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Unraveling lipopolysaccharide-induced behavioral and molecular effects in *Lymnaea stagnalis*, an emerging model organism for translational neuroscience

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ABSTRACT

In this study, we employed a reductionist (yet not simplistic) approach utilizing the established invertebrate model system of the pond snail, *Lymnaea stagnalis*, to investigate the behavioral and molecular effects of systemic administration of lipopolysaccharide (LPS)—a bacterial endotoxin—on the snails' central ring ganglia. Snails received injections of either a low dose (2.5 μg) or a high dose (25 μg) of LPS, and their behavioral and molecular responses were assessed at 2, 6, and 24 h post-injection. With the high dose, snails exhibited a significant increase in homeostatic aerial respiration lasting for at least 24 h, consistent with a sickness-like state induced by the immune challenge. Additionally, we found that when administered 2, 6, or 24 h before operant conditioning training, the high dose of LPS, impaired memory formation. To further explore the underlying molecular mechanisms, we examined the transcriptional effects of the two doses of LPS in the snails' central ring ganglia. Our analysis showed a dose- and time-dependent upregulation of immune and stress-related genes, including key enzymes involved in the kynurenine pathway (KP), toll-like receptor 4 (TLR4), and heat shock protein 70 (HSP70). Metabolomic analysis suggested that the high LPS dose shifted KP metabolism toward the production of neurotoxic metabolites within the ganglia, indicating a LPS-induced neuroinflammatory state. Together, our findings provide valuable insight into the conserved mechanisms of neuroinflammation in this invertebrate model, offering a simplified yet effective tool to further explore the molecular interactions between the immune and central nervous systems.

1. Introduction

Neuroinflammation is the response of the reactive central nervous system (CNS) components to altered homeostasis, regardless of the endogenous or exogenous nature of the trigger [1]. Despite its recognized role in the etiology of neurodegenerative and neuropsychiatric diseases, research into neuroinflammation remains extremely difficult [2]. This is in part due to the inherent complexity of the human CNS and the multifaceted interactions between neural and immune signaling pathways [3,4]. Complementary animal models can help to

Abbreviations: 3-HK, 3-hydroxykynurenine; 5HT, serotonin; ACMSD, 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase; AIF, allograft inflammatory factor; ANA, anthranilic acid; CNS, central nervous system; HAAO, hydroxy anthranilate 3,4-dioxygenase; HSP, heat shock protein; IDO, indoleamine 2,3 dioxygenase; KMO, kynurenine 3-monooxygenase; KP, kynurenine pathway; KYAT, kynurenine aminotransferase isoenzymes; KYN, kynurenine; KYNA, kynurenic acid; KYNU, kynureninase; LPS, lipopolysaccharide; MDM, molluscan defense molecule; QUIN, quinolinic acid; TBT, total breathing time; TDO, tryptophan 2,3-dioxygenase; TLR, toll-like receptor; TRP, tryptophan.

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elucidate the underlying mechanisms of neuroinflammation and facilitate the development of therapeutic interventions [5]. In fact, as the inflammatory response has been highly conserved during evolution [6], in the last two decades, neuroscientists have increasingly turned to invertebrate models to understand molecular physio-pathological mechanisms related to neurological or psychiatric disorders [7–9]. A well-established model for studying neuroinflammation in a preclinical setting is the acute immune stimulation with the bacterial toxin lipopolysaccharide (LPS) [10], which activates the innate immune system, promoting a highly conserved crosstalk between the immune and the central nervous systems [3,11]. After binding to toll-like receptor (TRL) 4, LPS triggers the secretion of pro-inflammatory cytokines, which increase the expression and activity of many molecular cascades, including the kynurenine pathway (KP) [12,13], a complex, multi-step cascade responsible for degrading tryptophan into several neuroactive metabolites known as kynurenines [14–17].

In this study, we investigated the behavioral and molecular effects in the central ring ganglia induced by an immune challenge (i.e., LPS injection) into the pond snail *Lymnaea stagnalis* [18]. *L. stagnalis* is particularly well-suited for such studies due to its relatively simple CNS, open circulatory system, and well-characterized homeostatic behaviors [19]. Here, we focused on a highly tractable behavior—aerial respiration [20]—which can be operantly conditioned using a standardized behavioral protocol [21]. By administering either 2.5 μg and 25 μg of LPS, (i.e. low and high doses, respectively), we examined the effects of this immune challenge on homeostatic breathing behavior and the memory forming capability as well as their underlying molecular mechanisms. Thus, we evaluated the time- and dose-related effects of LPS on the expression levels of immune and stress-related targets involved in the innate immune response in the central ring ganglia of snails sacrificed 2, 6, or 24 h post-injection.

We first focused our attention on Toll-like receptor 4 (Lym TLR4), a key component for the innate immunity of invertebrates [12], allograft inflammatory factor (Lym AIF) [22], molluscan defense molecule (Lym MDM), an Ig-superfamily member which allows mollusks to mount an effective immune response and ensure their survival [23], and the heat-shock protein 70 (Lym HSP70), which plays a key conserved role in stress response [24,25]. Additionally, as we recently characterized the enzymes of the KP in our model organism [26], we investigated the transcriptional effects induced by this immune challenge on the expression levels of known KP enzymes in *Lymnaea*.

Changes in the levels of metabolites of the KP pathway were also evaluated in the ganglia of *L. stagnalis* following the systemic administration of LPS as studies from rodents demonstrated that inflammation can disrupt the balance between neurotoxic and neuroprotective KP metabolites, affecting the crosstalk between the immune and central nervous systems [27,28]. This disruption can impact metabolic function, cognition, pain, and emotion, potentially increasing the risk of psychiatric disorders [29–34]. The conservation of the KP across species, from yeast to humans, suggests that simpler model organisms, such as *Lymnaea*, could provide an efficient way to unravel KP complexity while offering insights applicable to mammals [35].

To our knowledge, this is the first study investigating the relationship between immune stimulation, stress response, KP activation, and behavioral responses in an invertebrate model organism following an immune challenge. The use of *L. stagnalis* in LPS-induced neuroinflammation and stress research provides a powerful tool for understanding the conserved molecular and behavioral effects of these processes.

2. Materials and methods

2.1. Animals

Laboratory-bred *L. stagnalis* (Linnaeus, 1758), originally derived by the Vrije University in Amsterdam (The Netherlands), were housed at the University of Modena and Reggio Emilia (Italy) in aquaria with well-oxygenated dechlorinated tap water at the temperature range of 21–22 °C. Animals were subjected to a 12/12-h light/dark cycle (lights activated at 08:00 a.m.). Adult snails with shell lengths between 20 and 25 mm were selected for the experiments. The diet consisted of pesticide-free romaine lettuce, provided twice a week.

2.2. LPS treatment

Snails were divided into three groups receiving either:

1) snail saline solution (41.15 mM NaCl; 0.54 mM KCl; 3.55 mM $CaCl_2$; 2.61 mM $MgCl_2$; 5 mM Tris; pH 7.5 [19,36]);
2) 2.5 μg of *E. coli*-derived LPS serotype O127:B8 (L3129, Merck KGaA; Darmstadt, Germany) dissolved in snail saline solution;
3) 25 μg of *E. coli*-derived LPS serotype O127:B8 dissolved in snail saline solution.

For each snail, a single intramuscular injection of 40 μL was administered into the foot using a 31G syringe. We calculated that a snail with a 20-mm shell length has a hemolymph volume of about 400 μL and the weights approximately of 3 g. Thus, the doses were estimated to be 0.625 μg/mL and 6.25 μg/mL or 0.83 mg/kg and 8.3 mg/kg respectively.

2.3. Aerial respiration

In hypoxic conditions, snails move to the water's surface and breathe through the respiratory orifice (i.e., the pneumostome) [37–39]. To induce a hypoxic environment, 100 % nitrogen (N_2) was continuously bubbled into a 1-L beaker for 20 min before placing the animals [40,41]. Snails were then moved to the beaker and bubbling was reduced to maintain the hypoxic environment without disturbing the animals. Snails were given a 10-min acclimation period, after which we recorded the total breathing time (i.e., the total time the pneumostome is open - TBT) over 30 min. This is the homeostatic breathing time.

2.4. Operant conditioning of aerial respiration

Operant conditioning training consisted of a single 30-min training session (TS) during which a tactile stimulus (a hand-held sharpened wooden stick) was delivered to the pneumostome area each time it began to open in the hypoxic environment [42]. The same procedure was performed during the 30-min memory test (MT), performed 3 h after the training session. Great care was taken to use approximately the same stimulus strength within and between TS and MT, as well as between each snail. During both the TS and MT, the number of 'pokes' (i.e., attempted pneumostome openings) was recorded [41,43]. Memory was formed if there was a significant reduction in the number of attempted pneumostome openings during the MT compared to the TS [44]. It is well-established that in laboratory-bred snails, such as those used in this study, a single 30-min TS induces intermediate-term memory (ITM) lasting for at least 3 h but 24 h.

