ability during 0–14 days after amputation. The statistical significance of differences of the pharynx-extension rate was determined between the data of intact planarians at Day 0 to Day 14, and the data of head-less fragments of planarians on each of the respective days. *, $P < 0.05$; **, $P < 0.005$; NS, not significantly different; $t = 120$ sec; n (number of animals) = 16–20.

Table 2. Pharynx extension rate of planarians with RNAi of the genes coding for limiting enzymes for synthesis of monoamine and amino acid neurotransmitters. t = 120 sec; n = 31–72. Distance moved. t = 300 sec; n = 11–24.

Planarians	No. of planarians that extended pharynx/Number of planarians					Avg. pharynx extension rate ± s.e.m.	Distance moved ± s.e.m.
	Ex.1	Ex.2	Ex.3	Ex.4	Total		
GFP(*RNAi*)	21/24	10/12	19/24	9/12	59/72	81.9 ± 2.6%	144.5 ± 9.5 mm
TH(*RNAi*)	11/12	9/11	10/11	–	30/34	88.1 ± 3.2%	114.1 ± 13.9 mm
TBH(*RNAi*)	9/11	10/11	7/9	–	26/31	83.5 ± 3.5%	108.2 ± 18.7 mm
TPH(*RNAi*)	11/12	11/12	11/12	–	33/36	91.7 ± 0.0%	118.1 ± 14.6 mm
ChAT(*RNAi*)	11/11	10/12	12/12	–	33/35	94.4 ± 5.6%	148.8 ± 12.6 mm
GAD(*RNAi*)	12/12	11/11	7/8	–	30/31	95.8 ± 4.2%	164.8 ± 9.9 mm
glutaminase(*RNAi*)	9/12	11/12	10/11	–	30/35	85.9 ± 5.4%	96.3 ± 17.2 mm

Table 3. Pharynx extension rate of *syt*(*RNAi*) and *PC2*(*RNAi*) treated planarians. $^{**}P < 0.005$; t = 120 sec; n = 55–60. Distance moved. $^{**}P < 0.005$; t = 300 sec; n = 12–60.

	No. of planarians that extended pharynx/Number of planarians						Avg. pharynx extension rate ± s.e.m.	Distance moved ± s.e.m.
	Ex.1	Ex.2	Ex.3	Ex.4	Ex.5	Total		
GFP(*RNAi*)	12/12	11/12	12/12	10/12	9/12	54/60**	90.0 ± 4.9%	121.3 ± 6.9 mm**
syt(*RNAi*)	8/10	8/10	9/12	8/11	10/12	43/55**	78.2 ± 1.9%	106.5 ± 8.0 mm**
PC2(*RNAi*)	0/11	2/12	0/12	3/10	1/12	6/57**	11.0 ± 5.7%	10.4 ± 1.4 mm**

Although the locomotion activity in planarians treated with RNAi against for each of these genes was unaffected (Table 2), *GAD*(*RNAi*) planarians did show defects in phototactic behavior, in agreement with previously reported findings (data not shown) (Nishimura et al., 2008b). These results suggested that dopamine-, octopamine-, GABA- and glutamic acid-producing neurons may be not involved in pharynx extension.

