Comparative Biochemistry and Physiology, Part C 297 (2025) 110291

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# Effects of the inhibition of miRNA biogenesis in the central ring ganglia of a widely used invertebrate model species, *Lymnaea stagnalis*

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ARTICLE INFO

Edited by Martin Grosell

*Keywords:*
Serotonin
Dopamine
HSP70
MicroRNA
Learning
Feeding
Stress

ABSTRACT

MicroRNAs (miRNAs) are key regulators of gene expression, shaping neuroplasticity, stress responses, and neuromodulation. In the pond snail *Lymnaea stagnalis*, inhibition of the miRNA-processing enzyme Dicer with Poly-L-Lysine (PLL) has been shown to impair long-term memory (LTM) formation, yet the molecular pathways affected remain unclear. Here, we examined PLL injection's transcriptional and cellular/neurochemical effects in untrained (i.e., non-associatively conditioned) snails. We focused on genes involved in neuroplasticity (LymGRIN1, LymCREB1), stress response (LymHSP70), and serotonergic/dopaminergic signaling (LymTPH, LymSERT, LymDDC), along with measurements of the levels of serotonin, dopamine, and HSP70 protein. We found that PLL did not alter the expression of memory-related genes in the non-associatively conditioned snails. However, we observed a marked downregulation of LymTPH and LymSERT, which was accompanied by a significant reduction in the level of serotonin. In contrast, the expression of LymDDC and the level of dopamine remained unchanged. Although we detected a significant upregulation in the expression of LymHSP70, indicating a strong stress response, the level of HSP70 protein did not change significantly. These findings suggest that PLL-induced Dicer inhibition primarily disrupts serotonin homeostasis, potentially altering motivation and feeding behaviour rather than directly impairing memory processes. This study highlights the broader role of miRNA pathways in regulating stress resilience and neuromodulation. By linking miRNA activity to serotonergic signaling, our findings suggest that miRNAs influence behavioural states beyond synaptic plasticity, with potential implications for understanding how miRNA dysregulation affects mood, motivation, and cognitive function across species.

## 1. Introduction

In 1991, the laboratory of Nobel Laureate Gary Ruvkun made a seminal discovery in *Caenorhabditis elegans*. They found that deleting two small sequences in the 3′ untranslated region (3′ UTR) of lin-14 mRNA resulted in the unexpected accumulation of LIN-14 protein (Wightman et al., 1991). These deletions did not affect the proteins stability or function, suggesting the existence of a post-transcriptional regulatory mechanism. This led to the hypothesis that an unidentified factor bound these sequences to repress translation. Two years later, this

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https://doi.org/10.1016/j.cbpc.2025.110291
Received 7 May 2025; Received in revised form 4 July 2025; Accepted 14 July 2025
Available online 15 July 2025

factor was identified as a small non-coding RNA (Lee et al., 1993), marking the discovery of the first microRNA (miRNA).

Subsequent studies expanded our understanding of miRNAs, revealing their roles in a wide range of biological functions, including learning and memory (Cannell et al., 2008; O'Hara et al., 2009; Rashidi et al., 2023). This concept was later extended to the great pond snail *Lymnaea stagnalis*, a well-established invertebrate model in neuroscience (Fodor et al., 2020; Rivi et al., 2021b, 2023).

Korneev et al. (2018) demonstrated that the miRNA pathway is essential for long-term memory (LTM) formation, particularly during the post-training consolidation window. Specifically, they showed that inhibition of Dicer, an enzyme critical for miRNA maturation, via poly-L-lysine (PLL) injection (Watashi et al., 2010), shortly after classical conditioning, blocks LTM formation. The conditioning protocol involved pairing sucrose (an appetitive stimulus) with amyl acetate (a novel, non-food chemostimulus) (Alexander et al., 1984). Under normal conditions, this results in a long-term feeding response to amyl acetate. However, PLL-induced Dicer inhibition significantly impaired this response, providing direct evidence that miRNAs are crucial for memory consolidation. At the molecular level, Lym-miR-137 was shown to target Lym-CREB2, a transcriptional repressor known to negatively regulate memory (Ribeiro et al., 2003). Following training, Lym-miR-137 levels increased while Lym-CREB2 expression decreased. Conversely, inhibition of Lym-miR-137 before training resulted in elevated Lym-CREB2 levels and impaired LTM formation. Both Lym-miR-137 and Lym-CREB2 were found to be co-expressed in the cerebral giant cells (CGCs), key neurons involved in memory formation (Kemenes et al., 2006).

These findings suggest that Lym-miR-137 promotes memory consolidation by downregulating Lym-CREB2, thereby removing a molecular "brake" and enabling the activity of Lym-CREB1, a transcriptional activator that drives gene expression essential for LTM (Finkbeiner et al., 1997).

While these studies established the role of miRNAs in memory consolidation, growing evidence indicates that miRNAs also influence a broader spectrum of behavioural and emotional processes. For instance, Kagan et al. (2022) demonstrated that Dicer inhibition in *L. stagnalis* disrupted reward processing, leading to anhedonia. Although specific miRNAs were not identified, the findings suggested that miRNA pathways may modulate non-associative behaviors. Supporting this, mammalian studies have linked miR-137 to the regulation of anxiety, reward sensitivity, and affective behaviour (Jia et al., 2016; Yan et al., 2019), pointing to a conserved, cross-species role.

Beyond Lym-miR-137, several other miRNAs have been implicated in learning and memory in *L. stagnalis* and other invertebrate models. Among these, miR-124 is one of the most extensively studied, with well-established roles in neuronal development and synaptic plasticity. In *Aplysia*, miR-124 has been shown to regulate genes involved in synaptic remodeling and memory formation (Rajasethupathy et al., 2009). Similarly, miR-9 affects neuronal differentiation and learning-related plasticity in various species, including *Drosophila melanogaster*, where it modulates synaptic growth. In *C. elegans*, miR-58 and members of the let-7 family have been linked to neural development and learning, influencing both synaptic connectivity and memory retention. Together, these studies highlight the conserved roles of miRNAs such as miR-124, miR-9, and let-7 in regulating gene expression critical for behavioural plasticity across invertebrate species (Korneev et al., 2025).