2.5. Study design

To characterize *L. stagnalis* as a model system for studying the conserved mechanisms underlying LPS-induced neuroinflammation, we performed 4 experiments. The aim of *Experiment 1* was to investigate whether the two selected doses of LPS altered the snails' aerial respiratory behavior. Thus, we recorded snails' TBT in hypoxic artificial pond water for 30 min. Three hours later, the snails were then divided randomly into 2 groups, one group received the high LPS dose while the other received the low dose. We then recorded the total time the pneumostome was open (i.e., TBT) for each snail was recorded during each of the 30-min observation periods, performed at 2-, 6-, 24-, and 48

h post-LPS injection [41]. Following each breathing observation period, snails were returned to their home eumoxic (i.e., normal atmospheric oxygen) aquaria. In *Experiment 2*, we investigated the effects of the selected doses of LPS on the learning and memory abilities of *Lymnaea*. Thus, snails were injected with the low and/or the high dose of LPS 2, 6, or 24 h before training. Memory was then assessed 3 h after the single 0.5 h training session. If there was not a significant difference in the number of attempted pneumostome openings between the training and memory test sessions we concluded that there was memory impairment [37]. In *Experiment 3,* we investigated the transcriptional effects induced by LPS on key targets for immune and stress response in snails' central ring ganglia. Thus, animals were injected with the low or the high dose of LPS or snail saline and sacrificed either 2-, 6-, or 24 h after injection. Finally, *Experiment 4* aimed to investigate the impact of a 25 μg dose of LPS on metabolites within the KP in the central ring ganglia of *L. stagnalis*. To achieve this, snails received an injection of either 25 μg LPS or a control saline solution and were subsequently sacrificed at 2, 6, or 24 h post-treatment to assess temporal changes in KP metabolite levels.

2.6. RNA extraction and Retrotranscription

Snails were anesthetized on ice for 10 min, and subsequently, the central ring ganglia were dissected out and preserved at −80 °C for later analysis. Total RNA extraction was carried out from a single ganglion. The extraction process, including DNAse treatment, utilized the GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck KGaA; Darmstadt, Germany), following established protocols [45–47]. For reverse transcription, 500 ng of total RNA was utilized in a 20 μL reaction mix with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation). Quantitative Real-Time PCR (qRT-PCR) was performed on 20 ng of cDNA using a Bio-Rad® CFX ConnectTM Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The cycling parameters included an initial step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Cycle threshold (Ct) values were determined using CFX Maestro™ Software (Bio-Rad). Specific forward and reverse primers, generating an amplicon between 100 and 200 bp, were used at a final concentration of 300 nM (Table 1).

The mRNA levels of each target were normalized on two reference genes, elongation factor 1α and β-tubulin. The stability of mRNA expression for these endogenous controls was evaluated using Normfinder® [48,49], considering both intra- and inter-group variation. The geometric mean of Cycle Quantification values of the endogenous genes emerged as the most stable gene across groups and was utilized for gene normalization. The comparative $2^{-\Delta\Delta Ct}$ method was employed for the quantitative evaluation of changes [50], using the average expression levels of control animals (i.e., saline-receiving animals sacrificed 2 h after injection) as the calibrator.

2.7. Liquid chromatography

The concentration of 5HT, TRP, KYN, KYNA, ANA, 3-HK, and QUIN was measured in the central ring ganglia of snails receiving snail saline or 25 μg of LPS (high-dose) and sacrificed either 2-, 6-, or 24 h after treatment as previously described with some modification [51]. Each ganglion was homogenized in 100 μL of an ascorbic acid solution of 0.1 % by sonication. Protein concentration of homogenates was determined using the Bradford assay (Merck KGaA, Darmstadt, Germany). To each 50 μL of the sample was added an equal volume of ice-cold 1 M perchloric acid ($HClO_4$) fortified with a mix of 5HT-d_4 (Cayman Chemical, USA), KYN-d_4, KYNA-d_5, QUIN-d_3 (Buchem BV, Netherlands) and TRP-d_5 (Merck KGaA, Darmstadt, Germany) each one at the final concentration of 1 μM. Samples were centrifuged (15,000 ×*g*, 15 min), and the supernatants were collected and directly injected into LC-MS/MS.

The analysis of KP metabolites was 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

Table 1
The forward (FW) and reverse (RV) primer nucleotide sequences utilized in qRT-PCR are provided, along with the accession number for each target and the size (bp) of the PCR product obtained through the amplification of cDNA (mRNA).

Gene bank accession	Target	Product length (bp)	Type sequence (5'-3')
DQ206432.1	*L. stagnalis* heat-shock protein 70	199 bp	**FW:** AGGCAGAGATTGGCAGGAT
	Lym HSP70		**RV:** CCATTTCATTGTGTCGTTGC
U58769.1	*L. stagnalis* molluscan defense molecule	104 bp	**FW:** CGGGTACACACACAGATGGA
	Lym MDM		**RV:** TGACTGAACATTGGGCACAC
DQ278446.1	*L. stagnalis* allograft inflammatory factor	116 bp	**FW:** CGTTTATGGTAAGCTGGAAAGA
	Lym AIF		**RV:** CTGGGAGCAAAGTCAAGCAT
AY577328.1	*L. stagnalis* lipopolysaccharide binding protein-like protein	134 bp	**FW:** CTGAGGTCAAGAGGGTGCAG
	Lym TLR4		**RV:** GATCCTGTCGCGGATCATGT
FX225637	*L. stagnalis* tryptophan 2,3 dioxygenase	120 bp	**FW:** CTCTAGAATGTCGATTTGGT
	Lym TDO-like		**RV:** TGTCAGAGATATGTTCATGC
FX190660.1	*L. stagnalis* indoleamine 2,3-dioxygenase	179 bp	**FW:** ACTTAGGAAGAGTTTCAGCA
	Lym IDO-like		**RV:** TTAAACCTAATCCCACAGAC
FX191423.1	*L. stagnalis* kynurenine formamidase	138 bp	**FW:** ACAAAATTAGGTTCCGTAAG
	Lym AFMID-like		**RV:** ATGATCCTCCAGAGTTTAGA
FX185910	*L. stagnalis* kynurenine 3-monooxygenase	194 bp	**FW:** ATTGTCCAATTTCTTCCTAA
	Lym KMO-like		**RV:** GGTATGGAGGACTGTATTGT
FX191915.1	*L. stagnalis* kynurenine aminotransferase	118 bp	**FW:** GGAGGTATCACTGTCAATTT
	Lym KYAT I/III-like		**RV:** TACTTCATGATGGCTGATTA
FX183988.1	*L. stagnalis* kynurenine/alpha-aminoadipate aminotransferase	189 bp	**FW:** GTCAGCCTATAAGGAAAGAA
	Lym AADAT-like		**RV:** GTAAACACCTGGAAGAAAAC
FX188572.1	*L. stagnalis* kynureninase	178 bp	**FW:** TTCCTTCAGATCATTACACA
	Lym KYNU-like		**RV:** CTGTACACCAGAAAAACAAA
FX195327.1	*L. stagnalis* 3-hydroxyanthranilate 3,4-dioxygenase	124 bp	**FW:** GTCCAACTCTTTGGAGATAA
	Lym HAAO-like		**RV:** TGAGTAACAACTGACTCACC
FX187039.1	*L. stagnalis* 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	138 bp	**FW:** TTTAGGAAATCTCTCAAGGA
	Lym ACMSD-like		**RV:** AAGAGCACAACTGTGTGATA
X15542.1	Snail, beta-tubulin	100 bp	**FW:** GAAATAGCACCGCCATCC
	LymTUB		**RV:** CGCCTCTGTGAACTCCATCT
DQ278441.1	*L. stagnalis* elongation factor 1-alpha	150 bp	**FW:** GTGTAAGCAGCCCTCGAACT
	LymEF1α		**RV:** TTCGCTCATCAATACCACCA

column (3 μm particle size, 150 × 2.1 mm, Supelco, Milan, Italy) using 0.1 % 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 0.5 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 171–>160, 181–>164, 205–>188, 210–>192, 190–>144, 193–>147, 209–>192, 213–>196, 138–>120, 225–>208, 168–>78, and 171–>153, for 5HT, 5HT-d_4, TRP, TRP-d_5, KYNA, KYNA-d_5, KYN, KYN-d_4, ANA, 3-HK, QUIN, and QUIN-d_3 respectively. The calibration curves were constructed using calibration standards and were linear over the concentration range of 0.0391–10.000 μM with a correlation coefficient (r^2) of 0.999 and an accuracy within acceptable range (100 % ± 20 %). KP metabolite concentrations were normalized to protein content. Metabolite concentrations were used to evaluate the activity of TDO/IDO, KAT, KYNU, and KMO by calculating KYN/TRP ratio, KYNA/KYN ratio, ANA/KYN ratio, and 3-HK/KYN ratio respectively. The 5HT/TRP and 3-HK/KYNA ratio were calculated using the following formula: ([5HT]/[TRP])x100 and ([3−HK]/[KYNA])x100.

2.8. Statistical analysis

Statistical analyses were conducted using SPSS version 29 (SPSS Inc., Chicago, USA) and graphs were created using GraphPad Prism v.10. First, we confirmed that our data were normally distributed using a Kolmogorov–Smirnov test (KS distance and *P*-value).

