© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

RESEARCH ARTICLE

Pharmacological effects of cannabinoids on learning and memory in *Lymnaea*

Hiroshi Sunada[1,2], Takayuki Watanabe[3], Dai Hatakeyama[2], Sangmin Lee[1], Jeremy Forest[1], Manabu Sakakibara[4], Etsuro Ito[2,5,*] and Ken Lukowiak[1,*]

ABSTRACT

Cannabinoids are hypothesized to play an important role in modulating learning and memory formation. Here, we identified mRNAs expressed in *Lymnaea stagnalis* central nervous system that encode two G-protein-coupled receptors (*Lymnaea* CBr-like 1 and 2) that structurally resemble mammalian cannabinoid receptors (CBrs). We found that injection of a mammalian CBr agonist WIN 55,212-2 (WIN 55) into the snail before operant conditioning obstructed learning and memory formation. This effect of WIN 55 injection persisted for at least 4 days following its injection. A similar obstruction of learning and memory occurred when a severe traumatic stimulus was delivered to *L. stagnalis*. In contrast, injection of a mammalian CBr antagonist AM 251 enhanced long-term memory formation in snails and reduced the duration of the effects of the severe traumatic stressor on learning and memory. Neither WIN 55 nor AM 251 altered normal homeostatic aerial respiratory behaviour elicited in hypoxic conditions. Our results suggest that putative cannabinoid receptors mediate stressful stimuli that alter learning and memory formation in *Lymnaea*. This is also the first demonstration that putative CBrs are present in *Lymnaea* and play a key role in learning and memory formation.

KEY WORDS: Aerial respiratory behaviour, Cannabinoid, Long-term memory, *Lymnaea stagnalis*, Operant conditioning

INTRODUCTION

Stress alters learning and memory formation. Roger Bacon suggested this in 1620 (Bacon, 1620) and it is codified in the Yerkes–Dodson inverted-U law (Yerkes and Dodson, 1908). Yerkes and Dodson (1908) stated that 'an easily acquired habit may be readily formed under strong stimulation, whereas a difficult habit may be acquired only under relatively weak stimulation' (see also Ito et al., 2015). Donald Hebb (1955) was probably the first to portray this as an inverted-U in his presidential address to the American Psychological Association in the 1950s, where he discussed stress and memory. Succinctly put: there is an optimal level of stress that results in the 'best' memory; too much or too little stress results in poorer memory formation and/or its recall.

In our model system, the pond snail *Lymnaea stagnalis*, ecologically relevant stressors such as predator detection, crowding, low environmental calcium and thermal shock all alter the ability of snails to learn associatively and form long-term memory (LTM) following operant conditioning of aerial respiratory behaviour (Lukowiak et al., 2010, 2014a,b). For example, following crowding, LTM formation is blocked (de Caigny and Lukowiak, 2008), while following exposure to a thermal shock, LTM formation is enhanced (Teskey et al., 2012). When more than one stressor is experienced by the animal, there are emergent effects arising from the combination of the stressors that result in unpredictable consequences on the snails' ability to learn and form memory (Dalesman and Lukowiak, 2012; Dalesman et al., 2013; Lukowiak et al., 2014a; Ito et al., 2015). The mechanisms by which the various stressors affect memory formation are not entirely clear. However, it has been hypothesized that cannabinoids play a key role in mediating stressor effects on adaptive behaviours (Morena and Campolongo, 2014; Wamsteeker-Cusulin et al., 2014; Goodman and Packard, 2015).

In mammals, the endocannabinoid system is composed of endogenous ligands, such as 2-arachidonoyl glycerol (2-AG) and anandamide (AEA), and mainly pre-synaptically localized receptors. These receptors, e.g. cannabinoid receptor type 1 (CB_1), exert important neuromodulatory roles, such as how stress alters learning and memory (Campolongo et al., 2009; Hauer et al., 2011; Atsak et al., 2012; Tan et al., 2014). The endocannabinoid system also plays a key role in the neuronal regulation of anxiety and fear responses (Ruehle et al., 2012; Mechoulam and Parker, 2013). We hypothesize that cannabinoid receptors (CBrs) in *Lymnaea* are involved in a similar role and the activity of the cannabinoid system also plays a key role in how stress modifies LTM formation.

Although the endocannabinoid system is phylogenetically ancient, having been shown to occur in most phyla with the notable exception of insects (McPartland et al., 2001; Salzet and Stefano, 2002; McPartland, 2004; Elphick, 2012), it has not yet been identified in *Lymnaea*. Importantly, demonstrating the presence of CBrs would allow us to make use of a number of advantages offered to us by our model system for the study of the role of cannabinoids in memory formation in *Lymnaea* (Elphick, 2012). A chief advantage of our model system is that an identified neuron, right pedal dorsal 1 neuron (RPeD1), is a necessary site for LTM formation, its reconsolidation, extinction and forgetting following operant conditioning of aerial respiration (Scheibenstock et al., 2002; Sangha et al., 2003a,b, 2005). It would therefore be possible to begin to determine exactly how the cannabinoids alter activity in this neuron that is causal to memory formation.

It was shown in the gastropod mollusc *Aplysia californica* in the 1970s that the main psychoactive ingredient of *Cannabis sativa*, Δ^9-tetrahydrocannabinol, caused a depression in neuronal excitability (Acosta-Urquidi and Chase, 1975). Presumably, this effect was mediated by CBrs although until now the presence of such receptors in *Aplysia* has not been confirmed. The endocannabinoid system has also been reported to be involved in

[1]Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada T2N 4N1. [2]Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa 769-2193, Japan. [3]Research Institute for Electronic Science, Hokkaido University, Sapporo, Hokkaido 060-0811, Japan. [4]School of High-Technology for Human Welfare, Tokai University, Numazu, Shizuoka 410-0321, Japan. [5]Department of Biology, Waseda University, Shinjuku, Tokyo 162-8480, Japan.

