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Expression Level Changes in Serotonin Transporter Are Associated With Food Deprivation in the Pond Snail *Lymnaea stagnalis*

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In the pond snail *Lymnaea stagnalis*, serotonin (5-HT) plays an important role in feeding behavior and its associated learning (e.g., conditioned taste aversion: CTA). The 5-HT content in the central nervous system (CNS) fluctuates with changes in the nutritional status, but it is also expected to be influenced by changes in the serotonin transporter (SERT) expression level. In the present study, we identified SERT in *Lymnaea* and observed its localization in 5-HTergic neurons, including the cerebral giant cells (CGCs) in the cerebral ganglia and the pedal A cluster neurons and right and left pedal dorsal 1 neurons in the pedal ganglia by in situ hybridization. Real-time PCR revealed that the SERT mRNA expression level was lower under severe food deprivation than under mild food deprivation in the whole CNS as well as in a single CGC. These results inversely correlated with previous data that the 5-HT content in the CNS was higher in the severely food-deprived state than in the mildly food-deprived state. Furthermore, in single CGCs, we observed that the 5-HT level was significantly increased in the severely food-deprived state compared with the mildly food-deprived state. Our present findings suggest that changes in the SERT expression level associated with food deprivation may affect 5-HT signaling, probably contributing to learning and memory mechanisms in *Lymnaea*.

Key words: 5-HT, conditioned taste aversion, food deprivation, *Lymnaea*, serotonin transporter

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter in the pond snail *Lymnaea stagnalis*. 5-HT involvement is implicated in feeding rhythms (Elekes et al., 2018), decision-making (Aonuma et al., 2020), behavioral changes associated with nutritional conditions (Dyakonova et al., 2015a, b; Totani et al., 2023), and behavioral changes associated with feeding (Aonuma et al., 2018a, b). Furthermore, Ivashkin and colleagues discovered that the 5-HTergic system plays a critical role in transmitting non-genetic instructive signals from the mother to the offspring that modulate the dynamics of growth, feeding behavior, and locomotion (Ivashkin et al., 2015).

A pair of 5-HTergic interneurons, the cerebral giant cells (CGCs) (Croll and Chiasson, 1989; Hatakeyama and Ito, 1999), in the central nervous system (CNS) are primarily responsible for several behavioral changes (Yeoman et al., 1996; Korneev et al., 1998; Elliott and Vehovszky, 2000; Straub and Benjamin, 2001; Scutt et al., 2015; Hatakeyama et al., 2022a). In particular, the CGCs act as regulatory neurons for the feeding central pattern generator (Kojima et al., 1997). That is, the amount of 5-HT released from the CGCs directly affects feeding behavior and learning and memory formation of conditioned taste aversion (CTA), a classical conditioning task acquired by *Lymnaea* (Nakai et al., 2020a, b). For example, Sunada et al. investigated whether the CGCs play a key role in CTA and showed that the CGC somata are necessary for both memory formation and memory retrieval (Sunada et al., 2017). The 5-HT receptor types in neurons receiving synaptic inputs from the CGCs were also examined (Kawai et al., 2011).

Previous studies demonstrated that different food deprivation states of *Lymnaea* are associated with changes in 5-HT levels throughout the CNS. The CNS 5-HT content after 5 days of food deprivation (Day 5 snails) is higher than that after 1 day of food deprivation (Day 1 snails) (Aonuma et al., 2018a). Inversely correlated with this variability, Day 1 snails exhibit a stronger CTA than Day 5 snails (Aonuma et al., 2018b). Totani and colleagues recently reported that the reason for the increase in the CNS 5-HT content in Day 5 snails is that snails heavily deprived of food use an autophagy mechanism to restore tryptophan in the CNS, resulting in an increase in 5-HT (Totani et al., 2023). That is, following prolonged food deprivation, autophagy in the CNS is enhanced to provide amino acids (e.g., tryptophan) by degrading proteins in the neurons, thereby ameliorating the lack of CNS tryptophan. As a result, CNS 5-HT concentrations are increased and the enhanced CTA learning ability is reversed.

