# Genes Upregulated by Operant Conditioning of Escape Behavior in the Pond Snail Lymnaea stagnalis

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# Genes Upregulated by Operant Conditioning of Escape Behavior in the Pond Snail *Lymnaea stagnalis*

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**The pond snail *Lymnaea stagnalis* is capable of learning by both classical conditioning and operant conditioning. Although operant conditioning related to escape behavior with punishment has been examined by some research groups, the molecular mechanisms are not known. In the present study, we examined changes in the expression levels of cAMP-response element binding protein 1 (CREB1), CREB2, CREB-binding protein (CBP), and monoamine oxidase (MAO) in the *Lymnaea* central nervous system (CNS) using real-time PCR following operant conditioning of escape behavior. CREB1 and CREB2 are transcription factors involved in long-term memory in *Lymnaea*; CBP is a coactivator with CREB1; and MAO is a degrading enzyme for monoamines (e.g., serotonin) with important roles in learning and memory in *Lymnaea*. In operant conditioning, the punishment cohort, in which snails escaping from the container encountered aversive KCl, exhibited significantly fewer escape attempts than the control cohort, in which snails escaping from the container encountered distilled water, during both the training and memory test periods. After the operant conditioning, CREB1 and CREB2 were upregulated, and the ratio of CREB1/CREB2 was also increased, suggesting that the operant conditioning of escape behavior involves these factors. MAO was also upregulated, suggesting that the content of monoamines such as serotonin in the CNS decreased. The upregulated genes identified in the present study will help to further elucidate learning and memory mechanisms in *Lymnaea*.**

**Key words:** CBP, CREB, escape behavior, *Lymnaea*, MAO, operant conditioning

## INTRODUCTION

Both classical and operant conditioning are reported in the pond snail *Lymnaea stagnalis* (Kemenes et al., 2022; Nakai et al., 2022; Kagan et al., 2023; Rivi et al., 2023; Wingrove et al., 2023). One type of classical conditioning is conditioned taste aversion (CTA) (Kojima et al., 1996; Nakai et al., 2020), and a type of operant conditioning is escape behavior suppression learning (EBSL) (Kobayashi et al., 1998; Benatti et al., 2020; Fujimoto et al., 2023). In the EBSL memory test, snails spontaneously suppress their tendency to escape from a container because they have learned that an aversive stimulus (KCl) exists outside the container. Some of the molecules involved in CTA have been identified and characterized; e.g., research is progressing on the transcription factors cAMP-response element binding proteins (CREB1 and CREB2) as well as their co-factor CREB-binding protein (CBP) (Sadamoto et al., 2004; Hatakeyama et al., 2022a). On the other hand, the molecules involved in EBSL in *Lymnaea* are unknown, except for adiponectin and its receptor (Fujimoto et al., 2023).

Serotonin (5-HT) in the central nervous system (CNS) is involved in learning and memory in the pond snail *Lymnaea stagnalis* (Lukowiak et al., 2014; Aonuma et al., 2018a). Variability in the 5-HT levels is due in part to the serotonin transporter and autophagy (Sadamoto et al., 2008; Chikamoto et al., 2023; Totani et al., 2023), but also likely due to the degradation of 5-HT by monoamine oxidase (MAO) (Jones and Raghanti, 2021). MAO is responsible for the degradation of 5-HT as well as dopamine and noradrenaline in the CNS of vertebrates and invertebrates (Sloley, 2004). Although MAO is known to have a role in neuropsychiatric, neurodevelopmental, and neurodegenerative diseases, especially in the human CNS (Jones and Raghanti, 2021), few studies have examined the involvement of MAO in learning and memory mechanisms (Zhukovsky et al., 2017). To the best of our knowledge, no studies have evaluated the role of MAO in learning and memory in *Lymnaea*.

In the present study, we examined the changes in gene expression induced by EBSL using real-time PCR. We first identified MAO in *Lymnaea* (referred to as LymMAO). The LymCREB1, LymCREB2, LymCBP, and LymMAO genes were analyzed. Changes in the expression levels of these genes indicate that EBSL is useful for further investigation of the molecular mechanisms underlying learning and memory in *Lymnaea*.

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## MATERIALS AND METHODS

### Snails

*Lymnaea stagnalis* with a 20–25 mm shell length were used. The 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 *Lymnaea stagnalis* culture was derived from stocks originally maintained at Vrije Universiteit Amsterdam.