*Authors for correspondence (eito@waseda.jp; lukowiak@ucalgary.ca)

E.I., 0000-0002-1877-6566

Received 7 March 2017; Accepted 7 June 2017

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

List of symbols and abbreviations

2-AG	2-arachidonoyl glycerol
CBr	cannabinoid receptor
CNS	central nervous system
LTM	long-term memory
MT	memory test session
TS1	initial training session
TS2	second training session

the regulation of synaptic efficacy in the terrestrial snail *Helix lucorum* (Lemak et al., 2007). CBr genes have been identified in some other invertebrates such as the leech *Hirudo medicinalis* (Stefano et al., 1997; Elphick, 1998; Matias et al., 2001; McPartland and Glass, 2003), the nematode *Caenorhabditis elegans* (McPartland and Glass, 2003; Oakes et al., 2017), the ascidian *Ciona intestinalis* (Elphick et al., 2003) and the lancelet *Branchiostoma floridae* (Elphick, 2007). We show here mRNA expression of two putative CBr genes in the central nervous system (CNS) of *L. stagnalis*. We also show pharmacologically that these putative CBrs are involved in a key role in modulating learning and memory formation. We then further demonstrate that experiencing an extreme stressor blocks learning and memory. This effect of extreme stress can be mimicked by the injection of a CBr agonist, and blockage of these receptors by a CBr antagonist mitigates the effects of the severe stressor. Moreover, the CBr antagonist itself can enhance LTM formation.

MATERIALS AND METHODS

Animals

The pond snail *Lymnaea stagnalis* (Linnaeus 1758) (20–30 mm shell length) was used. Snails were maintained on an 8 h light:16 h dark cycle at room temperature (20–23°C) in well-aerated pond water and fed lettuce *ad libitum* (Yamagishi et al., 2015; Aonuma et al., 2016, 2017; Sunada et al., 2017). Artificial pond water was made from deionized water containing 0.26 g l^{-1} Instant Ocean (Aquarium Systems Inc., Mentor, OH, USA) and calcium sulphate dihydrate to provide a calcium concentration of 80 mg l^{-1} (Dalesman and Lukowiak, 2010).

Molecular cloning of putative CBrs in *L. stagnalis*

Database search

We conducted a tblastn search on the *Lymnaea* CNS transcriptome shotgun assembly (TSA) sequence database (Sadamoto et al., 2012) to search for *Lymnaea* homologues of the invertebrate-type CBr. We found two TSA contigs (FX183817 and FX186161), both of which encode rhodopsin-like G-protein-coupled receptors with sequence similarity to *Ciona intestinalis* CBr (NP_001027653).

cDNA cloning

Total RNA was isolated from *L. stagnalis* CNS with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and treated with DNase I (TaKaRa, Shiga, Japan) at 37°C for 1 h. Total RNA was then reverse-transcribed using Superscript III reverse transcriptase (Thermo Fisher Scientific) with the 3′-RACE adapter primer from the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). cDNAs encoding the full-length *L. stagnalis* CBr-like proteins were amplified using gene-specific primers designed to the 5′ and 3′ untranslated regions (UTRs) of two putative *Lymnaea* CBr-like genes (*Lymnaea* CBr-like 1, 5′-CGT TAC CAA ATT TAC CGA CCA C-3′ and 5′-CCG GAT GAA CTC ACG ATT CG-3′; *Lymnaea* CBr-like 2, 5′-GGA CAC GAC CAC CAC CG-3′ and 5′-CAA GGG AAG ATA CAT TCA TAG CCA G-3′). PCRs were carried out using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Tokyo, Japan). The amplified DNA fragments were subcloned into the plasmid vector pGEM-T easy (Promega, Madison, WI, USA), and the nucleotide sequences were determined.

Structural analysis

The deduced amino acid sequences of the *Lymnaea* CBr-like proteins were aligned with those of molluscan CBr-like proteins by the MAFFT algorithm (Katoh and Standley, 2013) in Geneious (v8.1.6; http://www.geneious.com/) and visualized with BoxShade (v3.21; http://www.ch.embnet.org/software/BOX_form.html). Transmembrane helices of *Lymnaea* CBr-like proteins were predicted using the GPCRHMM algorithm (Wistrand et al., 2006).

Phylogenetic analysis

The deduced full-length protein sequences of *Lymnaea* CBr-like proteins and those of the G-protein-coupled receptors related to the CBr family were aligned as described above. A maximum likelihood tree was constructed from the aligned sequences using the MEGA 6 program with default settings (Tamura et al., 2011, 2013). One thousand bootstrap replications were conducted to evaluate the reliability of the reconstructed tree. Vertebrate G-protein-coupled receptors closely related to the CBr (Wolf and Grüneweld, 2015) [melanocortin receptors (*Homo sapiens* MC1-5), sphingosine 1-phosphate receptors (*Homo sapiens* S1P1-5) and lysophosphatidic acid receptors (*Homo sapiens* LPA1-6)] were included in the analysis as outgroups to root the tree. The obtained tree was visualized with FigTree (v1.4.2; http://tree.bio.ed.ac.uk/software/figtree/). GenBank IDs of the proteins used for phylogenetic analysis are listed in Table 1.

