Different combinations of serotonin receptors regulate predatory and bacterial feeding behaviors in the nematode Pristionchus pacificus

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Abstract

Feeding behavior is one of the most fundamental behaviors in animals, and regulation of this behavior is critical for proper food intake. The nematode Pristionchus pacificus exhibits dimorphism in feeding behavior, bacterial feeding and predatory feeding on other nematodes, and the latter behavior is assumed to be an evolutionarily novel behavior. Both types of feeding behavior are modulated by serotonin; however, the downstream mechanism that modulates these behaviors is still to be clarified. Here, we focused on serotonin receptors and examined their expression patterns in P. pacificus. We also generated knockout mutants of the serotonin receptors using the CRISPR/Cas9 system and examined feeding behaviors. We found that Ppa-ser-5 mutants and the Ppa-ser-1; Ppa-ser-7 double mutant decreased predation. Detailed observation of the pharyngeal movement revealed that the Ppa-ser-1; Ppa-ser-7 double mutant reduces tooth movement, which is required for efficient predatory feeding. Conversely, Ppa-ser-7 and Ppa-mod-1 mutants decreased bacterial feeding. This study revealed that specific combinations of serotonin receptors are essential for the modulation of these distinct feeding behaviors, providing insight into the evolution of neural pathways to regulate novel feeding behavior.

Keywords: feeding behavior; Pristionchus pacificus; serotonin receptor; predation

Introduction

Animals have evolved to change their diet to adapt to their surrounding environment. To achieve this, it is necessary to acquire new feeding behaviors by altering morphological structures such as feeding apparatus, physiological features such as composition of digestive juice, and behavioral characteristics. Alteration in behavioral features should be accompanied by changes in the nervous system related to behaviors.

Nematodes have extremely diverged in feeding behavior (Yeates et al. 1993). To adapt to food sources such as bacteria, fungi, and plants, they have developed numerous types of feeding structures and regulatory systems of feeding behavior. The predatory nematode Pristionchus pacificus is an intriguing model to explore the mechanisms of feeding regulation and its evolution. P. pacificus belongs to the family Diplogastridae that has been established as a satellite model animal compared to Caenorhabditis elegans (Sommer et al. 1996). Various genetic tools such as annotated genome sequences (Dieterich et al. 2008; Rödelsperger et al. 2017), genome editing methods using the CRISPR/Cas9 system (Witte et al. 2015; Nakayama et al. 2020) and transformation of exogenous genes (Schräler et al. 2009; Namai and Sugimoto 2018) are available for P. pacificus. Moreover, neuronal connectomics in pharyngeal neurons and amphid sensory neurons have been identified (Bumbarger et al. 2013; Hong et al. 2019), increasing the potential for detailed behavioral analysis.

P. pacificus exhibits polyphenism in its feeding structure in response to the surrounding environment during development. One form is called the eury stomatous morph, which is characterized by a wider and shallow buccal cavity, a claw-like dorsal tooth, and several subventral teeth. The other is a sten stomatous morph, with a narrow, deep buccal cavity and a single flint-like tooth (Bento et al. 2010). P. pacificus with either mouth form feeds on bacterial food, while predatory feeding behavior is only seen in eury stomatous worms (Wilecki et al. 2015). While bacterial feeding is common in a broader range of taxonomic clades, predatory feeding behavior is observed in Diplogastridae nematodes (Blaxter et al. 1998). Thus, predatory feeding behavior is assumed to be an evolutionarily novel behavior and provides a suitable model for understanding the neural evolution of feeding behaviors.

The predatory feeding behavior in P. pacificus is characterized by the coordinated movement of pharyngeal pumping and tooth movement, enabling the efficient killing of prey (Wilecki et al. 2015; Okumura et al. 2017). A neuromodulator, serotonin, is a key molecule that regulates this behavior (Wilecki et al. 2015). Mutants of two enzymes essential for serotonin synthesis, Ppa-tpH-1, and Ppa-bas-1, fail to induce efficient feeding on other
nematodes. These mutants decrease tooth movement during feeding on other nematodes, disrupting coordinated feeding rhythms (Okumura et al. 2017). In contrast, exogenous serotonin solely induces the coordinated movement of pharyngeal pumping and tooth movement (Willeck et al. 2015), suggesting that serotonin plays an essential role in the regulation of predatory feeding behavior. Serotonin also modulates the bacterial feeding rate in *P. pacificus* (Okumura et al. 2017). Compared with pharyngeal movements during predation, pharyngeal movement during bacterial feeding lacks tooth movements, and the pumping rate is faster (Willeck et al. 2015). However, how serotonin modulates these different feeding modes and how the nervous systems evolve to modulate predatory feeding behavior remains unclear.

In this study, we present serotonin receptors that regulate predatory and bacterial feeding behaviors in *P. pacificus*. In the model nematode *C. elegans*, five serotonin receptors play a role in feeding behavior (Olde and McCombie 1997; Ranganathan et al. 2000; Hamdan et al. 2019; Hobson et al. 2003; Tsalik et al. 2003; Hapiak et al. 2009; Ishita et al. 2020). We examined the functions and expressions of all five serotonin receptors in *P. pacificus*. We found serotonin receptors were expressed in some orthologous cells between *C. elegans* and *P. pacificus*, while some differences in the expression patterns were observed between these species. We generated serotonin receptor mutants using CRISPR/Cas9 system and found that *Ppa-ser-1*, *Ppa-ser-7*, and *Ppa-ser-5* have significant roles in the regulation of predatory feeding behavior. We also determined that *Ppa-ser-7* and *Ppa-mod-1* play major roles in the modulation of bacterial feeding behavior. This study offers downstream genetic mechanisms of serotonin in the regulation of predatory and bacterial feeding behaviors in *P. pacificus* and insights into the evolution of a novel feeding behavior.