Furthermore, 5-HT actions depend on the extracellular levels of this amine, which are regulated by its synthetic

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enzymes and the plasma membrane serotonin transporter (SERT) (Carvajal-Oliveros and Campusano, 2021). SERT is monoamine transporter protein that transports 5-HT from the synaptic cleft back to the presynaptic neuron in a process known as serotonin reuptake. This transport of 5-HT by the SERT protein terminates the action of 5-HT and recycles it in a sodium-dependent manner (Pelliccioni et al., 2001). Studies of SERT to date have focused mainly on depression (Campos et al., 2023). Although there are a few studies in *Drosophila* and rodents on the relationship between SERT expression and 5-HT levels (Kim et al., 2005; Homberg et al., 2007; Borue et al., 2010), there are not many studies available. The details of the function of SERT in *Lymnaea* (hereafter referred to as LymSERT) for 5-HT levels remain unclear. Sadamoto and colleagues (Sadamoto et al., 2008) performed a preliminary study of SERT localization in *Lymnaea*, but they did not describe whether or not the full length was determined and in situ hybridization was performed using only a part of the sequences. No functionality for LymSERT has yet been clarified.

In the present study, we identified LymSERT, confirmed its localization in the CNS, and examined changes in the LymSERT mRNA expression level in different food deprivation states. We studied these changes not only in the whole CNS but also in individual CGCs.

MATERIALS AND METHODS

Snails

Lymnaea stagnalis with a 20- to 25-mm shell length were used. Snails were maintained in dechlorinated tap water under a 12-h light: 12-h dark cycle at 20°C–23°C and fed Japanese mustard spinach (*Brassica rapa* var. *peruviridis*, known as komatsuna in Japanese). The culture of *Lymnaea stagnalis* was originally derived from stocks maintained at Vrije Universiteit Amsterdam. Food deprivation status was defined as follows: 'Day 1 snails' were mildly food-deprived for 1 day and 'Day 5 snails' were severely food-deprived for 5 days.

Identification of the LymSERT gene transcript

LymSERT sequences were identified by a standard nucleotide BLAST (BLASTn) search (https://blast.ncbi.nlm.nih.gov/Blast.cgi [accessed on 12 March 2023]) using the transcriptome shotgun assembly (TSA) database for *Lymnaea stagnalis* (Sadamoto et al., 2012) with *Aplysia californica* serotonin transporter cDNA (ApSERT: accession No. NP_001191502.1) as a query sequence. The domain sequences of the identified amino acid sequences were predicted with the database Pfam in InterPro (http://www.ebi.ac.uk/interpro/ [accessed on 12 March 2023]). The neighbor-joining tree of SERT in various species was generated using MEGA 10 software (https://www.megasoftware.net/ [accessed on 12 March 2023]). Accession numbers for the amino acid sequences designed for the phylogenetic tree are listed in Table 1.

CNS and CGC sample preparations

For in situ hybridization, immunohistochemistry, and real-time PCR experiments, samples were prepared in the same way as in the previous studies (Hatakeyama et al., 2022a; Fujimoto et al., 2023; Totani et al., 2023). For CGC isolation, the CGCs were isolated using a glass capillary in high osmolality medium (Leibovitz's L-15 medium [Gibco BRL, Gaithersburg, MD, USA] and *Lymnaea* saline [10 mM HEPES at pH 7.9, 50 mM NaCl, 1.6 mM KCl, 2.0 mM $MgCl_2$ and 3.5 mM $CaCl_2$], with a 1:1 mixing ratio). Isolated cells were transferred into a CGC lysis solution (0.45 μL DEPC-treated water [Thermo Fisher Scientific, Waltham, MA, USA] and 0.25 μL RNase inhibitor [Applied Biosystems, Foster City, CA, USA]), frozen in liquid nitrogen, and stored at −80°C.

In situ hybridization and immunohistochemistry

The protocol for the in situ hybridization experiments was the same as that used in the previous studies (Hatakeyama et al., 2004; Fujimoto et al., 2023). Sections used in the in situ hybridization experiments were cut at 10-μm thickness. For probe synthesis, regions of probes for LymSERT (288 bp) were amplified from *Lymnaea* cDNA with Ex Taq (Takara Bio, Shiga, Japan), and the primers are listed in Table 2. The PCR products were cloned into pTAC-2 plasmids. To prepare the template DNA for in vitro transcription, the inserted regions were amplified with KOD FX (Toyobo, Osaka, Japan) and forward and reverse M13 primers, and then purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). The sense and antisense probes were synthesized at 37°C for 2 h with a MAXIscrip SP6/T7 Transcription Kit (Invitrogen-Thermo Fisher Scientific) and RNA labeling mix (DIG-UTP; Roche, Basel, Switzerland).

The immunohistochemistry protocol was the same as that used in the previous studies (Croll and Chiasson, 1989; Hatakeyama and Ito, 1999). The whole mount CNS was used. Anti-5-HT rabbit antibody (Immunostar, Hudson, WI, USA; 1:1000 dilution) was used as the primary antibody. Alexa Fluor 488-labeled goat anti-rabbit IgG H&L (Abcam, Cambridge, MA, USA; 1:1000 dilution) was then used as the secondary antibody. DAPI was used for counterstaining.