SYBR Green-based qRT-PCR

The procedures of primer extension analyses were performed as described previously with modifications (Hatakeyama et al., 2013). To determine the expression level of *Lymnaea* CBr-like 1 and 2 mRNA, we dissected the CNS, buccal mass, penis, ovotestis, gut and mantle from 12 snails. Four samples of each tissue were allocated into three groups. Total RNA samples of tissues were purified using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA). Reverse transcription (RT) was performed using 20 µl of each total RNA preparation, SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific), RNaseOUT (Thermo Fisher Scientific) and oligo $d(T)_{12–18}$ primer (Thermo Fisher Scientific) following the product manuals. RT samples were diluted 1/20 with distilled water, then mixed with SYBR Green Realtime PCR Master Mix (ToYoBo, Osaka, Japan) and a primer set selectively amplifying *Lymnaea* CBr-like 1 and 2 or *Lymnaea* actin. We designed two primer sets for each *Lymnaea* CBr-like 1 and 2 using Primer Express Software v3.0 (Thermo Fisher Scientific). We used three different reference genes: actin, tubulin and *Lymnaea* elongation factor α (LEFα). The nucleotide sequence of each primer was as follows: *Lymnaea* CBr-like-1-F (1st set) 5′-TCT CCA CTG AGA TCT AAA CGA ATC TG-3′; *Lymnaea* CBr-like-1-R (1st set) 5′-ACA CCA GTA GGC CTT GAA TAC CTT-3′; *Lymnaea* CBr-like-1-F (2nd set) 5′-CAT CTA TGG GAT ACT GTT CGA GTC ATA C-3′; *Lymnaea* CBr-like-1-R (2nd set) 5′-ATG GTG CCC CGT GTA CTT GTA G-3′; *Lymnaea* CBr-like-2-F (1st set) 5′-CCG ACG GAC TCA AGG AAA AC-3′; *Lymnaea* CBr-like-2-R (1st set) 5′-TGA CCA GTG CGT TGC CAA T-3′; *Lymnaea* CBr-like-2-F (2nd set)

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

Table 1. GenBank accession numbers of G-protein-coupled receptors used for phylogenetic analysis

GenBank ID	Organism	Protein
Q17594	*Caenorhabditis elegans*	C02H7.2
CDQ00588	*Brugia malayi*	CBr-like
KHN77693	*Toxocara canis*	CBr-like
KKA72247	*Pristionchus pacificus*	CBr-like
XP_003376943	*Trichinella spiralis*	CBr-like
KHJ42684	*Trichuris suis*	CBr-like
XP_005096075	*Aplysia californica*	CBr-like
XP_013079859	*Biomphalaria glabrata*	CBr-like
XP_013413429	*Lingula anatina*	CBr-like
XP_009045672	*Lottia gigantea*	CBr-like
NP_001027653	*Ciona intestinalis*	Cannabinoid receptor
Branchiostoma floridae genomic scaffold 332 (bases 475179–476558; reverse strand)	*Branchiostoma floridae*	Cannabinoid receptor (see Elphick, 2007)
NP_149421	*Homo sapiens*	CB_1
XP_003971857	*Takifugu rubripes*	CB_{1A}
XP_003962753	*Takifugu rubripes*	CB_{1B}
NP_001832	*Homo sapiens*	CB_2
NP_001179232	*Bos taurus*	CB_2
XP_003965706	*Takifugu rubripes*	CB_2
AF153436	*Homo sapiens*	Melanocortin 1 receptor (MC_1)
AAO67714	*Homo sapiens*	Melanocortin 2 receptor (MC_2)
NP_063941	*Homo sapiens*	Melanocortin 3 receptor (MC_3)
NP_005903	*Homo sapiens*	Melanocortin 4 receptor (MC_4)
NP_005904	*Homo sapiens*	Melanocortin 5 receptor (MC_5)
NP_001392	*Homo sapiens*	Lysophosphatidic acid receptor 1 (LPR_1)
NP_004711	*Homo sapiens*	Lysophosphatidic acid receptor 2 (LPR_2)
NP_036284	*Homo sapiens*	Lysophosphatidic acid receptor 3 (LPR_3)
NP_001264929	*Homo sapiens*	Lysophosphatidic acid receptor 3 (LPR_4)
NP_001136433	*Homo sapiens*	Lysophosphatidic acid receptor 3 (LPR_5)
NP_001155969	*Homo sapiens*	Lysophosphatidic acid receptor 3 (LPR_6)
NP_001391	*Homo sapiens*	Sphingosine 1-phosphate receptor 1 ($S1P_1$)
NP_004221	*Homo sapiens*	Sphingosine 1-phosphate receptor 2 ($S1P_2$)
NP_005217	*Homo sapiens*	Sphingosine 1-phosphate receptor 3 ($S1P_3$)
NP_003766	*Homo sapiens*	Sphingosine 1-phosphate receptor 4 ($S1P_4$)
NP_001159687	*Homo sapiens*	Sphingosine 1-phosphate receptor 5 ($S1P_5$)
NP_001008533	*Mus musculus*	Adenosine receptor A1 (A_1)

5′-ATG CGC AGT ATC ACC AAT ATA TTT CT-3′; *Lymnaea* CBr-like-2-R (2nd set) 5′-CTG CAG GTA GTG GAC AGC CTT AC-3′; *Lymnaea* actin-F 5′-TCC CTT GAG AAG AGC TAC GAG C-3′; *Lymnaea* actin-R 5′-GAG TTG TAG GTG GTT TCG TGG-3′. The nucleotide sequences of primers for tubulin and LEFα were obtained from van Nierop et al. (2006). The reaction was carried out at 95°C for 1 min followed by 50 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s each using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Each preparation of CBr-like 1, CBr-like 2 and actin was applied to a 96-well plate in triplicate. Relative levels of CBr-like 1 and 2 mRNA were calculated by the comparative C_T ($\Delta\Delta C_T$) method (Thermo Fisher Scientific) (Livak and Schmittgen, 2001; Hatakeyama et al., 2013) using actin mRNA as a reference to give normalized CBr-like 1 and 2 mRNA expression levels.