### Identification of LymMAO

The LymMAO sequence was identified by a BLAST 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) based on *Aplysia californica* probable flavin-containing monoamine oxidase A (accession No. XP_005098472.1). 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 MAO in various species was generated with MEGA 10 software (https://www.megasoftware.net/ [accessed on 12 March 2023]). The amino acid sequences used for the phylogenic tree are listed in Table 1.

### EBSL

EBSL experiments were carried out according to previous studies (Kobayashi et al., 1998; Fujimoto et al., 2023). Briefly, a tray was lined with paper towels soaked with distilled water (DW, control) or 100 mM KCl (punishment), and 35-mm dish lids were placed on the tray. The lids were filled with DW to a depth of 3 mm and *Lymnaea* was placed in the center of each lid. The experiments comprised 2 steps: step 1 was a 60-min training period. The punishment (100 mM KCl) and control (DW) cohorts were placed on the lids, which were positioned on paper towels soaked with KCl or DW, respectively. The second step was a 60-min memory test. Both cohorts were placed on the lids positioned on paper towels soaked with DW. During both the training and the memory test, the number of escapes from the lid was recorded for 60 min. The timing of the escape was defined as the moment when *Lymnaea* leaned out of the lid and put its head on the paper towel. When the escape was confirmed, the snail was relocated to the center of the lid. Besides recording the number of escapes, the latency to the first escape was recorded during training and during the memory test. For individuals that did not escape within 60 min, the first escape was considered to have a 60-min latency. The CNS was dissected from *Lymnaea* 60 min after the memory test and subjected to real-time PCR experiments. The snails used to obtain the real-time PCR were different from those used for the behavioral data. Furthermore, control experiments were performed to confirm that changes in the expression levels of the target mRNAs were specific to the association with EBSL and not solely due to KCl exposure. Two cohorts were prepared. One cohort was used with the following protocol. A 100-mM KCl solution was applied to the snails remaining on the lids and shortly thereafter the KCl was changed to DW. The snails were kept on the lids by covering them with a 35-mm dish to prevent them from escaping for 60 min. Then, the DW was changed to fresh DW for another 60 min. The other cohort was exposed to the following protocol. DW was applied to snails on the lids and shortly thereafter the DW was changed to fresh DW. The snails were kept on the lids by covering them with 35 mm dishes to prevent their escape for 60 min. Then, the DW was changed to fresh DW again for another 60 min.

### Real-time PCR

The protocol for the real-time PCR experiments was the same as that used in previous studies (Hatakeyama et al., 2022b; Fujimoto et al., 2023). The relative mRNA levels were quantified using the comparative Ct method. The whole CNS of the snails was dissected and stored at – 80°C before the real-time PCR measurements. Total RNA was extracted using ISOGEN II (311-07361; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). THUNDERBIRD Next SYBR qPCR Mix (Toyobo) was used to perform real-time PCR (StepOnePlus Real-Time PCR System; Applied Biosystems, Waltham, MA, USA). The Ct values of the target genes (i.e., LymCREB1, LymCREB2, LymCBP, and LymMAO) were normalized by dividing by the mean of the Ct values of elongation factor 1 alpha (EF1$\alpha$) and $\beta$-tubulin as reference genes. The mean EF1$\alpha$ and $\beta$-tubulin values were stable under the measured conditions. The primer sequences are shown in Table 2. Efficiency values for the real-time PCR primers ranged from 90% to 110%. The PCR conditions were as follows: 1 cycle at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s; and annealing at 60°C for 10 s. Melting curve analysis was performed from 60°C to 95°C with a heating rate of 0.3°C/s. In the control experiments to confirm that the changes in the expression levels of target mRNAs were specific to the association with EBSL but not with KCl exposure, the expression levels of the target genes (i.e., LymCREB1, LymCREB2, LymCBP, and LymMAO) and reference genes (i.e., EF1$\alpha$ and $\beta$-tubulin) were measured.