**Materials and methods**

**Strains**

All strains used in the experiments were maintained at 20°C on standard NGM plates with Escherichia coli OP50 (Brenner 1974; Stiemagle 2006). The strains are listed in Supplementary Table S1.

**Molecular cloning**

The *P. pacificus* orthologs of five *C. elegans* serotonin receptors were predicted as previously described (Baskaran et al. 2015). A 5' and 3' RACE was performed to determine the mRNA sequences of the serotonin receptors. The cDNA for RACE was prepared using the SMART^®^ RACE 5'/3' Kit (Clontech, 634858), and the target sequences were amplified using PrimerSTAR Max DNA polymerase I (TaKaRa, 505A) or KOD-Fx-Neo (TOYOBO, KFX-201). The amplicons were purified and sequenced using Sanger sequencing.

**Generation of transgenic animals**

The promoter regions of the serotonin receptors were amplified using PrimerSTAR Max DNA polymerase I or KOD One PCR Master Mix (TOYOBO, KMM-101). The lengths of the promoter sequences were as follows: *Ppa-ser-1*: 3.5 kb; *Ppa-ser-4p*: 4.5 kb; *Ppa-ser-5p*: 5.7 kb; *Ppa-ser-7p*: 7.1 kb; *Ppa-mod-1p*: 3.8 kb. The promoters for *Ppa-ser-4* and *Ppa-ser-7* include intergenic regions flanked by the end of predicted 3' untranslated regions (UTRs) of upstream genes on the same strand and the start codon of genes of interest. For *Ppa-ser-1p* and *Ppa-ser-5p*, we used shorter sequences because of the difficulty of PCR amplification. *Ppa-mod-1p* has a longer sequence containing 151 bp of 3' UTR of a predicted upstream gene due to the difficulty of plasmid construction. The primers used to amplify the promoters are shown in Supplementary Table S2. To examine the expression patterns of serotonin receptors, complex arrays were generated as previously described (Schlager et al. 2009). Transgenes and genomic DNA of PS312 were digested using appropriate digest enzymes (*Ppa-eq-20p*: Venus, *Ppa-ser-4p*: turboRFP, *Ppa-ser-7p*: turboRFP, *Ppa-mod-1p*: turboRFP. FastDigest HindIII (Thermo Fischer, FD0504); *Ppa-ser-1p*: turboRFP. FastDigest HindIII and FastDigest BamHI (Thermo Fischer, FD054); *Ppa-ser-5p*: turboRFP. FastDigest KpnI (Thermo Fischer, FD0524). The mixture containing *Ppa-eq-20p*: Venus (15 ng/µL) as an injection marker, the digested genomic DNA (60 ng/µL), and reporter genes for serotonin receptors (*Ppa-ser-1p*: turboRFP. 10 ng/µL, *Ppa-ser-4p*: turboRFP. 2 ng/µL, *Ppa-ser-5p*: turboRFP. 10 ng/µL, *Ppa-ser-7p*: turboRFP. 10–15 ng/µL, *Ppa-mod-1p*: turboRFP. 3 ng/µL) was injected in the gonads of young adults. The transgenic animals were screened using a fluorescent stereoscope (Leica).

The fluorescent images of the transgenic animals were obtained using an LSM microscope (Zeiss). Expressing cells were examined by manual scans of Z-stack images using ImageJ software (RRID: SCR_003070). The images in the head region of adult worms were analyzed for at least six animals for each mouth form per gene. The mouth form was evaluated as previously described (Soborbyan et al. 2013).

**Quantification of serotonin receptor expression**

To quantify the expression levels of serotonin receptors, quantitative reverse transcript PCR (qRT-PCR) was performed as previously described (Schuster and Sommer 2012). The wild-type PS312 strain was used as the eurytomatous-rich strain, and the *Ppa-eud-1* mutant was adopted as the all-stenostomatous strain (Ragsdale et al. 2013). A total of 100–120 young adult worms were manually selected, and the total RNA was extracted using an RNeasy Mini Kit (Qiagen, 74104). The CDNA was synthesized with a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, RR047A) and qRT-PCR was performed with a CFX Connect Real-Time PCR Detection System (Bio-Rad, #1855201J1). PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, 600882) as a DNA polymerase. Three technical replicates and three biological replicates were examined for each gene. We used *Ppa-cdc-42* gene expression as a reference gene (Schuster and Sommer 2012). Data were processed and plotted using CFX Manager 3.1 (RRID: SCR_017251). Primers for qRT-PCR are listed in Supplementary Table S3.