Real-time PCR

The real-time PCR protocol was the same as that used in the

Table 1. List of SERTs for neighbor-joining tree.

Species	Accession number
Mus musculus	CAA70092.1
Homo sapiens	AAW80933.1
Aplysia californica	NP_001191502.1
Crassostrea gigas	XP_034298937.1
Drosophila melanogaster	NP_523846.2
Caenorhabditis elegans	AAK84832.1
Strongylocentrotus purpuratus	XP_790949.4
Danio rerio	XP_031750749.1
Xenopus laevis	XP_018104283.1
Schmidtea mediterranea	AHB51758.1

Table 2. List of PCR primers used for preparation of in situ hybridization probes.

Primer		Sequence (5'–3')
LymSERT	Forward	AAG TGG ATG CTT AAA GTT GG
	Reverse	TCA ACT AAG AGC CGG AAC A
M13	Forward	GTA AAA CGA CGG CCA GT
	Reverse	CAG GAA ACA GCT ATG AC

Table 3. List of PCR primers for real-time PCR.

Primer		Sequence (5'–3')
EF1α	Forward	TCC AAA GAA GGC CAG ACC C
	Reverse	TAT GGT GGT GAG GTG CTG TC
β-tubulin	Forward	CAA GCG CAT CTC TGA GCA GTT
	Reverse	TTG GAT TCC GCC TCT GTG AA
LymSERT	Forward	GGC AAC CAT CTT CAC CCG TA
	Reverse	TAG TGC TTT GCT CCG TGA GG

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previous study (Fujimoto et al., 2023). Relative mRNA levels were quantified using the comparative Ct method. The Ct values of the target genes were normalized by dividing by the mean of the Ct values of elongation factor 1 alpha (EF1α) and β-tubulin as reference genes. Mean EF1α and β-tubulin values were stable under the measuring conditions. The primer sequences are shown in Table 3.

ELISA

The 5-HT ELISA protocol was the same as that used in the previous study (Totani et al., 2023). Briefly, a 5-HT ELISA kit (KA2518, Abnova, Taipei, Taiwan) was used. Isolated CGCs were added into 5-HT stabilizer solution (1.66 μL DEPC-treated water [R0603, Thermo Fisher Scientific] + 0.26 μL 10% 5-HT stabilizer solution contained in ELISA kit), and ELISA was performed accord-