Aerial respiratory operant conditioning

Lymnaea is a pulmonate bimodal breather. In the eumoxic environment (i.e. normal levels of O_2), snails mainly absorb oxygen through their skin, whereas in the hypoxic environment, they move up to the water surface and open the pneumostome to inhale atmospheric air. The experimental procedures for aerial respiratory operant conditioning were described previously (Orr et al., 2009; Lukowiak et al., 2000). Briefly, a 1 litre beaker containing 500 ml pond water at room temperature was bubbled with N_2 for 20 min to make it hypoxic. Snails were numbered 24 h before training, and then placed in the hypoxic training beaker for 10 min for acclimation, followed by training either with a single 0.5 h training session or with two 0.5 h training sessions separated by a 1 h interval. In both training procedures, LTM was tested 24 or 48 h later. Memory formation has been operationally defined (Orr et al., 2009; Lukowiak et al., 2000). When two 0.5 h training sessions are used, memory formation is indicated if the number of attempted pneumostome openings in the memory test session (MT) is significantly lower than the number of attempted openings in the initial training session (TS1) and not significantly greater than the number of attempted openings in the second training session (TS2). However, when only a single 0.5 h training session procedure is used, as in the data shown in Results, ‘Trauma and LTM’, the number of attempted openings in the memory test session has to be significantly lower than that in the initial training session. In the training and memory test sessions, a gentle tactile stimulus was applied to the pneumostome area using a sharpened wooden stick (approximately 0.8 mm diameter sharpened end) every time the snail began to open its pneumostome to perform areal respiration. This tactile stimulus evoked only pneumostome closure; it did not cause the snail to withdraw its foot and mantle (i.e. the whole-body withdrawal response). Snails were returned to the eumoxic water after the training sessions. Twenty-four hours after the final training session (TS1 or TS2), a memory retention test was carried out following the same procedure as in the training session, for 30 min. The time sequence of operant conditioning is shown in Fig. 4A. It

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

should be noted that individual snails in each cohort were only used in a single experiment; a cohort was not used in multiple experiments and each snail was only tested for memory once.

Noxious mechanical/tactile stress

We traumatized snails using repeated presentations (~50 times) of a noxious tactile stimulus to the foot or mantle cavity with a hand-held sharp wooden stick (as for the operant conditioning procedure, described above). This stimulation procedure did not break the skin of the snail. Such stimulation always elicited the whole-body withdrawal response (Sunada et al., 2012). As *Lymnaea* do not possess an operculum, the foot of the snail was always visible and could thus receive continued stimulation, even though the snails withdrew as much as possible into their shell with this procedure. Snails did not have to be repositioned to enable us to present this traumatic stimulus.

