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RESEARCH ARTICLE

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Behavioral and transcriptional effects of carnosine in the central ring ganglia of the pond snail *Lymnaea stagnalis*

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Abstract

Carnosine is a naturally occurring endogenous dipeptide with well-recognized anti-inflammatory, antioxidant, and neuroprotective effects at the central nervous system level. To date, very few studies have been focused on the ability of carnosine to rescue and/or enhance memory. Here, we used a well-known invertebrate model system, the pond snail *Lymnaea stagnalis*, and a well-studied associative learning procedure, operant conditioning of aerial respiration, to investigate the ability of carnosine to enhance long-term memory (LTM) formation and reverse memory obstruction caused by an immune challenge (i.e., lipopolysaccharide [LPS] injection). Exposing snails to 1 mM carnosine for 1 h before training in addition to enhancing memory formation resulted in a significant upregulation of the expression levels of key neuroplasticity genes (i.e., glutamate ionotropic receptor *N*-methyl-D-aspartate [NMDA]-type subunit 1—LymGRIN1, and the transcription factor cAMP-response element-binding protein 1—LymCREB1) in snails' central ring ganglia. Moreover, pre-exposure to 1 mM carnosine before an LPS injection reversed the memory deficit brought about by inflammation, by preventing the upregulation of key targets for immune and stress response (i.e., Toll-like receptor 4—LymTLR4, molluscan defense molecule—LymMDM, heat shock protein 70—LymHSP70). Our data are thus consistent with the hypothesis

Abbreviations: CNS, central nervous system; CREB1, cAMP-response element-binding protein; GRIN1, glutamate *N*-methyl-D-aspartate receptor subunit 1; HSP70, heat shock protein 70; ITM, intermediate-term memory; LPS, lipopolysaccharide; LTM, long-term memory; MDM, molluscan defense molecule; MT, memory test; TLR4, Toll-like receptor 4; TS, training session.

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that carnosine can have positive benefits on cognitive ability and be able to reverse memory aversive states induced by neuroinflammation.

KEYWORDS
inflammation, invertebrates, learning, memory, stress

1 | INTRODUCTION

It is becoming progressively evident that memory decline and cognitive impairment linked to various central nervous system (CNS) pathologies are significantly influenced by neuroinflammation, oxidative stress, and the abnormal accumulation of proteins within the brain (Ahmad et al., 2022; Singh et al., 2022; Tangestani Fard & Stough, 2019; Wang et al., 2023). In light of this, the recognized antioxidant, anti-inflammatory, and antiaggregation properties of carnosine, a naturally occurring endogenous dipeptide, have been more closely examined in order to gain deeper insights into its therapeutic potential in the treatment of cognitive disorders (Aloisi et al., 2013; Berezhnoy et al., 2019; Caruso et al., 2019; Schön et al., 2019).

Since its discovery more than 100 years ago by Gulewitsch and Amiradžibi (1900), a plethora of publications have been dedicated to the description of carnosine structure and biological activity in different body areas, showing that this dipeptide can be found at high concentrations (i.e., millimolar order) in the brain, as well as in cardiac and skeletal muscles (up to 20 mM), where it exerts a multimodal mechanism of action (Bae et al., 2013; Bonfanti et al., 1999; Calabrese et al., 2005; Mannion et al., 1992). Additionally, in the last decade, the procognitive effects of carnosine supplements in patients suffering from mild cognitive impairment (Afshin-Majd et al., 2015; Bae & Majid, 2013; Masuoka et al., 2019), diabetes, subcortical ischemic vascular dementia (Ahshin-Majd et al., 2016; Bakardjiev, 1998; Bauer et al., 1982; Corona et al., 2011; Ma et al., 2012), and Alzheimer's disease emerged (Berezhnoy et al., 2016; Boldyrev et al., 1997, 2004, 2007; Boldyrev & Severin, 1990; Caruso et al., 2019; Privitera et al., 2023). However, although carnosine has become an appealing supplement for developing new therapeutic approaches to prevent or reduce cognitive impairment affecting various neurodegenerative and chronic disorders (Bellia et al., 2011; Cesak et al., 2023; Corona et al., 2011; Hisatsune et al., 2016; Santiago & Potashkin, 2023; Schön et al., 2019), to date the molecular mechanisms through which carnosine exerts its procognitive effects and rescues cognitive deficit remain almost unexplored. This is in part due to the complexity of mammalian brains and behaviors, together with the complex etiopathogenesis of neurodegenerative disorders.

To overcome some of the aforementioned obstacles, we made use of our model system the pond snail *Lymnaea stagnalis* (Linnaeus, 1758) to examine at both the behavioral and molecular levels how carnosine may enhance cognitive ability and suppress inflammatory effects. *Lymnaea stagnalis* has been shown to possess highly reproducible associative learning procedures that are negatively impacted by inflammatory processes (Amorim et al., 2019; Audesirk et al., 1985; Rivi, Batabyal, Benatti, et al., 2022; Rivi, Batabyal, Benatti, Blom, et al., 2023a; Rivi, Batabyal, Lukowiak, Benatti, et al., 2023). Thus, this model system would appear to be well suited to explore at both the behavioral and molecular levels how carnosine acts to enhance cognition and be an anti-inflammatory agent. Adopted as a model organism for learning and memory studies beginning in the 1960s (Coutellec & Lagadic, 2006; Dalesman, 2018; Fodor et al., 2020; Rivi et al., 2024; Rivi, Batabyal, Benatti, Blom, et al., 2023b), *L. stagnalis* provides a valid tool in which to investigate the action of carnosine on learning and memory, as well as its anti-inflammatory effects.

Significance

To the best of our knowledge, this is the first study showing carnosine-mediated enhancement of long-term memory formation in an invertebrate model, the pond snail *Lymnaea stagnalis*, after an operant-conditioning procedure. This work also provides the first support for carnosine to prevent the sickness state and memory block induced by an immune challenge (i.e., bacterial lipopolysaccharide). These findings pave the way for mammalian experiments, offering insights into the therapeutic potential of carnosine as a pharmacological tool for cognitive disorders marked by immune overactivation and inflammation.

First, operant conditioning (a form of associative learning) of aerial respiration has been studied in *L. stagnalis* since 1996 (Lukowiak et al., 1996). In addition, a single identified neuron is necessary for long-term memory (LTM) formation in the nervous system of this snail (Syed & Winlow, 1991).

Moreover, memory formation can be altered by environmental stressors and bioactive compounds, allowing us to assess how different factors alter memory formation (Batabyal et al., 2024; Gust et al., 2013; Kagan et al., 2022; Pyatt et al., 1997; Rivi et al., 2024; Rivi, Batabyal, Benatti, Tascedda, et al., 2023c, 2023d; Rivi, Benatti, Rigillo, & Blom, 2023).

Second, pond snails are aquatic invertebrates with an open circulatory system, allowing the use of membrane-permeant drugs that can be easily absorbed—like carnosine—to unravel the complexity of various signaling pathways and to provide new insights on how bioactive compounds can modulate different neuronal functions and behaviors (Rivi et al., 2022; Rivi, Batabyal, Benatti, Tascedda, et al., 2023e; Rivi, Batabyal, Wiley, et al., 2022; Rivi, Benatti, Rigillo, & Blom, 2023).

Third, *L. stagnalis* responds to several bioactive compounds that can alter memory formation (Rivi, Batabyal, Benatti, Blom, et al., 2023b; Rivi, Batabyal, Lukowiak, Benatti, et al., 2023; Rivi, Benatti, Actis, et al., 2022) and—even more importantly—the effects of these compounds often mirror those seen in vertebrates, strengthening the case for *L. stagnalis* as a model to unravel the conserved effects of carnosine at both behavioral and molecular levels.

To comprehensively examine whether and how carnosine may alter memory formation and a sickness state in *L. stagnalis*, we designed and executed a series of experiments, each addressing specific questions.

Experiment 1, studied the effects of various doses of carnosine on snails' feeding behavior, a fundamental homeostatic behavior, assuming that a significant reduction in feeding behavior after carnosine exposure would be indicative of a sickness state (Benjamin, 1983).

In *Experiment 2*, we investigated whether exposure to carnosine before a .5-h training session (TS) for the operant conditioning of aerial respiration (which typically results in a 3-h lasting intermediate-term memory—ITM) in laboratory-inbred snails (Lukowiak et al., 2000) would be able to enhance LTM (lasting for at least 24 h) formation. Moreover, as carnosine has been found to exert anti-inflammatory effects in many animal models (Caruso et al., 2019; Fleisher-Berkovich et al., 2009; Kubota et al., 2020), we explored whether exposure to carnosine would be able to prevent the lipopolysaccharide (LPS)-induced inflammation in snails' central ring ganglia. We recently demonstrated that the injection of 25 mg (6.25 μg/mL) of *Escherichia coli*-derived LPS serotype O127:B8—which is a potent activator of the immune system—induces a "sickness state" that alters the ability of *Lymnaea* to form or recall LTM for operant conditioning of aerial respiration (Rivi, Batabyal, Benatti, et al., 2022; Rivi, Batabyal, Benatti, Blom, et al., 2023a).

Thus, we hypothesize that exposure to carnosine before the LPS injection would prevent the LPS-induced sickness at both behavioral and transcriptional levels (*Experiments 3* and *4*). In particular, we focused our attention on specific molecular targets known to play pivotal roles in inflammatory and stress responses, as well as neuroplasticity. These included Toll-like receptor 4 (LymTLR4) (Ciesielska et al., 2021; Escoubet-Lozach et al., 2011; Lu et al., 2008), molluscan defense molecule (LymMDM) (Hoek et al., 1996), heat shock protein 70 (LymHSP70) (Fei et al., 2007; Swinton et al., 2018), glutamate ionotropic receptor *N*-methyl-D-aspartate (NMDA)-type subunit 1 (LymGRIN1) (Dhar & Wong-Riley, 2009; Luscher & Malenka, 2012), and the transcription factor cAMP-response element-binding protein 1 (LymCREB1) (Rivi et al., 2024). This allowed us to study the transcriptional effects induced by LPS injection, carnosine exposure, and the exposure to carnosine before the LPS injection in snails' central ring ganglia.

Finally, in *Experiment 5*, we investigated whether the transcriptional effects on the abovementioned targets in the central ring ganglia of snails were consistent with the observed behavioral effects. This experiment allowed us to test the hypothesis that carnosine exposure before an immune challenge would be able to reverse the memory block induced by LPS.