**Generation of serotonin receptor mutants using the CRISPR/Cas9 system**

Serotonin receptor mutants were generated using the CRISPR/Cas9 genome editing system, as previously described (Witte et al. 2015). The guide RNA targets were selected using the CHOPCHOP web tool (https://chopchop.cbu.iubio.io) (last accessed: 2021, Feb. 4th, RRID: SCR_015723). Target sequences for CRISPR/Cas9 mutagenesis are listed in Supplementary Table S4. The tracrRNA and target-specific crRNA (IDT), or synthesized single-guide RNAs (sgRNA) using the EnGen™ sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322S) were utilized as guide RNAs. In the latter case, single-stranded DNA oligos (Eurofins) were used as templates. A guide RNA and Cas9 protein (IDT) were mixed in a 2:1 molar ratio and incubated at 37°C for 10 min to make a ribonucleaseprotein (RNP) complex and diluted with nuclelease-free water or TE buffer. The RNP complex was injected into the gonads of 1-day adult hermaphrodites. To generate *Ppa-ser-5* (cbh23) and *Ppa-ser-1* (cbh58)
mutants, the Ppa-prl-1 RNP complex was also injected as a co-injection marker (Nakayama et al. 2020). FO worms were discarded 12–24 h after microinjection.

For mutation screening, the heteroduplex mobility assay (HMA) was performed as previously described (Nakayama et al. 2020). The primers for HMA are listed in Supplementary Table S5. The mutants were back-crossed at least three times with the original wild-type strain (PS312).

Assays for feeding behaviors

Predatory behavioral assays were performed using previously established methods (Wilecki et al. 2015; Lightfoot et al. 2016; Okumura et al. 2017) with some modifications.

To perform the bite assay, C. elegans (N2) larvae were collected with M9 buffer and filtered by double 20-µm nylon filters. After concentrating C. elegans larvae via centrifugation, 2 µL of the larva were placed on fresh NGM plates without E. coli food and the larvae were allowed to spread. One- to two-day-old P. pacificus was placed on the plates and allowed to recover for 30 min–1 h. Bite, kill, and feed events were counted for 10 min using a stereo-microscope. Each predatory event was determined as follows: bites, restriction of prey movement, kills, rupture of prey cuticles, and feeds, consuming prey body fluid (Figure 3A). The assay was performed blindly on at least two different days at 20°C in total, more than 15 animals per strain were examined.

The pumping rate was examined during predatory feeding, bacterial feeding, and exposure to exogenous serotonin. Thirty-five mm NGM plates were prepared for each experiment. The N2 larvae prepared as described above were placed on the plates for predatory feeding, and E. coli OP50 was seeded for bacterial pumping. One- to two-day-old P. pacificus adults were manually selected on the plates and allowed to recover for more than 30 min. To examine pharyngeal movements in response to exogenous serotonin, 30 µL of 10 mg/mL of serotonin (Sigma-Aldrich, H7752) in M9 buffer was applied onto the worms and a cover glass was loaded onto the agar plate. The pharyngeal movement was recorded for 15 s at 30 frames per second using a CCD camera with a DIC microscope (Leica). Tooth movement together with pharyngeal pumping during predation and exogenous serotonin exposure were recorded at 400× magnification, while bacterial pumping was recorded at 200× magnification. The video was manually scanned frame-by-frame using ImageJ software, and the numbers of pharyngeal pumping and tooth movements were recorded.

Egg-laying assay

Egg-laying assays were performed using methods described previously (Okumura et al. 2017). Two- to three-day-old adult worms were loaded into 50 µL M9 buffer or 4 mg/mL serotonin solution in 96-well culture plates. After 2 h, the number of eggs was manually counted. The number of eggs in the uterus was counted in 3-day-old adult worms. The investigator was not blinded during this test.

Locomotion assay

Paralyzing test for the Ppa-ser-4 mutant was performed as previously described (Gürel et al. 2012). Two- to three-day-old adult worms were put into 50 µL M9 buffer with or without 30 mM serotonin in 96-well culture plates. After 1 h, the numbers of paralyzed worms and moving worms were counted. Worms bending the whole body smoothly were classified as the moving animals. Worms with movements only in the head region or with jerky movements in partial body parts were categorized into the paralyzed worms. For body bending assay, the number of body bending was examined in worms loaded into M9 buffer for 10 s. Worms that were not moving were excluded from this assay. The investigator was not blinded for this assay.

Statistical analysis

The data were processed and analyzed using GraphPad Prism 7 (RRID: SCR_002798) and in the case of Figure 2H, Microsoft Excel (RRID: SCR_016137). Error bars represent the SEM, and all statistical tests used, and meanings of symbols can be found in the figure legends.

Data availability

All animal strains shown in Supplementary Table S1 and plasmids used in this study are available upon request. The sequences of primers and guide RNA targets for CRISPR/Cas9 genome editing used in this study can be found in Supplementary Tables S2–S5. Supplementary Figures and Tables are available at figshare: https://doi.org/10.25387/g3.13413503.

Results

Gene structures and expression patterns of serotonin receptors in P. pacificus

To reveal the serotonergic modulation of feeding behaviors in P. pacificus, we first conducted molecular cloning of serotonin receptors. The orthologs of serotonin receptors were predicted using best-reciprocal hits, and coding sequences were determined by 5’ and 3’ RACE (Figure 1A). The following gene annotations were predicted as orthologs of serotonin receptors: PPA38517 for Ppa-ser-1, PPA14349 for Ppa-ser-4, PPA26151 for Ppa-ser-5, PPA39471 for Ppa-ser-7, and PPA01915 for Ppa-mod-1. The amino acid sequences of serotonin receptors were highly conserved between P. pacificus and C. elegans (50.59% identity for SER-1, 60.71% for SER-4, 72.35% for SER-5, 51.86% for SER-7, 74.72% for MOD-1). We confirmed that there were no Pristionchus-specific paralogs of those serotonin receptors by BLASTP searches (data not shown). Thus, we considered those genes encoding orthologs of serotonin receptors identified in C. elegans.