### Statistics

The data for behavioral experiments are represented by boxplots, and the data for real-time PCR are expressed as mean ± SEM. Statistical analyses were performed with IBM SPSS Statistics soft-

**Table 1.** List of MAOs for neighbor-joining tree.

| Species | Accession number |
|---|---|
| *Mus musculus* MAO A | AAH29100.1 |
| *Mus musculus* MAO B | EDL35718.1 |
| *Homo sapiens* MAO A | AAA59547.1 |
| *Homo sapiens* MAO B | AAA59551.1 |
| *Xenopus laevis* | NP_001088354.1 |
| *Danio rerio* | AAO16681.3 |
| *Oncorhynchus mykiss gairdneri* | AAA64302.1 |
| *Crassostrea gigas* | CAD89351.1 |
| *Haliotis discus hannai* | UXK97412.1 |
| *Aplysia californica* | XP_005098472.1 |
| *Salpingoeca rosetta* | EGD74218.1 |

**Table 2.** List of PCR primer sets for real-time PCR.

| Primer | | Sequence (5'–3') |
|---|---|---|
| EF1$\alpha$ | Forward | TCC AAA GAA GGC CAG ACC C |
| | Reverse | TAT GGT GGT GAG GTG CTG TC |
| $\beta$-tubulin | Forward | CAA GCG CAT CTC TGA GCA GTT |
| | Reverse | TTG GAT TCC GCC TCT GTG AA |
| LymCREB1 | Forward | GCT CAA GGT GTT GTT ATG ACA GGT |
| | Reverse | TTC GAG AGC CTT CTT CAG ACA TG |
| LymCREB2 | Forward | CCT AGC TAC GGC TGC TAT ATC TAC AAA |
| | Reverse | GTC AAC AAG TCC AGG TCC CAT T |
| LymCBP | Forward | GCC CTC CGG CCA ACA AGA A |
| | Reverse | TAT TAT CGC TGG GTG TAT TGA GAG AT |
| LymMAO | Forward | TGC CCA GGT TAG CCA GTT TG |
| | Reverse | TAG GGC AGC AAC TGA GGT TC |

ware (version 28) with $P < 0.05$ considered significant. For comparison between two groups, Student's *t*-test or Welch's *t*-test was used. For multiple comparisons among three or more groups, a repeated measures 2-way ANOVA and post-hoc Bonferroni test were used.

## RESULTS

### Identification of LymMAO

A putative MAO in *Lymnaea*, LymMAO, was identified from *Aplysia californica*, namely, probable flavin-containing monoamine oxidase A (XP_005098472.1), referred to as ApMAO. The Blastn (Standard Nucleotide BLAST) search resulted in hits for *Lymnaea stagnalis* mRNA TSA (contig: Lym-stCNS_TSA_9496, mRNA sequence FX189614.1). Examination of this mRNA sequence revealed that it contained the full length of its open reading frame. The sequence was translated for the open reading frame and aligned with ApMAO (Fig. 1). The predicted LymMAO comprises 523 amino acids, and LymMAO and ApMAO display 76.8% amino acid similarity. LymMAO contains an amine oxidase domain.

A molecular phylogenetic tree of MAO-like proteins deduced from various animals was generated using the neighbor-joining method (Fig. 2). LymMAO was most similar to ApMAO, and these two were closely related to oyster and abalone MAOs, forming an Invertebrata group. The MAO of choanoflagellates (*Salpingoeca rosetta*) was used as an outgroup.

### Behavioral change in EBSL

The number of escapes during the memory test period using the control (DW) stimulation significantly decreased compared with that during the training period ($P = 0.007$, $n = 20$/group; Fig. 3A). The escape numbers did not differ significantly between the training and memory test periods using the punishment (KCl) ($P = 0.710$). In the training period, the escape numbers using the punishment (KCl) were significantly lower than those using the control (DW) stimulation ($P < 0.001$). In the memory test period, the escape numbers using the punishment (KCl) were significantly lower compared with those using the control (DW) stimulation ($P = 0.021$).

The latency of the first escape was higher in the memory test period than during the training period for the control (DW) cohort ($P = 0.003$) as well as for the punishment (KCl) cohort ($P < 0.001$; Fig. 3B). The escape latencies of the punishment (KCl) cohort and that of the control (DW) cohort were not significantly different from each other during the training period ($P = 0.299$). The escape latencies of the punishment (KCl) cohort and that of the control (DW) cohort were also not significantly different from each other during the memory test period ($P = 0.052$).