Data from *Experiment 1* were analyzed using repeated measures (RM) analysis of variance (ANOVA) combined with *post-hoc* Tukey tests. Data from *Experiment 2* were analyzed using paired *t*-tests. The main effects and interactions from *Experiment 3 and 4* were assessed through two-way ANOVA (treatment x time).

Interaction effects were further analyzed by *post-hoc* contrasts of estimated marginal means, employing Bonferroni's correction implemented in SPSS (with a significance level set at $p < 0.05$). Extreme outliers were excluded before statistical analysis using the boxplot tool in SPSS (instances more than 3 times the interquartile range outside the end of the interquartile box).

2.9. Ethics

Ethical approval was not required for this study, as pond snails (*Lymnaea stagnalis*) are invertebrates and do not fall under the jurisdiction of the Institutional Animal Care and Use Committee (IACUC) (Italian Legislative Decree D.L. 4 marzo 2014, n. 26, "Attuazione della Direttiva n. 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici"). Nevertheless, every effort was made to minimize the number of animals used and to ensure their well-being by providing proper nutrition, clean, oxygenated water, and low-density housing conditions. The LPS treatment used in this study has no long-term effects on snails. Therefore, this study fully adheres to the 3R principles (Replacement, Reduction, Refinement) in biomedical research.

3. Results

3.1. Experiment 1: Effects of the two doses of LPS on snails' aerial respiration

First, we investigated whether the snails' homeostatic aerial respiratory behavior would be altered by either of two doses of LPS. To assess this, we measured the TBT in hypoxic artificial pond water 3 h before (i.e., pre-LPS) and at 2, 6, 24, and 48 h after the LPS injection (Fig. 1). Injecting the snails with a low dose of LPS (2.5 μg) did not significantly alter their aerial respiratory behavior [$F(2.627, 23.65) = 2.23$; $p = 0.12$] (Fig. 1A). In contrast, a significant main effect of the high dose of LPS

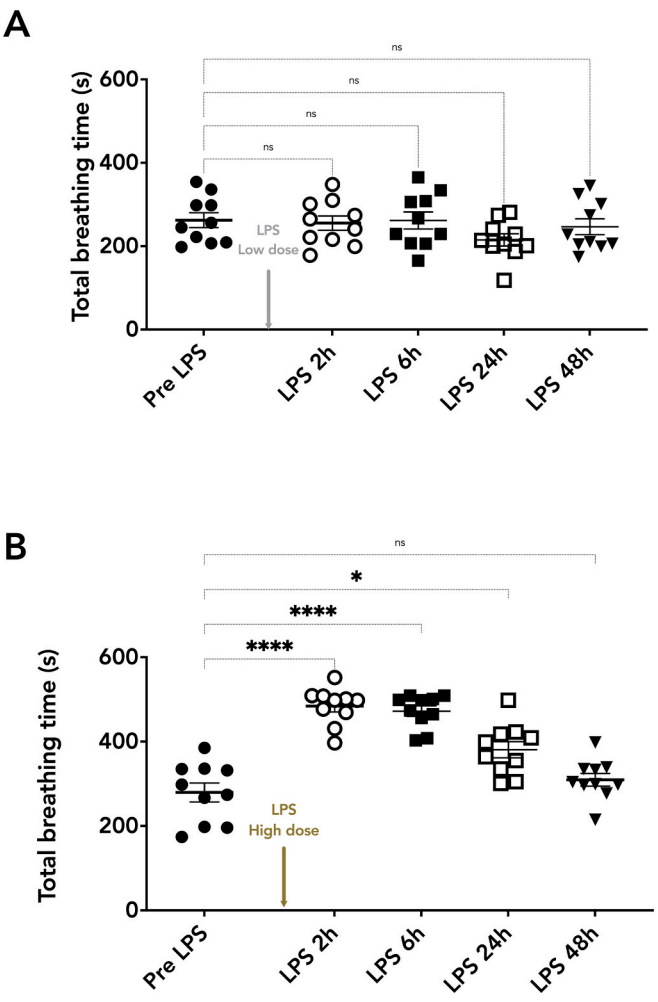

Fig. 1. Behavioral effects induced by two doses of LPS on the duration of total breathing in hypoxic artificial pond water. (A) Snails total breathing time was recorded in a naive cohort of 10 snails ($N = 10$) in hypoxic artificial pond water 3 h before the injection with 2.5 μg of LPS (Pre LPS-closed circles) and 2 h (LPS 2 h – open circles), 6 h (LPS 6 h – closed squares), 24 h (LPS 24 h – open squares), and 48 h (LPS 48 h– downward triangles). **(B)** Snails total breathing time was recorded in a naive cohort of 10 snails (N = 10) in hypoxic artificial pond water 3 h before the injection with 25 μg of LPS (Pre LPS-closed circles) and 2 h (LPS 2 h – open circles), 6 h (LPS 6 h – closed squares), 24 h (LPS 24 h – open squares), and 48 h (LPS 48 h– downward triangles). Data are represented as means ± S.E.M. and were analyzed with repeated measure ANOVA followed by Tukey's *post-hoc* test. **** $p < 0.00001$ and * $p < 0.05$; ns, not significant as $p > 0.05$.

(25 μg) on TBT was observed [$F(3.167, 28.50) = 32.85$; $p < 0.0001$] (Fig. 1B). Specifically, Tukey's *post-hoc* analysis revealed a significant increase in TBT at 2-, 6-, and 24-h post-injection compared to the pre-LPS levels ($p < 0.0001$ for 2 and 6 h post-injection; $p = 0.02$ for 24 h post-injection). No significant differences were found between TBT recorded at 48 h post-injection and the pre-LPS levels ($p = 0.54$). Additionally, there were no significant differences in TBT recorded before the injection between the low and high LPS doses ($t = 0.60$, df = 18, $p = 0.55$). Thus, only the high dose of LPS significantly altered homeostatic breathing behavior and those effects persisted for 24 h but not 48 h. These data are consistent with our previous studies [37].

3.2. Experiment 2: Effects of the two doses of LPS on snails' learning and memory abilities

Building on our findings that the high dose of LPS significantly

elevated homeostatic breathing behavior for at least 24 h, we next examined whether those doses would impact the snails' ability to form intermediate-term memory (ITM) following a single 30 min operant conditioning training session (Fig. 2). Here we used a total of 90 snails divided up into 3 separate cohorts ($N = 30$ in each cohort). In each individual cohort snails ($n = 10$) received either an injection of saline, a low dose of LPS, or a high dose of LPS. One cohort was operantly conditioned 2 h post injection (A,B,C); whilst the other two cohorts were trained 6 (D,E,F) and 24 h post injection (G,H,I). As can be seen, the saline and low-dose LPS injections did not alter the ability of snails to form memory (i.e., MT significantly less than TS) in the 3 cohorts (2 h – snail saline: $t = 7.49$, df = 9, $p < 0.0001$ – Fig. 2A; LPS low dose: $t = 7.86$, df = 9, $p < 0.0001$ – Fig. 2B; 2 h – snail saline: $t = 7.66$, df = 9, $p < 0.0001$ – Fig. 2D; LPS low dose: $t = 8.51$, df = 9, $p < 0.0001$ – Fig. 2E; 24 h – snail saline: $t = 7.49$, df = 9, $p < 0.0001$ – Fig. 2G; LPS low dose: $t = 5.58$, df = 9, $p = 0.0003$ – Fig. 2H). In contrast, the high dose of LPS consistently impaired memory formation, with no significant reduction in attempted pneumostome openings in any of the time points tested (2, 6, or 24 h post-injection: $t = 0.28$, df = 9, $p = 0.78$ – Fig. 2C; $t = 0.15$, df = 9, $p = 0.88$ – Fig. 2F; $t = 0.001$, df = 9, $p > 0.99$– Fig. 2I). These data show that the immune challenge caused by the high dose of LPS blocked the ability of snails to form ITM following the single 30 min operant conditioning training session for at least 24 h. Having shown in *Experiment 1* that homeostatic breathing behavior returned to baseline levels 48 h after a high-dose LPS injection, we hypothesized that snails trained 48 h post-injection would regain the ability to form ITM. To test this, we injected a further 10 naïve snails with 25 μg of LPS and then trained them 48 h later.