We generated reporter lines containing the upstream region of each serotonin receptor gene and RFP to examine the expression patterns of these serotonin receptors in P. pacificus (Figure 1, B–F). Ppa-ser-1 was expressed in the anterior pharyngeal muscles and several head neurons (Figure 1B, arrows). Notably, Ppa-ser-1 expression was prominent in the pm1 pharyngeal muscle, where the dorsal tooth denticle attaches. Ppa-ser-1 was weakly expressed in the anus (Supplementary Figure S1A). Ppa-ser-4 was expressed in the M1 pharyngeal neuron, and head neurons including several amphid neurons (Figure 1C, open arrowheads) and labial neurons (Figure 1C, white arrowheads). Ppa-ser-4 was also expressed in nonneuronal cells in the head region (Figure 1C, asterisks) and several tail neurons (Supplementary Figure S1B). Ppa-ser-5 was expressed in several head neurons including amphid neurons (Figure 1D, open arrowhead), labial neurons (Figure 1D, white arrowheads), and the M1 pharyngeal neuron (Figure 1D). Ppa-ser-4 and Ppa-ser-5 seemed to be expressed redundantly in some of these cells. Ppa-ser-5 was also expressed in the vulval muscles (Supplementary Figure S1C). Ppa-ser-7 was expressed in some pharyngeal neurons, including M4, M1, M2, and M6 neurons, which were identified by their cell body positions and morphologies of neuronal processes (Figure 1E). Ppa-ser-7 was also expressed in the vulval muscles, which are different
from those expressing Ppa-ser-5 (Supplementary Figure S1D). Ppa-
mod-1 was expressed in several head neurons, including a pair of
amphid sensory neurons (Figure 1F, open arrowhead), but not
expressed in any pharyngeal cells (Figure 1F). It was also
expressed in gonadal cells, tail neurons, and slightly expressed in
vulval neurons (Supplementary Figure S1, E–G). We compared
the expression patterns and expression levels of serotonin recep-
tors in eurystomatous and stenostomatous worms using micro-
scopic observations and qRT-PCR. Although the expression level
of all serotonin receptors tended to be higher in the Ppa-eud-1
mutant, an all-stenostomatous strain (Supplementary Figure S2A),
we could not find apparent differences in the expression
patterns of serotonin receptors between the two mouth forms by
microscopic observations (Supplementary Figure S2, B–F).

**Generation of serotonin receptor mutants using the CRISPR/Cas9 system**

To examine the functions of serotonin receptors, we generated
serotonin receptor mutants using the CRISPR/Cas9 system. We
obtained mutants with insertion or deletion mutations in the

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**Table 1 The mutants of serotonin receptors in *P. pacificus***