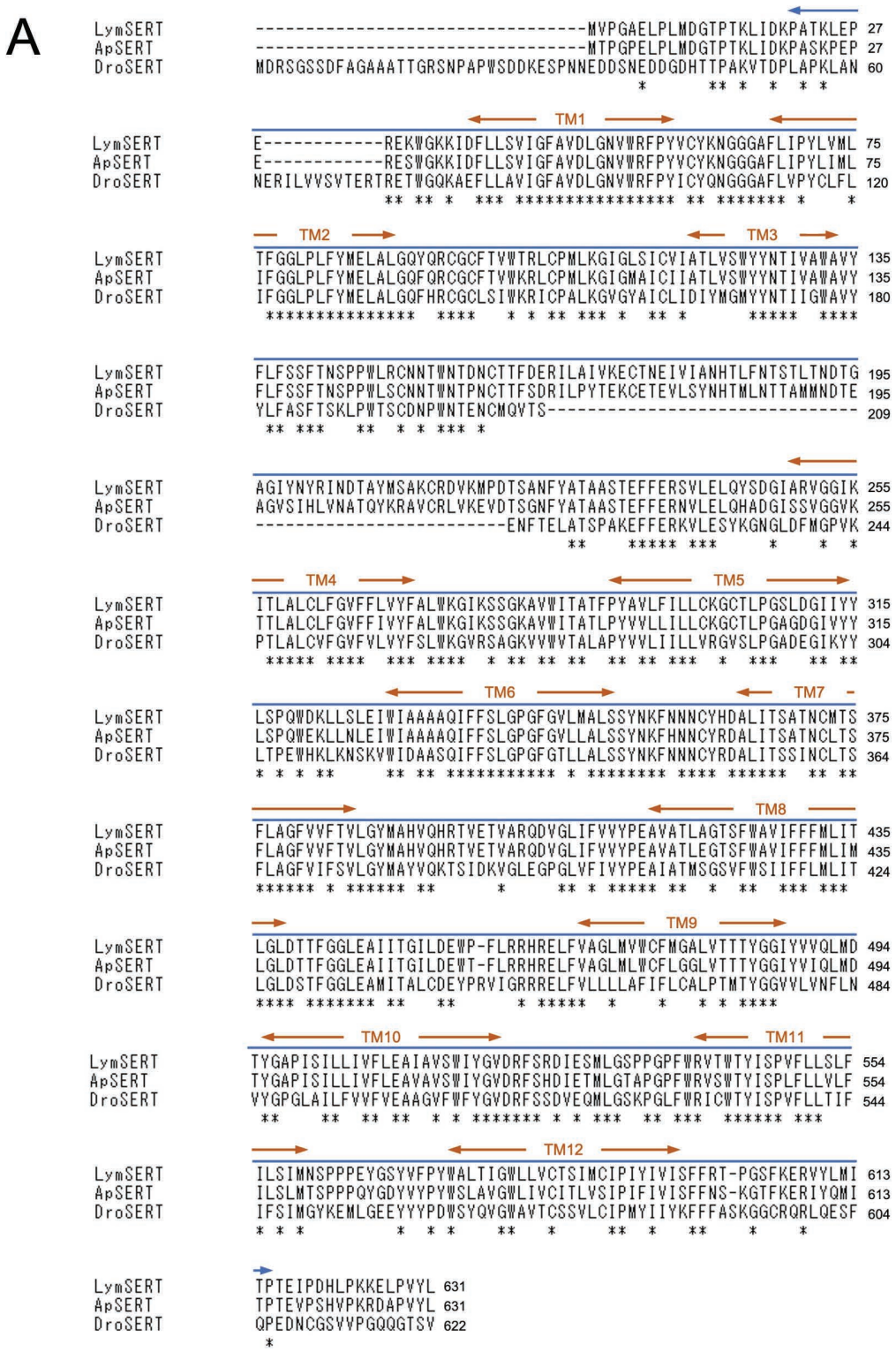

Fig. 1. Continued.

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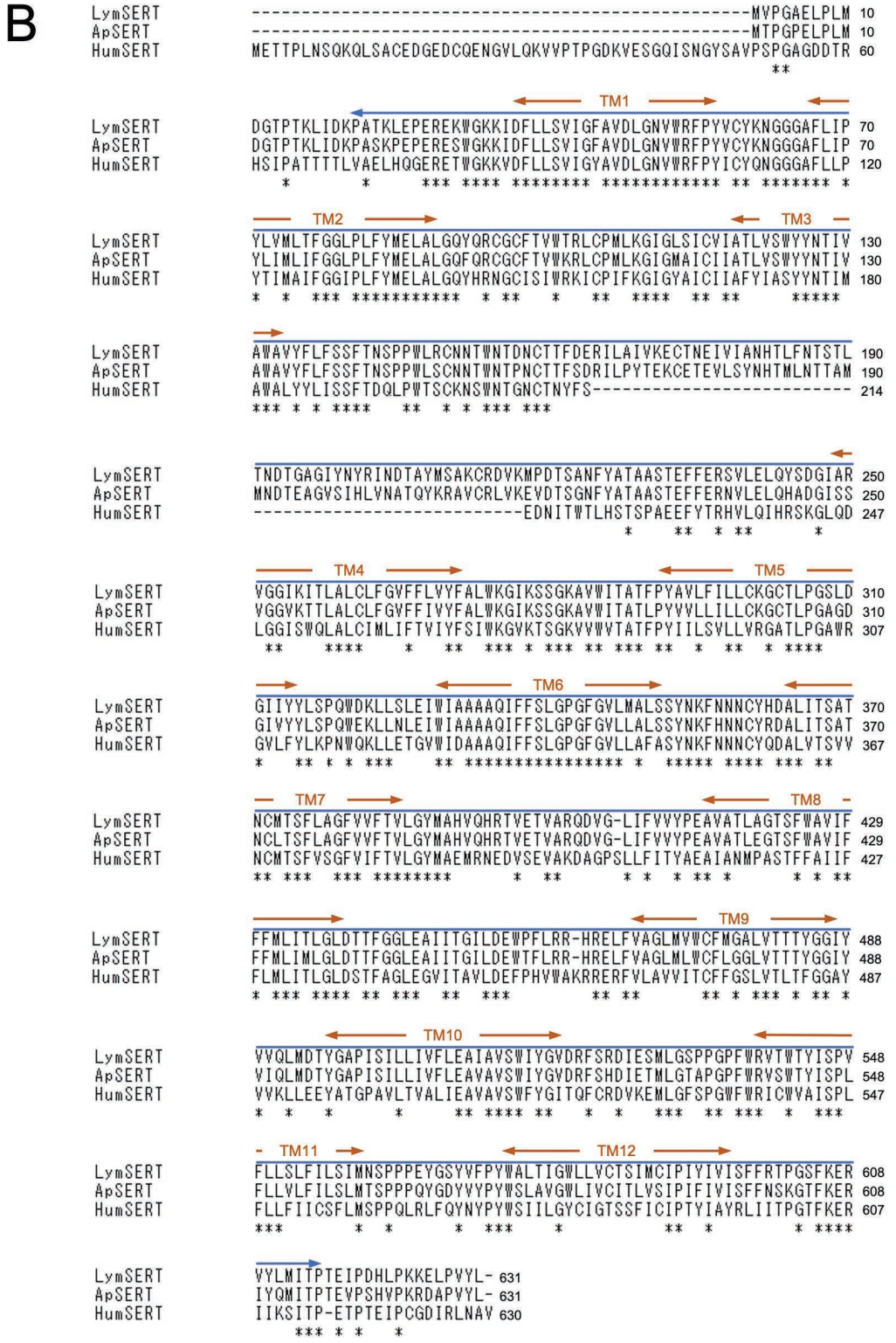