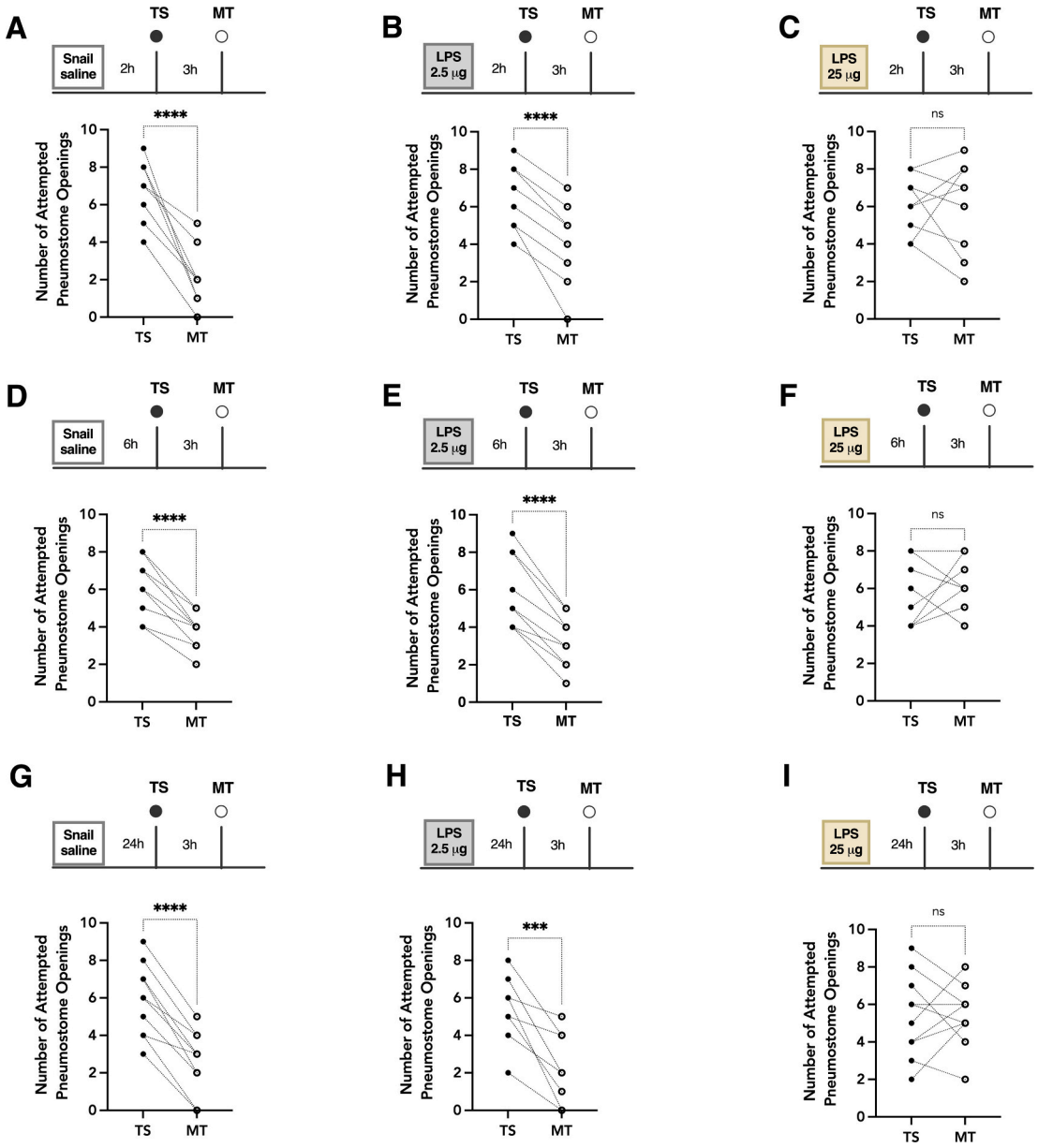

Fig. 2. Effects induced by two doses of LPS on snails' memory abilities. The timeline for each experiment is presented above the data. Snails were injected with snail saline (A, D, and G), LPS low dose (B, E, and H), or LPS high dose (C, F, and I) and were trained (TS—closed circles) 2, 6, or 24 h post-injection. Memory test (MT—open circles) was performed 3 h post-training. Snails injected with snail saline and the low dose of LPS formed ITM as a significant reduction in the attempted number of pneumostome openings was found. On the other hand, the high dose of LPS prevented memory formation as no significant differences in the number of attempted pneumostome openings were found.
Comparisons were made by paired *t*-test. *** $p < 0.001$ and **** $p < 0.0001$; ns, not significant as $p > 0.05$.

Our results supported this hypothesis, as there was a significant reduction in attempted pneumostome openings from the TS to the MT ($t = 7.33$, df = 9, $p < 0.0001$ – **Supplementary Fig. 1**), suggesting that once the "sickness state" subsides, snails can successfully form ITM.

3.3. Experiment 3: Effects of two different doses of LPS on the expression levels of immune- and stress-related targets in the central ring ganglia of Lymnaea

We evaluated the transcriptional effects of the two selected doses of LPS on the expression levels of immune- and stress-related target genes in the central ring ganglia of *L. stagnalis* (Figs. 3 and 4). Time points were chosen based on previous data obtained in immortalized cell lines and rodents exposed to LPS [52–54]. A main effect of treatment [F (2;78) =

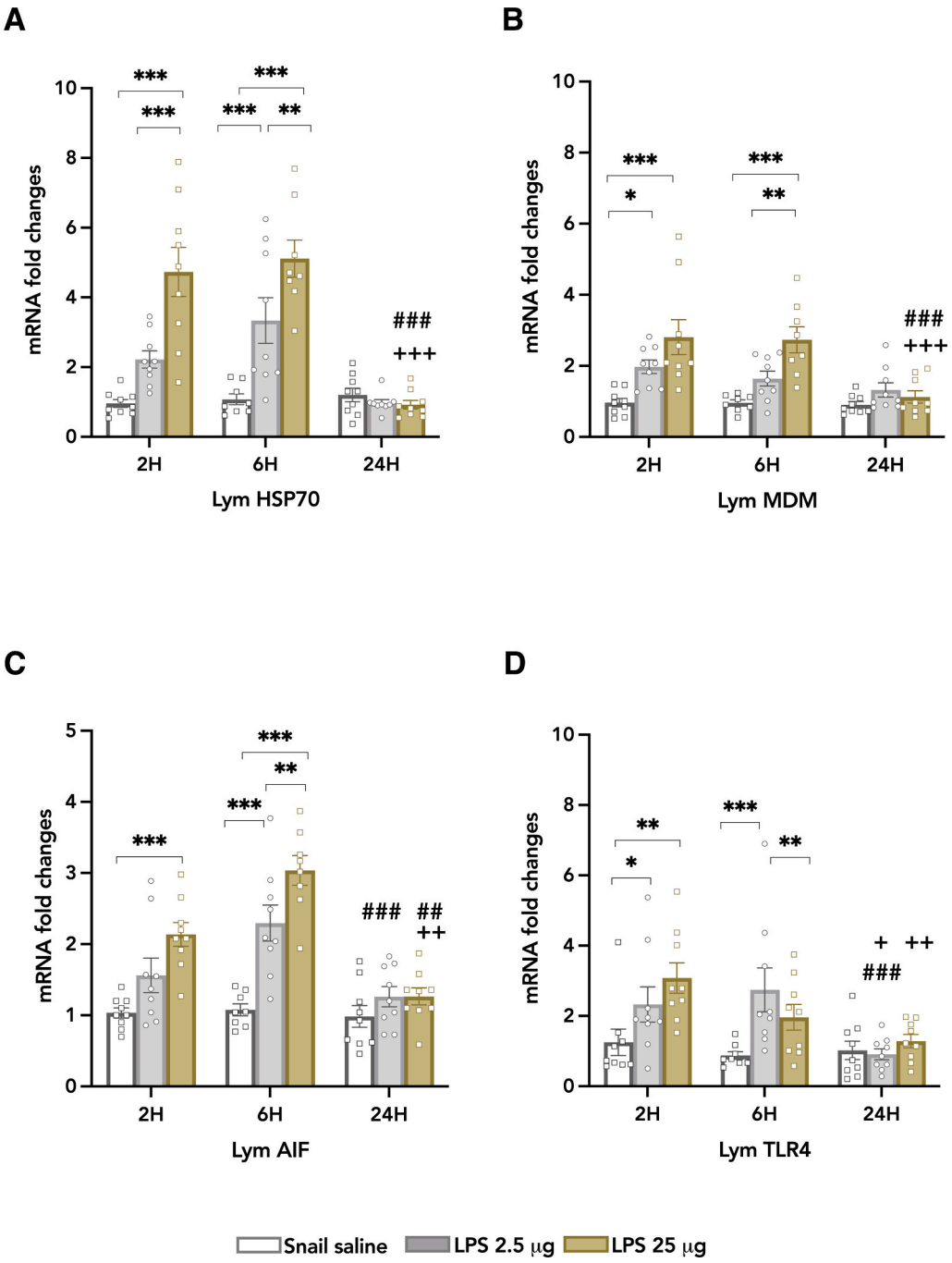

Fig. 3. Effect of an immune challenge on expression levels of immune- and stress-related target genes in the central ring ganglia of *Lymnaea stagnalis*. Adult snails were injected with either snail saline, 2.5 or 25 µg of LPS, and sacrificed 2 ($n = 9$ for saline, LPS low-dose, and LPS-high dose), 6 ($n = 8, 9, 8$ for saline, LPS low-dose, and LPS-high dose respectively), or 24 h later (n = 9 for each group). **(A)** Lym HSP70, **(B)** Lym MDM, **(C)** LymAIF, and **(D)** Lym TLR4 mRNA expression in the ganglia, with LymTUB/EF-1α as endogenous controls, were measured by qRT-PCR. Open circles indicate the individual data points. Data are represented as means ± S.E.M. and were analyzed with two-way ANOVA followed by Bonferroni *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$. Time-dependent differences are reported as: +++ $p < 0.0001$; ++ $p < 0.01$; + $p < 0.05$ vs matching treatment at 2 h; ### $p < 0.0001$; ## $p < 0.01$ vs matching treatment at 6 h.