This multifaceted approach, with each experiment addressing specific questions, allowed for a nuanced exploration of the memory-enhancing and anti-inflammatory proprieties of carnosine and its ability to reverse a learning and memory deficit brought about by an immune challenge.

2 | METHODS AND MATERIALS

2.1 | Snails and animal maintenance

Laboratory-reared *L. stagnalis*, originally derived from a stock generously donated by Prof. Lukowiak (University of Calgary, the W-strain), was used in this study. The "Canadian snails" stock originated from an inbred colony maintained at the Vrije University of Amsterdam and were originally bred from animals collected in the 1950s in polders near Utrecht, The Netherlands. Animals were maintained in aquaria at the University of Modena and Reggio Emilia (Italy) at 21–23°C in well-aerated dechlorinated tap water on a 12/12-h light/dark cycle (lights on at 08:00 a.m.). Six-month-old snails having shell lengths of 20–25 mm were used in these experiments. Animals were fed pesticide-free lettuce twice a week. In this study, we used 176 snails. The sample size and the power analysis for each experiment were a priori calculated using G power 3.1.9.7 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany—http://www.gpower.hhu.de/) (Faul et al., 2007). The optimal sample size, given a predicted effect (*f*) of .7, α of .05, and the power ($1-\beta$) of .8, was calculated a priori to be $N=7$ per group in G*Power 3.1.

2.2 | Liquid chromatography

The concentration of carnosine was measured in the ganglia of control *L. stagnalis* ($N=4$). Each ganglion was homogenized in 100 μL of a solution of ascorbic acid .1% by sonication. Protein concentrations of homogenates were determined using the Bradford assay (Merck KGaA, Darmstadt, Germany). To each 50 μL of sample, an equal volume of ice-cold 1 M perchloric acid ($HClO_4$) fortified with L-carnosine-d_4 (final concentration 1 μM, Cayman Chemical, USA) was added. Samples were centrifuged (15,000 *g*, 15 min), and the supernatants were collected and directly injected into LC–MS/MS. The analyses of carnosine in the supernatant were performed using an Agilent HP 1200 liquid chromatograph (Agilent, Milan, Italy) consisting of a binary pump, an autosampler, and a thermostated column compartment.

Chromatographic separations were carried out using a Discovery HS-F5 column (3 μm particle size, 150 × 2.1 mm, Supelco, Milan, Italy) using .1% formic acid in water and acetonitrile (ACN) as mobile phase. The HPLC analyses were carried out using a linear elution profile of 15 min from 5% to 90% of ACN.

The column was washed with 90% ACN for 3.5 min, then equilibrated for 5 min with 5% ACN. The flow rate was .5 mL/min. The injection volume was 20 μL. An Agilent 6410 triple quadrupole mass spectrometer with an electrospray ion source operating in positive mode was used for detection. The SRM pairs were 227 to >110 and 231 to >156 for L-carnosine and L-carnosine-d4, respectively. The calibration curves were constructed using calibration standards (L-carnosine, Cayman Chemical, USA) and were linear over the concentration range of .0391–10.000 μM, with a correlation coefficient (r^2) of .999. Carnosine concentrations were normalized to protein content.

2.3 | Carnosine treatment

For each carnosine experiment, snails were exposed to carnosine (100 μM, 1 mM, or 10 mM) for 1 h. A 500 mM stock of carnosine (β-alanyl-L-histidine; Merck KGaA, Darmstadt, Germany; MW: 226.23) was prepared in phosphate-buffered saline (PBS 1×). Then, we prepared carnosine solutions at different concentrations by dissolving the stock in artificial pond water.

2.4 | LPS treatment

For each LPS experiment, snails were randomly divided into groups of 7–8 animals for each. One group was injected with 25 μg of *E. coli*-derived LPS serotype O127:B8 (Merck KGaA, Darmstadt, Germany; L3129), which corresponds to approximately 8 mg/kg (Rivi, Batabyal, Lukowiak, Benatti, et al., 2023). LPS solution was prepared by dissolving 625 μg of LPS in 1 mL of snail saline solution (51.3 mM NaCl; 1.7 mM KCl; 5.0 mM $MgCl_2$; 1.5 mM $CaCl_2$; 5.0 mM HEPES; pH 7.9–8), as previously described (Straub et al., 2004). We injected 40 μL of LPS into the abdominal body cavity of each snail. Snails used as sham-injected control were injected with 40 μL of snail saline. Once injected, snails were kept in an upside-down position without immersion in artificial pond water for 10 min, consistent with previous studies (Rivi, Batabyal, Benatti, Blom, et al., 2023a).

As the calculated volume of hemolymph in a snail with a 20-mm shell length was 400 μL (Murakami et al., 2013), a single injection of 40 mL was performed intramuscularly in the foot of the snail using a 31G syringe.

2.5 | Study design

2.5.1 | Experiment 1: Carnosine dosing

The number of rasps elicited by a "familiar taste"—lettuce slurry—was measured 1, 3, and 24 h before snails were exposed to carnosine 100 μM, 1 mM, or 10 mM for 1 h. Lettuce slurry was prepared by blending two medium leaves of romaine lettuce (approximately 20 g) along with 500 mL of artificial pond water, as previously described (Rivi, Batabyal, Benatti, Blom, et al., 2023a).

Following blending and straining the mixture, a lettuce slurry was obtained without any observable pieces of lettuce. To record the number of raps elicited by the lettuce slurry, snails were placed into a 14 cm diameter Petri dish with enough lettuce slurry to be partially submerged. The snails were given a 5-min acclimation period before their rasping behavior was monitored. Each snail was monitored for 2 min and the number of rasps was counted; the average number of rasps per minute was then calculated.

Snails were then returned to their home aquarium for 1 h before being exposed to carnosine. The same procedure was repeated at 3 and 24 h postcarnosine exposure.

Thus, 32 snails were used in *Experiment 1* and were randomly divided into the four experimental groups:

1. In eight snails we recorded the rasping behavior in the presence of lettuce slurry 1 h before and 3 and 24 h after being exposed to artificial pond water for 1 h.
2. In eight snails the rasping behavior in lettuce slurry was recorded 1 h before and 3 and 24 h after being exposed to carnosine 100 μM for 1 h.
3. In eight snails the number of rasps elicited by the lettuce slurry was recorded 1 h before 3 and 24 h after being exposed to carnosine 1 mM for 1 h.
4. In eight snails the rasping behavior elicited by the lettuce slurry was recorded 1 h before and 3 and 24 h after being exposed to carnosine 10 mM for 1 h.

2.5.2 | Experiment 2: Behavioral effects induced by the exposure to different doses of carnosine on the ability of snails to form ITM and/or LTM following the operant conditioning of aerial respiration training procedure

Artificial pond water was made hypoxic (≤.1 mL O_2 L^{-1}) by vigorously bubbling N_2 gas through the water for 20 min before the operant-conditioning TS. The hypoxic environment caused snails to move to the water surface and attempt to open their pneumostome more frequently (Lukowiak et al., 1996). Following these 20 min, the intensity of bubbling was reduced and the snails to be trained were placed in the beaker for a 10-min acclimation period before training began. The reduced bubbling maintained the established hypoxia without disturbing the snails. The operant-conditioning procedure consisted of applying a tactile stimulus (i.e., a poke) to the edge of the pneumostome with a wooden stick every time a snail attempted to perform aerial respiration. The stimulus was strong enough to cause the pneumostome to close, but was gentle enough that the snails did not complete a full-body withdrawal response. The total number of pokes per snail was recorded. Between sessions, snails were returned to their home aquarium where they had ad libitum access to food (Lukowiak et al., 1996). The memory test (MT) session

was performed in hypoxic artificial pond water at 3, 24, or 48 h post-training. As in the TS, during the MT, snails received a tactile stimulus each time they attempted to open their pneumostome in the .5-h MT. Memory has been operationally defined as significantly fewer attempted pneumostome openings in the MT session than in the TS.

Thus, four naïve cohorts of snails ($N = 64$) were used in this study:

1. A naïve cohort of snails ($N = 16$) was first exposed to carnosine 100 μM for 1 h, and 3 h later was trained with a .5-h TS. In half of these snails ($N = 8$), ITM was tested 3 h later, whereas in the remaining group, LTM enhancement was tested 24 h post-TS.
2. A naïve cohort of snails ($N = 16$) was exposed to 1 mM carnosine for 1 h, and 3 h later was trained with a .5-h TS. In half of these snails ($N = 8$), ITM was tested 3 h later, whereas in the remaining group, LTM enhancement was tested 24 h post-TS.
3. A naïve cohort of snails ($N = 16$) was exposed to 10 mM carnosine for 1 h, and 3 h later was trained with a .5-h TS. In half of these snails ($N = 8$), ITM was tested 3 h later, whereas in the remaining group, LTM enhancement was tested 24 h post-TS.
4. Control snails ($N = 16$) were exposed to artificial pond water for 1 h instead of carnosine. Three hours later, snails trained with a .5-h TS. In half of these snails ($N = 8$), ITM was tested 3 h later, whereas in the remaining group, LTM enhancement was tested 24 h post-TS.

Sixty-four snails were used in *Experiment 2* and were randomly divided into the four experimental groups.

To minimize subjective bias, behavioral experiments were performed blindly as the experimenter performing the MT did not know the previous treatment. Only after all the results were tabulated did we know the outcome of the various experiments.

2.5.3 | Experiment 3: Behavioral effects induced by carnosine, LPS, and their paired presentation on snails' aerial respiration

Snails were placed in a 1-L beaker filled with 500 mL of artificial pond water made hypoxic (≤5% O_2) by vigorously bubbling with N_2 for 20 min. The amount of time the pneumostome was open for each snail without any tactile stimulation (i.e., total breathing time, TBT) was recorded for .5 h (i.e., observation period 1). Three hours later, snails ($N = 24$) were randomly divided into three groups and were subjected to different treatments:

1. Eight naïve snails were injected with LPS.
2. Eight naïve snails were exposed to carnosine for 1 h.
3. Eight naïve snails were exposed to carnosine for 1 h and immediately after were injected with LPS.

Then, snails were returned to their home aquaria for 3 h, before the TBT was again recorded in hypoxic pond water.

2.5.4 | Experiment 4: Transcriptional effects induced by carnosine, LPS, and their paired presentation in snails' central ring ganglia

Twenty-eight snails were used in *Experiment 4* and were randomly divided into the four experimental groups ($N = 7$, each group):

1. Snails that were injected with snail saline.
2. Snails that were injected with 25 μg of LPS.
3. Snails that were exposed to carnosine for 1 h.
4. Snails that were exposed to carnosine for 1 h and immediately after were injected with LPS.