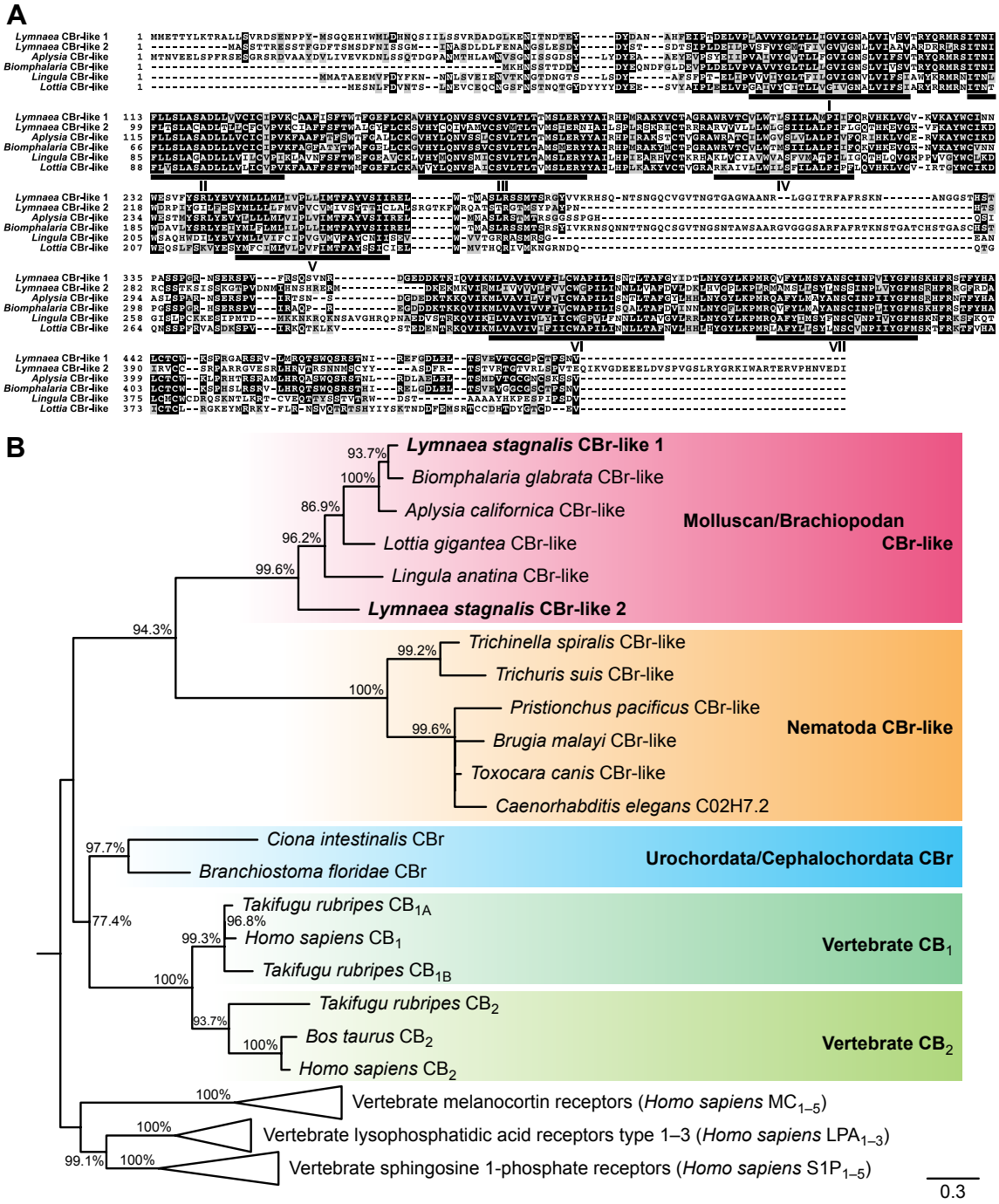

Fig. 1. Molluscan cannabinoid receptor (CBr)-like proteins. (A) Sequence comparison of two *Lymnaea* CBr-like proteins with other molluscan CBr-like proteins. Transmembrane regions (I–VII) predicted by the GPCRHMM algorithm are indicated by black bars under the alignment. (B) Molecular phylogenetic tree of *Lymnaea* CBr-like proteins and other CBr/CBr-like proteins. The scale bar indicates 0.3 substitutions per site. Bootstrap values are placed on the nodes. G-protein-coupled receptors related to CBr (vertebrate melanocortin receptors, sphingosine 1-phosphate receptors and lysophosphatidic acid receptors) were used as outgroups. The GenBank accession numbers of proteins used for the analysis are listed in Table 1.

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

Effects of CBr agonist and antagonist on behaviour changes

The mammalian CBr agonist WIN 55,212-2 (WIN 55) and its antagonist AM 251 (both from Tocris Bioscience, Bristol, UK) were dissolved in DMSO as a stock solution at 10 mmol l^{-1} concentration. Stock solutions were diluted with *Lymnaea* saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 4.0 mmol l^{-1} $CaCl_2$ and 10.0 mmol l^{-1} Hepes, pH 8.0) for both the behavioural experiments. For control experiments, 0.1% DMSO was dissolved in the saline as the vehicle solution. For behavioural experiments, snails were anaesthetized with ice-cold pond water for 30 min before injection. The CBr agonist or antagonist (10 µmol l^{-1}) in 100 µl *Lymnaea* saline was injected into the abdominal cavity of a snail, giving an estimated concentration of the drug in the *Lymnaea* body of about 2 µmol l^{-1}. In the experiment described in Results, 'Trauma and LTM', AM 251 was dissolved in 0.1% DMSO and 0.1% Tween 80 in *Lymnaea* saline. This stock solution was diluted in the saline to give a final concentration of 18 µmol l^{-1} AM 251; 100 µl of this solution was injected into the snails to give a final concentration in the body of approximately 3.6 µmol l^{-1} AM 251. Snails were prepared as independent groups in each behavioural experiment.

Data analysis

The data are represented as means±s.e.m. The mRNA expression levels of *Lymnaea* CBr-like genes were analysed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. As stated above, memory was operationally defined as a significant decrease in the number of attempted pneumostome openings in MT compared with TS1, where the number was not significantly greater than that in TS2. A one-way ANOVA and Tukey's *post hoc* test were used to determine whether the agonists or antagonists altered homeostatic aerial respiratory behaviour. Two-way repeated measures ANOVA (rmANOVA) was used to analyse the behavioural response of the snails receiving operant conditioning of aerial respiratory behaviour, with the various endocannabinoid agonists, antagonists and trauma applications (Figs 4–7) as the between-subjects factor and the training and memory test sessions (TS1, TS2, MT) as the within-subject factors. Where significant interactions were found ($P<0.05$), Tukey's *post hoc* test was used for comparisons to determine significant differences between the responses. In the naive and vehicle conditions, a paired *t*-test was used to determine whether LTM was formed; in the AM 251 condition, an ANOVA followed a Tukey's test was used to determine whether memory was formed 24 and 48 h after training. In all cases, for significance to be declared, $P<0.05$ had to be achieved. Data were analysed using Prism 6 software for Macintosh.

RESULTS

Putative CBr-like genes in *L. stagnalis*

We found two transcriptome shotgun assembly contigs (*Lymnaea* CBr-like 1, GenBank: FX183817; and *Lymnaea* CBr-like 2, GenBank: FX186161), both of which encode rhodopsin-like G-protein-coupled receptors with sequence similarity to *Ciona intestinalis* CBr (NP_001027653) (Elphick et al., 2003). We next performed RT-PCR to ensure that the mRNAs of two *Lymnaea* CBr-like genes are expressed in the *Lymnaea* CNS, using gene-specific primers designed to the 5′- and 3′-UTRs of the genes, and successfully amplified their cDNA fragments: a 1622 bp cDNA fragment of *Lymnaea* CBr-like 1 (GenBank: LC093511) and a 1537 bp cDNA fragment of *Lymnaea* CBr-like 2 (GenBank: LC093512). The cDNA fragment of *Lymnaea* CBr-like 1 contains an open reading frame (ORF) spanning 97–1581 bp with a predicted protein product of 494 amino acids. The cDNA fragment of *Lymnaea* CBr-like 2 contains an ORF spanning 46–1482 bp with a predicted protein product of 478 amino acids. The amino acid sequence of *Lymnaea* CBr-like 1 is 37.8% identical to that of *Lymnaea* CBr-like 2. The transmembrane regions of *Lymnaea* CBr-like proteins are conserved (57.5% identity).