Fig. 1. Comparison of deduced amino acids of LymSERT with other SERTs. For easier understanding of identity and preservability, two panels are provided. **(A)** Alignment of LymSERT, ApSERT, and DroSERT. **(B)** Alignment of LymSERT, ApSERT, and HumSERT. * indicates the conserved amino acid. TM shows the transmembrane regions numbered 1 through 12. Blue line indicates sodium: neurotransmitter symporter superfamily domain.

ing to the manufacturer's protocols. As the left and right CGCs have the same functionality (Goldschmeding et al., 1981), we selected only the left CGCs for the 5-HT ELISA.

Statistics

All data are expressed as mean ± SEM. Statistical analyses were performed with IBM SPSS Statistics software (version 28)

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with $P < 0.05$ indicating a significant difference. Student's *t*-test was used for comparison between two groups.

RESULTS

Identification of the LymSERT gene transcript

A putative SERT in *Lymnaea*, LymSERT, was searched for using *Alysia* SERT (ApSERT) (NP_0011911502.1) as a query sequence. The BLASTn search resulted in a hit in the *Lymnaea stagnalis* mRNA TSA database (contig: Lym-stCNS_TSA_4904, mRNA sequence FX185022.1). This TSA sequence was predicted to contain an open reading frame (ORF). The predicted amino acid sequence was aligned with ApSERT, *Drosophila melanogaster* (DroSERT), and *Homo sapiens* (HumSERT) (Fig. 1). LymSERT comprises 631 amino acids. LymSERT and ApSERT display 82.4% amino acid identity; LymSERT and DroSERT display 49.8% amino acid identity; and LymSERT and HumSERT display 46.0% amino acid identity. The domain sequence and transmembrane region predictions suggest that LymSERT has the sodium:neurotransmitter symporter superfamily domain (Schmidt et al., 2022), which occupies a substantial part of the ORF. Furthermore, LymSERT has 12 transmembrane regions, as does human SERT (Yamashita et al., 2005; Coleman et al., 2016).

A molecular phylogenetic tree of SERT-like proteins deduced from various animals was generated using the neighbor-joining method (Fig. 2). LymSERT was most closely related to ApSERT, and these two together with *Crassostrea gigas* SERT were clustered into a single family, which can be called a Mollusca group. This cluster forms an Invertebrata group with Arthropoda. Echinodermata belong to the Vertebrata group and not the Invertebrata group, consistent with the fact that Echinodermata and Vertebrata are both subgroups of Deuterostomia.

Co-localization of LymSERT mRNA and 5-HT in the CNS

The localization of LymSERT mRNA and the immunore-

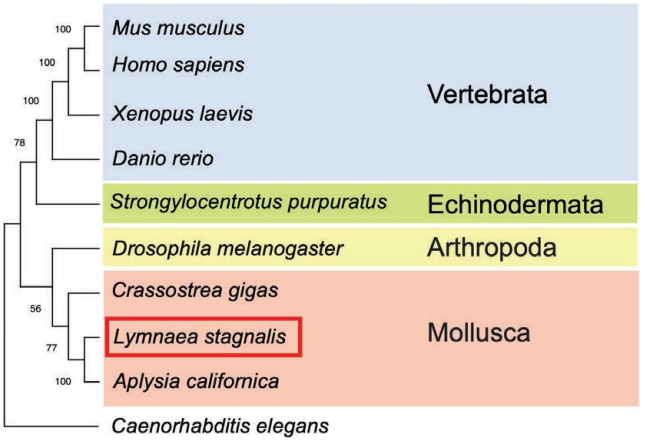

Fig. 2. A molecular phylogenetic tree of 10 SERT-like proteins inferred using the neighbor-joining method. The bootstrap value for each branch was calculated by testing the phylogenetic tree 1000 times and is expressed as a percentage.