Three hours later, snails were euthanized in ice for 10 min, and the central ring ganglia were dissected (buccal ganglia were excluded) and stored at −80°C before analysis.

2.5.5 | Experiment 5: Behavioral and transcriptional effects induced by carnosine exposure before LPS injection on snails' memory abilities

Twenty-eight snails were used in *Experiment 4* and were randomly divided into the four experimental groups ($N = 7$, each group):

1. Snails that were injected with snail saline 3 h before the .5 h TS.
2. Snails that were injected with 25 μg of LPS 3 h before the .5 h TS.
3. Snails that were exposed to carnosine for 1 h and 3 h before the .5 h TS.
4. Snails that were exposed to carnosine for 1 h before being injected with LPS and 3 h later were trained for the operant conditioning of aerial respiration.

Immediately after the MT at 3 h post-TS, snails were euthanized in ice for 10 min, and the central ring ganglia were dissected (buccal ganglia were excluded) and stored at −80°C before analysis.

Behavioral experiments were performed blindly, without the experimenter being aware of the previous treatment during the MT. The outcomes of the different experiments were only revealed after all the results were recorded and tabulated.

2.6 | Total RNA extraction, reverse transcription, and real-time polymerase chain reaction

Total RNA extraction and DNAse treatment were performed on snails from Experiments 4 and 5 using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck KGaA, Darmstadt, Germany) as previously described (Cristina et al., 2022). A single, central ring ganglion was used for total RNA extraction. Seven samples were analyzed for each group. A 200-ng sample of total RNA was reverse transcribed with a High-Capacity

cDNA Reverse Transcription Kit (Thermo Fisher). Real-time quantitative PCR was carried out on 20 ng mRNA using a Bio-Rad® CFX Connect™ Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The cycling parameters were 95°C for 2 min and 95°C for 10 s, 60°C for 30 s for 40 cycles, and a dissociation curve analysis followed the amplification. Cycle threshold (Ct) values were determined by CFX Maestro™ Software (Bio-Rad). Specific forward and reverse primers were used at the final concentration of 300 nM (Table 1). The mRNA levels of each target were normalized to two reference genes, elongation factor 1α and tubulin. The stability of mRNA expression of these endogenous controls was assessed using Normfinder®, considering intra- and intergroup variations. The mean between the two endogenous genes was found to be the most stable gene across groups and was used for gene normalization. The endogenous control mRNA levels were not affected by any procedure (one-way analysis of variance [ANOVA]) and the amplification efficiency of the target genes and endogenous control genes was approximately equal. For quantitative evaluation of changes, the comparative $2^{-\Delta\Delta Ct}$ method was performed using the average levels of expression of control animals as a calibrator. Before statistical analysis, we searched for extreme outliers using the boxplot tool in SPSS (more than 3× the interquartile range outside of the end of the interquartile box). No outliers were found.

2.7 | Statistical analyses

First, we confirmed that our data were normally distributed using a Kolmogorov–Smirnov test (KS distance and *p* value). Behavioral data from Experiment 1 were analyzed using a repeated measures (RM) ANOVA combined with post hoc Tukey's tests to compare the number of rasps elicited by lettuce slurry before and after the carnosine exposure for 1 h. In Experiment 2, we compared the number of attempted pneumostome openings between the .5-h TS and the MTs performed at 3 and 24 h post-TS using a one-way ANOVA followed by Tukey's post hoc test. Significant differences between the two groups were examined by Student's paired *t* test. Behavioral data from Experiment 3 were analyzed using a paired *t* test to compare the differences between the TBT before and after the treatment (i.e., carnosine alone, LPS alone, or carnosine before LPS). Behavioral data of Experiment 5 were analyzed using a paired *t* test to compare the differences between the number of attempted pneumostome openings recorded during the .5-h TS and the MT performed 3 h after the treatment (i.e., carnosine alone, LPS alone, or carnosine before LPS). For the molecular data of Experiments 4 and 5, one-way ANOVA was used to compare the expression levels of each target in the central ring ganglia of snails of the different groups. Significant changes were determined by Tukey's post hoc test. In all analyses reported here, a type I error rate of .05 was used. Data were presented as mean ± standard error (SEM). All statistical analyses were performed using SPSS software ver. 26.0 (IBM Corp., Armonk, NY, United States), whereas graphs were created using GraphPad Prism v. 9.00e for Windows® (GraphPad Software, Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | L-Carnosine levels in the ganglia of *L. stagnalis*

The levels of carnosine were measured by liquid chromatography in the ganglia of control snails. Basal level of carnosine in the ganglia was ~.0035 μM/μg protein (±.0026 *SD*) (i.e., ~.783 μmole/μg protein).

TABLE 1 Nucleotide sequence of the forward and reverse primers used for real-time PCR.

Gene bank accession	Target	Product length (bp)	Type sequence
X15542.1	*Snail, beta-tubulin LymTUB*	100 bp (92–192)	FW: GAAATAGCACCGCCATCC RV: CGCCTCTGTGAACTCCATCT
DQ278441.1	*Lymnaea stagnalis elongation factor 1alpha LymEF1α*	150 bp (7–157)	FW: GTGTAAGCAGCCCTCGAACT RV: TTCGCTCATCAATACCACCA
AY577328.1	*Lymnaea stagnalis Toll-like receptor 4 LymTLR4*	100 bp (74–174)	FW: GGAGGGTCAAGCATAAAGTGT RV: CATCAAGGTCAACGCCAAT
U58769.1	*Lymnaea stagnalis molluscan defense molecule precursor LymMDM*	104 bp (1614–1718)	FW: CGGGTACACACACAGATGGA RV: TGACTGAACATTGGGCACAC
DQ206432.1	*Lymnaea stagnalis heat-shock protein 70 LymHSP70*	199 bp (134–333)	FW: AGGCAGAGATTGGCAGGAT RV: CCATTTCATTGTGTCGTTGC
AY571900.1	*Lymnaea stagnalis NMDA-type glutamate receptor subunit 1 LymGRIN1*	140 bp (831–917)	FW: AGAGGATGCATCTACAATTT RV: CCATTTACTAGGTGAACTCC
AB041522.1	*Lymnaea stagnalis cAMP-responsive element-binding protein LymCREB1*	180 bp (49–229)	FW: GTCAGCAGGGAATGGTCCTG RV: ACCGCAGCAACCCTAACAA

Note: For each target, the accession number and the size (bp) of the PCR product obtained by the amplification of the cDNA (mRNA) are given.

3.2 | Experiment 1: The exposure to carnosine 100 μM and 1 mM does not affect snails' feeding behavior, whereas the exposure to carnosine 10 mM results in significant suppression of feeding

In Experiment 1, we investigated whether exposure to 100 μM, 1 mM, or 10 mM carnosine for 1 h would affect snails' feeding behavior. We first ascertained that the repeated handling procedure (i.e., from the home aquaria to the Petri dish where the feeding behavior was recorded and back, and from the home aquaria to the beaker used for the treatment and back) would not affect snails' feeding behavior. Thus, in a naïve cohort of eight snails, we measured the number of rasps elicited by a "familiar taste," lettuce slurry, 1 h before and 3 and 24 h after the exposure to artificial pond water (20°C) (Figure 1a). We found that the response to the lettuce slurry was not affected by the handling of snails (RM ANOVA, $F[1.678, 11.74] = .014$, $p = .971$), as no significant differences in the number of rasps elicited by the lettuce slurry were found. Then, the effect of this behavior of three different doses of carnosine was evaluated.

Eight snails per group were exposed for 1 h to either 100 μM, 1 mM, or 10 mM carnosine in their home aquaria. For all the animals, the number of rasps elicited by the lettuce slurry was recorded for 2 min, 1 h before, 3 h, and 24 h after the treatment with carnosine. We found that the response to the lettuce slurry was not affected by the exposure to lower doses of carnosine (100 μM) (RM one-way ANOVA, $F[1.760, 12.32] = .573$, $p = .551$ for 100 mM and $F[1.630, 11.41] = 1.762$, $p = .211$ for 1 mM), as no significant differences were found in the number of rasps elicited by the lettuce slurry 1 h before and 3 h and 24 h after the treatment (Figure 1b,c). A main effect of the treatment was found after exposing snails to 10 mM carnosine (RM ANOVA, $F[1.104, 7.729] = 21.38$, $p = .002$). In particular, Tukey's post hoc analysis revealed a significant reduction in the number of rasps elicited by the lettuce slurry 3 h after the exposure to carnosine compared to that recorded before ($p = .007$). When we recorded the number of rasps elicited by the lettuce slurry 24 h postcarnosine 10 mM exposure, we found that snails returned to normal feeding as no significant differences were found in the number of rasps elicited by the lettuce slurry 1 h before and 24 h after the exposure to carnosine 10 mM ($p = .0002$) (Figure 1d).

3.3 | Experiment 2: Memory effects of different doses of carnosine

In Experiment 2, we investigated the possible memory-enhancing effects induced by different doses of carnosine in snails trained using the operant conditioning of aerial respiration procedure (Lukowiak et al., 1996). First, we wanted to confirm that a single .5-h TS only resulted in 3 h lasting memory (i.e., ITM), but was not sufficient to cause LTM to form. Thus, in a naïve cohort of snails ($N = 16$) in artificial pond water, snails received a single .5-h TS. Memory was then tested for 3 h ($N = 8$; MT 3 h) and 24 h later ($N = 8$; MT 24 h). A main effect of the training procedure was found (one-way ANOVA, $F[2, 29] = 22.33$, $p < .0001$). Tukey's post hoc test revealed a significant reduction in the number of attempted pneumostome openings between TS and MT at 3 h ($p < .0001$), consistent with ITM formation. However, consistent with previous studies (Rivi, Batabyal, Benatti, et al., 2022), a significant reduction was not found between TS and MT at 24 h ($p = .764$), indicative that LTM did not form (Figure 2a). We next tested three different concentrations of carnosine (100 μM, 1 mM, and 10 mM) in naïve cohorts of snails in the operant conditioning of aerial respiration procedure. For each dose, a cohort of naïve snails ($N = 16$) was exposed to carnosine for 1 h and then trained 3 h later in pond water with a .5-h TS. In each cohort, half of the snails ($N = 8$) ITM were tested 3 h later (MT 3 h), and the remaining cohort ($N = 8$) 24 h later (MT 24 h). In Figure 2b, the lowest dose of carnosine (100 μM) was used. A one-way ANOVA revealed a main effect of the training procedure ($F[2, 29] = 13.45$, $p = .0001$). In the 3-h MT (MT 3 h), a significant reduction ($p < .001$) in the number of attempted pneumostome openings between was found; however, these snails did not form LTM, as the number of attempted openings in the 24 h MT (MT 24 h) was not significantly reduced ($p = .732$). Thus, the exposure to 100 μM carnosine for 1 h did not block ITM formation, but also did not enhance LTM formation.