Neuropeptide-producing neurons may be involved in pharynx extension

Given this surprising result, what types of neurons are involved in the regulation of pharynx extension? We conducted RNAi experiments of two pan-neural genes, the *syt* and *PC2* genes, and confirmed the reduction of the level of the target mRNAs by qPCR (Supplementary Figure S3B). Although both of these genes are widely expressed in the CNS and are used as pan-neural markers, it has been reported that *syt*(*RNAi*) and *PC2*(*RNAi*) respectively do not accumulate in small synaptic vesicles or large neurosecretory vesicles in presynaptic regions (Oosaki and Ishii, 1965; Takeuchi et al., unpublished observation). *syt*(*RNAi*) planarians showed almost normal pharynx-extension behavior, and the locomotion activity of *syt*(*RNAi*) planarians was unaffected (Table 3). In contrast, pharynx extension was dramatically reduced in *PC2*(*RNAi*) planarians (Table 3). The enzyme PC2 is known to be involved in the first step of processing neuropeptide precursors to mature forms in pig and many other species (Seidah et al., 1992; Zhou et al., 1999). The active site sequence of PC2 family proteins is conserved in the predicted planarian PC2 protein, although no direct functional analysis has been reported in planarian. The reduction of pharynx extension of *PC2*(*RNAi*) planarians suggested that neuropeptide-producing neurons may be involved in pharynx extension.

Neuropeptide-coding genes in *D. japonica*

Next, we searched for genes coding for neuropeptides and peptide-hormones in our EST database and in our transcriptome database that was obtained by next-generation sequencing using a Roche 454 system (Nishimura et al., 2012; Kashima et al., unpublished). The DNA sequence data of genes that had been identified as genes coding for neuropeptides and peptide-hormones of another planarian species, *Schmidtea mediterranea* (Collins et al., 2010), were used as queries for BLAST searches. The amino acid sequences of BLAST hits were compared, and conservation of landmark sequences of neuropeptides, such as GKR and signal peptides, was confirmed (Table 4). We identified 21 neuropeptide and peptide-hormone coding genes from *D. japonica* as homologs of related genes in *S. mediterranea*. Two of these cDNA clones (*Dj_aH_019_P02* and *Dj_aH_401_P19*) were found in our EST library (Nishimura et al., 2012). The remaining 19 cDNA clones were obtained by PCR using specific primers (Supplementary Table S1). We also added the following genes, which had previously been identified in our laboratory as neuropeptide-coding, to the list of neuropeptide genes: *1020HH* (Inoue et al., 2004), *eye53* (Inoue et al., 2004) and *Dj_aH_308_M24* (Takatsu et al., unpublished data). A total of 24 neuropeptide-coding genes were thus taken to be candidates for further analysis as putative neuropeptide neurons involved in pharynx extension (Table 4).

Candidate genes possibly involved in feeding behavior were narrowed down using a simple assay

In order to select from the above 24 candidates, we utilized a simple feeding-behavior assay using planarians treated with RNAi for each candidate gene. Ten RNAi-treated planarians were placed at the periphery of a Petri dish (90 mm diameter), and a piece of chicken liver was placed at the center of the dish (Supplementary Figure S2B). The number of planarians that started eating the food was counted after 20 minutes. Of the 24 candidate genes, we selected the 13 genes that caused reduction of feeding activity to < 60% of the control level in this assay as candidates for secondary screening (Supplementary Figure S4).

Identification of five genes as genes required for pharynx extension

After thus narrowing the field of *D. japonica* neuropeptide-coding gene candidates to 13, we used these to conduct a full pharynx-extension assay using RNAi planarians nine days after amputation (Table 5, Supplementary Figure S3C). The five candidates (*Dj_aH_308_M24* (*DjNpM24*), *Dj_aH_019_P02* (*DjNpP02*), *DjNp19*, *DjNp42* and *DjNp47*)

Table 4. Neuropeptide-coding genes reported previously and newly identified here in *D. japonica*.