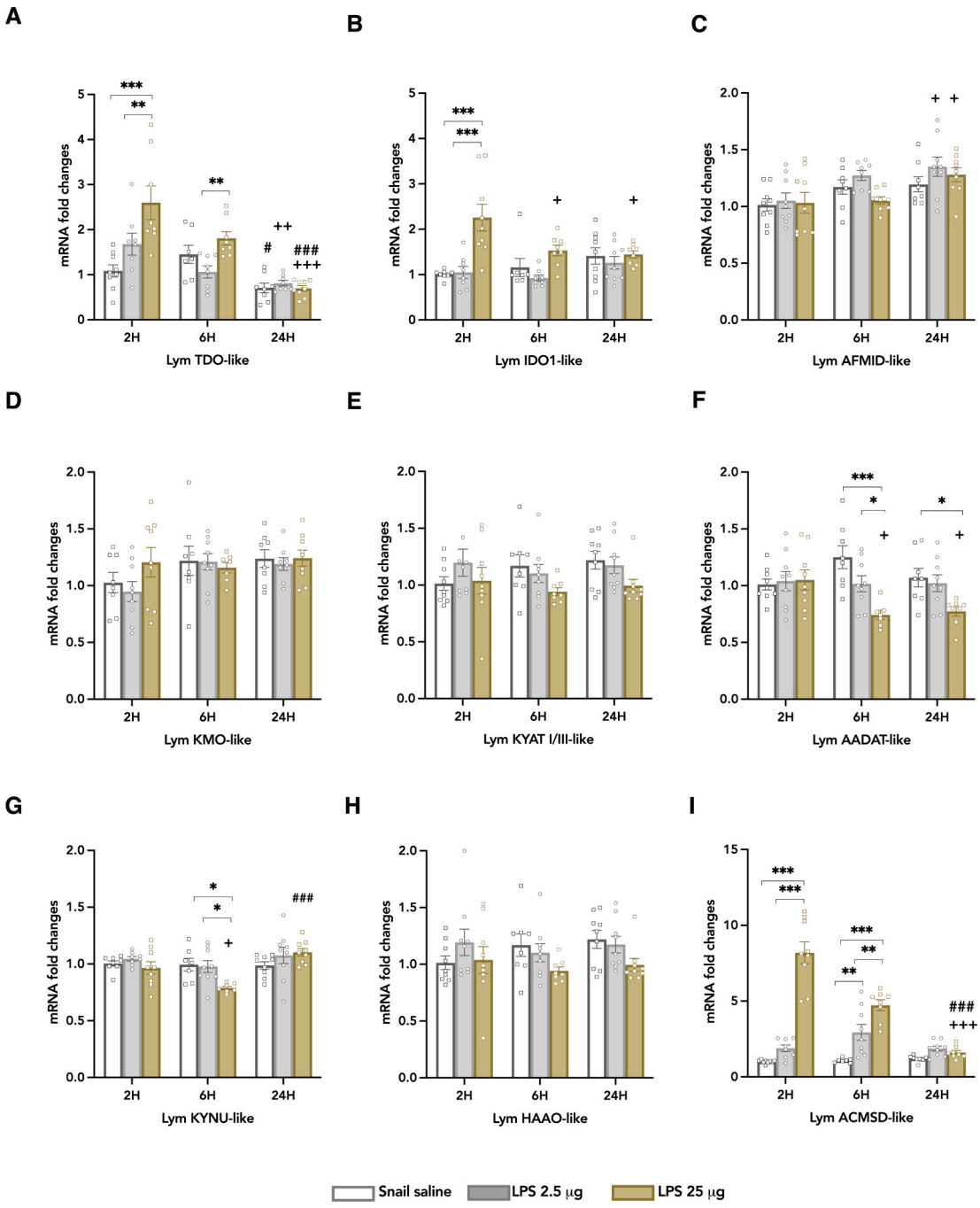

Fig. 4. Effects of an immune challenge on the transcriptional levels of putative KP enzymes in the central ring ganglia of *Lymnaea stagnalis*. Adult snails were injected with either snail saline, 2.5 or 25 µg of LPS, and sacrificed 2 (n = 9 for saline, LPS low-dose, and LPS-high dose), 6 (n = 8, 9, 8 for saline, LPS low-dose, and LPS-high dose respectively), or 24 h later (n = 9 for each group). **(A)** Lym TDO-like, **(B)** Lym IDO-like, **(C)** Lym AFMID-like, **(D)** Lym KMO-like, **(E)** Lym KYAT I/III-like, **(F)** Lym AADAT -like, **(G)** Lym KYNU-like, **(H)** Lym HAAO-like, and **(I)** Lym ACMSD-like mRNA expression in the ganglia, with LymTUB/EF-1α as endogenous controls, were measured by qRT-PCR. Open circles indicate the individual data points. Data are represented as means ± S.E.M. and were analyzed with two-way ANOVA followed by Bonferroni *** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$. Time-dependent differences are reported as: +++ $p < 0.0001$; ++ $p < 0.01$; + $p < 0.05$ vs matching treatment at 2 h; ### $p < 0.0001$; # $p < 0.05$ vs matching treatment at 6 h.

33.111; $p < 0.0001$], time after injection [F (2;78) = 26.821; $p < 0.0001$], and an interaction between the two terms [F (4;78) = 9.252; $p < 0.0001$] was observed on the expression levels of Lym HSP70. Two hours after the immune challenge, the higher LPS dose significantly increased Lym HSP70 mRNA levels in the ganglia above those of animals receiving either the lower dose of LPS ($p < 0.0001$) or saline ($p < 0.0001$). Similar data were obtained in animals sacrificed 6 h after treatments: both doses of the immune challenge increased Lym HSP70 gene expression with respect to saline-exposed animals ($p < 0.0001$). However, the effect evoked by the higher dose remained significantly higher with respect to that of the lower dose ($p = 0.005$). No difference was observed in Lym HSP70 mRNA levels between animals treated with saline or LPS and sacrificed 24 h later (Fig. 3A). For Lym MDM mRNA levels, two-way ANOVA revealed a main effect of treatment [F (2;78) = 19.93; $p < 0.0001$], time after injection [F (2;78) = 9.07; $p < 0.0001$] and interaction between the two terms [F (4;78) = 3.890; $p = 0.007$]. An injection of 25 µg of LPS resulted in a significant increase in Lym MDM mRNA levels in the ganglia with respect to their saline-injected

counterparts sacrificed 2 or 6 h later ($p < 0.0001$ at both time points). The lower dose of LPS caused a similar effect 2 h after treatment ($p = 0.014$ vs saline), which did not last up to the six-hour time point when the expression levels of this target returned to control levels while being significantly lower than their counterparts treated with 25 μg of LPS ($p = 0.007$). Again, no difference was present in Lym MDM gene expression in animals sacrificed 24 h after injection, irrespective of the treatment received (Fig. 3B).

For Lym AIF expression levels, a two-way ANOVA revealed a main effect of treatment [$F(2;78) = 31.88$; $p < 0.0001$], time after injection [$F(2;78) = 23.70$; $p < 0.0001$] and interaction between the two terms [$F(4;78) = 6.06$; $p < 0.0001$]. Two hours after an injection of 25 μg of LPS a significant increase in Lym AIF mRNA levels was observed compared to their saline-injected counterparts ($p < 0.0001$). The lower dose of LPS caused an increase in the transcriptional levels of this target that reached statistical significance only 6 h after injection ($p < 0.0001$ vs saline), as observed for Lym HSP70. At this time point, Lym AIF mRNA levels were significantly increased by the higher immune stimulus with respect to both saline-injected animals ($p < 0.0001$) and snails receiving 2.5 μg of LPS ($p = 0.008$). No difference was revealed between animals sacrificed 24 h after the injection irrespective of the treatment administered (Fig. 3C).

A two-way ANOVA revealed a main effect of treatment [$F(2;77) = 11.59$; $p < 0.0001$], time after injection [$F(2;77) = 8.54$; $p < 0.0001$], and an interaction between the two terms [$F(4;77) = 5.97$; $p < 0.0001$] on the transcriptional levels of Lym TLR4. After 2 h, in the ganglia of animals exposing to immune challenge, the mRNA levels of Lym TLR4 were significantly increased when compared to saline-exposed animals, irrespective of the dose ($p = 0.044$ and $p = 0.001$ for 2.5 and 25 μg of LPS respectively). At the 6-h time point, the gene expression of Lym TLR4 remained significantly high only in the group exposed to the lower dose of LPS compared to both saline-exposed animals ($p < 0.0001$) and the group receiving the higher dose of LPS ($p = 0.007$). No difference in the expression levels of this target was still present between the groups sacrificed 24 h after treatment (Fig. 3D).