Similarly (Figure 2c), a carnosine concentration of 1 mM was used and the obtained results were different from the outcome with 100 μM carnosine. That is, with pretreatment with 1 mM carnosine, ITM formation occurred, but so did LTM formation (Figure 2c). A main effect of the training procedure was found for the 1 mM dose (ANOVA, $F[2, 29] = 22.39$, $p < .0001$). Tukey's post hoc test revealed a significant reduction in the number of attempted pneumostome openings at the 3-h MT (MT 3 h; $p = .0001$). That is, ITM was formed. Moreover, these snails also showed LTM as MT 24 h was significantly less than TS ($p < .0001$). Thus, in snails exposed to 1 mM carnosine enhancement of LTM formation was shown.

Finally, we examined the effect produced by the highest concentration of carnosine used (i.e., 10 mM; Figure 2d). With this concentration of carnosine, neither ITM nor LTM formed. That is an ANOVA followed by Tukey's post hoc test showed that no memory formation occurred following the operant-conditioning training procedure ($F[2, 29] = 1.31$, $p = .293$; TS vs. MT 3 h and MT 24 h, respectively, $p = .944$ and .221) later. Having shown that a carnosine concentration of 1 mM enhanced LTM formation, we next determined (Figure 3) if the enhancement also resulted in a longer lasting LTM. Thus, a naïve cohort ($N = 7$) was first exposed to 1 mM carnosine for 1 h and 3 h later was trained with a .5-h TS. A MT (MT 48 h) was performed 48 h later and no significant difference in the number of attempted pneumostome openings between TS and the 48-h MT was found ($t = 2.20$, $df = 6$, $p = .072$). Thus, the exposure to carnosine 1 mM for 1 h before the TS resulted in an LTM lasting at least 24 h, but not 48 h (Figure 3). Based on these data, we focused our attention on the behavioral and transcriptional effects induced by the exposure to 1 mM of carnosine in snails' central ring ganglia.

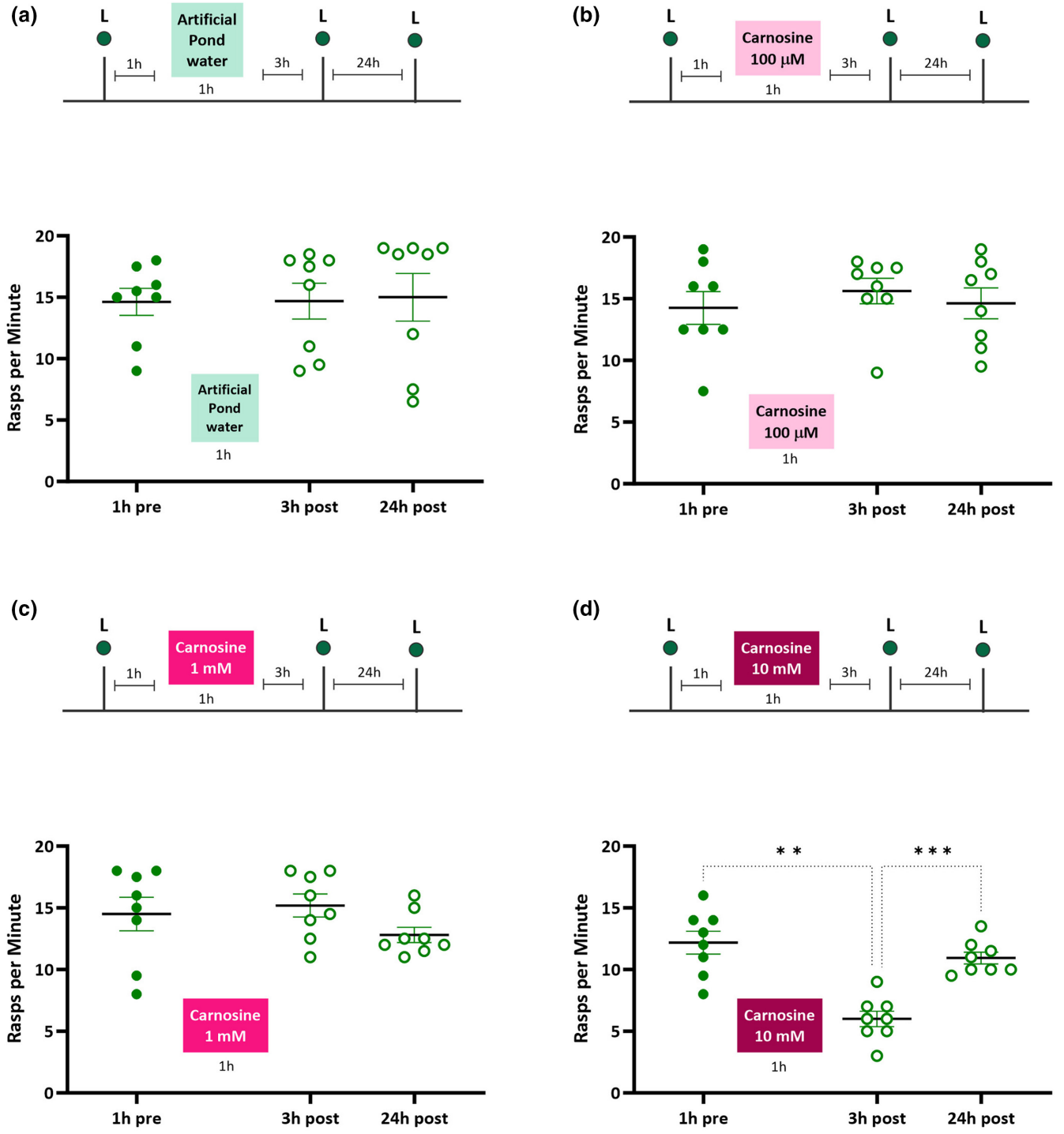

FIGURE 1 Exposure to 100 µM or 1 mM carnosine does not affect snails' feeding behavior, whereas exposure to 10 mM carnosine causes a significant suppression of feeding. The timeline for the experiment is presented above the data. The number of rasps elicited by the lettuce slurry (L) was recorded in a naïve cohort of eight snails 1 h before (closed circles) and 3 and 24 h after (open circles) the exposure to artificial pond water (a), 100 µM carnosine (b), 1 mM carnosine (c), and 10 mM carnosine (d). Comparisons were performed using RM ANOVA followed by Tukey's post hoc test. The solid line is the mean and the error bars are the SEM. ***$p < .001$; **$p < .01$.

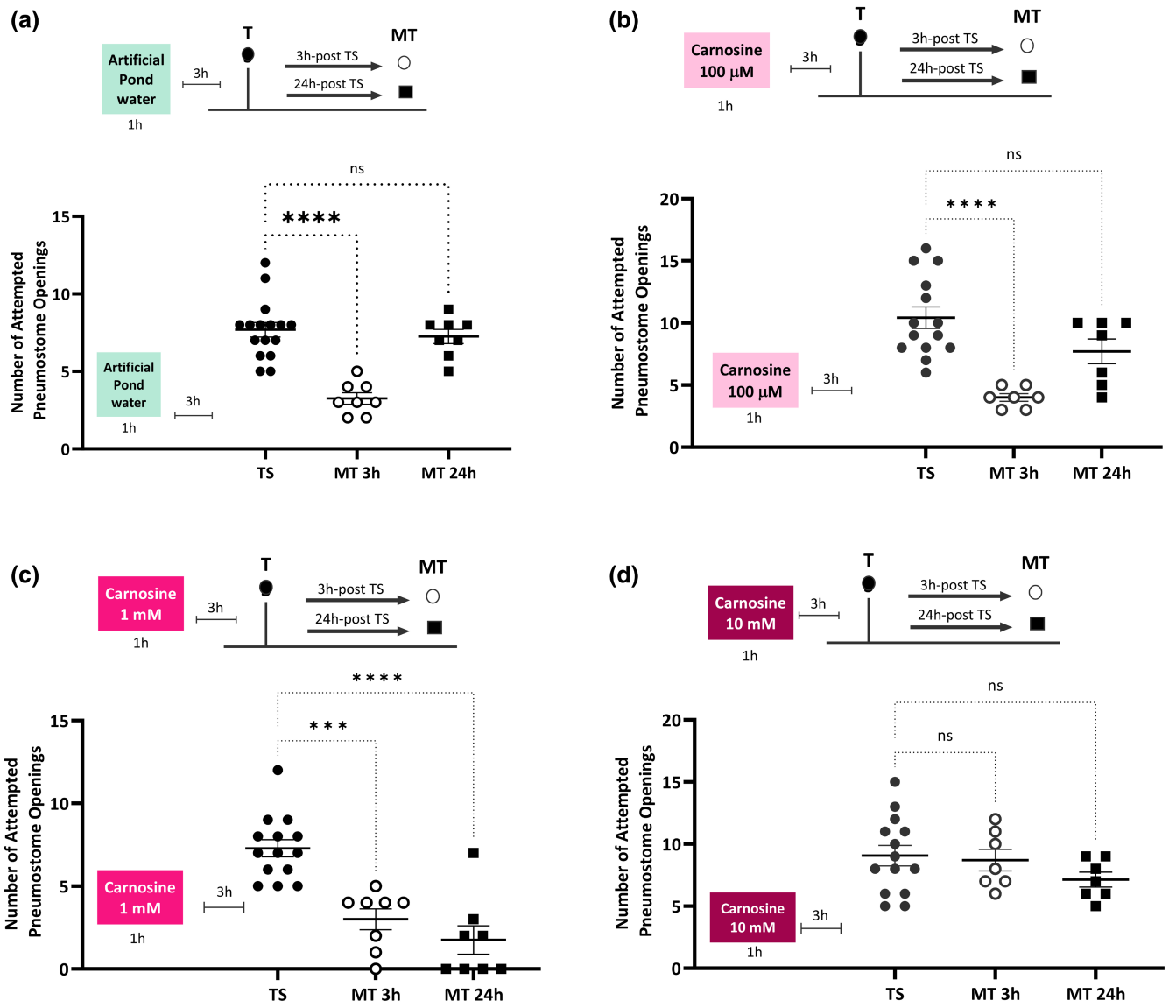

FIGURE 2 Effects of different doses of carnosine on memory formation for the operant conditioning of aerial respiration. The timeline for each experiment is presented above the data. Snails ($N = 16$ for each group) were exposed to either artificial pond water (a), 100 µM carnosine (b), 1 mM (c), and 10 mM (d) for 1 h and, 3 h later, were trained with a .5-h training session (TS—black circles). In eight snails, ITM was tested 3 h later (MT 3 h—white circles) and in the remaining eight snails, LTM was tested 24 h post-training (MT 24 h—black squares). Memory was formed when a significant reduction in the number of attempted pneumostome openings was found between TS and MT. Comparisons were made by RM ANOVA followed by Tukey's post hoc test. The solid line is the mean and the error bars are the SEM. ****$p < .01$; ***$p < 0.01$; ns = not significant as $p > .05$.