### Changes in expression levels of LymCREB1, LymCREB2, LymCBP, and LymMAO by EBSL

We examined the changes in expression levels of

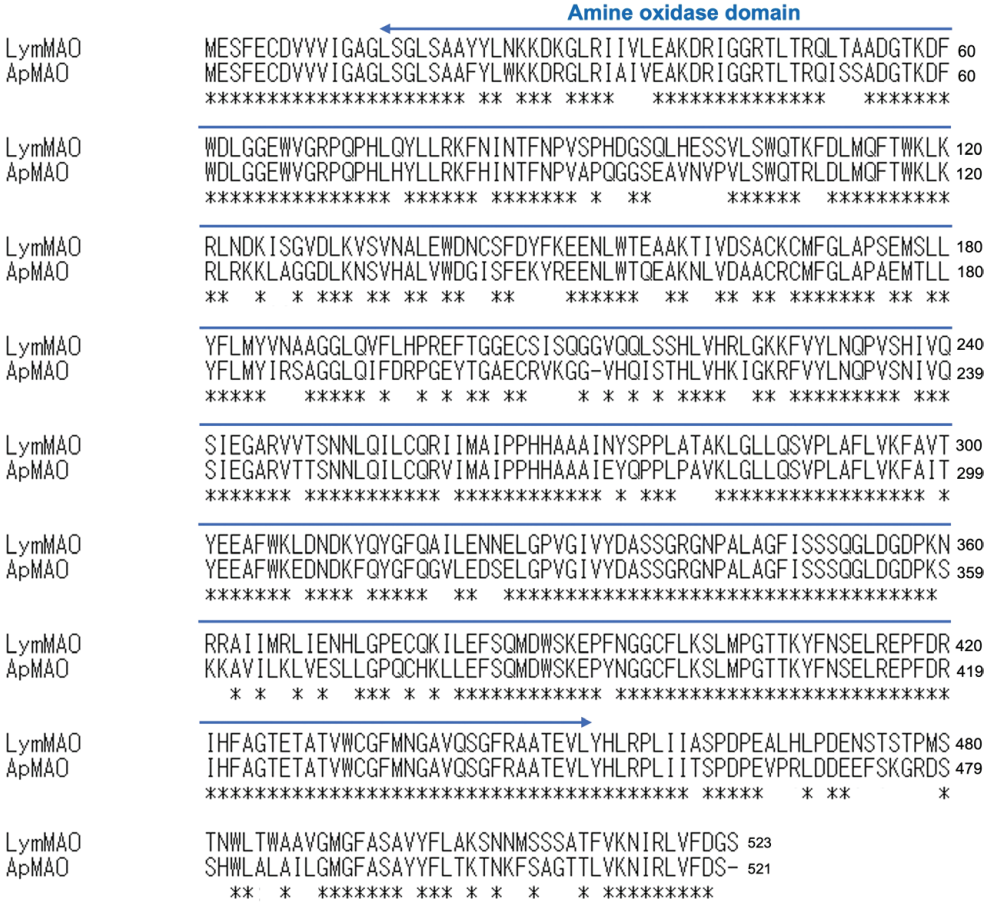


**Fig. 1.** Comparison of deduced amino acids of LymMAO with ApMAO. * indicates conserved amino acids. The blue line indicates an amine oxidase domain.

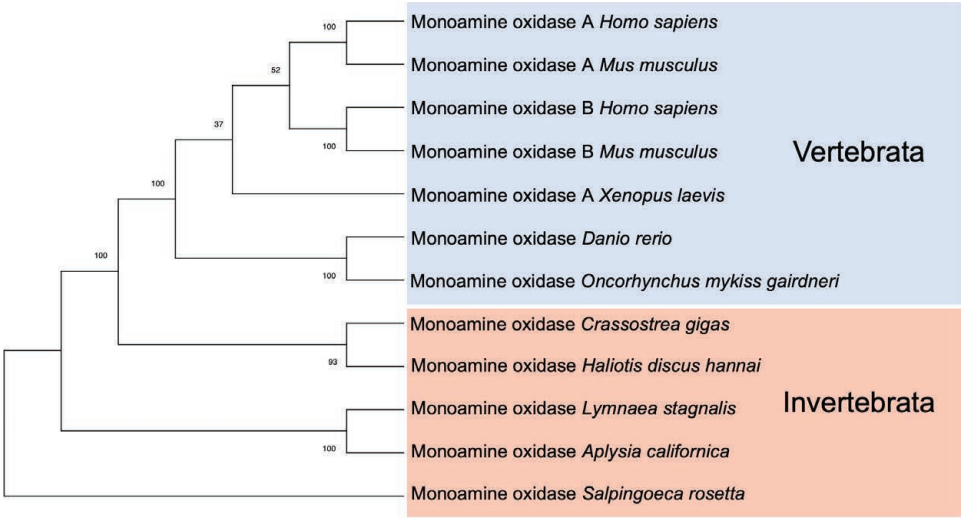


**Fig. 2.** Molecular phylogenetic tree of 12 MAO-like proteins using the neighbor-joining method. The bootstrap value for each branch was calculated by testing the phylogenetic tree 1000 times and is expressed as a percentage.