Next, we focused our attention on the transcriptional effects induced by the two doses of LPS on the expression levels of the KP enzymes genes. For Lym TDO-like expression levels (Fig. 4A), a main effect of treatment [$F(2;72) = 9.90$; $p < 0.0001$], time after injection [$F(2;72) = 26.01$; $p < 0.0001$] and an interaction between the two terms [$F(4;72) = 6.086$; $p < 0.0001$] emerged. The higher dose of LPS (25 μg) increased significantly Lym TDO-like mRNA levels compared to saline-injected animals ($p < 0.0001$) and to snails exposed to the lower dose of LPS ($p = 0.002$) at 2 h. At 6 h, a significant difference between animals receiving the different doses of the endotoxin was still present ($p = 0.017$). No difference was revealed between groups of animals sacrificed 24 h after the injection irrespective of the treatment.

Lym TDO-like mRNA levels at this time point were significantly lower with respect to animals sacrificed at the earlier time points receiving a matching treatment. Only for this target, we observed a significant difference between saline-receiving animals sacrificed at 6 and 24 h ($p = 0.043$). No other difference was reported between saline-exposed animals sacrificed at different time points for any of the targets evaluated. Concerning Lym IDO-like gene, two-way ANOVA revealed a main effect of treatment [$F(2;73) = 12.383$; $p < 0.0001$] and an interaction between treatment and time [$F(4;73) = 3.058$; $p = 0.023$] (Fig. 4B).

Again, an upregulation of Lym IDO-like mRNA levels was present in animals receiving the higher dose of LPS induced and sacrificed 2 h later with respect to saline-injected animals ($p < 0.0001$), and to snails receiving 2.5 μg of LPS ($p < 0.0001$). No difference was revealed between the groups sacrificed 6 or 24 h after the injection, irrespective of the treatments. For Lym AFMID-like mRNA levels (Fig. 4C), two-way ANOVA revealed a main effect of time after injection [$F(2;76) = 10.446$; $p < 0.0001$]. No difference was observed between animals sacrificed at the same time-point irrespective of the treatments, while 24 h after the exposure to either dosage of LPS an upregulation of Lym AFMID-like gene expression was observed within the groups receiving a matching treatment sacrificed 2 h later ($p = 0.005$ for the lower dose, $p = 0.027$ for the higher dose).

Two-way ANOVA for the time of injection and treatment revealed no main effects in transcriptional levels for Lym KMO-like (Fig. 4D) and Lym KYAT-like (Fig. 4E) in our experimental conditions. A main effect of treatment [$F(2;75) = 8.694$; $p < 0.0001$] and an interaction between treatment and time [$F(4;75) = 3.575$; $p = 0.011$] was revealed for Lym AADAT-like mRNA levels.

In animals receiving 25 μg of LPS a significant decrease in expression levels of Lym-AADAT was observed with respect to their saline-injected counterparts sacrificed 6 h ($p < 0.0001$) or 24 h later ($p = 0.024$) (Fig. 4F). This effect was present even when comparing the two doses of the immune challenge: Lym AADAT-like gene expression was significantly lower in animals receiving the higher dose of LPS with respect to the lower one 6 h after injection ($p < 0.0001$), and a similar trend was observed at the 24-h time point ($p = 0.063$).

For Lym KYNU-like gene, we found a main effect of time after injection [$F(2;74) = 6.412$; $p = 0.003$] and an interaction between treatment and time [$F(4;74) = 3.112$; $p = 0.021$]. Six hours after the immune challenge, the higher dose of LPS decreased significantly Lym KYNU-like ganglia expression with respect to snails receiving either the lower dose of LPS ($p = 0.017$) or saline ($p = 0.014$). No effects were reported in cohorts of animals sacrificed 2 or 24 h after injection (Fig. 4G).

For Lym HAAO-like expression, two-way ANOVA only revealed a main effect of treatment [$F(2;78) = 3.397$; $p = 0.04$] (Fig. 4H). Regarding Lym ACMSD-like gene, two-way ANOVA statistical analysis revealed a main effect of treatment [$F(2;74) = 84.26$; $p < 0.0001$], time after injection [$F(2;74) = 27.59$; $p < 0.0001$] and interaction between the two terms [$F(4;74) = 30.40$; $p < 0.0001$] (Fig. 4I). The higher dose of LPS induced in snails a significant increase in mRNA levels of Lym ACMSD-like compared to animals exposed to either 2.5 μg of LPS ($p < 0.0001$) or saline ($p < 0.0001$) and sacrificed after 2 h. No difference was present between animals receiving saline and the lower dose of LPS at this time point. When considering animals sacrificed 6 h after treatment, Lym ACMSD-like expression was significantly increased by both doses of the endotoxin with respect to snails injected with saline ($p = 0.002$ and $p < 0.0001$ for the low and high doses respectively). Following 25 μg of LPS, the upregulation of Lym ACMSD-like expression remained significantly higher concerning the group exposed to 2.5 μg ($p = 0.002$). At the 24-h time point, the three groups have similar expression levels of Lym ACMSD-like.

3.4. *Experiment 4: Effects of LPS on the levels of the metabolites of the KP in the central ring ganglia of Lymnaea*

Given that the most significant transcriptional effects were observed following treatment with the higher dose of LPS, we only measured the concentrations of key metabolites from the KP enzyme activity in the ganglia of *L. stagnalis* receiving that dose (Fig. 5). Data normalized to μmol/μg protein are presented in **Supplementary Table 1**. Serotonin to tryptophan levels were not affected by the immune challenge: no main effects for the time of injection and treatment were revealed in our experimental conditions (Fig. 5A). To assess enzyme activity, we analyzed the ratios KYN/TRP, KYNA/KYN, ANA/KYN, and 3-HK/KYN, which reflect the activity of TDO/IDO, KAT, KYNU, and KMO, respectively. We found an interaction between treatment and time for the KYN/TRP ratio [$F(2;33) = 4.092$; $p = 0.028$]: KYN/TRP in saline-exposed animals was significantly higher concerning their LPS-receiving counterparts ($p = 0.006$) (Fig. 5B). A similar effect was observed for the KYNA/KYN ratio that failed to reach statistical significance (Fig. 5C). Although no main effect was reported for the ANA/KYN ratio (Fig. 5D), two-way ANOVA revealed a main effect of treatment [$F(1,35) = 8.524$; $p = 0.007$] and an interaction between treatment and