Integrating findings from *L. stagnalis*, *D. melanogaster*, and *C. elegans* suggests that miRNAs are central players in the regulation of synaptic efficacy and behaviour. They appear to exert their effects not only by facilitating memory consolidation (e.g., via CREB2 suppression) but also by modulating broader neuromodulatory systems involved in motivation, reward, and stress.

Building on these insights, the current study aimed to investigate whether PLL-induced Dicer inhibition elicits molecular changes beyond those related to associative learning. Specifically, we asked: *Does inhibition of miRNA biogenesis affect the expression of genes and neurochemicals involved in motivation and stress regulation under baseline, non-learning conditions?*

To address this, we examined the molecular and neurochemical consequences of PLL treatment in untrained snails, focusing on conserved pathways related to stress, plasticity, and reward. First, we assessed the serotonergic and dopaminergic systems, which are integral to feeding and reward behaviors (Kemenes et al., 1997; Chaouloff et al., 1999; Meneses and Liy-Salmeron, 2012; Benatti et al., 2017a; Bacqué-Cazenave et al., 2020; Baik, 2020). We measured the expression of LymTPH (tryptophan hydroxylase), LymSERT (serotonin transporter), and LymDDC (DOPA decarboxylase), as well as central serotonin and dopamine levels. Next, we tested whether PLL induces a stress response by quantifying LymHSP70 mRNA and protein levels in the central ring ganglia. HSP70 proteins are molecular chaperones upregulated under cellular stress, and their induction could signal that PLL disrupts cellular homeostasis (Mayer & Bukau, 2005a; Song et al., 2014), potentially contributing to altered behavioural responses to appetitive stimuli. Finally, we examined LymGRIN1 (NMDA receptor subunit 1) and LymCREB1, two genes critical for synaptic plasticity and long-term neuronal adaptation (Ribeiro et al., 2003; Farber, 2003; Ha et al., 2006; Morris, 2013).

Our rationale for using untrained animals was to isolate the direct molecular consequences of Dicer inhibition, without the confounding transcriptional changes induced by associative learning. While previous studies established the behavioural and molecular impacts of PLL in the context of memory (Korneev et al., 2018; Kagan et al., 2022), we aimed here to dissociate these effects from experience-dependent plasticity. This approach enabled us to characterize the baseline neuromodulatory disruptions arising from impaired miRNA biogenesis. By expanding our investigation beyond memory-related genes, we sought to determine whether Dicer inhibition impacts stress and reward pathways as well.

If so, this would support the idea that miRNAs play a broader regulatory role in modulating motivation and affective state, even in the absence of learning. Such insights are critical to advancing our understanding of miRNAs as integrators of cognitive and emotional function across species.

## 2. Materials and methods

### 2.1. Snails and animal maintenance

Two inbred strains of *Lymnaea stagnalis*, both derived from the original Amsterdam laboratory stock and maintained under controlled laboratory conditions for multiple generations, were used in this study. Due to the collaborative nature of this work, molecular analyses were conducted on the W-strain housed at the University of Calgary, while proteomic analyses and neurotransmitter measurements were performed on a parallel strain maintained at the HUN-REN Balaton Limnological Research Institute (BLRI). Both populations were fed romaine lettuce *ad libitum* and kept at 20 ± 1 °C. Five-month-old adult, mature specimens were used in all experiments. Importantly, experimental procedures were conducted during comparable time periods to minimize potential variability due to seasonal or circadian influences. To reduce strain-related variability, both strains were matched for age, environmental conditions, and shared a common genetic background. Although no direct statistical comparison was performed between the two strains, their functional equivalence was assumed based on these shared parameters.

### 2.2. Saline and PLL injection

A 25 μmol $L^{-1}$ solution of poly-L-lysine hydrobromide (PLL, #SLBZ6281, Merck, Germany) was prepared in *Lymnaea* saline (51.3 mmol $L^{-1}$ NaCl, 1.7 mmol $L^{-1}$ KCl, 1.5 mmol $L^{-1}$ $MgCl_2$, 4.0 mmol $L^{-1}$ $CaCl_2$, and 10.0 mmol $L^{-1}$ Hepes, pH 8.0), following the method

described by Kagan et al. Snails in the control group were injected with 0.1 mL of *Lymnaea* saline, while animals in the treated group were injected with 0.1 mL PLL solution. Before all injections, snails were anesthetized in cold artificial snail water (4 °C) for 2 min. In the case of the gene expression measurements, snails were returned to their home tank for 3 h before being sacrificed.

The 3-h interval was selected based on previous studies (Kagan et al., 2022). The time intervals for measurements were 12 h for neurotransmitters analy and 6 h for HSP70 protein levels.

### 2.3. RNA extraction, retrotranscription, real-time quantitative PCR (qPCR), and gene expression analyses

Snails (N = 8 per group) were randomly assigned to two treatment groups: (1) injected with snail saline (i.e., Sal group) and (2) injected with PLL (i.e., PLL group). Three hours after the PLL or saline injection, animals were sacrificed, and the central ring ganglia were dissected and stored at −80 °C before analysis. Total RNA extraction and DNase treatment were performed using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck KGaA; Darmstadt, Germany) as previously described (Benatti et al., 2017b; Rivi et al., 2021a). Two hundred ng of total RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation) in 20 μL of the reaction mix. qPCR was performed on 20 ng of mRNA with SYBR Green Master Mix (Bio-Rad). Specific forward and reverse primers were used at a final concentration of 300 nmol $L^{-1}$ (Table 1). Primers of LymDCC were designed using the contig annotation recently generated by Cristina et al. (2022), which further confirmed previous studies from Sadamoto et al. (2012). Single PCR products were subjected to a heat dissociation protocol (StepOne Real-Time PCR System, Applied Biosystems). Cycle threshold (Ct) values were determined by CFX Maestro™ Software (Bio-Rad). For quantitative evaluation of changes in mRNA expression, the comparative ΔΔCt method was performed, using average expression levels of controls (i.e., saline-injected snails) as a calibrator. The mRNA levels of each target were normalized to two reference genes, elongation factor 1α (LymEF1α) and tubulin (LymTUB). The stability of mRNA expression of these reference genes and their arithmetic mean was assessed using Normfinder® (https://moma.dk/normfinder-software) (Wang et al., 2012), considering intra and intergroup variation. The arithmetic mean of Cqs of the reference genes was used as a normalizer. For an appropriate application of the comparative ΔΔCt method, we verified that the amplification efficiency of the target genes and endogenous control gene was approximately equal.