| Gene     | Allele | Mutation            | Types of mutation |
|----------|--------|---------------------|-------------------|
| Ppa-ser-1| cbh3   | 5 bp deletion       | Frame-shift       |
|          | cbh58  | 17 bp deletion      | Frame-shift       |
| Ppa-ser-4| cbh12  | 10 bp deletion      | Frame-shift       |
| Ppa-ser-5| cbh11  | 10 bp deletion      | Frame-shift       |
|          | cbh23  | 5 bp deletion       | Frame-shift       |
| Ppa-ser-7| cbh1   | 4 bp insertion, 1 bp SNP | Frame-shift     |
|          | cbh2   | 4 bp deletion       | Frame-shift       |
| Ppa-mod-1| cbh4   | 11 bp deletion      | Frame-shift       |
|          | cbh5   | 13 bp deletion      | Frame-shift       |
Figure 2 Phenotypic analysis of serotonin receptor mutants in egg-laying behavior and locomotion. (A) The number of eggs in the uterus of wild type and Ppa-ser-1 mutants. Wild type, \( n = 48 \). Ppa-ser-1 (cbh3), \( n = 47 \). Ppa-ser-1 (cbh58), \( n = 46 \). Error bars represent the SEM. One-way ANOVA with Dunnett’s multiple comparison tests. **\( P < 0.01 \). ****\( P < 0.0001 \). (B)–(G) The number of eggs laid in M9 buffer and serotonin solution within 2 h in serotonin receptor mutants. (B) Wild type, \( n = 47 \). Ppa-ser-1 (cbh3), \( n = 48 \). (C) Wild type, \( n = 143 \). Ppa-ser-7 (cbh1), \( n = 96 \). Ppa-ser-7 (cbh2), \( n = 95 \). (D) Wild type, \( n = 48 \). Ppa-ser-5 (cbh11), \( n = 48 \) for M9 buffer, \( n = 46 \) for serotonin solution. Ppa-ser-5 (cbh23), \( n = 48 \). (E) Wild type, \( n = 95 \). Ppa-ser-1 (cbh3), Ppa-ser-7 (cbh1), \( n = 96 \). (F) Wild type, \( n = 48 \). Ppa-ser-4 (cbh12), \( n = 48 \) for M9 buffer, \( n = 47 \) for serotonin solution. (G) Wild type, \( n = 48 \). Ppa-mod-1 (cbh5), \( n = 48 \) for M9 buffer, \( n = 45 \) for serotonin solution. Δ serotonin and + serotonin represent M9 buffer and serotonin solution, respectively. Error bars represent the SEM. Two-way ANOVA with Tukey’s multiple comparison tests. n.s., not significant. *\( P < 0.05 \). **\( P < 0.01 \). ***\( P < 0.001 \). ****\( P < 0.0001 \). (H) Proportions of moving versus paralyzed animals in M9 buffer and serotonin solution in wild type and Ppa-ser-4 (cbh12) animals. All conditions, \( n = 75 \). Fischer’s exact tests. n.s., not significant. ****\( P < 0.0001 \). (I) Number of body bending within 10 s in M9 buffer in wild type and Ppa-ser-4 (cbh12) animals. Wild type, \( n = 29 \). Ppa-ser-4 (cbh12), \( n = 30 \). Error bars represent SEM. Student’s t-test. ***\( P < 0.001 \).
coding regions, resulting in premature stop codons (Table 1; Supplementary Figure S3). We observed that the Ppa-ser-1 mutants carried more eggs in the uterus than the wild type (Figure 2A), which is consistent with a previous report that egg-laying is one of the serotonin-related behaviors in P. pacificus (Okumura et al. 2017). We also examined the number of eggs laid over 2 h in M9 buffer with or without serotonin. In the wild type, the number of eggs laid in M9 buffer containing serotonin was slightly decreased compared with that in normal M9 buffer (Figure 2B), although previous studies showed that the number of eggs laid in serotonin solution was almost half of that in M9 buffer (Gutierrez and Sommer 2007; Okumura et al. 2017). This difference may be caused by slight differences in experimental conditions. With the Ppa-ser-1 mutant, the number of eggs laid in M9 buffer declined to half of that laid by the wild type, and exogenous serotonin did not affect the number of eggs in the Ppa-ser-1 mutant (Figure 2B). This data suggests that Ppa-ser-1 has a function to promote egg laying. Since Ppa-ser-7 and Ppa-ser-5 were expressed in the vulval muscles, we also examined egg-laying behavior in these mutants. We found that Ppa-ser-7 mutants exhibited a decreased number of eggs laid in M9 buffer, and egg-laying was strongly suppressed by exogenous serotonin (Figure 2C). This finding indicates that egg-laying is enhanced via the Ppa-ser-7 serotonin receptor. Similar to the Ppa-ser-1 mutants, Ppa-ser-5 mutants decreased egg-laying in M9 buffer, and the number of eggs laid was not altered by exogenous serotonin (Figure 2D). The Ppa-ser-1; Ppa-ser-7 double mutant showed phenotypic features of both of the serotonin receptors; the number of eggs laid in M9 buffer was similar to the Ppa-ser-1 mutant and exogenous serotonin further decreased the number of eggs like Ppa-ser-7 mutants (Figure 2E). This suggest that Ppa-ser-1 and Ppa-ser-7 function in parallel in the modulation of egg-laying. Since previous studies showed that Cel-ser-4 and Cel-mod-1 have inhibitory roles in egg laying in C. elegans (Carnell et al. 2005; Hapiak et al. 2009), we wondered whether those serotonin receptors also suppress egg laying in P. pacificus. However, Ppa-ser-4 and Ppa-mod-1 mutants rather decreased the number of eggs laid both in M9 buffer and serotonin solution (Figure 2, F and G). These results suggest that all of the serotonin receptors play a role in egg-laying behavior. As the exogenous serotonin suppressed egg-laying greatly in the Ppa-ser-7 mutants, we could not exclude a possibility that some of the serotonin receptors might have both stimulating and suppressive functions in egg-laying in different cells. Because Ppa-ser-4 was expressed in head and tail neurons whose neurites extended toward the body wall, we suspected that the Ppa-ser-4 mutant may play a role in locomotor behavior. We examined the locomotor behavior in M9 buffer containing 30 mM of serotonin (Figure 2H). Both wild type and the Ppa-ser-4 mutant strains were moving in M9 buffer without exogenous serotonin. While 89.3% of the wild-type animals were paralyzed by the serotonin solution, only 33.3% of the Ppa-ser-4 mutant worms were not moving in the same condition (Figure 2H). This result indicates that the Ppa-ser-4 mutant is resistant to the paralyzing effect of serotonin, similar to Cel-ser-4 mutants in C. elegans as previously reported (Gürel et al. 2012). Also, we investigated the body bending rate in M9 buffer without serotonin in the Ppa-ser-4 mutant. The mutants showed body bending more frequently than wild-type animals (Figure 2I). These data suggest that Ppa-ser-4 has an inhibitory role in locomotion in response to serotonin. Together, these serotonin receptor mutations are likely to be

![Figure 3](https://example.com/figure3)

**Figure 3** Ppa-ser-1, Ppa-ser-5, and Ppa-ser-7 are required for predatory feeding behaviors. (A) Schematic of the three predatory events in P. pacificus. (B)–(I) The number of bites, kill, and feed events in 10 min in single mutants (B–F) and multiple serotonin receptor mutants (G–H). (B, C, and E), n = 20. (D and F), n = 16. (G and H), n = 19. WT, wild type. Error bars represent the SEM. Student’s t-tests were performed for (B, C, G, and H). One-way ANOVA with Dunnett’s multiple comparisons was used for other single mutants. n.s., not significant. *P < 0.05. **P < 0.01. ***P < 0.001.
loss-of-function alleles and can be used for behavioral analysis (see also Table 2).