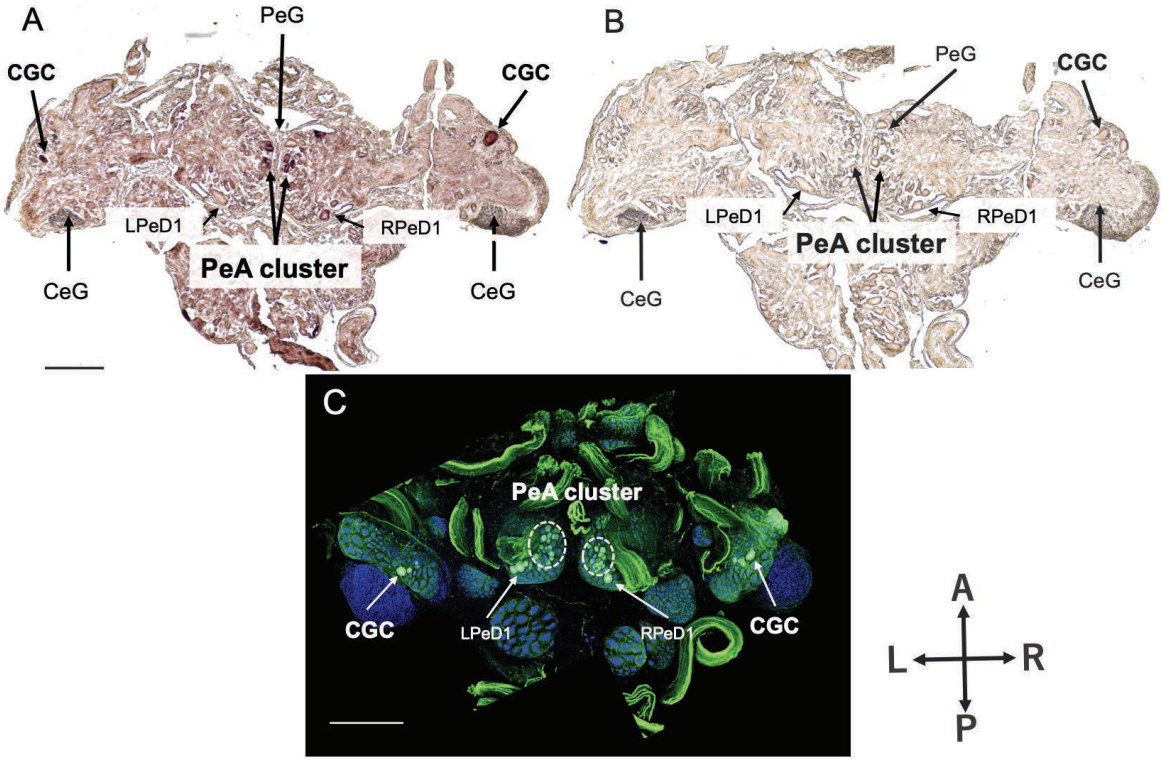

Fig. 3. In situ hybridization of LymSERT and immunohistochemistry of 5-HT in *Lymnaea* CNS. **(A)** Staining of the antisense probe for LymSERT. **(B)** Staining of the sense probe (control) for LymSERT. The sections of **(A)** and **(B)** are adjacent to each other. **(C)** Immunostaining for 5-HT. CeG: cerebral ganglion, PeG: pedal ganglion, CGC: cerebral giant cell, RPeD1 and LPeD1: right and left pedal dorsal 1 neurons, PeA cluster: pedal A cluster neurons. Scale bars: 0.3 mm.