### 2.4. Sample preparation and LC-MS measurement of neurotransmitters

The whole CNS was dissected from both the saline and PLL-injected snails (N = 15 animals/group, 3 independent replicates (i.e., total animal N = 90) 12 h after the injection). The samples were pooled per replicate and then homogenized in 700 μL acetonitrile:water 60:40 with a Dounce homogenizer and then further extracted with ultrasonication. After centrifugation (10,000 ×*g* for 10 min at 4 °C), 600 μL supernatants were placed in a new tube and concentrated with a SpeedVac Concentrator Plus (Eppendorf AG, Hamburg, Germany) vacuum concentrator device. Finally, the samples were reconstituted with 100 μL ultra-pure water containing 0.1 % formic acid, induced by vortex mixing and ultrasonication. The goodness of the extraction method was checked with serotonin-d4 (#747521, Merck) and dopamine-d4 (#73483; Merck) internal standards at a final concentration of 100 ng/mL. LC-MS analysis was performed using a Thermo Scientific Orbitrap Exploris 120 MS instrument (Thermo Scientific, Germany) coupled with a Thermo Vanquish UPLC system (Thermo Fisher Scientific, USA). Chromatography was performed with a Kinetex C18 reversed-phase column (1.7 μm, 3 mm × 100 mm i.d., 100 Å) (Phenomenex, USA) at 35 °C, and the injected volume was 5 μL. The eluent composition comprised Solvent A (water/formic acid, 99.9/0.1, v/v) and Solvent B (acetonitrile/formic acid, 99.9/0.1, v/v). The flow rate of the mobile phase was set to 400 μL/min. The multistage gradient elution compositions were as follows: 0–2 min - 0 % B, 2–5 min - 45 % B, 5–7 min – 100 % B, 7–14 min - 100 % B, 14–15 min - 0 % B, and 15–20 min - 0 % B. The mass spectrometer was operated in positive mode, utilizing parallel tMS2 mode for data acquisition. The ESI capillary voltage was 3500 V, and the vaporizer temperature was 200 °C. The scanning mass-to-charge range was 20–200 *m/z* at a 1 Hz acquisition rate. The quadrupole isolation window was set to one Dalton, and the most intense precursor-to-fragment transitions were used for analysis: 177.09 → 160.07 m/z for serotonin (Rt: 5.18) and 154.08 → 137.05 m/z for dopamine (Rt: 4.08). The transitions and the optimal ionization conditions were derived from our recorded mass spectra using 10 μg/mL solutions containing the individual analytes (prepared from stock solutions with distilled water containing 0.1 % formic acid) directly introduced into the MS by a syringe pump. Peak detection and quantification were achieved using the Thermo Scientific Chromeleon™ Chromatography Data System 7.3.2 software.

The ions under observation were verified and quantified based on specific criteria: accurate MS1 mass, retention time, MS2 masses, and the pattern of fragmentation. The production was used for the quantitation of both neurotransmitters. A six-point calibration curve was

**Table 1**
Nucleotide sequence of the forward and reverse primers used for Real-Time PCR. For each target, the accession number and the size (bp) of the PCR product obtained by the amplification of the cDNA (mRNA) are given.

| Gene bank accession | Target | Product length (bp) | Sequence |
|---|---|---|---|
| AF129815.1 | *Lymnaea stagnalis* tryptophan hydroxylase mRNA **LymTPH** | 179 bp (238–417) | FW 5′–AGGATACAGTCTACCGACAG-3′ RV 5′-TGAGTTCACGGAAAACTATT-3′ |
| FX185022 | *Lymnaea stagnalis* serotonin transporter **LymSERT** | 177 bp (726–903) | FW 5′-ATACCGTACCTTGTCATGTT-3′ RV 5′-TGTTGTAGTACCAGGAGACA-3′ |
| DQ278441.4 | predicted *Lymnaea stagnalis* DOPA decarboxylase **LymDDC** | 99 bp (134–233) | FW 5′-CACTGAGCTAGAAGTCTCCA-3′ RV 5′-TATAACACCTCCACCTTTTC-3′ |
| DQ206432.1 | *Lymnaea stagnalis* heat-shock protein 70 **LymHSP70** | 199 bp (134–333) | FW 5′-AGGCAGAGATTGGCAGGAT-3′ RV 5′-CCATTTCATTGTGTCGTTGC-3′ |
| AY571900.1 | *Lymnaea stagnalis* NMDA-type glutamate receptor **LymGRIN1** | 140 bp (831–971) | FW 5′-AGAGGATGCATCTACAATTT-3′ RV 5′-CCATTTACTAGGTGAACTCC-3′ |
| AB041522.1 | *Lymnaea stagnalis* cAMP responsive element binding protein **LymCREB1** | 180 bp (49–229) | FW 5′-GTCAGCAGGGAATGGTCCTG-3′ RV 5′-AACCGCAGCAACCCTAACAA-3′ |
| X15542.1 | Snail, beta-tubulin **LymTUB** | 127 bp (92–219) | FW 5′-GAAATAGCACCGCCATCC-3′ RV 5′-CGCCTCTGTGAACTCCATCT-3′ |
| DQ278441.1 | *Lymnaea stagnalis* elongation factor 1-alpha **LymEF1α** | 150 bp (7–157) | FW 5′-GTGTAAGCAGCCCTCGAACT-3′ RV 5′-TTCGCTCATCAATACCACCA-3′ |

generated for the quantitative analysis using 10, 100, 1000, 2500, 5000, and 10,000 ng/mL standard mixtures, where serotonin (#H9523, Merck) and dopamine (#H8502, Merck) were in equivalent concentrations. The correlation coefficients (R2) for all acceptable calibration curves were between 0.97 and 0.99 (not shown).