3.4 | Experiment 3: Behavioral effects induced by carnosine, LPS, and their paired presentation on snails' aerial respiration

We previously demonstrated that injecting snails with LPS altered their homeostatic aerial respiratory behavior in hypoxic pond water for at least 24 h, suggesting that the observed significant increase in TBT following the LPS injection was consistent with the hypothesis that the LPS injection resulted in *sickness state* (Rivi, Batabyal, Benatti, et al., 2022). In Experiment 3 (Figure 4), we investigated whether exposing snails to carnosine for 1 h would prevent the LPS-induced *sickness state* as evidenced by a significant increase in TBT in hypoxic conditions.

Thus, in a naïve cohort of snails ($N = 7$), we recorded the TBT in hypoxic artificial pond water for .5 h. One hour later, snails were injected with 25 mg of LPS, and 4 h later the TBT was again recorded.

A significant increase in the TBT ($t = 5.33$, $df = 6$, $p = .002$) was found (Figure 4a). These data confirm the earlier findings that the LPS injection significantly increased TBT. Next, we asked whether exposure to 1 mM carnosine for 1 h altered TBT in hypoxic pond water.

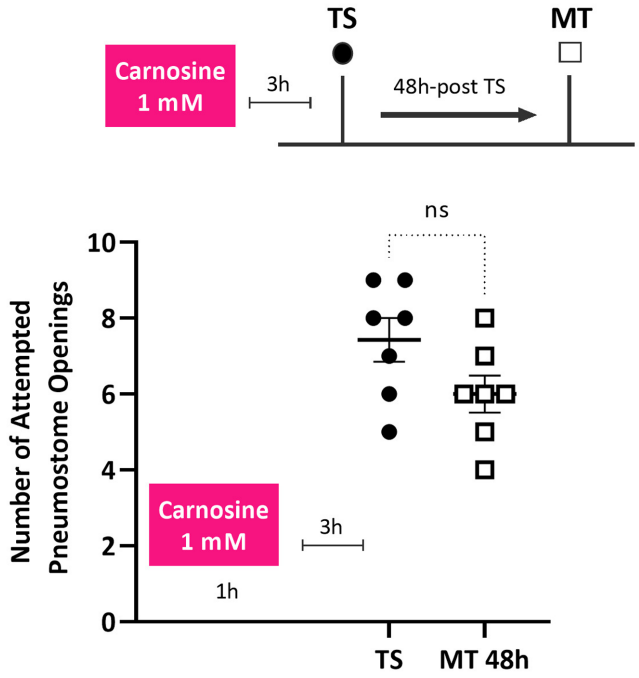

FIGURE 3 When tested 48 h post-training, snails exposed to 1 mM carnosine before training did not show LTM. The timeline for the experiment is presented above the data. Seven naïve snails were exposed to carnosine 1 mM for 1 h, and 3 h later were trained with a .5-h training session (TS—black circles). When LTM was tested 48 h later (MT 48 h—white squares), no significant reduction was found. Comparisons were made by paired *t* test. The solid line is the mean and the error bars are the SEM. ns = not significant as $p > .05$.

Exposure to carnosine did not significantly alter TBT ($t = .27$, $df = 6$, $p = .793$; Figure 4b). Finally, we assessed the effect of the combination of 1 mM carnosine exposure followed by the LPS injection on TBT in another naïve cohort of snails ($N = 7$; Figure 4c). As can be seen, the exposure to carnosine mitigated the effect that LPS had on TBT. That is, where previously LPS significantly increased TBT, now the LPS injection had no discernable effect on TBT in hypoxic pond water ($t = 1.18$, $df = 6$, $p = .281$). Thus, these data are consistent with the hypothesis that exposure to carnosine prevented the *sickness state* induced by LPS (Figure 4c).

3.5 | Experiment 4: Transcriptional effects induced by carnosine, LPS, and their paired presentation in snails' central ring ganglia

We next ascertained the transcriptional effects induced by carnosine, LPS, and their paired presentation on the expression levels of key targets involved in the immune and stress response (i.e., LymTLR4, LymMDM, and LymHSP70) and neuroplasticity (LymGRIN1 and LymCREB1) in snails' central ring ganglia. A main effect of the treatments was observed (Figure 5a–c) for LymTLR4 ($F[3, 24] = 46.86$, $p < .001$), LymMDM ($F[3, 24] = 7.65$, $p = .0009$), and LymHSP70 ($F[3, 24] = 18.45$, $p < .0001$). Tukey's post hoc multiple comparison tests showed significant upregulation of the expression levels of these targets in LPS-injected snails compared to the other groups (LymTLR4: $p < .0001$, for all; LymMDM: LPS vs. saline: $p = .0075$, vs. carnosine: $p = .004$, vs. carnosine before LPS: $p = .0016$; LymHSP70:

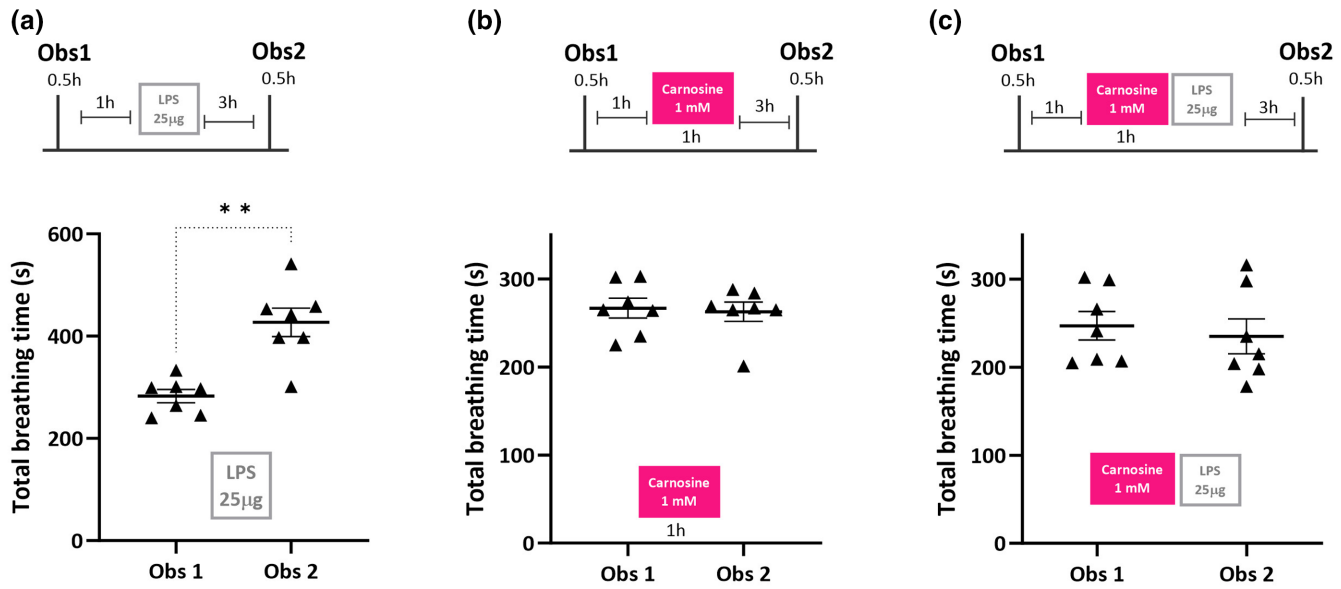

FIGURE 4 Exposure to 1 mM carnosine before the LPS injection prevents the LPS-induced sickness status. The timeline for the experiment is presented above the data. The total breathing time (TBT) was recorded 1 h before and 4 h after the LPS injection (a), the exposure to carnosine 1 mM (b), and the exposure to 1 mM carnosine followed by the LPS injection (c). The solid line is the mean and the error bars are the SEM. Comparisons were made by paired *t* test. $^{**}p < .01$; ns = not significant as $p > .05$.

LPS vs. saline: $p < .0001$, vs. carnosine: $p = .0002$, vs. carnosine before LPS: $p < .0001$). No significant differences were found between snails injected with saline, those exposed to only carnosine, and those exposed to carnosine before the LPS injection (LymTLR4: saline vs. carnosine: $p = .682$, saline vs. carnosine before LPS: $p = .997$, carnosine vs. carnosine before LPS: $p = .534$; LymMDM: saline vs. carnosine: $p = .999$, saline vs. carnosine before LPS: $p = .911$, carnosine vs. carnosine before LPS: $p = .971$; LymHSP70: saline vs. carnosine: $p = .981$, saline vs. carnosine before LPS: $p = .451$, carnosine vs. carnosine before LPS: $p = .258$). We also examined two different neuroplasticity targets: LymGRIN1 and LymCREB1 (Figure 5d,e). No main effects of the treatment were observed for these neuroplasticity targets: LymGRIN1 ($F[3, 24] = 1.57$, $p = .222$) and LymCREB1 ($F[3, 24] = .098$, $p = .961$) (Figure 5d,e). Thus, our data suggest that exposure to 1 mM carnosine for 1 h before the LPS injection prevents the LPS-induced upregulation of key targets involved in immune (LymTLR4 and LymMDM) and stress (LymHSP 70) responses, but did not cause significant upregulation of two neuroplasticity targets.

3.6 | Experiment 5: Behavioral and transcriptional effects induced by carnosine exposure before LPS injection on snails' memory abilities

We previously demonstrated that exposure to LPS 3 h before the single .5 h TS for the operant conditioning of aerial respiration obstructs memory formation (Rivi, Batabyal, Benatti, et al., 2022). Experiment 5 was performed to investigate—both at the behavioral and transcriptional levels—whether the exposure to 1 mM carnosine for 1 h before the LPS injection would prevent the LPS-induced memory impairment (Figure 6). Consistent with our previous studies (Rivi, Batabyal, Benatti, et al., 2022), we first showed (Figure 6a) that the injection of snail saline into a naïve cohort of snails ($N = 7$) 3 h before the .5 h TS did not prevent the ability of snails to form ITM ($t = 6.97$, $df = 6$, $p = .0004$).