Gene name	Predicted Peptide(s)	Query for BLAST search	Reference	Accession number
1020HH	YSYLKGGVRW, PNYRNNRYLKGGIRW		Inoue et al., 2004	AB126830
eye53	LSIPTYWDEMDPN, LSVPTYYDEWDAR, LSVPSYYEDWDNK		Inoue et al., 2004	AB126831
Dj_aH_308_M24	RGLI(× 6)		Nishimura et al., 2012	FY943272
Dj_aH_019_P02	KHIGHQIFRL, GYHFFRL	*ssp-18,19*	Collins et al., 2010 Nishimura et al., 2012	FY929204
Dj_aH_401_P19	AYWASRM	*spp-1*	Collins et al., 2010 Nishimura et al., 2012	FY949649
DjNp3	LPRHGDNLRTYDSVLEELNNYEPIY, QSYLTGGIRYKKREL,YLTGGIRY	*Sm1020HH-2*	Collins et al., 2010	LC085450
DjNp4	LNYLTGGIRY	*Sm1020HH-2*	〃	LC085451
DjNp9	ALVPDAWDDWEL,AVVPDAWDDWDI	*eye53-2*	〃	LC085452
DjNp12	YDTGHDIFRL, GYHYFRLRRTLNQMKCSSDPKAIMSFIE	*grh-1*	〃	LC085453
DjNp17	AKYFRL(× 3), SYDSSALD	*npp-22*	〃	LC085454
DjNp19	AIFLTRF	*npp-3*	〃	LC085455
DjNp23	FDYPFQF(× 4), FDPIMF(× 4), FDYPFQF	*spp-15*	〃	LC085456
DjNp25	SAWRDMPW(× 4), NAWRDMPW	*npp-5*	〃	LC085457
DjNp28	DSRVDIYRKSIFSSPEARRYLQQMNEYLAIVARPRY	*npy-5*	〃	LC088229
DjNp34	YFSPRM(× 2)	*ppl-1*	〃	LC085458
DjNp35	RSYYDPIGGSLL, SYYDPIGGSLL, SYYDPIGGSLLK	*ppp-2*	〃	LC085459
DjNp40	GLRILRM, DELFRLLN, GMRHMRL	*spp-5*	〃	LC085460
DjNp41	GLRLMRL, NLEDDNVIQIRDM	*spp-5*	〃	LC085461
DjNp42	TMGFGLNSNYRLY, LLE	*spp-8*	〃	LC085462
DjNp47	NQKSHENSQYPLVFRE	*spp-10*	〃	LC085463
DjNp49	NYMDFFGLNGDMQRF, QQFHRNHRPEFEWN	*spp-12*	〃	LC085464
DjNp51	FDPIMF(× 3), FDPIQF(× 5), FDPIMF	*spp-15*	〃	LC085465
DjNp52	VRSGVQRYYVTRGENFRDYI, QFDPIMY(× 2), QHNPSYYNRIGL	*spp-16*	〃	LC085466
DjNp56	GLRLMRL, NLEDDNVIQIRDM	*spp-5*	〃	LC085467

Table 5. Identification of genes required for pharynx extension. **P* < 0.05; ***P* < 0.005; *t* = 120 sec; *n* = 54–131. Distance moved. ***P* < 0.005; *t* = 300 sec; *n* = 12–108.