Predatory feeding behavior was decreased in Ppa-ser-5 mutants and the Ppa-ser-1; Ppa-ser-7 double mutant

We analyzed the predatory feeding behavior in serotonin receptor mutants using the "bite assay" to quantify predatory feeding events (Wilecki et al. 2015). We counted the number of the three types of predatory feeding events exhibited. Bite, kill, and feed events were characterized by the restriction of prey movement, breaking open the prey cuticle, and consuming the prey body fluid, respectively (Figure 3A). Among the five serotonin receptor mutants, Ppa-ser-5 mutants showed decreased "kill" and "feed" events, while the number of "bite" event was not altered compared with wild-type worms (Figure 3D). This is a specific phenotype in the Ppa-ser-5 mutants; Ppa-tph-1 and Ppa-bas-1 mutants showed decreased numbers of all the predatory events (Okumura et al. 2017). Single mutants of other serotonin receptors did not show a decrease in the number of predatory feeding events, except for one of the two alleles of Ppa-ser-7 mutants (Figure 3, B, C, E, and F). While the Ppa-ser-7 (cbh1) allele brought a stop codon immediately after the native amino acid sequence, there were 55 extra amino acids before the premature stop codon in the Ppa-ser-7 (cbh2) allele (Supplementary Figure S3). This difference might cause different phenotypes between the two alleles.

In C. elegans, the pharyngeal pumping rate is modulated via the Cel-ser-7 serotonin receptor and partially via the Cel-ser-1 serotonin receptor. The Cel-ser-7, Cel-ser-1 double mutant abolishes the upregulation of the bacterial feeding rate (Hobson et al. 2006). We hypothesized that serotonin receptors work redundantly, and the double mutant for these serotonin receptors might decrease predatory feeding behavior in P. pacificus. The Ppa-ser-1, Ppa-ser-7 double mutant decreased predatory feeding events, especially the bite event (Figure 3G). The Ppa-ser-4, Ppa-mod-1 double mutant did not show significant changes in the number of predatory events (Figure 3H), implying that these serotonin receptors are not required for the regulation of predatory feeding behavior. These data suggest that Ppa-ser-5 is important for prey killing and Ppa-ser-1 and Ppa-ser-7 have redundant roles in biting and killing prey during predation.

Ppa-ser-1 and Ppa-ser-7 are required for tooth movement during predatory feeding and the response to exogenous serotonin

To evaluate the role of serotonin receptors in predatory feeding events, we counted pharyngeal pumping and tooth movement events during predation in Ppa-ser-5, Ppa-ser-1, and Ppa-ser-7 mutants (Figure 4A). During predation, wild-type animals showed almost the same rate of pharyngeal pumping and tooth movement as previously described (Wilecki et al. 2015). Strikingly, the Ppa-ser-1; Ppa-ser-7 double mutant showed a significant decrease in tooth movements, whereas the single mutants did not. Likewise, tooth movements in response to exogenous serotonin were much lower in the Ppa-ser-1, Ppa-ser-7 double mutant compared with wild-type worms (Figure 4B). In this context, the Ppa-ser-1 mutant also exhibited decreased tooth movements, while the reduction was weaker than that observed with the Ppa-ser-1; Ppa-ser-7 double mutant. Unexpectedly, the Ppa-ser-5 mutants, which showed reduced kill and feed events in the bite assay, did not show any reduction in the number of pharyngeal pumping and tooth movements during predatory feeding or following exposure to serotonin (Figure 4). These results suggest that Ppa-ser-1 and Ppa-ser-7 redundantly enhance tooth movement in response to serotonin, while Ppa-ser-5 is not related to tooth movement or feeding rhythms.

Ppa-ser-7 and Ppa-mod-1 mutants exhibit decreased pharyngeal pumping during bacterial feeding

Serotonin also contributes to the upregulation of pharyngeal pumping during bacterial feeding in both P. pacificus and C. elegans (Sze et al. 2000; Okumura et al. 2017; Ishita et al. 2020). We investigated whether the downstream pathway regulating pumping during bacterial feeding is conserved between these species. We examined the pharyngeal pumping rate in serotonin receptor mutants during bacterial feeding (Figure 5). Single mutants of Ppa-ser-7 and Ppa-mod-1, and the Ppa-ser-4; Ppa-mod-1 double mutant showed a significant decrease in the pharyngeal pumping rate. Other single mutants and multiple mutants of serotonin receptors did not show any alteration in the pumping rate during bacterial feeding. Curiously, the Ppa-ser-1; Ppa-ser-7 double mutant did not decrease the pumping rate on OP50, even though it carried mutation in Ppa-ser-7. The Ppa-ser-1 mutation may mask the effects of the Ppa-ser-7 mutation. Together, these data suggest that Ppa-ser-7 and Ppa-mod-1 play a major role in the upregulation of the pumping rate during bacterial feeding in P. pacificus.

**Discussion**

Although serotonin regulates feeding behaviors, the downstream neuronal mechanisms have not been elucidated previously in P. pacificus. In this study, we demonstrated the functions of Ppa-ser-1, Ppa-ser-5, and Ppa-ser-7 in predatory feeding behavior and Ppa-ser-7 and Ppa-mod-1 in bacterial feeding behavior by utilizing CRISPR/Cas9 knockout mutants. We also identified cells expressing serotonin receptors. These cells are candidates for the target of serotonin in modulating feeding behaviors in this animal. This study suggests putative downstream mechanisms of serotonergic modulation in predatory and bacterial feeding behaviors (Figure 6).