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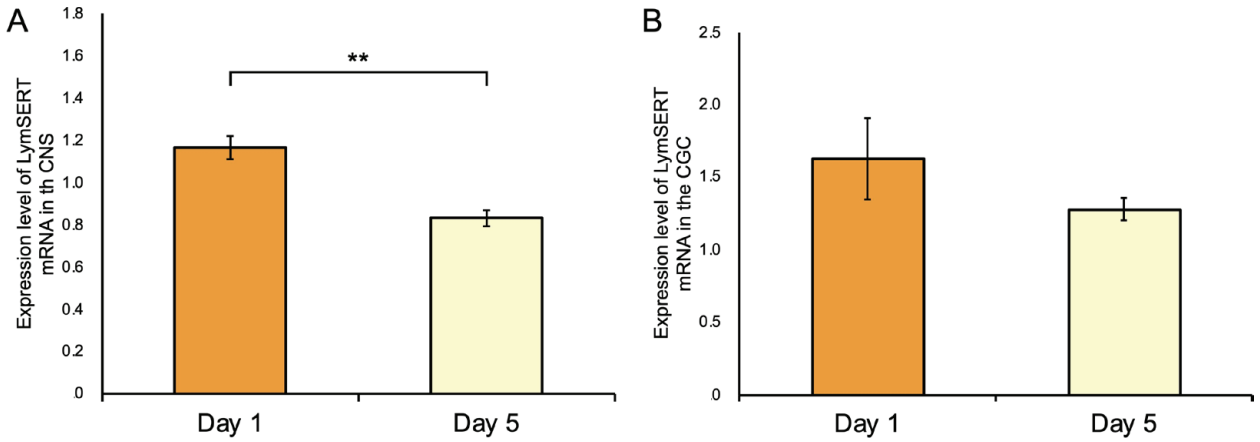

Fig. 4. Changes in the LymSERT mRNA expression level associated with food deprivation. **(A)** LymSERT expression level in the CNS. A significant difference was observed between Day 1 and Day 5. $n = 7$ for Day 1 snails and $n = 5$ for Day 5 snails. ** indicates $P < 0.01$. **(B)** LymSERT expression level in a single CGC. $n = 26$ for Day 1 snails and $n = 22$ for Day 5 snails. $P = 0.570$.

activity of 5-HT in the *Lymnaea* CNS were examined by in situ hybridization and immunohistochemistry, respectively (Fig. 3). Studies of 5-HT immunostaining in the *Lymnaea* CNS have been reported (Croll and Chiasson, 1989; Hatakeyama and Ito, 1999). The co-localization of LymSERT and 5-HT was observed in the CGC in the cerebral ganglia, the pedal A (PeA) cluster neurons, and the right and left pedal dorsal 1 neurons (RPeD1 and LPeD1) in the pedal ganglia. That is, the putative SERT identified in *Lymnaea* in the present study probably functions as a 5-HT reuptake transporter.

Comparison of LymSERT mRNA expression levels between Day 1 and Day 5 snails

We examined changes in the LymSERT mRNA expression levels between a mildly food-deprived state (Day 1 snails) and a severely food-deprived state (Day 5 snails) (Fig. 4). Comparison of these different food deprivation states was performed at both the whole CNS and the single CGC levels (Hatakeyama et al., 2022a). In the CNS, the LymSERT mRNA expression level was lower in Day 5 snails than in Day 1 snails ($P = 0.002$, $n = 7$ for Day 1 snails and $n = 5$ for Day 5 snails; Fig. 4A). In the single CGC, a decreasing trend in LymSERT mRNA expression was also observed ($P = 0.570$, $n = 26$ for Day 1 snails and $n = 22$ for Day 5 snails; Fig. 4B).

Change in the 5-HT content in a single CGC after 5 days of food deprivation

Previous studies demonstrated that the 5-HT content in the whole CNS of severely food-deprived snails (Day 5 snails) is increased compared with that in mildly food-deprived snails (Day 1 snails) (Aonuma et al., 2018a, b). We thus examined the 5-HT content in a single CGC of both Day 1 and Day 5 snails (Fig. 5). As in the whole CNS, the 5-HT content in the single CGC was increased in Day 5 snails ($P = 0.04$, $n = 16$ for Day 1 snails and $n = 12$ for Day 5 snails). That is, the 5-HT content negatively correlated with the LymSERT expression level.

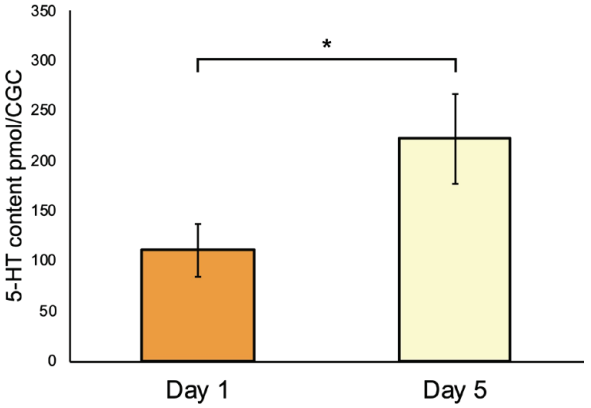

Fig. 5. Change in 5-HT content in a single CGC associated with food deprivation. $n = 16$ for Day 1 snails and $n = 12$ for Day 5 snails. * indicates $P < 0.05$.