Planarians	No. of planarians that extended pharynx/Number of planarians							Avg. pharynx extension rate ± s.e.m.	Distance moved ± s.e.m.
	Ex.1	Ex.2	Ex.3	Ex.4	Ex.5	Ex.6	Total		
GFP(*RNAi*)	20/23	12/12	33/36	33/36	11/12	11/12	117/131	88.1 ± 3.5%	118.8 ± 4.8 mm
syt(*RNAi*)	9/11	8/10	8/10	9/12	8/11	10/12	43/66	78.8 ± 1.7%	106.5 ± 8.0 mm
PC2(*RNAi*)	2/12	0/11	2/12	0/12	3/10	1/12	8/69**	11.9 ± 4.7%	10.4 ± 1.4 mm**
1020HH(*RNAi*)	10/12	10/12	11/12	12/12	8/10	–	51/58	87.7 ± 3.6%	115.9 ± 6.3 mm
DjNpM24(*RNAi*)	7/12	8/12	7/12	7/12	9/12	10/12	48/72**	66.7 ± 4.3%	138.9 ± 8.2 mm
DjNpP02(*RNAi*)	5/12	9/12	7/12	8/12	10/12	–	39/60**	65.0 ± 7.2%	105.1 ± 6.5 mm
DjNp4(*RNAi*)	8/12	10/12	12/12	9/10	8/8	–	47/54	88.0 ± 6.2%	104.2 ± 6.8 mm
DjDjNp9(*RNAi*)	8/11	8/12	10/12	10/12	9/10	–	45/57	79.2 ± 4.2%	100.6 ± 7.0 mm
DjNp17(*RNAi*)	9/12	10/12	9/12	10/12	8/10	–	46/58	79.3 ± 1.9%	103.1 ± 5.3 mm
DjNp19(*RNAi*)	5/12	6/12	9/12	8/12	11/11	–	39/59**	66.7 ± 10.2%	132.8 ± 7.1 mm
DjNp23(*RNAi*)	8/12	9/12	10/12	10/12	8/9	–	44/57	77.8 ± 3.8%	109.4 ± 6.6 mm
DjNp40(*RNAi*)	11/12	11/12	8/12	9/11	10/12	–	49/59	83.0 ± 4.6%	106.9 ± 10.0 mm
DjNp42(*RNAi*)	5/12	6/12	7/11	9/12	5/12	8/10	40/69**	58.7 ± 6.8%	102.8 ± 5.8 mm
DjNp47(*RNAi*)	6/12	8/12	10/12	6/11	7/10	–	37/57**	64.9 ± 5.9%	119.2 ± 7.7 mm
DjNp51(*RNAi*)	10/12	8/12	8/12	9/11	9/11	9/11	44/58*	76.1 ± 3.9%	106.0 ± 6.0 mm
DjNp52(*RNAi*)	11/12	11/12	12/12	11/12	10/10	–	55/58	95.0 ± 2.0%	114.2 ± 8.7 mm

causing a statistically significant effect ($P < 0.005$) were selected as genes putatively required for pharynx extension, and subjected to further analyses to identify the cells/neurons in which they are expressed (Table 5). Importantly, there was almost no difference among the results of several independent experiments (Table 5). We did not observe any significant defect in locomotion ability in any of these five RNAi-treated planarians (Table 5).

Expression patterns of the five neuropeptide-coding genes found to be required for pharynx extension

We then examined the expression patterns of the five neuropeptide-coding genes found to be required for pharynx extension by whole-mount in situ hybridization. To investigate the detailed expression patterns of these genes in the pharynx, we conducted both general whole-mount in situ hybridization and in situ hybridization using the isolated pharynx (see Materials and Methods), since RNA probes sometimes did not soak into the pharynx in whole-body specimens. Ventral views of whole-mount in situ hybridization staining of planarians are shown in Fig. 3A. Figure 3B shows the whole-mount in situ hybridization staining patterns of the corresponding isolated pharynxes.

DjNpM24- and *DjNp19*-positive in situ hybridization signals were detected in a minor population of CNS cells, including in the brain. Higher-magnification views of *DjNpM24* and *DjNp19* signals in the head region are shown in Fig. 3C. Expression of *DjNp19* was not detected in the pharynx, while *DjNpM24*-positive cells were detected in the pharynx, in addition to the CNS. The number of *DjNpM24*-positive cells was larger in the distal part of the pharynx than in the basal part (Fig. 3B, D). In the case of *DjNp47*, a clear signal was not detected in the whole-mount specimen, but qPCR analysis suggested that *DjNp47* may also be expressed in the head and pharynx (Fig. 3E).