In this study, we showed that two serotonin receptors, Ppa-ser-1 and Ppa-ser-7, redundantly induce tooth movement during predation and in response to exogenous serotonin. Cel-ser-1 is

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**Table 2 Phenotypes observed in the serotonin receptor mutants**

| Genes    | Predation | Bacterial feeding | Egg-laying | Serotonin sensitivity in locomotion |
|----------|-----------|-------------------|------------|-------------------------------------|
| Ppa-ser-1 | + (defective in double mutant with Ppa-ser-7) | + | Defective | N/A |
| Ppa-ser-4 | + | + | Defective | Negative |
| Ppa-ser-5 | defective | + | Defective | N/A |
| Ppa-ser-7 | + (defective in double mutant with Ppa-ser-1) | Defective | Defective | N/A |
| Ppa-mod-1 | + | + | Defective | N/A |

All of the serotonin receptor mutants we generated showed some defects in behaviors. +, same phenotype as wild type. N/A, not examined.
expressed throughout the pharyngeal muscles of *C. elegans* (Tsalik et al. 2003; Carnell et al. 2005), while *Ppa-ser-1* is expressed only in the anterior parts in *P. pacificus* (Figure 1B). In addition, the expression of the *Ppa-ser-1* RFP reporter was prominent in the dorsal tooth muscle (pm1) compared with the other pharyngeal muscles (Figure 1B). This finding suggests that the alteration of expression patterns of serotonin receptors may play a role in the evolutionary acquisition of predatory feeding behavior. Furthermore, a molluscan study showed a correlation between the expression of serotonin receptors in a homologous neuron and species-specific swimming behavior, implying that the expression of serotonin receptors in specific cells may be important for the presence of a specific behavior (Tamvacakis et al. 2018).

Supporting this idea, the *Ppa-ser-1* mutant showed the greatest defect in tooth movements in response to exogenous serotonin among single serotonin receptor mutants. We should note that the promoter fragments used in this study might overlap with the potential regulatory regions of adjacent genes. To verify *bona fide* expression patterns, evaluation of the expression patterns using knock-in of tag sequences in genomic loci or other methods such as single-molecule fluorescence in situ hybridization (smFISH) might be useful in future studies.

*Ppa-ser-7* was expressed in the pharyngeal neurons (Figure 1E), whose expression patterns were different from those of *Ppa-ser-1*. Among *Ppa-ser-7*-expressing neurons, the M1 pharyngeal neuron is the most probable candidate for the regulation of tooth movement (Figure 6). There are four empirical reasons for this hypothesis. First, the M1 neuron has synaptic connectivity with the anterior parts of the pharynx, including pm1 and g1D gland cells that may be involved in predation (Bumbarger et al. 2013; Riebesell and Sommer 2017). Second, a previous *C. elegans* study revealed that the M1 neuron is responsible for “spitting” behavior, which opens the anterior tip of the pharynx and expels the contents of the pharynx, implying that the M1 neuron is capable of stimulating anterior tip muscles (Bhatia et al. 2015). Third, the M1 neuron also has synaptic connectivity with I1 and I2 neurons, which are also presynaptic to the anterior pharyngeal muscles. These connections are not seen in *C. elegans*, implying that the functions of these neurons change to regulate the predatory feeding in *P. pacificus* (Bumbarger et al. 2013). Finally, *Cel-ser-7* is not expressed in the M1 neuron in *C. elegans* (Hobson et al. 2006). Therefore, the M1 pharyngeal neuron may have direct or indirect roles in stimulating the dorsal tooth muscles, and serotonin regulates the tooth movement via the M1 neuron. To prove this model, cell-aboration or neuron-specific rescue experiments for those neurons should be conducted in future studies. Conversely, the M4 neuron, another *Ppa-ser-7* expressing cell, was previously described as a neuron regulating peristalsis in the posterior pharyngeal muscles and partial pharyngeal pumping (Chiang et al. 2006), suggesting a role for serotonin in the peristalsis of the posterior pharynx. In *C. elegans*, the M4 neuron modulates isthmus peristalsis via Cel-SER-7 (Song and Avery 2012), suggesting that the role of SER-7 in the M4 neuron might be conserved between the two species.

We also revealed that *Ppa-ser-5* decreased the number of “kill” and “feed” events, but not “bite” event during predatory feeding behavior (Figure 3D). This feature is not observed in serotonin synthesis mutants or other serotonin receptor mutants (Okumura et al. 2017 and Figure 3, B, C, E, and F). In addition, *Ppa-ser-5* mutants did not decrease the number of pharyngeal pumping and tooth movements during predatory feeding behavior or in response to serotonin (Figure 4). Interestingly, *Ppa-ser-5* was expressed in some sensory neurons, including amphid neurons and labial neurons (Figure 1D). If *Ppa-ser-5* is involved in predation via these sensory neurons, serotonin might modulate the activity of sensory neurons during prey-sensing. Serotonergic modulation of the activity of sensory neurons has been reported in *C. elegans* studies. The pair of ASH amphid neurons are activated by nose touch only when the amount of serotonin is abundant (Hilliard et al. 2005), and it seems to be modulated via Cel-SER-5 (Harris et al. 2009). Although we could not specify the amphid neurons that express *Ppa-ser-5*, similar activation mechanisms might be related to prey sensing and the subsequent predatory feeding behavior in *P. pacificus*.