### 2.5. In silico searches and bioinformatic analysis

The neural transcriptome data obtained in our previous study (Pirger et al., 2024) were used to search for the full-length coding sequence of the previously identified partial *Lymnaea* HSP70 sequence (DQ206432.1). We also compared the obtained sequences with the public *Lymnaea* CNS transcriptome shotgun assembly (sequence read archive: #DRR002012) (Sadamoto et al., 2012) and genome data (Koene et al., 2024). A conserved domain search using NCBI CDD/SPARCLE (Lu et al., 2020) was performed to check if the key regions were present in the deduced protein sequence, while the EMBL-EBI tool (Madeira et al., 2024) was used to check the conservation level of the amino acid sequence.

### 2.6. Sample preparation and LC-MS measurement of HSP70

The whole CNS was dissected from both the saline and PLL-injected snails (N = 15 animals/group, 3 independent replicates (i.e., total animal number = 90)) 12 h after the injection. The samples were pooled per replicates in Protein LoBind Eppendorf tubes and then homogenized in 200 μL of cell lysis prepared for the efficient protein extraction, stabilization, and preservation: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 % Triton X-100, and Halt™ Protease Inhibitor Cocktail (#78429, Thermo Fisher Scientific, USA). The samples were homogenized with a Dounce homogenizer and then further extracted with ultrasonication on ice. Next, 1 mL of ice-cold chloroform/methanol 1:1, v/v was added to each sample.

After thorough vortexing, the samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C. The separated protein pellets were transferred into fresh tubes and dried for 10 min using an Eppendorf Concentrator Plus device (Eppendorf GmbH, Germany). The proteins were then resuspended in 200 μL rehydration buffer. To promote re-dissolution, each sample was sonicated on ice for 0.5 min at 20 % power using a micro-tip probe with a 50 % pulse setting. Protein concentrations were determined using the Micro BCA™ Protein Assay Kit (#23235, Thermo Fisher Scientific), and all samples were normalized to a protein mass of 150 μg before enzymatic digestion. The in-solution overnight digestion was performed using a Trypsin/Lys-C enzyme mixture (#V5072, Promega, USA). Before digestion, protein extracts were reduced and alkylated, following the manufacturer's technical bulletin. The digested peptides were purified using an Oasis HLB 96-well solid-phase extraction (SPE) plate, and the eluted samples were concentrated with an Eppendorf Concentrator Plus system. Finally, the purified peptides were redissolved in 100 μL of water containing 0.1 % formic acid. Nano-scale proteomic separation was conducted using a Bruker EASY-nLC system coupled with a Bruker Maxis 4G UHR-QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). A 5 μL aliquot of each sample was injected and separated using a homemade C18 analytical column (3 μm, 75 μm × 150 mm) with a 120-minute multistep gradient elution at a flow rate of 350 nL/min. Two solvents were used for separation: Solvent A (water/formic acid, 99.9/0.1, v/v) and Solvent B (water/formic acid/acetonitrile, 4.9/0.1/95, v/v/v). The mass spectrometer operated in positive ion mode with a scanning range of 300–2200 *m/z*. The nebulizer gas flow rate was set to 1 L/min at a pressure of 0.8 bar, and the temperature was maintained at 160 °C. The capillary voltage was 3.8 kV, and the 30 most intense peptide peaks were selected for collision-induced dissociation (CID) fragmentation. To ensure greater accuracy in quantitative concentration determinations, we confirmed the results using targeted multiple reaction monitoring (MRM) analysis of the peptides identified during qualitative studies, applying the ion source parameters detailed above. In this case, the quadrupole isolation window was set to two daltons. For protein identification, data were processed with Data Analysis 4.4 software. Peak area-based relative quantification was based on the most characteristic peptide fragment of the *Lymnaea* HSP70 protein (i.e., HSP1A1) (Supplementary information).

Searching parameters were set to allow one missed cleavage site, with a mass tolerance of 50 ppm for $MS^1$ and 0.05 Da for $MS^2$. For protein/polypeptide identification, we used variable modifications, including methionine oxidation, and carbamidomethylation on cysteine as a fixed modification.

### 2.7. Statistical analyses

First, normality and homogeneity of variance were verified using Shapiro-Wilk and Levene's tests, respectively. All data were normally distributed. Analyses and figures were then performed using Prism version 9.0.1 for Mac (GraphPad Software, San Diego, California, USA). Statistical analyses were performed using separate unpaired *t*-tests with $P < 0.05$ as the significance level.

## 3. Results

### 3.1. Effects of PLL on memory-related and stress-associated gene expression

We first examined whether PLL injection influenced the expression of key memory-related genes. Gene expression analyses were performed with 8 snails per group (saline- and PLL-injected snails). First, we focused on the impact of PLL on neuromodulatory pathways and analyzed the expression of genes involved in serotonergic and dopaminergic signaling. Our results revealed a significant downregulation of LymTPH ($t = 3{,}54$, $df = 14$; $p = 0.003$ – Fig. 1A) and LymSERT ($t = 5.58$, $df = 14$; $p < 0.0001$ – Fig. 1B) in PLL-injected snails compared to saline-injected controls. In contrast, LymDDC mRNA levels, which influence dopamine synthesis, remained unchanged ($t = 2.08$, $df = 14$, $p = 0{,}06$ - Fig. 1C). Next, we investigated whether PLL affected stress-related gene expression by measuring LymHSP70 mRNA levels (Fig. 1D). An unpaired t-test showed a significant upregulation of LymHSP70 in PLL-injected snails compared to saline-injected controls ($t = 2.88$, $df = 14$; $p = 0.01$ - Fig. 1D), suggesting that PLL induces a physiological stress response.