In contrast to the expression patterns of the above three genes, *DjNpP02*-positive cells were detected sparsely in the body surface region (Fig. 3A). Examining the expression pattern of this gene in transverse sections revealed that *DjNpP02*-expressing cells are present in both the ventral and dorsal sides of the body surface region (Fig. 3D, arrowheads; Supplementary Figure S5). *DjNp42*-expressing cells were detected only in the pre-pharyngeal region of the ventral side, which is defined as the body

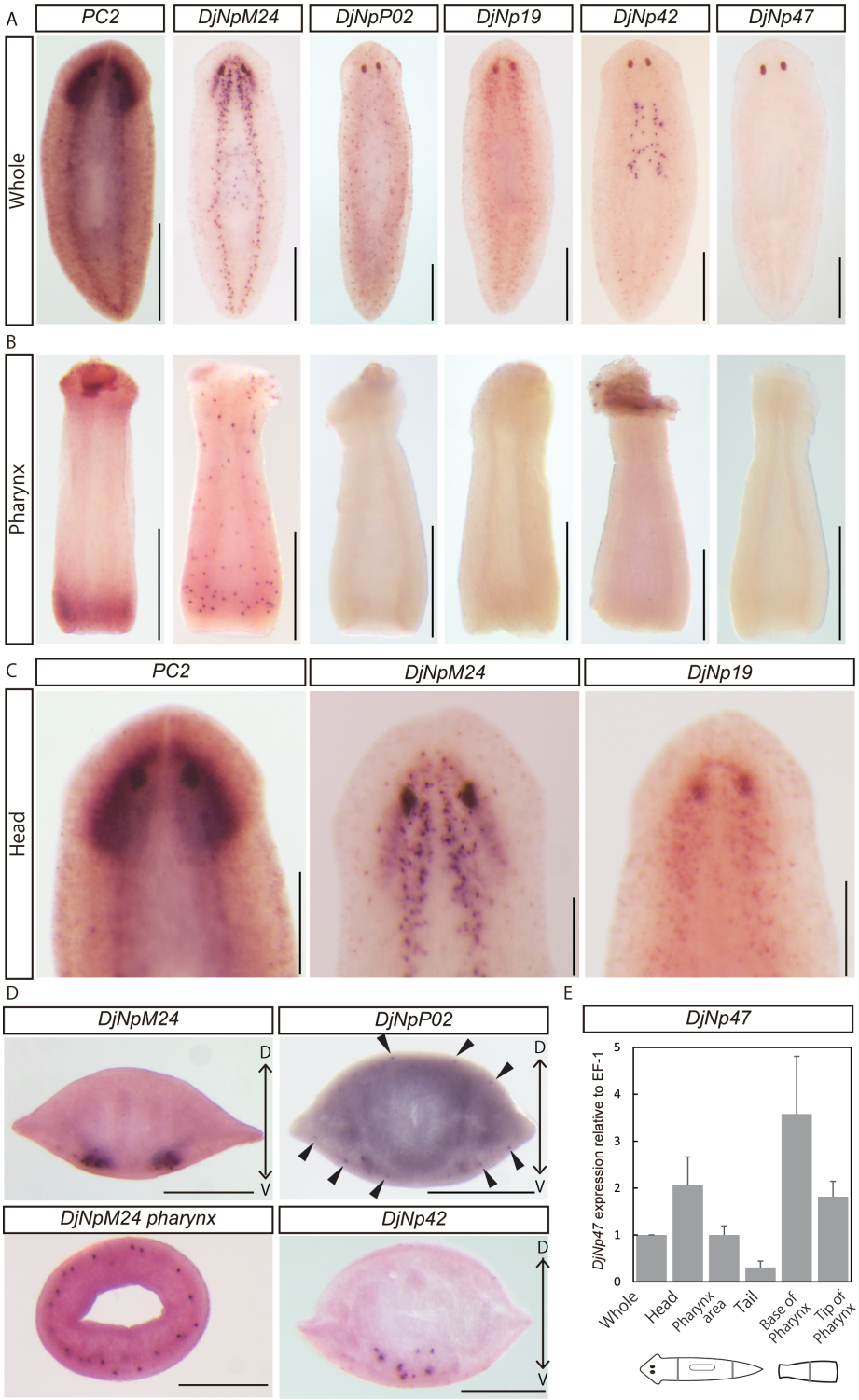

Fig. 3. Expression pattern analysis of genes encoding neuropeptides involved in pharynx extension. **(A)** Expression pattern of neuropeptide genes detected by in situ hybridization in the whole body. Ventral views. Scale bar 500 μm. **(B)** Expression pattern of neuropeptide genes in the pharynx detected by in situ hybridization Scale bar 500 μm. **(C)** Higher-magnification view of the expression in the head region of the *PC2*, *Dj_aH_308_M24* (*DjNpM24*) and *DjNp19* genes. Scale bar 250 μm. **(D)** Expression of indicated genes by in situ hybridization in transverse sections of body cut at the pre-pharyngeal region (*DjNpM24*, *DjNpP02* and *DjNp42*) and in the tip of the pharynx (*DjNpM24*). Arrowheads indicate *DjNpP02*-positive cells. Scale bar 250 μm. **(E)** Relative expression level of *DjNp47* measured by qPCR in each indicated portion of the body. Relative expression level of the whole body was taken as = 1. Error bars indicate s.e.m.

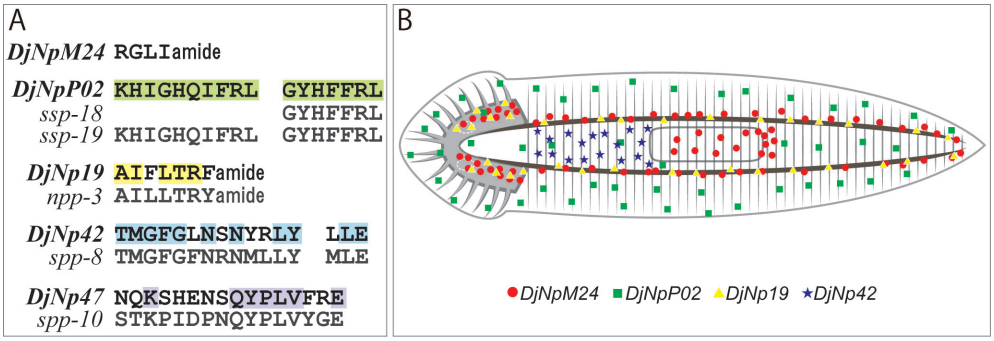

Fig. 4. Predicted amino acid sequences and expression patterns of five putative neuropeptide-coding genes shown in this study to be involved in pharynx extension. **(A)** Comparison of the predicted peptide(s) between *S. mediterranea* and *D. japonica*. Predicted peptide(s) sequence of *DjNpM24*, *DjNpP02*, *DjNp19*, *DjNp42* and *DjNp47* (each upper line) are aligned with the corresponding predicted peptide sequences (each lower line) of *spp-18*, *spp-19*, *npp-3*, *spp-8* and *npp-10* in *S. mediterranea*, respectively (Collins et al., 2010). Amino acid residues in the *D. japonica* sequence that match those in *S. mediterranea* are shaded with color. **(B)** Diagram of expression patterns of four neuropeptide-coding genes in the planarian whole body. Symbols show the expression patterns of the four neuropeptide-coding genes.

region between the brain and pharynx, suggesting that *DjNp42*-positive neurons may be involved in the connection between the brain and pharynx in the ventral side of the body (Fig. 3A, D). The predicted amino acid sequences and the expression patterns of these five genes are summarized in Fig. 4. In conclusion, five genes were identified here as genes involved in regulating pharynx extension, each of which showed a distinct expression pattern, suggesting that the control of pharynx extension may involve a complex neural circuit.

DISCUSSION

In the present study, we focused on pharynx extension behavior in planarian and sought to identify types of neurons involved in the regulation of pharynx extension. Our amputation experiments clearly showed that the head region is indispensable for pharynx extension, suggesting that pharynx extension is regulated by the brain. The time-course of recovery was nearly the same as the reported time-course of recovery of phototactic and thermotactic behaviors from planarian trunk fragments (Inoue et al., 2004, 2014), suggesting that brain function is required for pharynx extension, as it is for other brain-regulated behaviors. Thus, it seems that certain interconnection(s) between the brain and the regenerating pharynx may be necessary for complete recovery of pharynx-extension ability.