Consistent with the studies in *C. elegans*, *P. pacificus* also requires serotonin for fast pharyngeal pumping during bacterial...
Figure 5  Ppa-ser-7 and Ppa-mod-1 play a major role in bacterial feeding. The number of pharyngeal pumping events during feeding on E. coli OP50 in 15 s in single and multiple serotonin receptor mutants. Wild type, $n = 20$. Ppa-ser-1, $n = 10$. Ppa-ser-4, $n = 15$. Ppa-ser-5 (cbh11), $n = 15$. Ppa-ser-5 (cbh23), $n = 10$. Ppa-ser-7, $n = 10$. Ppa-mod-1, $n = 10$. Ppa-ser-1, Ppa-ser-7, $n = 10$. Ppa-ser-4, Ppa-mod-1, $n = 10$. WT, wild type. Error bars represent the SEM. One-way ANOVA with Dunnett’s multiple comparisons. n.s., not significant. **$P < 0.01$. ***$P < 0.001$.

Figure 6 A hypothetical model for serotonergic modulation of predatory and bacterial feeding. For the regulation of predation, serotonin released from serotonergic neurons (shown in green) activates the M1 neuron via the Ppa-ser-7 serotonin receptor. The M1 neuron innervating dorsal tooth muscle cells transmits neuronal signals to induce tooth movement. The M1 neuron may also activate I1 and I2 neurons, resulting in indirect stimulation of the anterior pharyngeal muscles. In parallel, the anterior pharyngeal muscles (shown in pink), including the dorsal tooth muscles (shown in red), can be activated by serotonin directly via Ppa-SER-1. These neural pathways may work redundantly; thus, only the Ppa-ser-1, Ppa-ser-7 double mutant showed tooth movement defects. While the functional roles of Ppa-ser-5 are not clear, it may function in some head neurons to promote the efficient killing of prey. For the modulation of bacterial feeding, serotonin activates the M4 pharyngeal neuron via Ppa-SER-7, resulting in stimulating posterior parts of the pharynx, promoting peristalsis of the posterior pharynx and partially pharyngeal pumping. Ppa-MOD-1 may have a role in the head neurons and indirectly upregulate pharyngeal pumping, perhaps via weakening the activation of feeding inhibitory neurons.
feeding (Okumura et al. 2017). We showed that single mutants of Ppa-ser-7 and Ppa-mod-1 failed to increase pharyngeal pumping during bacterial feeding (Figure 5). These results are different from the results of C. elegans studies in three aspects. First, the P. pacificus Ppa-ser-7 mutants exhibited a lower pumping rate during bacterial feeding on agar plates. While a study using the HB101 strain as a bacterial food showed similar results for C. elegans (Song and Avery 2012), most of the C. elegans studies showed that the single Cel-ser-7 mutations do not decrease the bacterial feeding rate. Second, in P. pacificus, Ppa-mod-1 mutants decreased the pumping rate during bacterial feeding. This defect has not been reported in C. elegans Cel-mod-1 mutants, even in a study that revealed that Cel-mod-1 functions in the upregulation of pharyngeal pumping by suppressing feeding downregulation circuits (Liu et al. 2019). Third, while the Cel-ser-1; Cel-ser-7 double mutant significantly decreased the bacterial feeding rate in C. elegans (Hobson et al. 2006), the pumping rate on bacterial food in the Ppa-ser-7; Ppa-ser-7 mutant was not significantly different from that of the wild type. This result is unexpected because a single mutation of Ppa-ser-7 significantly decreased the bacterial feeding rate, implicating the possibility that the mutation in Ppa-ser-1 blocks the effects of Ppa-ser-7 mutation in an unknown manner. These differences between the two species may reflect the alteration of the functions of serotonin receptors during the acquisition of predatory feeding behavior. Ppa-ser-1 shifted to specialize in regulating predatory feeding behavior, and the functions of Ppa-ser-7 and Ppa-mod-1 in bacterial feedings became more pronounced. To confirm this working hypothesis, functional and expression analysis in evolutionarily related species should be performed in future studies.

This study demonstrated that different types of feeding behaviors in P. pacificus are under the control of distinct combinations of serotonin receptors. One of the remaining questions is how to switch bacterial feeding movements into predatory feeding movements. The switching mechanism is likely to require other neural regulatory systems; thus, studies with mutations of other neurotransmitters and neuropeptides are necessary. This switching may also be dependent on the sensory input received from the food source. While Ppa-self-1, a small peptide required to avoid cannibalism, has been recently identified (Lightfoot et al. 2019), the neuronal mechanisms of food recognition are largely unknown in P. pacificus. Future neurogenetic studies of food sensing will reveal how food stimuli are converted to behavioral output via the serotonergic nervous system. Another question is how the polyphenism of feeding behaviors, depending on the two mouth forms, is regulated in P. pacificus. We could not find marked differences in the expression patterns of serotonin receptors between predatory eurystomatous and nonpredatory stenostomatous morphs. Exploration of neuronal activity, synaptic connectivity, or transcriptional profiles might enable us to determine the differences in the nervous system between these morphs to understand the behavioral polyphenism in P. pacificus.

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