DISCUSSION

We deduced the presence of a SERT (LymSERT) in *Lymnaea* by a BLASTn search. LymSERT has a sodium: neurotransmitter symporter superfamily domain and 12 transmembrane regions. LymSERT mRNA is expressed in the 5-HTergic CGC in the cerebral ganglia and the 5-HTergic PeA cluster neurons and RPeD1 and LPeD1 in the pedal ganglia. The co-localization of 5-HT and LymSERT mRNA suggests that LymSERT achieves selective reuptake of 5-HT into the neurons of snails as well as in other species such as mammals (Hoffman et al., 1998). Taken together, these findings indicate that the molecule identified in the present study may function as a SERT.

Focusing on the function of the SERT mRNA localization site, the pedal ganglia have been shown to regulate locomotion (Aonuma et al., 2020). Furthermore, it is important to note that that we confirmed the co-expression of LymSERT mRNA and 5-HT, especially in the CGCs. CGCs innervate multiple interneurons and motor neurons involved in feeding behavior and CTA (Yeoman et al., 1996; Kojima et al., 1997; Sunada et al., 2017; Hatakeyama et al., 2022b;

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Nakai et al., 2022). The report by Dyakonova et al. (2015a) also showed that 5-HTergic neuron activity increases during fasting. We examined food-deprived Day 1 snails and Day 5 snails here to determine whether LymSERT mRNA expression in the pedal ganglia may be involved in behavioral changes. In CTA of *Lymnaea*, snails show approach and biting to a sucrose solution that is an appetitive stimulus, but they stop locomotion and biting when an aversive KCl stimulus is applied, and show a withdrawal response to this KCl solution (Kojima et al., 1996). When sucrose and KCl are repeatedly applied in the CTA training protocol, sucrose application does not result in locomotion or biting. Therefore, the pedal ganglia are strongly implicated in CTA.

Furthermore, LymSERT mRNA expression levels negatively correlated with the 5-HT content in a single CGC as well as in the whole CNS. Previous studies showed that the CNS 5-HT content in food-deprived Day 1 snails was low whereas that in Day 5 snails was high (Aonuma et al., 2018a). On the other hand, the contents of 5-HT catabolites, *N*-acetylserotonin (Nac-5-HT) and 5-hydroxyindole acetoaldehyde (5-HIAA), in the CNS did not differ significantly between Day 1 and Day 5 snails (Aonuma et al., 2018a). That is, the causal relationship between the 5-HT reuptake inability due to the decrease in SERT expression and the increase in 5-HT content remains unknown. The 5-HT in Day 1 snails may be degraded by monoamine oxidase (MAO) (Chikamoto et al., 2023), but future studies should examine the existence of MAO in *Lymnaea,* and whether it is present in the 5-HTergic neurons or other neuronal types.

Severely food-deprived (Day 5) snails had a high CNS 5-HT content, whereas mildly food-deprived (Day 1) snails had a low CNS 5-HT content (Aonuma et al., 2018a, b). Previous studies clarified that this difference is due to autophagic flux upregulated in severe food deprivation (i.e., tryptophan production by the destruction of proteins) (Totani et al., 2023). The downregulation of LymSERT in Day 5 snails is thought to also contribute to the increase in 5-HT. Even at the single CGC level, LymSERT in Day 1 snails was high whereas that in Day 5 snails was low. In addition, the 5-HT content was low in Day 1 snails and high in Day 5 snails at the single CGC level, as shown in the present study.

On the other hand, in classical conditioning (CTA), the learning scores are worse in Day 5 snails than in Day 1 snails (Aonuma et al., 2018a, b). Although we do not know the detailed mechanism underlying this phenomenon at present, we speculate that the 5-HT content affects the learning score. Our present findings suggest the involvement of uptake transporters of neurotransmitters in learning and memory mechanisms. This is important because many studies of uptake transporters have only been performed in the context of cognitive impairment as a human disease (van de Blaak and Dumont, 2022).

In conclusion, there are only a few studies on the relationship between SERT expression and 5-HT levels in some animals (Kim et al., 2005; Homberg et al., 2007; Borue et al., 2010). However, this relationship requires more scrutiny. The present study showed that the LymSERT expression level is inversely related to the 5-HT content in the *Lymnaea* CNS in association with food deprivation. A change in the LymSERT expression level may contribute to the CTA mechanism in *Lymnaea*.

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COMPETING INTERESTS

The authors declare no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

NC and EI designed experiments. NC, KF, JN, and YT conducted experiments. NC, DH, and EI interpreted the data. JN acquired funding. NC, DH, and EI wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY

All data that support the findings of this study are available from the corresponding author upon reasonable request.

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