Finally, our analysis revealed no significant changes in LymGRIN1 ($t = 1.35$, $df = 14$; $p = 0{,}19$ - Fig. 1E) or LymCREB1 ($t = 1.76$, $df = 14$, $p = 0{,}1$- Fig. 1F) in mRNA levels between PLL-injected and saline-injected snails. This indicates that, in the absence of associative conditioning, PLL does not affect these critical pathways underlying memory formation.

### 3.2. Impact of PLL on neurotransmitter levels and HSP70 protein expression

We then measured the neurotransmitter levels in 15 snails per group across 3 independent biological replicates. We found that PLL injection led to a significant decrease in serotonin levels when compared to the saline-injected controls ($p = 0.046$) (Fig. 2A). However, no significant changes were observed in dopamine levels between the PLL-and saline-injected control groups ($p = 0.58$) (Fig. 2B). We identified the full-length coding sequence of the previously identified partial *Lymnaea* HSP70 sequence (Supplementary information). The deduced protein sequence contained the key domain characteristic of *HSPA1A* protein of the HSP70 protein family and showed a high conservation with human HSPA1A protein (identity: 73.7 %; similarity: 85.4 %) (Supplementary information). Finally, we quantified HSP70 protein levels in the saline-injected controls and PLL-injected groups but found no significant difference ($p = 0.18$ – Fig. 3).

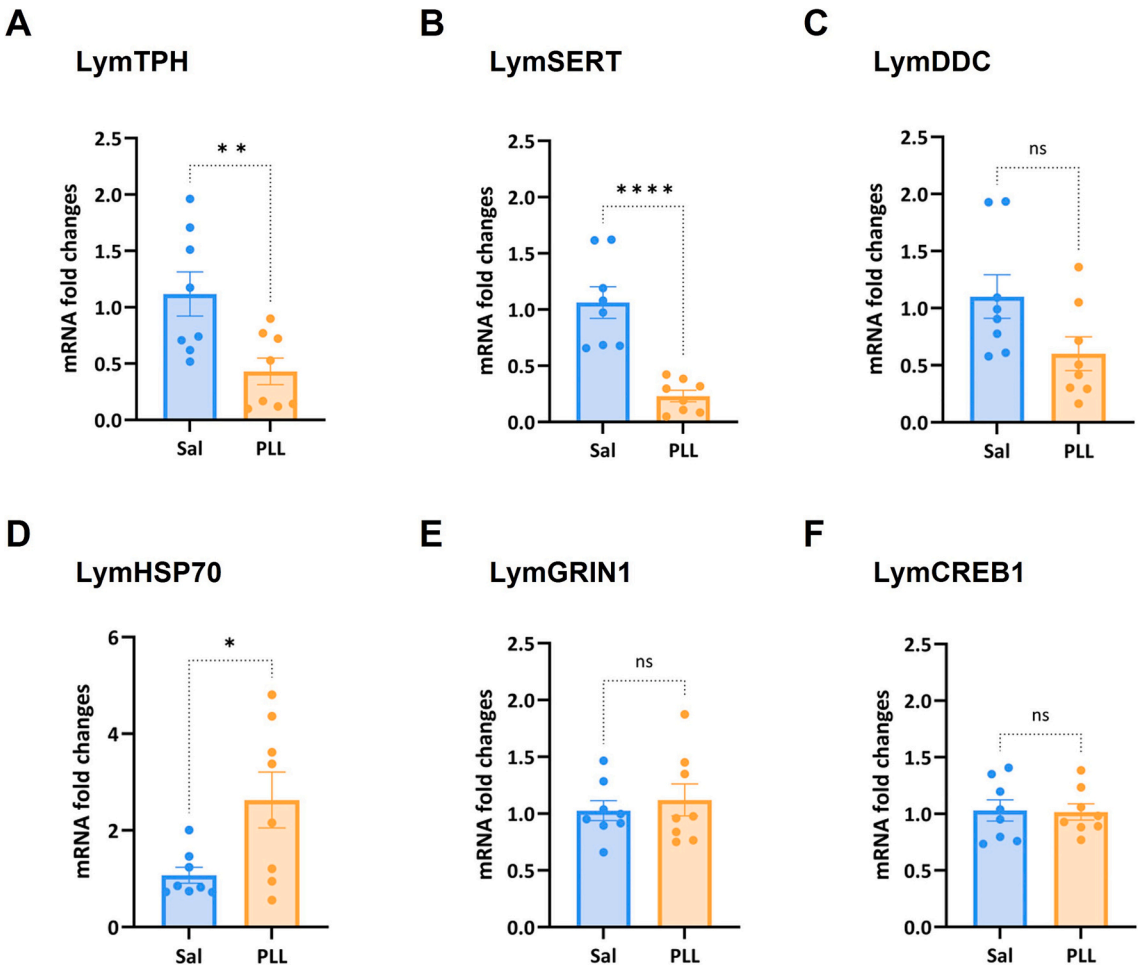


**Fig. 1.** PLL injection did not affect the expression levels of LymGRIN1 and LymCREB1 but upregulated the mRNA levels of LymHSP70 in the central ring ganglia of *Lymnaea*. Snails (N = 8 per group) were randomly assigned to two treatment groups: (1) injected with snail saline (i.e., Sal) and (2) injected with poly-L-lysine (i.e., PLL). Three hours after the PLL or saline injection, the central ring ganglia were extracted, and mRNA levels of LymTPH (A), LymSERT (B), and LymDDC (C), LymHSP70 (D), LymGRIN1 (E), and LymCREB1 (F) were measured. Data were analyzed using an unpaired *t*-test. The solid line is the mean, and the error bars are the SEM. ****$p < 0.0001$; **$p < 0.01$, and ns = not significant as $p > 0.05$.

## 4. Discussion

Building on our aim to explore miRNA functions beyond associative learning, one of the most compelling findings of this study is the effect of PLL-induced Dicer inhibition on the serotonergic system in *Lymnaea*'s central ring ganglia. At the transcriptional level, we found a significant downregulation of LymTPH, encoding tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis, and LymSERT, which encodes the serotonin reuptake transporter. mRNA levels may reflect a change in enzyme activity, while the downregulation of LymSERT, the serotonin transporter responsible for serotonin reuptake, may reflect a compensatory mechanism activated against the low levels of neurotransmitter. These transcriptional changes were accompanied by a significant decrease in serotonin levels compared to saline-injected controls. Importantly, these effects occurred in the absence of associative learning, indicating that miRNA-dependent regulation of serotonin homeostasis operates independently of cognitive demand and may represent a baseline modulatory function.