We then showed (Figure 6b) that ITM was blocked (i.e., no significant difference in the number of attempted openings in MT 3 h compared to TS) if a cohort of naïve snails ($N = 7$) were injected with LPS injection 3 h before the .5 h TS ($t = .00001$, $df = 6$, $p > .999$). Again,

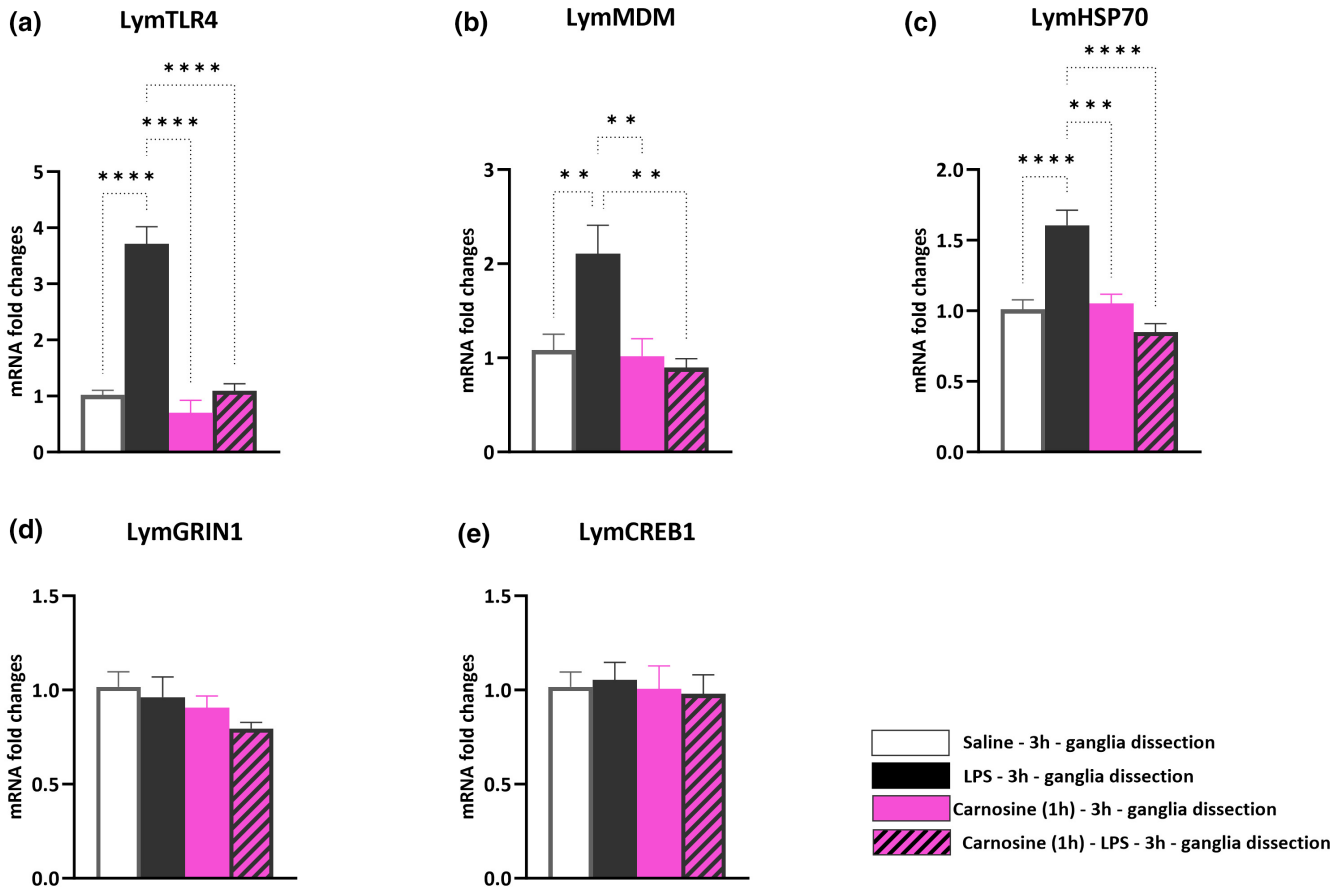

FIGURE 5 Transcriptional effects induced by LPS injection, 1 mM carnosine exposure, and their paired presentation in snails' central ring ganglia. The expression of LymTLR4 (a), LymMDM (b), LymHSP70 (c), LymGRIN1 (d), and LymCREB1 (e) has been measured in the central ring ganglia of snails injected with snail saline (white bars), snails injected with LPS (black bars), snails exposed to 1 mM carnosine for 1 h (pink bars), and snails exposed to 1 mM carnosine for 1 h before the LPS injection (pink bars with black diagonals). Three hours later, snails were sacrificed, the central ring ganglia were dissected, and the RNA was extracted. The mRNA levels were analyzed by real-time PCR. $N = 7$ for each group. Data are represented as means ± SEM and were analyzed with one-way ANOVA followed by Tukey's post hoc analyses. ****$p < .0001$, ***$p < .001$, **$p < .01$.

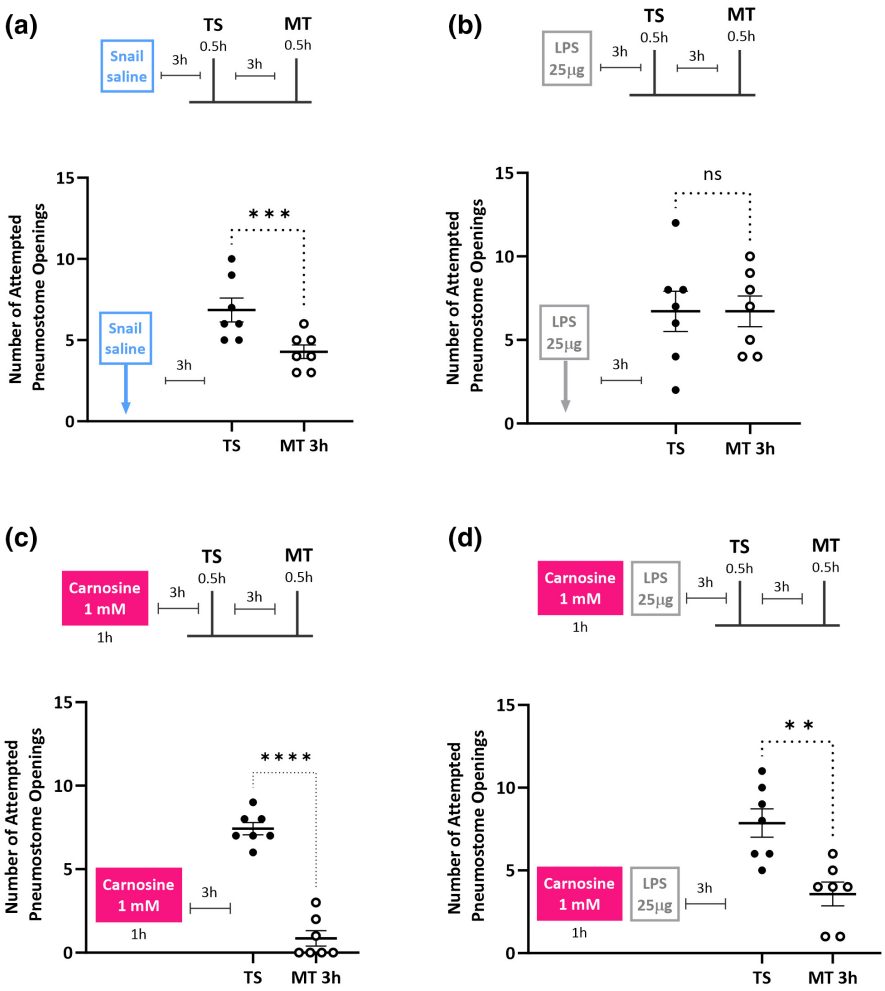

FIGURE 6 Behavioral effects induced by LPS injection, 1 mM carnosine exposure, and their paired presentation on memory formation for the operant conditioning of aerial respiration. The timeline for each experiment is presented above the data. Snails ($N = 7$ for each group) were injected with snail saline (a) and LPS (b), were exposed to 1 mM carnosine for 1 h (c), or were exposed to 1 mM carnosine for 1 h and then injected with LPS (d), and 3 h later, were trained with a .5-h training session (TS—black circles). Memory was tested 3 h later (MT 3 h—white circles). Memory was formed when a significant reduction in the number of attempted pneumostome openings was found between TS and MT. The solid line is the mean and the error bars are the SEM. Comparisons were made by paired *t* test. $^{****}p < .0001$; $^{***}p < .001$; $^{**}p < .01$; ns = not significant as $p > .05$.

the data shown in Figure 6a,b are consistent with previous findings. Next, we investigated whether exposure to 1 mM carnosine in a naïve cohort of snails ($N = 7$) altered ITM formation (Figure 6c). These data show that ITM formed; that is, there was a significant reduction of attempted openings in the MT (MT 3 h) compared to TS ($t = 11.51$, $df = 6$, $p < .0001$).

Finally, we investigated whether the exposure to 1 mM carnosine prevented the memory-obstructing effects caused by LPS. Thus, a naïve cohort of snails ($N = 7$) was exposed to 1 mM carnosine for 1 h and then immediately the snails were injected with LPS. The single .5-h TS was performed 3 h later (Figure 6d). Snails treated in this manner exhibited ITM; that is, there was a significant decrease in the number of attempted openings in MT 3 h compared to TS ($t = 4.03$, $df = 6$, $p = .007$). Thus, 1 mM carnosine prevented the memory block due to a sickness state brought about by the immune challenge evoked by the LPS injection. As shown in Figure 7, after each of the behavioral experiments shown in Figure 6 (i.e., immediately after MT 3 h), the snails were sacrificed and we examined the transcriptional effects in the central ring ganglia induced by the respective treatments on the expression levels of LymTLR4, LymMDM, LymHSP70, LymGRIN1, and LymCREB1. First, we examined the expression levels of transcripts (LymTLR4 and LymMDM) associated with the immune response (Figure 7a,b).