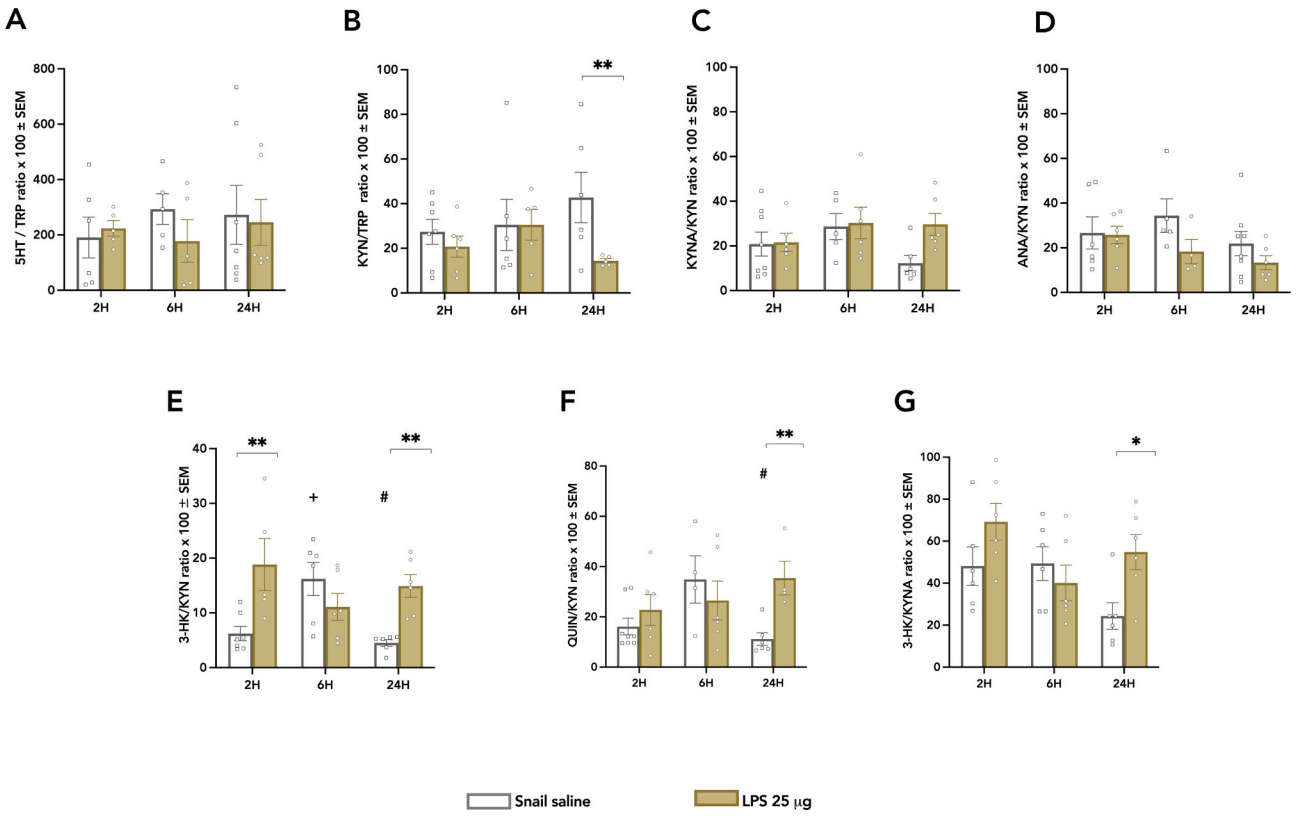

Fig. 5. Effects of an immune challenge on 5HT, TRP, and kynurenine metabolism in the central ring ganglia of *Lymnaea stagnalis*.
Adult snails were injected with either snail saline or 25 µg of LPS and sacrificed 2 (n = 8–6 for saline and LPS-high dose respectively), 6 ($n = 6$ for each group), or 24 h (n = 8–6 for saline and LPS-high dose respectively). The ratios **(A)** serotonin (5HT)/tryptophan (TRP), **(B)** kynurenine (KYN)/TRP, **(C)** kynurenic acid (KYNA)/KYN, **(D)** anthranilic acid (ANA)/KYN, **(E)** 3-hydroxykynurenine (3-HK)/KYN, **(F)** quinolinic acid (QUIN)/KYN, and **(G)** 3-HK/KYNA were measured by HPLC/MS in the ganglia; open circles indicate the individual data points. Data are represented as means ± S.E.M. and were analyzed with two-way ANOVA followed by Bonferroni: ** $p < 0.01$; * $p < 0.05$. Time-dependent differences are reported as: + $p < 0.05$ vs matching treatment at 2 h; # $p < 0.05$ vs matching treatment at 6 h.

time [F(2,35) = 7.474; p = 0.002] for 3-HK/KYN ratio. Specifically, LPS treatment significantly increased the 3-HK/KYN ratio compared to saline-exposed animals at 2- and 24-h post-treatment ($p = 0.001$ and p = 0.006, respectively). Additionally, saline-exposed animals sacrificed 6 h after injection showed a higher 3-HK/KYN ratio compared to those sacrificed at 2 or 24 h ($p = 0.019$ and p = 0.007, respectively) (Fig. 5E). For the QUIN/KYN ratio, two-way ANOVA revealed an interaction between treatment and time [F (2;34) = 3.373; $p = 0.049$]: animals exposed to the immune challenge and sacrificed after 24 h showed a significant increase in the ratio of QUIN to KYN compared to their saline-injected counterparts ($p = 0.011$). Moreover, snails injected with saline and sacrificed 6 h after injection showed a higher QUIN/KYN ratio than their counterparts sacrificed after 24 h ($p = 0.038$; Fig. 5F).

Finally, animals receiving LPS and sacrificed after 24 h showed a significant increase in the 3-HK to KYNA ratio ($p = 0.014$), two-way ANOVA revealed a main effect of treatment for this parameter in our experimental conditions [F (2;36) = 4.386; $p = 0.045$] (Fig. 5G).

4. Discussion

Over the past thirty years, LPS-induced neuroinflammation has been largely employed in rodents [55–58] to study the mechanisms underlying sickness behavior and to evaluate the effects of therapeutic interventions [3,24,54,59,60]. The present study is the first to explore at both the behavioral and molecular levels the effects induced by LPS on immune activation and KP modulation in the central ring ganglia of an invertebrate model organism, providing novel insights into the conserved mechanism underlying neuroinflammation and their possible effect on behavior. In particular, we found that the injection of a high dose of LPS induces dose-dependent distinct temporal behavioral and molecular changes.

First, we assessed the effects of the two doses of LPS on snails' aerial respiration (i.e., a key homeostatic behavior [20]) to determine the optimal dose to use to assess the conserved mechanisms involved in neuroinflammation. We found that only the high dose of LPS was robust enough to increase homeostatic aerial respiratory behavior. This heightened respiratory activity may reflect the snails' physiological attempt to manage immune-induced stress, potentially by increasing oxygen intake to support immune and metabolic processes [61,62]. These data are consistent with previous studies showing that the humoral immune response in *Lymnaea* is activated between 2 and 24 h after a parasite inoculation [63]. In line with our findings in *L. stagnalis*, organisms experiencing fever also exhibit an elevated respiratory rate [64,65].

Thus, the long-lasting increase in TBT rate induced by the injection of 25 µg of LPS may be indicative of a sickness state [66]. On the other hand, the low dose of LPS did not induce such respiratory changes, suggesting that mild immune activation does not demand the same physiological adjustment. The dose-dependent effects observed here mirror patterns seen in mammals [62], suggesting that once the immune response diminishes, homeostatic processes, including respiratory behavior, return to normal levels. Additionally, the finding that TBT returned to baseline at 48 h after high-dose LPS injection is consistent with a temporary "sickness state" induced by immune activation [37]. This recovery pattern reinforces the resilience of *Lymnaea*'s homeostatic systems, which, after coping with a high immune challenge, re-establish baseline physiological function. As the homeostatic respiration was altered by the LPS injection this led us to ask if cognitive ability of the

snails was altered as well.

Therefore, we investigated the effects of the two doses (low and high) on snails' learning and memory abilities, providing important insights into the conserved mechanisms through which immune challenges may impact cognitive behaviors such as memory formation. We found clear dose and time-dependent effects of LPS on the snails' ability to form ITM for operant conditioning of aerial respiration. These data are consistent with previous data from mammals [4,67–69]. Across all time points (2-, 6-, and 24-h post-injection), both the saline-injected and low-dose LPS groups were able to form ITM and this consistent performance suggests that the mild immune challenge does not block the ability of snails to form ITM. On the other hand, a significant memory block induced by the high dose of LPS was observed. This finding indicates that a high immune challenge interferes with the molecular mechanisms underlying memory formation following operant conditioning training. These results align with the hypothesis that the high-dose LPS induces a "sickness state," which appears to suppress cognitive functions related to memory, at least temporarily [66,70–72].

However, at 48 h post-injection, we found that both homeostatic aerial respiration and memory abilities were restored. This implies that the physiological effects of the immune challenge have subsided by this time and that the memory block observed after the high-dose LPS exposure is reversible and related to the duration of the immune-induced sickness state [73]. These findings corroborates the concept of a "cognitive cost" associated with high levels of immune activation [37,41,43,47,54,74,75], which may prioritize energy and resources for physiological homeostasis over cognitive functions such as memory formation. This temporary suppression of memory formation likely reflects an adaptive response to acute immune stress. Once the immune challenge and its physiological effects subside, cognitive functions are restored, as evidenced by the ability of snails to form ITM 48 h post-LPS injection. From a translational neuroscience perspective, these results underscore the potential impact of immune challenges on cognition, particularly under conditions of high immune activation.

Consistent with our previous studies, we found that the treatment with 25 μg of LPS induced a significant upregulation of the expression of immune- and stress-related genes [70,76]. In particular, the immune response of *L. stagnalis* was evaluated by assessing the gene expression of allograft inflammatory factor-1 (Lym AIF [8]), an interferon-like mediator, and the molluscan defense molecule (Lym MDM [23]), an immunoglobulin, that plays significant roles in immune defense reactions and different host responses to inflammatory stimuli. The exposure to 25 μg of LPS caused a significant upregulation of Lym MDM and Lym AIF transcriptional levels 2- and 6-h post-injection and returned to control levels after 24 h. As observed in rodents, the LPS-induced increase in their mRNA levels was positively correlated with its dose: the lower dose still evoked a similar but less marked effect [77,78]. An analogous trend was observed for the mRNA levels of Lym HSP70. We have previously shown that the transcription of this HSP is regulated by a variety of stressful situations in the ganglia of *L. stagnalis*, including LPS, acute heat shock, prolonged food deprivation, and exposure to the predator [19,45,79,80]. Here, we confirmed that the immune challenge induced a dose-dependent increase in mRNA levels of Lym HSP70 at the 2- and 6-h time points, which was no longer present 24 h after the injection.

Moreover, we confirmed that exposure to both doses of LPS was associated with an increased expression of the mRNA levels of Lym TLR4 in the first hours after the immune challenge [12]. Studies from mammals, including humans, have shown that the TLR family is capable of detecting various pathogen-associated molecular patterns [81]. Notably, TLR4 functions as a primary signaling receptor for LPS [12,82]. Upon activation, it engages key transcription factors, leading to the heightened synthesis of effector inflammatory genes [83]. Moreover, *in vitro* studies have demonstrated that LPS stimulation upregulates TRL4 expression [84,85]. Thus, these data from *L. stagnalis* suggest that the LPS-induced upregulation of TLR4, and the resulting inflammatory cascade activation, has been highly conserved over evolutionary history.

Importantly, LPS injection strongly induced the transcription of specific enzymes of the KP in the central ring ganglia of *L. stagnalis,* with this effect being particularly evident in animals subjected to the higher dose of LPS [86]. Because of that, we measured the central levels of serotonin, tryptophan, and KP metabolites in snails injected with this high dose of LPS [26]. At the transcriptional level, we found a significant upregulation of Lym IDO- and Lym TDO-like mRNA levels at 2 and 6 h post-high LPS dose. Despite their structural differences, IDO and TDO catalyze the same reaction: the conversion of tryptophan to N-formyl kynurenine, serving as the initial and rate-limiting enzymes in the KP [87–89].