To identify neurons in the head region involved in regulating pharynx extension, first we checked the possible involvement of monoaminergic and amino acidergic neurons, since in a previous study we found that certain of these neurons are involved in the regulation of fundamental behaviors, such as negative phototaxis and thermotaxis (Inoue et al., 2004, 2014). However, in the work reported here we did not obtain clear evidence showing that monoaminergic or amino acidergic neurons are involved in pharynx extension. RNAi of *syt* had almost no effect on pharynx extension in planarians (Table 3), but did inhibit chemotactic behavior (Table 3; Inoue et al., 2015), suggesting that chemotaxis and pharynx extension might involve different neural regulation.

We found that PC2, an enzyme involved in neuropeptide synthesis in various species (Seidah et al., 1992; Zhoue et al., 1999), is required for pharynx extension, suggesting the possible involvement of neuropeptide-producing neurons in pharynx extension. Fifty-one neuropeptide or peptide hormone-related genes have been reported in another planarian species, *S. mediterranea* (Collins et al., 2010), and it would thus be very difficult to screen candidate genes from the full set of such genes using our pharynx-extension assay. Thus, we first narrowed down the set of candidate genes by using a simpler "primary feeding behavior assay" that includes chemosensing, pharynx extension, and ingestion. Then we further analyzed 13 of the thus-identified genes using our specific full pharynx-extension assay system. Finally, five candidate genes were identified as involved in pharynx extension, and their expression patterns are summarized in Fig. 4. We concluded that planarian pharynx extension is regulated by a complex system involving a number of different types of neuropeptide-producing cells. Combinatory RNAi of several of these five coding genes may completely suppress pharynx extension in planarian, although we have not yet been able to conduct this experiment due to technical issues.

We cannot exclude the possibility that other neurons contribute the regulation of the pharynx extension in planarians. Recently, nearly the entire genome sequence of the planarian *D. japonica* was reported (Nishimura et al., 2015), and we expect to discover additional neuropeptide-coding genes using this genome sequence information.

In the case of mammals, several neuropeptides, such as neuropeptide Y (NPY), orexin and galanin, were identified as neuropeptides that promote feeding (Arora and Anubhti, 2006). It is well known that neuropeptide Y (NPY) is a highly conserved neuropeptide that promotes feeding in both invertebrate and vertebrate species (Stanley and Leibowitz, 1985; Blomqvi et al., 1992; Matsuda, 2009; Yokobori et al., 2012; Shimizu et al., 2013). The NPY homolog in invertebrates is called neuropeptide F (Nässel and Wegener, 2011). However, we did not find any contribution of NPY homologs to pharynx extension in planarian in this study. Although *DjNp28* encodes an NPY homolog with conservation of all of the consensus amino acid sequences of NPY (Table 4), *DjNp28*(*RNAi*) planarians showed normal pharynx-extension ability, suggesting that planarian pharynx extension may not be involved in the promotion of feeding behavior. However, as planarians possess a large number of NPY family genes (Collins et al., 2010; Matsuda et al., 2012), further study is needed to determine whether NPY is involved in feeding behavior in planarians.