Serotonin is a key neuromodulator that plays an essential role in feeding behaviour (Bacqué-Cazenave et al., 2020), reward processing, and motivation in *L. stagnalis* and other species (Tierney, 2020). The observed decrease in serotonin levels may account for the reduced rasping behaviour in response to a food stimulus that was previously associated with high hedonic value (Kagan et al., 2022). If serotonin signaling is compromised, snails may exhibit a decreased motivation to engage in food-seeking behaviors, leading to the observed reduction in feeding responses. In other words, the reduced serotonin levels we observed may thus contribute to the diminished feeding responses previously reported following PLL treatment, offering a mechanistic bridge between molecular disruption and behavioural phenotype.

From a translational perspective, this finding mirrors those in vertebrate models, where miRNA dysregulation—particularly involving miR-137, miR-16, and miR-135—has been linked to altered serotonergic signaling and behavioural outcomes, such as anhedonia, depression, and reduced reward sensitivity (Sun et al., 2018; Rosa et al., 2022; Yuan et al., 2023). In mammals, for instance, miR-16 directly regulates serotonin transporter expression, and its downregulation has been associated with depressive phenotypes and stress vulnerability (Yang et al., 2017; Martins and Schratt, 2021). Our data suggest a similar regulatory framework may be present in *L. stagnalis*, with miRNA pathways modulating key components of serotonergic function in a way that shapes motivational state and behavioural output.

Interestingly, while PLL injection significantly affected serotonin-related genes and serotonin levels, dopamine-related markers (LymDDC mRNA and dopamine levels) remained unchanged. The absence of changes in the expression levels of DOPA decarboxylase, the enzyme responsible for converting L-DOPA to dopamine, and in dopamine levels indicates that the dopaminergic system may not be as sensitive to PLL-induced Dicer inhibition as the serotonergic system. This selective effect on serotonin is particularly noteworthy given the close

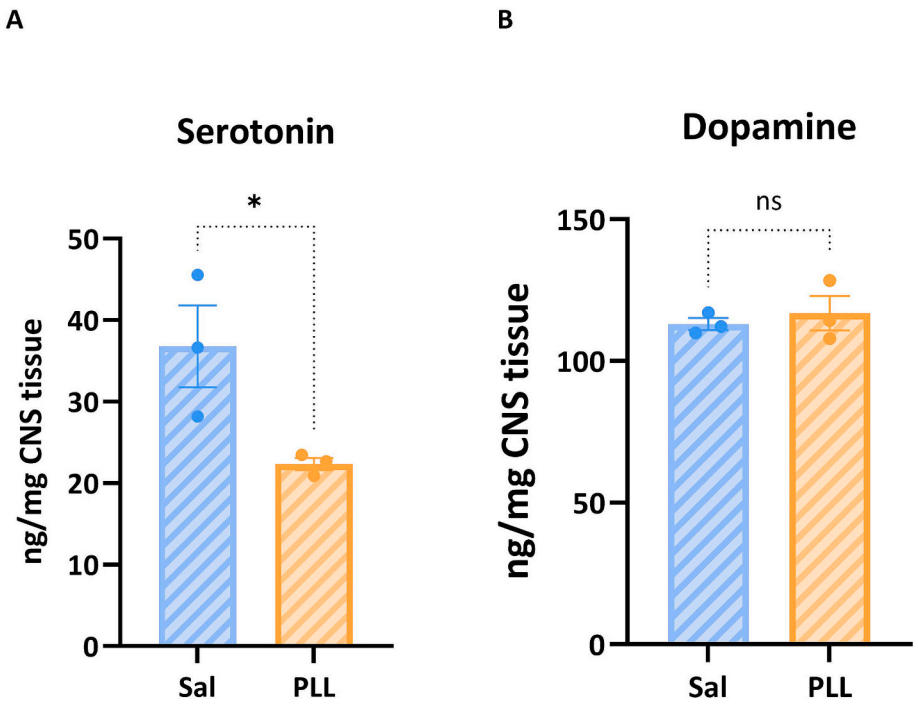


**Fig. 2.** PLL injection downregulates serotonin but not dopamine levels in the central ring ganglia of *Lymnaea*. Snails were randomly assigned to two treatment groups: (1) injected with snail saline (i.e., Sal) and (2) injected with poly-L-lysine (i.e., PLL). Twelve hours after the PLL or saline injection, the central ring ganglia were extracted, ganglia were pooled (N = 15 per group, 3 independent replicates), and neurotransmitter levels were analyzed by LC-MS. Data were analyzed using an unpaired t-test. The solid line is the mean, and the error bars are the SEM. *$p < 0.05$ and ns = not significant as $p > 0.05$.

functional interactions between the serotonergic and dopaminergic systems.

Both neurotransmitters play critical roles in feeding behaviour and reward processing, yet PLL appears to primarily impact serotonin-related pathways. This specificity suggests that miRNA regulation may be more tightly linked to serotonin homeostasis, at least in the context of stress and feeding behaviour.

The specificity of this effect, observed in serotonin but not dopamine pathways, suggests that the serotonergic system may be particularly vulnerable to disruptions in miRNA processing in this invertebrate model. This result is notable given serotonin's conserved role in modulating motivation, feeding, and reward-related behaviour across species. In *L. stagnalis*, serotonin is a key modulator of feeding circuits, influencing rasping frequency and motivational drive toward food stimuli.