A one-way ANOVA followed by Tukey's post hoc test showed a main effect of the behavioral procedure on the expression levels of LymTLR4 ($F[3, 24] = 13.34$, $p < .0001$) and LymMDM ($F[3, 24] = 15.40$, $p < .0001$). These data are similar to the data shown in untrained snails (Figure 5); that is, the injection of LPS exposure induced significant upregulation of the mRNA levels of these targets compared to the other groups (LymTLR4: LPS vs. saline: $p = .0008$, vs. carnosine: $p < .0001$, vs. carnosine before LPS: $p = .0016$; LymMDM: LPS vs. saline: $p = .0001$, vs. carnosine: $p < .0001$, vs. carnosine before LPS:

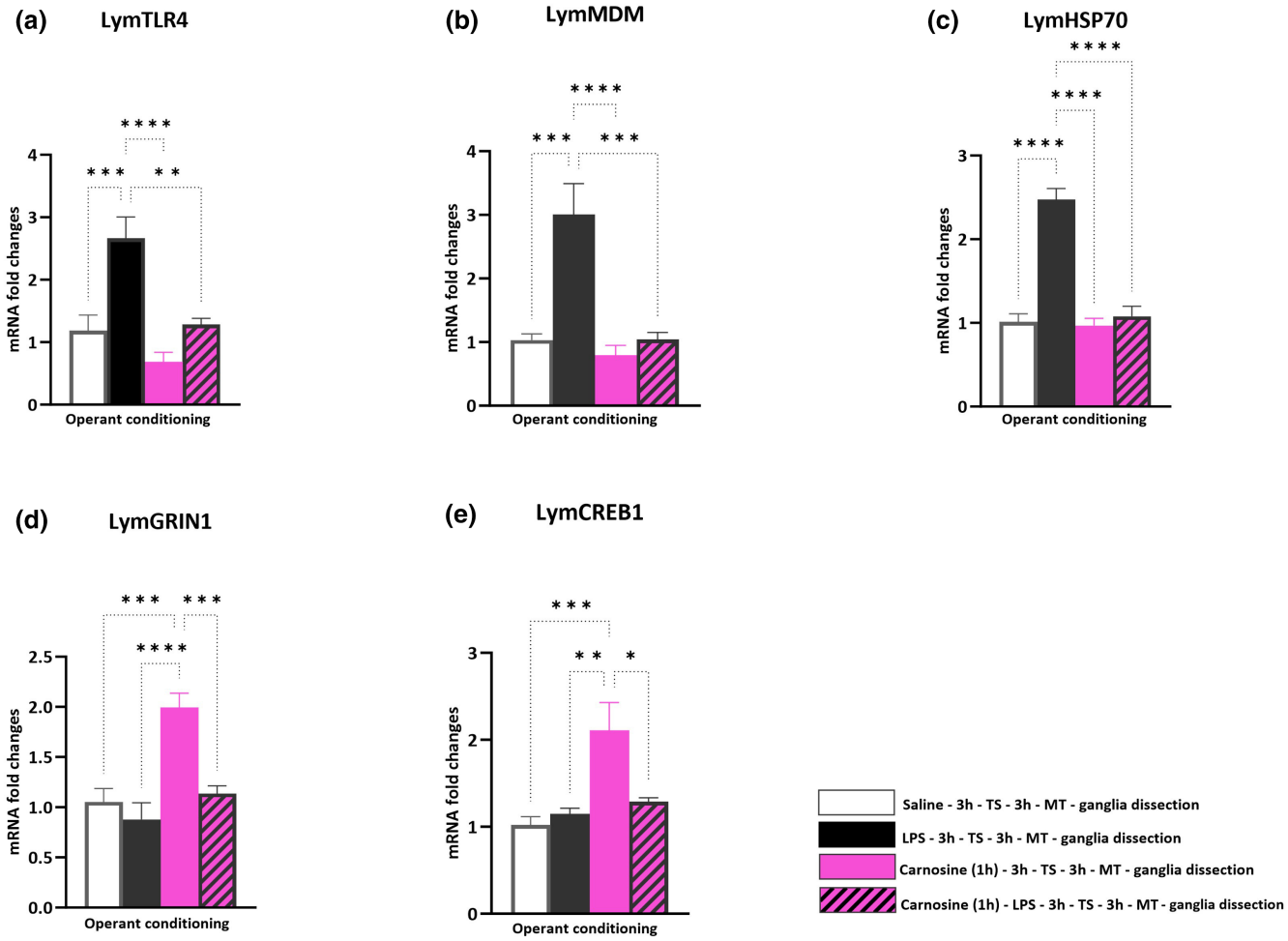

FIGURE 7 Transcriptional effects induced by LPS injection, 1 mM carnosine exposure, and their paired presentation in the central ring ganglia of snails trained for the operant conditioning of aerial respiration. The expression of LymTLR4 (a), LymMDM (b), LymHSP70 (c), LymGRIN1 (d), and LymCREB1 (e) has been measured in the central ring ganglia of snails injected with snail saline (white bars), snails injected with LPS (black bars), snails exposed to 1 mM carnosine for 1 h (pink bars), and snails exposed to 1 mM carnosine for 1 h before the LPS injection (pink bars with black diagonals). Three hours after the treatments, snails were trained for the operant conditioning of aerial respiration with a .5-h training session and 3 h later the memory test was performed. Immediately after the memory test, snails were sacrificed, the central ring ganglia were dissected, and the RNA was extracted. The mRNA levels were analyzed by real-time PCR. $N = 7$ for each group. Data are represented as means ± SEM and were analyzed with one-way ANOVA followed by Tukey's post hoc test. $^{****}p < .0001$, $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$.

$p = .0001$). No significant differences were found between snails injected with saline, those exposed to carnosine, and those exposed to carnosine before the LPS injection (LymTLR4: saline vs. carnosine: $p = .440$, saline vs. carnosine before LPS: $p = .999$, carnosine vs. carnosine before LPS: $p = .291$; LymMDM: saline vs. carnosine: $p = .923$, saline vs. carnosine before LPS: $p > .999$, carnosine vs. carnosine before LPS: $p = .912$).

Similarly, as shown in Figure 7c, the expression levels of LymHSP70 were upregulated ($F[3, 24] = 45.11$, $p < .0001$) in snails injected with LPS compared to the other groups following the behavioral procedure ($p < .0001$, for all) (Figure 7c). Again, no significant differences were found between snails injected with saline, those exposed to carnosine, and those exposed to carnosine before the LPS injection (saline vs. carnosine: $p = .999$, saline vs. carnosine before LPS: $p = .971$, carnosine vs. carnosine before LPS: $p = .892$).

Importantly, a main effect of the operant-conditioning procedure was found on the expression levels of LymGRIN1 ($F[3, 24] = 13.692$, $p < .0001$) (Figure 7d) and LymCREB1 ($F[3, 24] = 8.181$, $p = .0006$) (Figure 7e). Specifically, Tukey's multiple post hoc comparison test showed a significant upregulation of the expression levels of these targets only in snails exposed to 1 mM carnosine compared to the other groups (LymGRIN1: carnosine vs. saline: $p = .0003$, vs. LPS: $p < .0001$, vs. carnosine before LPS: $p = .0008$; LymCREB1: carnosine vs. saline: $p = .0008$, vs. LPS: $p = .003$, vs. carnosine before LPS: $p = .011$). No significant differences were found between snails injected with saline, those injected with LPS, and those exposed to carnosine before the LPS injection (LymGRIN1: saline vs. LPS: $p = .792$, saline vs. carnosine before LPS: $p = .971$, LPS vs. carnosine before LPS: $p = .53$; LymCREB1: saline vs. LPS: $p = .949$, saline vs. carnosine before LPS: $p = .689$, LPS vs. carnosine before LPS: $p = .94$).

4 | DISCUSSION

In this study, we used our well-grounded model system, the pond snail *L. stagnalis*, along with a well-understood associative learning procedure (i.e., operant conditioning of aerial respiration) to investigate the possible memory-enhancing and anti-inflammatory properties of carnosine. Carnosine is an endogenous dipeptide found in the brain and muscle (Cheng et al., 2011; Chez et al., 2002; De Marchis et al., 1997; Derave & Sale 2012; Fedorova et al., 2006, 2009, 2017), and is available as an over-the-counter food supplement (KL, personal observations). It is thought to possess potent antioxidant and anti-inflammatory properties, as well as neuroprotective benefits that may improve brain function (e.g., cognitive ability; Ahshin-Majd et al., 2016; Feng et al., 2009; Flancbaum et al., 1990; Kubota et al., 2020; Ma et al., 2012; Masuoka et al., 2019; Ouyang et al., 2016; Prokopieva et al., 2016).

As well, it may also play a role in the downregulation of mediators related to inflammation (Kubota et al., 2020), as well as the modulation of the release of molecules implicated in the pathophysiology of cognitive impairment by microglia (Caruso et al., 2019; Fresta et al., 2020; Gallant et al., 2000; Hipkiss et al., 1997; Hobart et al., 2004; Kulikova et al., 2016; Lopachev et al., 2016; Mehrazad-Saber et al., 2018; Rajanikant et al., 2007; Rokicki et al., 2015).

Here, we first demonstrated that carnosine is present in the ganglia of *L. stagnalis* at a concentration of .0035 μM/μg protein. Carnosine has been identified in the muscles of some invertebrates such as crabs (Cameron, 1989). While no previous report on the CNS of invertebrates is available, in the mouse brain carnosine concentration is usually ~.1 mM or lower (Boldyrev et al., 2013; Jain et al., 2020).

To determine if carnosine could play any role in the modulation of the immune system of the snail or impact cognitive ability, we first had to determine a concentration of carnosine that did not negatively impact important homeostatic behavior such as feeding but one that had a positive effect on cognition. We thus examine a range of carnosine concentrations (100 μM, 1 mM, or 10 mM carnosine). We first found that the exposure of snails to 100 μM carnosine for 1 h did not enhance LTM formation, whereas the exposure to 10 mM carnosine affected snails' feeding behavior and blocked learning and memory formation.

These data suggest that a dose of 100 μM is too low to induce any effect on snails' neuroplasticity, whereas 10 mM may be perceived as too high by snails, to which they respond by suppressing their feeding behavior. On the other hand, snails exposed to 1 mM carnosine for 1 h showed enhanced LTM for the operant conditioning of aerial respiration lasting for at least 24 h, but not 48 h. This is not surprising, as different studies are showing the importance of the selection of a specific concentration of carnosine in the models employed to obtain the maximal therapeutic effects. In particular, carnosine at the concentration of 20 mM, representing the gold standard in *in vitro* studies, and being the highest carnosine concentration at tissue levels, is more effective in murine models, while decreased cell viability and induced molecular alterations in human microglial cells (where 10 mM was instead protective) (Privitera et al., 2023). These results are also consistent with those obtained with other bioactive compounds, including the flavonoids quercetin and epicatechin (Rivi et al., 2022; Rivi, Batabyal, Benatti, Tascedda, et al., 2023e), further showing that *L. stagnalis* represents a well-founded model organism in which to investigate the effects of various compounds and molecules on learning and memory formation.