Next, we searched for molluscan genes encoding a protein that structurally resembles the *Lymnaea* CBr-like proteins. We found predicted genes encoding CBr-like proteins from three molluscan species (*Aplysia californica*, *Biomphalaria glabrata* and the owl limpet *Lottia gigantea*). We also searched for genes related to the *Lymnaea* CBr-like proteins in the gene database of Brachiopoda, which is a closely related phylum to Mollusca in the superphylum Lophotrochozoa, and found a gene encoding a putative CBr-like

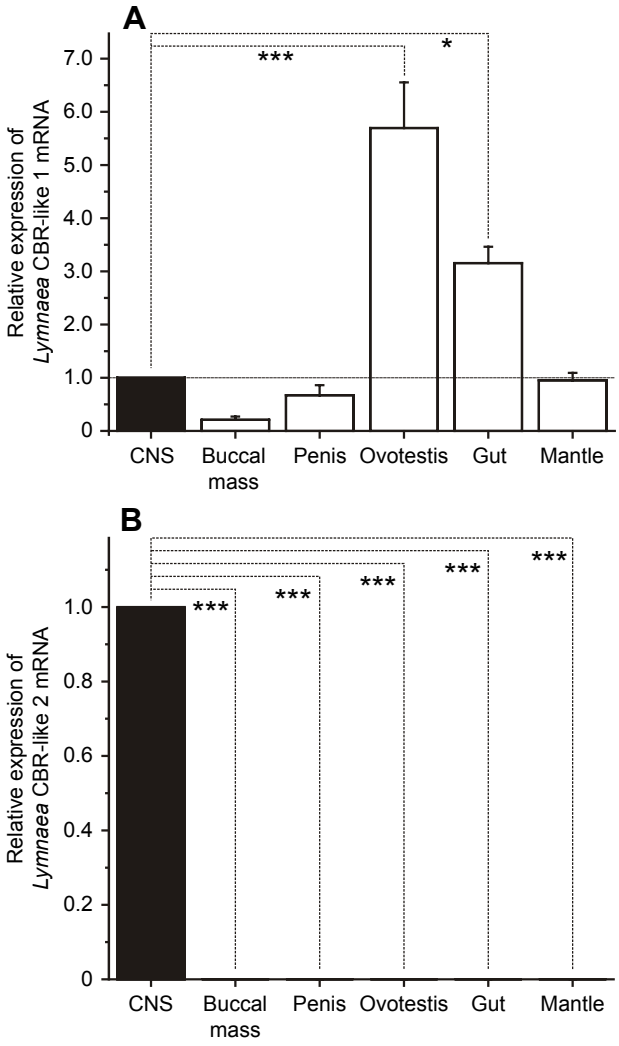

Fig. 2. Comparison of mRNA expression levels of *Lymnaea* CBr-like genes in snail CNS, buccal mass, penis, ovotestis, gut and mantle. (A) CBr-like 1 and (B) CBr-like 2 mRNA expression levels, normalized using actin, tubulin and LEFα as reference genes. The data show that *Lymnaea* CBr-like 1 gene was transcribed in all tested tissues, but the mRNA levels of this gene in ovotestis and gut were significantly higher than those in the CNS (one-way ANOVA followed by Tukey's *post hoc* test; $F_{5,19}=29.76$, $P<0.001$). *Lymnaea* CBr-like 2 gene was more highly transcribed in the CNS than in other tissues (one-way ANOVA followed by Tukey's *post hoc* test; $F_{5,13}=193{,}239.87$, $P<0.001$); levels in other tissues were too low to be quantified. Data are expressed as means±s.e.m. *$P<0.05$; ***$P<0.005$.

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

protein from the brachiopod *Lingula anatina*. We compared the amino acid sequences of *Lymnaea* CBr-like proteins and other molluscan/brachiopodan CBr-like proteins (Fig. 1, Table 1), and found that these CBr-like proteins can be classified into two subgroups according to the sequence and structure of the third cytoplasmic loop, the region between the fifth and sixth transmembrane helices. *Lymnaea* CBr-like 1 and *Biomphalaria* CBr-like protein have a long third cytoplasmic loop (104 amino acids and 112 amino acids, respectively) with high sequence homology (65.8% identity; Fig. 1A). *Lymnaea* CBr-like 2 and CBr-like proteins of *A. californica*, *L. gigantea* and *L. anatina* have a short third cytoplasmic loop (59–66 amino acids) with low sequence homology (14–32% identity; Fig. 1A). A molecular phylogenetic analysis of the CBrs with other G-protein-coupled receptors revealed that molluscan/brachiopodan CBr-like proteins are closely related to the vertebrate CBrs (CB_1 and CB_2) and other CBr-like proteins (Fig. 1B). Our phylogenetic analysis also revealed that gene duplication events of the CBr-like gene have independently occurred in molluscan and vertebrate lineages.