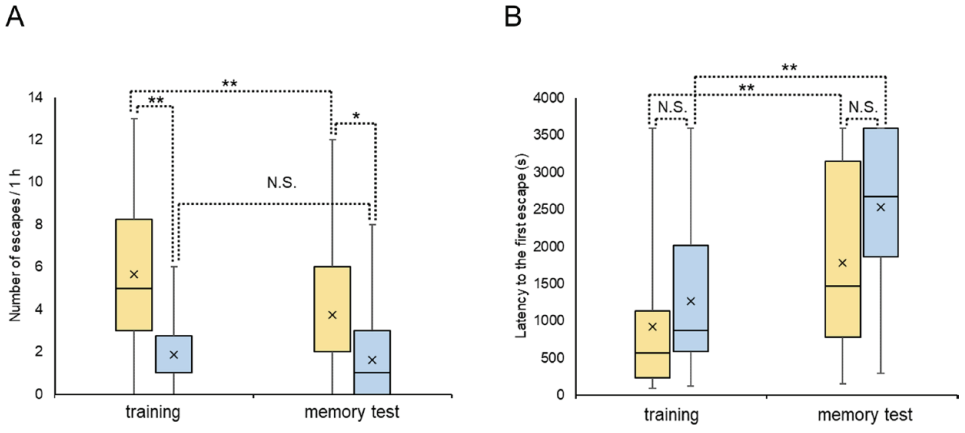


**Fig. 3.** Changes in escape behavior between training and memory test periods in operant conditioning using the control (DW) and punishment (KCl) stimulation. **(A)** The *y*-axis shows the number of escape attempts in 1 h. The *x*-axis shows the training and memory test periods. **(B)** Latency of first escape behavior. Individuals with a latency > 3600 s were assigned an escape time of 3600 s. The *y*-axis shows the latency of the first escape (s). The *x*-axis shows the training and memory test periods. The yellow boxes indicate the control (DW) cohorts and the blue boxes indicate the punishment (KCl) cohorts. The boxplots are indicated based on the 5-number summary: the minimum (0th percentile), the maximum (100th percentile), the sample median (50th percentile), and the first (25th percentile), and third (75th percentile) quartiles. Cross marks indicate the mean values. * $P < 0.05$. ** $P < 0.01$. $n = 20$/group.

LymCREB1, LymCREB2, LymCBP, and LymMAO in the CNS after EBSL between the control (DW) and punishment (KCl) conditions (Fig. 4). Expression levels of LymCREB1 and LymCREB2 mRNA significantly increased in the punishment (KCl) cohort compared with the control (DW) cohort ($P = 0.004$ and $P = 0.001$ in Fig. 4A, B, respectively; $n = 8$/group). The ratio between LymCREB1 and LymCREB2 mRNAs also increased in the punishment (KCl) cohort compared with the control (DW) cohort ($P = 0.012$, $n = 8$/group; Fig. 4C). The expression level of LymCBP mRNA after EBSL was significantly higher in the punishment (KCl) cohort than in the control (DW) cohort ($P = 0.012$, $n = 8$/group; Fig. 4D).

Previous studies showed that changes in monoamine content (e.g., 5-HT) in the CNS were associated with the formation of CTA (Aonuma et al., 2016, 2017, 2018b; Totani et al., 2019). We thus examined the expression level change in LymMAO mRNA following EBSL (Fig. 5). The LymMAO mRNA levels in the punishment (KCl) cohort were significantly increased compared with those in the control (DW) cohort ($P < 0.001$, $n = 8$/group). The increase in LymMAO by EBSL may decrease the 5-HT content in the CNS.