Importantly, we found that the pre-exposure to 1 mM carnosine before the LPS injection prevented the LPS effects both at the behavioral and molecular levels. Consistent with our previous studies (Rivi, Batabyal, Lukowiak, Benatti, et al., 2023), we found that the LPS injection induced a significant increase in the TBT (indicative of a sickness state). These data are also consistent with those of many mammalian studies showing that systemic inflammation affects sensory receptors that modulate breathing and can trigger inflammatory responses in the CNS, affecting various behaviors and cognition (Zhao et al., 2019). Moreover, many human clinical conditions associated with inflammation are characterized by strong activation of the respiratory control circuits which increase breathing to compensate for and maintain adequate ventilation (Hocker et al., 2017; Kugelberg, 2014; Peña-Ortega, 2019; Zwaag et al., 2022).

Therefore, our data suggest that in snails as in mammals, the increased aerial respiration following the LPS injection may be indicative of inflammatory/sickness status (Hocker et al., 2017; Saarentaus et al., 2023; Tzani et al., 2010). The ability of carnosine to counteract LPS-induced molecular alterations in numerous *in vitro* and *in vivo* studies has also been well documented (Caruso et al., 2019; Fresta et al., 2020; Ma et al., 2020; Tanaka et al., 2017).

Here, we also replicated many of our previous findings showing that injecting snails with LPS before a .5-h TS for the operant conditioning of aerial respiration blocks learning and memory formation (Rivi, Batabyal, Benatti, et al., 2022) Complementary to that finding, we found that at 3 h post-LPS injection, homeostatic respiration (i.e., TBT) is significantly increased due to the *sickness state*, suggesting that being sick at the time of training could either impair learning and/or obstruct memory formation.

Those behavioral data were congruent with our gene expression analyses, showing that the LPS injection upregulated the expression levels of LymTLR4 and LymMDM in snails' central ring ganglia, which are key mediators of the immune response (Rivi, Batabyal, Lukowiak, Benatti, et al., 2023). These data are also consistent with previous studies from mammals (including humans), showing that the stimulation of TLR4 by LPS induces the release of critical proinflammatory cytokines that are necessary for inducing a strong immune response, triggering sickness behavior (Dantzer, 2009). Furthermore, we found that the LPS injection induced a significant upregulation of LymHSP70, which plays a key role in stress response, suggesting that the LSP injection not only created a sickness status, but also acted as a stressor. On the other hand, 1 mM carnosine was effective in preventing the effects of LPS on snails' cognitive functions and respiratory rate. That is, when the LPS injection was preceded

by exposure to 1 mM carnosine snails exhibited normal homeostatic aerial respiration in a hypoxic environment and formed ITM following a single .5-h TS.

Although 1 mM carnosine per se did not affect the expression levels of LymTLR4, LymMDM, and LymHSP70, when 1 mM carnosine preceded the LPS injection, the LPS-induced upregulation of these targets was prevented. These data suggest that carnosine, by blocking LPS-induced activation of LymTLR4, LymMDM, and LymHSP70, may prevent downstream immune signaling in the nervous system and suppress the inflammatory cascade, the inflammatory state, and therefore, behaviorally, the *sickness behavior* and the memory block induced by LPS.

The suppression of the upregulation of TLR4 in the central ring ganglia of LPS-treated snails is in agreement with the ability of carnosine to downregulate the expression of this mediator of inflammation in an *in vitro* model of Parkinson's disease (Kubota et al., 2020), while the negative regulation of LymMDM, an Ig-superfamily member linked to effective immune response and survival (Hoek et al., 1996), is in line with the decreased response of immune cells such as macrophages (Caruso et al., 2019; Fresta et al., 2020) and microglia (Fleisher-Berkovich et al., 2009) under proinflammatory conditions (e.g., LPS stimulation). In a recent study, carnosine has also been shown to be able to downregulate HSP70 oxidative stress response marker in zebrafish larvae (Caruso et al., 2023), and this ability was also demonstrated in LPS-treated snails.

Finally, we found that while exposing snails to carnosine 1 mM alone did not induce any significant effect on the expression levels of targets for neuroplasticity (i.e., LymGRIN1 and LymCREB1), exposing snails to carnosine 1 mM for 1 h before the operant-conditioning procedure resulted in a significant upregulation of LymGRIN1 and LymCREB1. Our data suggest that the memory enhancement induced by 1 mM may be due to the upregulation of these targets in the snails' central ring ganglia. With regard to the CREB target, the data obtained in snails are corroborated by previous findings by Fujii and collaborators (Fujii et al., 2017), showing that carnosine activates the CREB pathway, augmenting the expression of CREB-regulated genes in Caco-2 cells, suggesting that an improvement of brain function could be the result of carnosine-induced activation of brain–gut interaction (Fujii et al., 2017). Carnosine has also been shown to modulate the glutamatergic system by upregulating the glutamate transporter 1 and reducing glutamate concentrations in the CNS (Ouyang et al., 2016). The abovementioned data regarding the preclinical efficacy of carnosine on cognitive status are also strengthened by a recent systematic review with meta-analysis giving preliminary evidence of the clinical efficacy of carnosine against cognitive decline in elderly subjects; in the four selected double-blind, randomized, placebo-controlled trials considered, the administration of carnosine in combination with its methylated analog anserine for 12 weeks, at a dose of 500 mg-1 g/day, was able to improve global cognitive function and verbal memory (Caruso et al., 2019).

To our knowledge, this is the first—albeit preliminary—study showing in an invertebrate model system that the exposure to carnosine not only enhances LTM, but can also prevent the LPS-induced effects both at the transcriptional and behavioral levels. Given the interesting results of this study, in the near future, we plan to perform proteomic and metabolomic analyses to correlate the effects of carnosine exposure and behavioral procedures on the homeostatic functions in *L. stagnalis* as well as on neuroplasticity.

The results obtained in this study raise several questions.

First, as *L. stagnalis* possesses an open circulatory system, an injection of LPS, as well as exposure to carnosine also affect the peripheral nervous system as well as other organs. Consequently, in our upcoming set of experiments, we intend to assess and compare the expression levels of specific targets across multiple tissues. Second, as many conditioning procedures have been validated in *L. stagnalis* and because this model can form high-order forms of learning (Ito et al., 1999; Kita et al., 2011), we plan to investigate the ability of carnosine to enhance memory formation in snails trained for different learning paradigms conditioned taste aversion, Garcia effect, operant conditioning of escape behavior, and configural learning.

To date, precise targets of carnosine action within the central ring ganglia of snails remain unknown, but represent an intriguing avenue for prospective studies. Previous studies demonstrated that aerial respiration is controlled by a central pattern generator, the neurons of which, as well as the motoneurons innervating the pneumostome, have previously been identified and their synaptic connections well characterized. In particular, right pedal dorsal 1 (RPeD1), which starts the activity within the circuit, plays a crucial role in memory formation, reconsolidation, and extinction for the operant conditioning of aerial respiration (Syed & Winlow, 1991). RPeD1 emerges as a pivotal neural component orchestrating various hierarchical facets of memory for the operant conditioning of aerial respiration (Syed & Winlow, 1991). Having shown in this study that exposure to carnosine before training enhances LTM for operant conditioning of aerial respiration, we asked: is it possible that carnosine may exert its effects on RPeD1 neuron? Future studies will aim to answer this question.

Finally, as carnosine has proven to prevent the LPS-induced upregulation of LymHSP70 mRNA levels, it may likely modulate the effects of other stressors, like heat shock, food deprivation, and predator scent, which have shown to upregulate the expression levels of LymHSP70. Thus, future experiments will aim to answer this question.

Of importance here, this is the first study showing that carnosine can prevent the *sickness state* and memory block induced by LPS in an invertebrate model system, opening new avenues of research into more detailed studies in mammals to elucidate the neuronal and molecular effects of this dietary supplement, as well as its role in modulating the complex interaction between the immune system and CNS, and the neuroplasticity processes.

5 | CONCLUSIONS

In summary, we provide the first evidence of the ability of 1 mM carnosine to enhance LTM formation following a single .5-h operant-conditioning TS in *L. stagnalis*. Interestingly, this effect was concentration specific as the exposure of snails to 100 μM carnosine did not lead to LTM formation, whereas 10 mM carnosine negatively affected snails' feeding behavior and blocked learning and memory formation. The memory-enhancing effect of 1 mM carnosine was paralleled by the upregulation of LymGRIN1 and LymCREB1 (markers of neuroplasticity) in snails' central ring ganglia 3 h after the 1 h exposure to carnosine. Carnosine at a concentration of 1 mM was also able to prevent the LPS-induced upregulation of LymTLR4, LymMDM, and LymHSP70 (markers of immune response and inflammatory state) in the central ring ganglia of snails and, behaviorally, counteract the sickness status and reversed the memory block induced by the immune challenge.

Despite its considerable evolutionary distance from humans, *L. stagnalis* exhibits both molecular and behavioral characteristics that make it a versatile model for investigating the pharmacological impacts of carnosine, paving the way for future studies in mammals aimed at further exploring the therapeutic potential of carnosine as a new pharmacological tool in the context of cognitive disorders characterized by immune overactivation and inflammation. From an ethical point of view, the use of snail models will limit as much as possible the use of mammals in preclinical studies and allow mammals to be involved only for the validation of the results obtained from invertebrates. This will reduce by several orders of magnitude the costs of numerous studies. Thus, *L. stagnalis* offers a translational approach that may help gain important knowledge and comprehension in the field of Translational Neuroscience and Pharmacology.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

AUTHOR CONTRIBUTIONS

Conceptualization, C.B. and G.C.; *Methodology*, V.R. and S.A.; *Investigation*, V.R.; *Validation*, V.R.; *Formal analysis*, V.R. and C.B.; *Resources*, F.T., K.L., J.M.C.B., G.C., and C.B.; *Supervision*, C.B.; *Writing—original draft*, V.R., C.B., and G.C.; *Writing—review & editing*, K.L., S.A., F.T., L.P., J.M.C.B., and F.C.; *Visualization*, J.M.C.B., F.T. and F.C.; *Funding acquisition*, F.T., L.P., and C.B.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no known or potential conflict of interest, including financial, personal, or other relationships, which could inappropriately influence or be perceived to influence the work presented here.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

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

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