Comparison of expression levels of CBr-like genes among several tissues of *Lymnaea*

We next examined tissue-specific mRNA expression of the two *Lymnaea* CBr-like genes by SYBR Green-based qRT-PCR. The levels of *Lymnaea* CBr-like 1 and 2 mRNA among snail CNS, buccal mass, penis, ovotestis, gut and mantle were compared using three different reference genes (actin, tubulin and LEFα) according to the $\Delta\Delta C_T$ method for qRT-PCR (Fig. 2). *Lymnaea* CBr-like 1 mRNA was detected in all tested tissues containing the CNS (Fig. 2A). Expression in the ovotestis and gut was significantly higher than that in the CNS (Fig. 2A, one-way ANOVA followed by Tukey's *post hoc* test). In contrast, *Lymnaea* CBr-like 2 mRNA expression in the CNS was significantly higher than that in other tissues (Fig. 2B, one-way ANOVA followed by Tukey's *post hoc* test). *Lymnaea* CBr-like 2 mRNA levels in the other tissues were too low (around 10^{-8} to 10^{-4}) to quantify. These results suggest that the *Lymnaea* CBr-like 1 gene was ubiquitously transcribed in tissues and the *Lymnaea* CBr-like 2 gene was specifically expressed in the CNS of *Lymnaea*.

Effects of injection of a mammalian CBr agonist or antagonist on aerial respiratory behaviour and aerial respiratory operant conditioning

To test whether the putative CBrs found in the CNS play a role in modulating adaptive behaviours in *Lymnaea*, we used a mammalian CBr agonist and antagonist to examine whether adaptive behaviour would be altered. We first determined whether the agonist or antagonist altered homeostatic aerial respiratory behaviour elicited in response to a hypoxic environment (Fig. 3A). We found that aerial respiratory behaviour of snails in hypoxic pond water was unaffected by injection of the mammalian CBr agonist WIN 55. Both the total number of breaths and the total breathing time during the 30 min session were unaltered. Likewise, we found that when we injected the mammalian CBr antagonist AM 251, there was no significant change in aerial respiratory behaviour (one-way ANOVA followed by Scheffé's *post hoc* test, $P>0.05$; Fig. 3B).

We found that injecting WIN 55 into a cohort of snails 1 h before operant conditioning resulted in the snails exhibiting neither learning nor LTM (Fig. 4A,B). To show that WIN 55 obstructed learning and memory, we injected snails with only the vehicle and found that learning and memory were not obstructed (Fig. 4D; $n=12$; one-way ANOVA followed by Tukey's *post hoc* test, $P<0.05$). We then injected WIN 55 into three cohorts and tested the snails' ability to learn and form LTM 2, 4 and 7 days later to allow us to determine the duration of WIN 55's effect (Fig. 4C,D). Learning and memory formation were obstructed when WIN 55 was injected either 2 days ($n=11$) or 4 days ($n=15$) before operant conditioning (Fig. 4D; one-way ANOVA followed by Tukey's *post hoc* test, $P>0.05$ in both cohorts). Learning and memory, however, were seen in the cohort of snails that received WIN 55 injection 7 days before training (Fig. 4D; $n=16$, one-way ANOVA followed by Tukey's *post hoc* test, $P<0.05$ in TS1, $P<0.01$ in MT).

Trauma and LTM

We next asked whether snails experiencing a severe traumatic stimulus exhibit a similar obstruction of learning and memory to that seen in the cohorts injected with WIN 55 2 and 4 days previously (Fig. 5A). We first trained a cohort of snails that did not receive the traumatic stimulus. These snails (Fig. 5B; $n=31$) showed learning

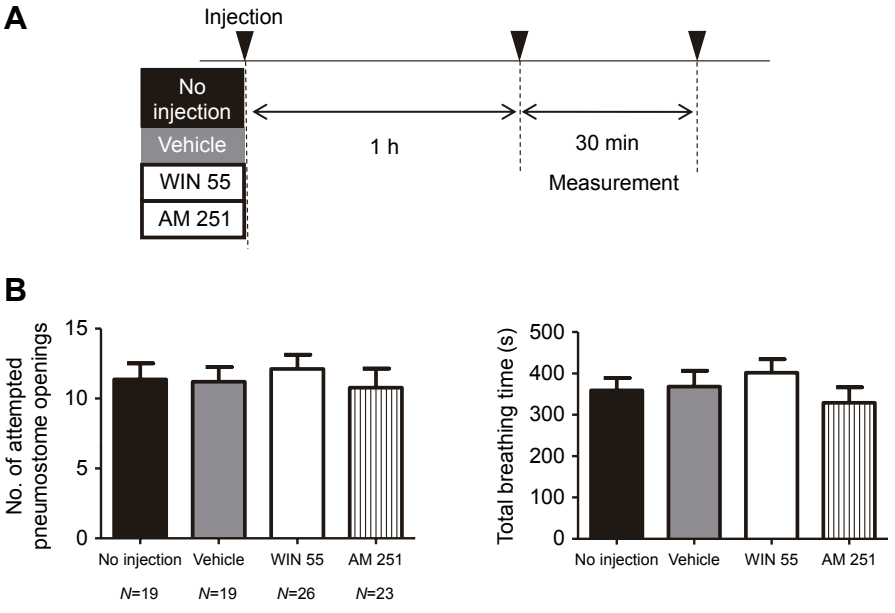

Fig. 3. Effects of a mammalian CBr agonist, WIN 55, and antagonist, AM 251, on aerial respiratory behaviour of snails. (A) Time line of the experiments. The following four groups were used: non-injection ($n=19$ snails); vehicle injection (0.1% DMSO, $n=19$ snails); WIN 55 injection (the estimated final concentration in the body was 2 µmol l^{-1}, $n=26$ snails); and AM 251 injection (the estimated final concentration in the body was 2 µmol l^{-1}, $n=23$ snails). (B) Total number of breaths (left) and total breathing time (right) in snails during the 30 min hypoxic session. No significance differences were observed among the groups. Total number of breaths: one-way ANOVA followed by Scheffé's *post hoc* test, $F_{3,83}=0.2565$, $P=0.8566$; total breathing time: one-way ANOVA followed by Scheffé's *post hoc* test, $F_{3,83}=0.8241$, $P=0.4843$.