We performed experiments in which a KCl solution was applied to snails remaining on the lids to confirm that the expression changes of target genes were not due to KCl exposure alone (Fig. 6). The relative expression level of the target mRNAs was not significantly different between KCl stimulation and DW stimulation, indicating that changes in the expression levels of the target mRNAs shown in Figs. 4 and 5 were associated with the EBSL and not solely due to KCl exposure.

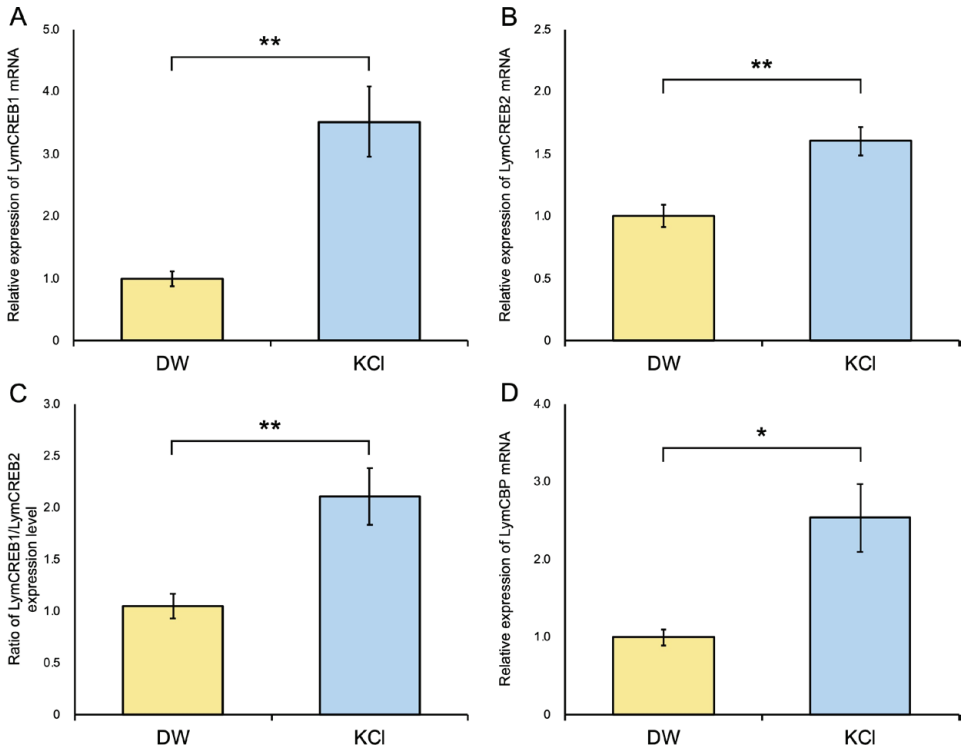


**Fig. 4.** Changes in the relative expression level of LymCREB1, LymCREB2, and LymCBP mRNAs associated with EBSL. The expression level of each gene is shown relative to that of the control (DW) cohort, which was set to 1. **(A)** Expression level of LymCREB1 in the CNS. A significant difference was observed between the control (DW) cohort and the punishment (KCl) cohort. **(B)** Expression level of LymCREB2 in the CNS. A significant difference was observed between the control (DW) cohort and the punishment (KCl) cohort. **(C)** Ratio of LymCREB1 and LymCREB2 calculated from relative expression level values. This ratio shows a significant upregulation of LymCREB1. **(D)** Expression level of LymCBP in the CNS. A significant difference was observed between the control (DW) cohort and the punishment (KCl) cohort. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. $n = 8$/group.

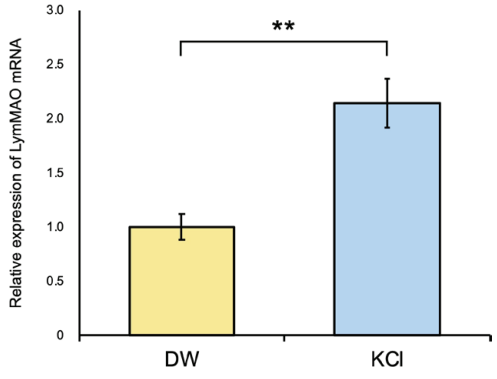


**Fig. 5.** Changes in the relative expression level of LymMAO mRNA associated with EBSL. Expression level of LymMAO in the punishment (KCl) cohort is shown relative to that in the control (DW) cohort, which was set to 1. ** indicates $P < 0.01$. $n = 8$ each.