Additionally, we found a significant upregulation of LymHSP70 mRNA in PLL-injected snails, which strongly suggests that Dicer inhibition induces a physiological stress response. HSP70 is a highly conserved molecular chaperone that plays a crucial role in protecting cells from damage under stressful conditions, including heat shock, oxidative stress, and metabolic challenges (Mayer and Bukau 2005b; Porto et al., 2018; Kim and Kim, 2023). Its high expression levels in the central ring ganglia of PLL-treated snails suggest that these animals experience a form of cellular stress, which may have widespread consequences for neural function. Stress is known to have profound effects on cognitive and motivational processes (Chaouloff et al., 1999; Dalesman and Lukowiak, 2012). In many species, including mammals, acute stress can modulate memory formation by altering neuroplasticity-related signaling pathways (Duman, 2004). Chronic stress, on the other hand, can impair cognitive function and lead to maladaptive behavioural changes (Radley et al., 2015). The fact that LymHSP70 expression is significantly increased suggests that PLL induces a stress-like state in *Lymnaea*, which could contribute to the behavioural effects observed in previous studies (Kemenes et al., 1997). However, despite its increased expression, the level of HSP70 protein did not change significantly. This observation is consistent with previous studies conducted in mammals showing that RNA-level regulation may be stronger than protein-level changes (Cheng et al., 2016).

The discrepancy between mRNA and protein results might be due to the applied time interval (6 h). Future studies will aim at applying multiple time intervals to determine whether the increased HSP70 expression also manifests at the protein level and to develop a general framework for more precise detection of PLL-induced changes at both the mRNA and protein levels. It is worth noting that the moderate correlation between mRNA and protein levels (reported as ~0.41 in mammalian cells; Schwanhäusser et al., 2011), may partly reflect temporal dynamics and post-transcriptional regulation. However, this value may not be generalizable to invertebrates, and further time-course studies are needed to clarify the relationship in *L. stagnalis*.

Importantly, stress responses are often linked to neuromodulatory changes, particularly within monoaminergic systems such as serotonin and dopamine signaling (Chaouloff et al., 1999; Bacqué-Cazenave et al., 2020; Baik, 2020). The interplay between stress and neuromodulation provides a potential mechanism through which PLL-induced Dicer inhibition could alter behaviour independently of direct effects on memory-related pathways. The results of this study highlight a novel aspect of PLL-induced Dicer inhibition in a molluscan model organism: its impact on stress-related markers and serotonergic signaling rather than direct memory impairment.

While these findings shed light on serotonergic vulnerability to Dicer inhibition, the use of PLL as a pharmacological agent introduces interpretive complexity that warrants consideration. As a cationic polymer, PLL can exert nonspecific biological effects beyond Dicer inhibition, Such effects may include interactions with negatively charged components of cell membranes, potential disruption of membrane integrity, and the induction of cellular stress or inflammatory responses. In the present study, the inclusion of saline-injected controls helps to differentiate specific effects attributable to PLL treatment from general injection-related stress. However, while PLL provides a useful experimental tool to probe the role of Dicer and miRNAs, complementary

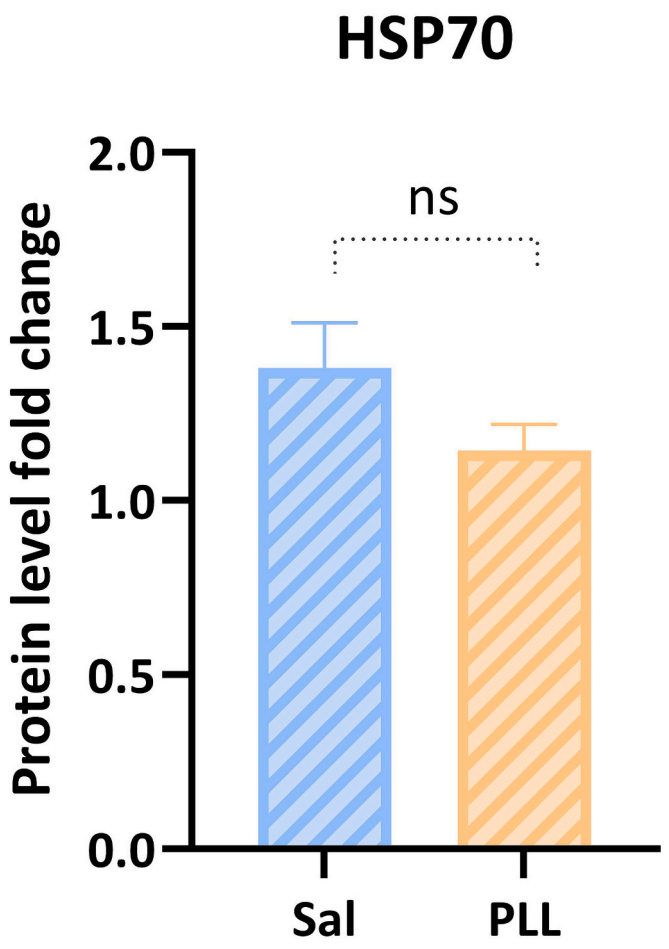


**Fig. 3.** PLL injection does not affect HSP70 protein levels in the central ring ganglia of *Lymnaea*. Snails were randomly assigned to two treatment groups: (1) injected with snail saline (i.e., Sal group) and (2) injected with poly-L-lysine (i.e., PLL group). Six hours after the PLL or saline injection, the central ring ganglia were extracted, the ganglia were pooled (N = 15 per group, 3 independent replicates), and protein levels were analyzed by LC-MS. Data were analyzed using an unpaired t-test. The solid line is the mean, and the error bars are the SEM. ns = not significant as $p > 0.05$.

approaches, such as genetic knockdown or knockout models, are necessary to definitively establish the causal relationship between Dicer inhibition and the neurobiological outcomes observed. Addressing these questions in future research will strengthen the mechanistic insights into miRNA regulation of neural plasticity and behaviour.

Finally, we found that PLL injection did not lead to significant changes in LymGRIN1 or LymCREB1 mRNA levels in snails that had not been associatively trained, suggesting that memory-related transcriptional networks are not directly affected by Dicer inhibition in untrained animals. LymGRIN1 encodes the NMDA receptor subunit 1, a key component of excitatory synaptic transmission that plays a central role in synaptic plasticity and memory formation (Li and Tsien, 2009; Rosenegger et al., 2010; Morris, 2013). Given the well-established role of NMDA receptors in activity-dependent synaptic modifications, their stability following PLL treatment suggests that basic excitatory signaling remains unaltered. Similarly, the fact that the mRNA levels of Lym-CREB1, a transcription factor that is critical for LTM formation (Batabyal et al., 2021), remained unchanged further supports that PLL, when animals are not challenged to learn, does not directly interfere with molecular processes required for memory storage.