Although the auricles and lateral branches of the brain

are thought to be involved in chemosensing in planarian, we did not identify neuropeptide genes expressed in the auricles or the lateral branches of the brain. It has been observed that some G-proteins are expressed in planarian in the tip of the pharynx as well as in lateral branches of the brain (Inoue et al., 2007). We speculate that G-protein coupled receptors may be involved in detecting food by chemosensory neurons located in the head or in the tip of the pharynx. However, one gene (*DjNpP02*) shown here to be required for pharynx extension had a dispersed expression pattern on the body surface region throughout the body in both the ventral and dorsal margins (Fig. 3A, D), suggesting that *DjNpP02*-expressing cells might work as one type of food-sensing cells in a short-range manner. The *DjNpM24*-expressing cells in the tip of the pharynx may also work as sensory neurons in responding to food signals. In addition, the possibility that chemosensory neurons located in the lateral branch region also play a role in pharynx extension has not been ruled out. If these chemosensory neurons are indeed involved in pharynx extension, that may account for the limited reduction of pharynx-extension ability in *DjNpP02*(*RNAi*) planarians (Table 5). We need to perform further screening to identify sensory neurons responsive to food at short range from the animal.

DjNpM24- and *DjNp19*-positive cells were detected in the brain. However, both were also distributed in the CNS in a dispersed pattern, suggesting that a subpopulation of CNS neurons may be involved in the regulation of pharynx extension. Why are brain neurons involved in regulating pharynx extension? Planarian's brain integrates various kinds of sensory information (Inoue et al., 2015). In order to eat, planarians must orient the extension of the pharynx onto the food source in a manner depending on their internal state and/or external stimuli. After ingestion of food, a planarian retracts its pharynx to its original position and detaches from the food, suggesting that the planarian may have a satiety center in the brain. For these reasons, it seems very likely that pharynx extension should be regulated by the brain. Identification of the satiety center in the brain and unraveling the relationship between the regulatory circuit of pharynx extension and the satiety center are goals for future study in our group.

The most interesting expression pattern was obtained with the *DjNp42* probe. *DjNp42*-expressing cells were located just between the brain and pharynx, suggesting that these neurons may work as interneurons connecting brain and pharyngeal neurons. Okamoto et al. have reported the existence of neurons directly connecting the brain and pharynx, as detected by DiI tracing (Okamoto et al., 2005). We thus plan to investigate how neurons connect brain and pharynx neurons by immunostaining using anti *DjNp42* antibody in a future study.

We typically use the *syt* and *PC2* genes as pan-neural genes to stain the planarian nervous system. In other species, a subpopulation of CNS neurons produces neuropeptides (Seidah et al., 1992; Zhou et al., 1999). However, in the case of planarian, we found here that all CNS neurons express *PC2*. In addition, in the synaptic region of another planarian, both small synaptic vesicles and large neurosecretory vesicles were observed (Oosaki and Ishii, 1965). Here we showed that neuropeptide-producing neurons have diverse expression patterns. However, we do not yet know whether each neuropeptide is expressed exclusively in a distinct subset of neurons or whether some neuropeptides are produced in the same neurons. It will be interesting to elucidate the functions of the neuropeptides and neural networks in various behaviors and physiological controls in planarians. For example, knockdown of eight of 13 candidate neuropeptide-coding genes examined here caused defects of feeding behavior, although the knockdown planarians showed normal pharynx extension. Thus, these eight genes appear to be involved in feeding behaviors other than pharynx extension, such as chemotaxis or ingestion. We expect that some may be involved in ingestion of food via the pharynx. To identify the functions of each neuropeptide-coding gene, in the future we will need to perform combinatory experiments using behavior assay systems and RNAi screening, which will enable us to clarify how different kinds of information are integrated to regulate feeding behavior in planarians.

ACKNOWLEDGMENTS

We sincerely thank Dr. Elizabeth Nakajima for critically reading the manuscript. This work was supported by a Grant-in-Aid for JSPS Fellows (14J01042) to SS, a Grant-in-Aid for JSPS Fellows (13J01064) to MK, Grants-in-Aid for Scientific Research (22700336, 15K07148) to TI, The Takeda Science Foundation funds to TI, and Grants-in-Aid for Scientific Research on Innovative Areas (22124001, 22124002) to KA.

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(Received October 13, 2015 / Accepted December 29, 2015)