Although, in mammals, TDO mainly acts under normal physiological circumstances, whereas IDO-dependent tryptophan metabolism is strongly activated in response to proinflammatory stimuli (i.e., including LPS) [90,91], in *L. stagnalis,* these enzymes are both induced by the LPS immune challenge. It is therefore possible to speculate that their actions are generalized. In other words, the complexity of vertebrate immune and nervous systems may have subjected them to selective pressures, resulting in the specialization of these enzymes in distinct tissues and under different conditions [7,92,93]. However, the time-dependent effects of LPS on inflammatory-related targets and IDO found here are consistent with data from rodents [31,60,88,94,95], as the same LPS serotype was demonstrated to induce a transient increase in the expression of IDO 6 h after treatment in selected brain areas and returned to basal levels after 24 h [96]. In mice, this effect was accompanied by a stronger overexpression of proinflammatory mediators that lasted up to 24 h after the immune challenge and was associated with an elevation in the kynurenine levels and kynurenine/tryptophan ratio also occurred (unpublished results; [86]). In the ganglia of snails that received the higher dose of LPS, we did not observe any changes in tryptophan levels or an increase in the KYN/TRP ratio.

The LPS treatment at the concentrations used in this study did not induce upregulation of the orthologues of the KP enzymes downstream of IDO. Notably, Lym KMO and Lym HAAO gene expression remained unaffected by LPS treatment at all the time points considered. KMO converts kynurenine into 3-hydroxykynurenine (3−HK), and HAAO further processes 3-hydroxy anthranilic acid into quinolinic acid (QUIN), both of which can be neurotoxic and contribute to neuroinflammation [96]. The higher ratio of 3-hydroxykynurenine to kynurenine (3-HK/KYN) at 2 and 24 h post-immune challenge in snails' ganglia suggests that KMO activity may increase, aligning with observations in mammals, where the activity of this enzyme can increase during inflammatory responses, potentially leading to a rise in neurotoxic metabolites [96]. Snails may have developed a more conservative metabolic response to stress and inflammation, possibly as an adaptation to their ecological niche where immediate survival may take precedence over rapid immune activation [39,45].

On the other hand, gene expression analysis revealed that Lym KYNU mRNA levels were downregulated 6 h after high-dose LPS treatment. A decrease in this enzyme – which catalyzes the conversion of kynurenine to anthranilic acid - could limit the production of anthranilic acid and potentially redirect the metabolic flow toward other branches of the KP, which might favor the production of neuroprotective metabolites instead of neurotoxic ones [57]. Thus, a decrease in KYNU expression could potentially redirect the metabolic flow toward other branches of the KP, which might favor the production of neuroprotective metabolites instead of neurotoxic ones. KYNU activity was assessed by measuring the ratio between anthranilic acid and kynurenine, as the levels of 3-hydroxy anthranilic acid were below detection limits in both hemolymph and ganglia of *L. stagnalis*. This ratio, however, showed no significant change under our experimental conditions suggesting that the immediate effects of LPS treatment may not be sufficient to alter this balance.

On the opposing metabolic branch, the expression levels of the orthologues of KAT enzymes (Lym KYAT and Lym AADAT) were differentially regulated by the treatment with the high dose of LPS.

While Lym KYAT remained unaffected, Lym AADAT was significantly downregulated in 6 and 24 h snails' central ring ganglia after the treatment with the high dose of LPS. Consistently with our results, in rodents at 24 h post-LPS, AADAT(KATII) expression was reduced in the hippocampus, enhancing the neurotoxic branch of the KP by increasing the 3-HK/KYNA ratio [95,97–99]. Thus, the downregulation of AADAT following LPS exposure in snails' central ring ganglia may contribute to an altered balance in KP metabolites, potentially favoring neurotoxic over neuroprotective metabolites, similar to findings in rodents. Consistent with findings in rodents, we also observed a significant increase in the ratio of 3-hydroxykynurenine to kynurenic acid 24 h after high-dose LPS treatment [100]. However, the KYNA/KYN ratio remained unaffected by the immune challenge.

Interestingly, in *L. stagnalis* CNS the mRNA levels of the orthologue of ACMSD were upregulated by the exposure to both doses of LPS. In particular, we observed an immediate increase at 2 h post-high dose that was still present at the 6-h time point and returned to basal levels after 24 h. The lower dose instead caused a gradual increase in the expression levels of Lym ACMSD at the 2- and 6-h time point, which was no longer present in snails sacrificed 1 day after treatment with either LPS or saline. As ACMSD converts 2-amino-3-carboxymuconic acid semialdehyde into the neuroprotective picolinic acid, its upregulation at 2 h post-injection suggests a compensatory mechanism aimed at mitigating neurotoxicity by shifting the KP toward protective pathways, potentially reducing the synthesis of harmful metabolites like quinolinic acid [101,102]. *In vitro* studies showed that under inflammatory conditions the overexpression of the *Acmsd* gene can reduce the expression levels of the *Ido1* gene and reduce TRP degradation [87,103,104]. Thus, future studies are needed to evaluate whether the increase in Lym ACMSD mRNA induced by LPS in snails' central ring ganglia may represent a compensatory mechanism that may switch the pathway toward the production of the neuroprotective KP metabolites [105].

To date, the role of QUIN as a neurotoxic mediator and precursor of NAD+ synthesis during an inflammatory insult is poorly understood [95,102].

Overall, our approach aligns with existing research that primarily utilizes gene expression analysis to estimate the expression of KP enzymes in both *in vitro* and *in vivo* studies [106,107]. However, there are limitations in measuring protein levels, especially in humans and rodents, and specific antibodies for KP enzyme orthologues in *L. stagnalis* are currently unavailable. Recent studies suggest that analyzing metabolite concentration ratios can serve as a more reliable metric for assessing KP enzyme activity in humans and rodents [108]. It is also crucial to recognize that the effects of LPS at transcriptional level may take time to manifest in KP metabolism. Additionally, the open circulatory system of L. *stagnalis* may alter the distribution of newly synthesized metabolites differently compared to more complex models, impacting how these metabolites are transported to and from the central ring ganglia.

Critical methodological considerations—including sensitivity, sample variability, and the translation of findings to mammalian systems—underscore the necessity for rigorous data collection and reporting to enable reproducibility and consistency across laboratories. While these physical and biological constraints present challenges, they also provide an invaluable opportunity to refine methodologies and establish benchmarks that enhance the robustness of the model. This study can serve as a foundational reference for other researchers investigating the KP in *L. stagnalis* and potentially in other invertebrate models. Such efforts are critical to broadening our understanding of the KP's evolutionary conservation and adaptability across species, ultimately facilitating its application in translational studies to mammalian systems. Systematic data sharing and collaborative standardization efforts will be essential to strengthen the model's reliability and utility in broader neurobiological and pharmacological research contexts.

5. Conclusion

The behavioral and molecular parallels drawn between *L. stagnalis* and mammalian models reinforce the utility of this invertebrate organism as a valid tool for exploring the evolutionary conservation of the mechanisms underlying neuroinflammation. The findings provided in this study reveal advantages that highlight *L. stagnalis* as a promising model organism and limitations that underscore areas for further exploration. One major advantage is that this study provides unique insights into invertebrate responses to immune challenges, specifically examining LPS-induced effects at behavioral and molecular levels. Our findings suggest that mechanisms of neuroinflammation, including KP modulation, may be conserved across species. This conservation is emphasized by the dose- and time-dependent immune responses in snails, which resemble those observed in mammals, especially in terms of dose-specific behavioral adaptations and memory suppression. Behavioral observations, such as increased aerial respiration in response to high-dose LPS, point to the relevance of *L. stagnalis* for modeling sickness-like behavior, as they suggest an adaptive physiological response to immune-induced stress.

We also demonstrated that the cognitive cost of an immune challenge, evidenced by reversible memory block, may mirror similar effects in mammals. Overall, the study illustrates the potential of *L. stagnalis* as a model for investigating immune-induced neuroinflammatory effects and their impact on cognition. At the same time, the findings underscore the importance of developing methods to quantify KP enzyme protein levels directly and to examine the broader evolutionary significance of KP modulation in invertebrates. However, our findings could refine *L. stagnalis*' utility as a model organism, and deepen our understanding of neuroinflammation across species. In conclusion, despite its evolutionary distance from humans, *L. stagnalis* exhibits molecular and behavioral properties that are conserved, rendering it a versatile platform for exploring new dimensions in understanding the intricate landscape of neuroinflammatory processes in the CNS.

Thus, although snails can never replace mammal models in preclinical research, the use of *L. stagnalis* aims to reduce as much as possible the use of rodents, with mammals involved only for result validation.

Institutional Review Board statement

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Informed consent statement

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CRediT authorship contribution statement

Veronica Rivi: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Giovanna Rigillo:** Writing – review & editing, Visualization. **Silvia Alboni:** Writing – review & editing, Visualization, Methodology. **Joris M. Koene:** Writing – review & editing. **Luca Pani:** Writing – review & editing, Funding acquisition. **Ken Lukowiak:** Writing – review & editing, Resources. **Fabio Tascedda:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Johanna M.C. Blom:** Writing – review & editing, Supervision. **Cristina Benatti:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2025.114418.

Data availability

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

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