These data may align with previous evidence demonstrating that miRNAs can regulate memory suppressor genes, such as Lym-CREB2 (Korneev et al., 2018), rather than directly enhancing memory. Although our findings suggest that, in untrained animals, Dicer inhibition does not significantly alter expression of memory-related genes such as GRIN1 and CREB1, though we cannot rule out effects in trained contexts where transcriptional activity is dynamically regulated during learning. In other words, the previously reported memory impairment in PLL-injected snails (Korneev et al., 2018; Kagan et al., 2022) may not result from direct disruptions to synaptic plasticity pathways but rather from secondary effects related to stress and neuromodulation.

Overall, this finding has significant implications for our understanding of miRNA function in behavioural regulation. Rather than acting solely as memory modulators, miRNAs may play broader roles in maintaining neuromodulatory balance, particularly in pathways related to motivation, reward, and stress resilience.

Future research will focus on several key questions. First, it will be important to determine whether the observed changes in serotonin-related gene expression and neurotransmitter levels are a direct consequence of Dicer inhibition or a secondary effect of PLL on stress-related molecular pathways. Additional experiments will examine whether pharmacological manipulation of serotonin levels can rescue the behavioural deficits observed in PLL-injected snails. Second, further investigation into the effects of PLL in associatively trained animals is necessary to determine whether similar transcriptional and neurochemical changes occur during memory consolidation. If PLL disrupts serotonin signaling in both trained and untrained animals, it would suggest a fundamental role for miRNA-dependent pathways in maintaining neuromodulatory stability during learning and memory processes.

Nonetheless, we acknowledge that comparing the effects of Dicer inhibition in both trained and untrained animals would yield deeper mechanistic insight into how miRNA-dependent processes are differentially engaged by experience-dependent plasticity.

Such a comparison could help clarify which transcriptional changes are specific to learning-related memory consolidation versus those reflecting broader, experience-independent miRNA functions.

Additionally, in this study, we used two closely related, yet separately maintained, *L. stagnalis* strains across different experimental assays. While both strains originate from the same Amsterdam genetic lineage and were kept under standardized and comparable environmental conditions, minor differences in housing water composition and maintenance protocols may introduce subtle strain-specific physiological or molecular variation. Although functional equivalence was assumed based on shared background and matched conditions, no direct statistical comparisons between strains were conducted. To expand upon these findings, future studies will replicate key molecular and neurochemical results within a single strain or include direct cross-strain comparisons. Such efforts will help determine the robustness and generalizability of the observed effects and could further refine *Lymnaea* as a model for investigating miRNA-mediated behavioural regulation.

Finally, these findings may have broader implications for understanding neuropsychiatric conditions in which dysregulated serotonin signaling contributes to cognitive and emotional disturbances. Given that miRNAs are highly conserved across species, their role in serotonin regulation may extend to mammals, influencing conditions such as depression, anxiety, and addiction. By expanding the focus beyond memory-related genes to include stress and neuromodulatory pathways, this study provides new insights into the complex interactions between miRNA activity, physiological stress, and behavioural regulation.

## 5. Conclusion

This study demonstrates that PLL-induced Dicer inhibition in *Lymnaea stagnalis* triggers a stress response and selectively disrupts serotonergic signaling—changes that likely contribute to the reduced motivation and altered behavioural responses observed in previous work. Although the specific miRNAs involved remain to be identified, the broad inhibition of miRNA biogenesis via Dicer suppression reveals a

previously underexplored role for miRNAs in maintaining neuromodulatory balance under non-learning conditions. These findings support the view that miRNAs contribute not only to synaptic plasticity and memory consolidation but also to the constitutive regulation of behavioural states related to stress, reward, and motivation. Given the evolutionary conservation of both serotonergic pathways and the miRNA machinery, our results suggest that miRNA-mediated regulation of neuromodulatory systems is a fundamental and widespread feature of nervous system function across phyla. By highlighting the broader role of miRNAs in shaping behavioural output beyond memory, this work opens new avenues for future studies focused on identifying specific miRNAs and their targets in neuromodulatory circuits. Such efforts will deepen our understanding of how molecular regulatory networks govern complex behaviors across diverse organisms.

## CRediT authorship contribution statement

**Veronica Rivi:** Writing – original draft, Visualization, Validation, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Istvan Fodor:** Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation. **Anuradha Batabyal:** Writing – review & editing, Project administration, Investigation. **Diana Kagan:** Writing – review & editing, Validation, Data curation, Conceptualization. **Johanna Maria Catharina Blom:** Writing – review & editing, Resources. **Fabio Tascedda:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Zsolt Pirger:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **János Schmidt:** Writing – review & editing, Validation, Methodology, Formal analysis. **Cristina Benatti:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Ken Lukowiak:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Funding

The current work was funded by FAR2024_Ricerca diffusa, provided by the University of Modena and Reggio Emilia. This work was also supported by the National Brain Project (#NAP2022-I-10/2022, ZP); the Hungarian Scientific Research Fund (#138039, ZP; #146787, IF) and Natural Sciences and Engineering Research Council of Canada (NSERC - 227993-2019). The work was also supported by the COST Action AFFECT-EVO (#CA23106; Z.P.).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

We would like to thank Dr. Petra Hermann from the University of Calgary for providing the experimental animals used in our experiments and Dr. Eleonora Daini for helping with the graphical abstract, which was created using MindTheGraph. We would also like to thank Consorzio Interuniversitario Biotecnologie (CIB, Trieste, Italy) for the research grant awarded to Dr. Veronica Rivi to work as a visiting researcher at the Hotchkiss Brain Institute (University of Calgary, AB, Canada).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110291.

## Data availability

The data presented in this study are available in the present article and on request from the corresponding author.

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