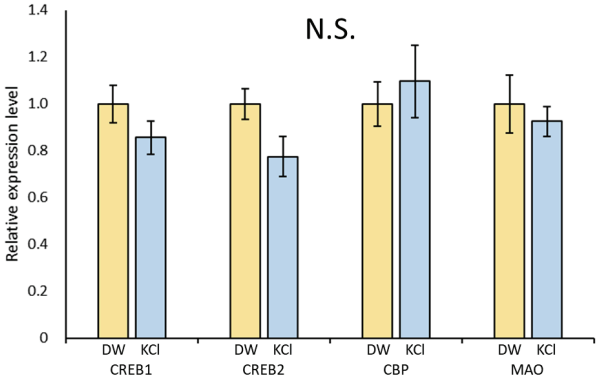


**Fig. 6.** Confirmation that the changes in the expression levels of target mRNAs were specific to the association with EBSL and not solely due to KCl exposure. Expression level of the target mRNA in the punishment (KCl) cohort is shown relative to that in the control (DW) cohort, which was set to 1. $n = 10$/group.

## DISCUSSION

A putative molecule of LymMAO was identified in the present study. The exact function of LymMAO is not yet clear, but considering its homology with the MAO of other species, it is assumed to have a similar function as MAO. Acquisition of EBSL increased the expression of transcription factors LymCREB1 and LymCREB2, as well as LymCBP (i.e., a cofactor of LymCREB1). Furthermore, because LymCREB1 is an activator isoform and LymCREB2 is a repressor isoform (Sadamoto et al., 2004; 2010), the ratio between them was examined, and the results showed that it was also increased by EBSL. This finding suggests that LymCREB1 is activated by the formation of EBSL.

On the other hand, the increase in LymMAO expression

after EBSL suggests that the 5-HT content in the CNS is likely to be decreased. This issue requires further investigation, but in the case of CTA, for example, the learning acquisition and CNS 5-HT content are inversely correlated (Aonuma et al., 2018b), so the same trend may be found in EBSL. Furthermore, if LymMAO is expressed intracellularly (e.g., mitochondrial outer membrane) like mammalian MAOs, 5-HT released into the synaptic cleft must be taken up into the neurons to be degraded by intracellular MAO (Bar-Am et al., 2016). This suggests that EBSL may also increase serotonin transporter expression. Taken together, these experimental results suggest that EBSL in *Lymnaea* may be a good model for performing molecular-level analysis of operant conditioning.

As shown in mammals (Table 1), we cannot deny the possibility of two or more different MAOs, even in invertebrates. In *Aplysia californica*, two different molecules have been reported as probable flavin-containing monoamine oxidase A (XP_005098472.1 for the 521-amino acid protein and XP_035825486.1 for the 545-amino acid protein). In the present study, XP_005098472.1 was used to identify LymMAO, and future studies will be needed to investigate the existence of multiple MAOs and the differences in their functions in *Lymnaea*.

The EBSL experiments produced two unexpected results. The first is that the escape numbers of the control (DW) cohort differed significantly between the training and memory test periods. As shown in Fig. 3A, the mean values look similar, and thus it seems that the number of escapes decreased uniformly although the reason for this is unclear (probably due to fatigue). The second unexpected result is the significant difference in the latency of the first escape between the training and the memory test periods in the control (DW) cohort. This might be due to the variability of the data during the memory test period, because in the control (DW) cohort, one of 20 snails did not escape within 3600 s in the training period, whereas four of 20 snails did not escape within 3600 s in the memory test period. This difference in snail numbers 'by chance' produced a significant difference in the comparison of the latency of the first escape between the training and memory test periods in the control (DW) cohort. Despite these unexpected results, it is important to note that the escape numbers were suppressed in the punishment (KCl) cohort during both the training and memory test periods, and the latency of the first escape was extended in the punishment (KCl) cohort during the memory test period compared with that during the training period. The longer latency to the first escape is thought to be due to suppression of the escape behavior.

In conclusion, few studies have examined the direct relationship between MAO and learning and memory mechanisms in any species. Most of the previous studies were performed to search for links to pathologic conditions such as dementia (Rinaldi et al., 2022). Clarification of the molecular mechanisms of learning and memory using *Lymnaea* EBSL will help to elucidate the mechanisms underlying operant conditioning.

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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, KN, and EI conducted experiments. NC, DH, and EI interpreted 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 authors upon reasonable request.

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