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

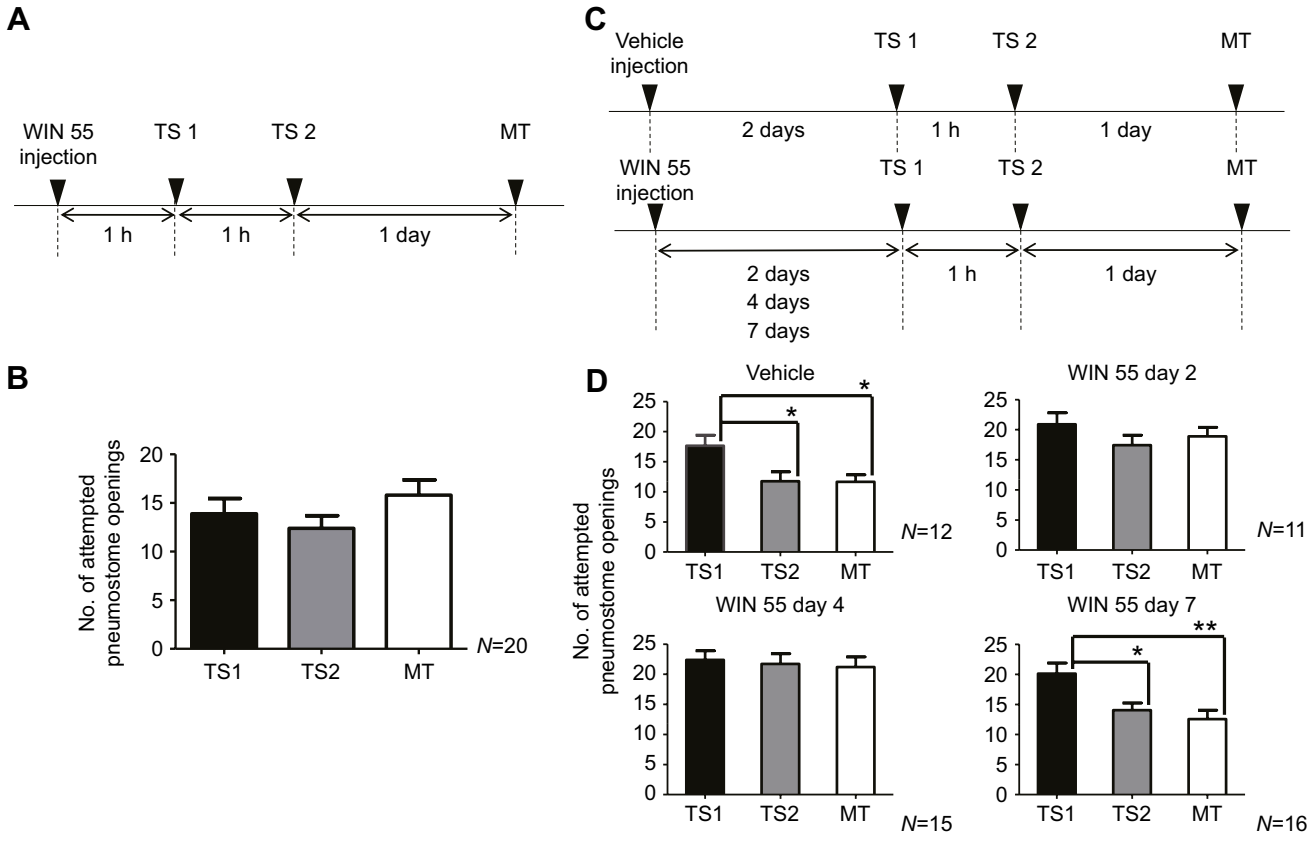

Fig. 4. WIN 55 blocks learning and long-term memory (LTM) formation for at least 4 days. (A) Time line of the experimental procedure of the pilot test, showing the time of injection of WIN 55 and the onset of the training procedure (TS, training session; MT, memory test). (B) Snails trained immediately (1 h) after WIN 55 injection exhibited neither learning nor LTM (i.e. MT was not significantly different from TS1; one-way ANOVA and Tukey's *post hoc* test; $F_{2,30}$=1.966, P=0.1649). Tukey's *post hoc* test revealed that there were no significant differences between any of the sessions. (C) Time line of the experimental procedure, showing the time of injection of vehicle or WIN 55, the interval between injection and the onset of the training procedure, the training procedure used and the time of testing for LTM. Four separate cohorts of snails were used. For the control group, snails were trained (i.e. TS1 and TS2) 2 days after injection of vehicle. For the experimental groups, snails were trained 2, 4 and 7 days after injection of WIN 55. LTM was tested 24 h after TS2. (D) The number of attempted pneumostome openings in each 0.5 h session. Data were analysed using a two-way rmANOVA ($F_{8,138}$=2.239, P=0.0281) followed by Tukey's *post hoc* test for multiple comparisons. Snails injected 2 days previously with vehicle exhibited LTM (top left). In these vehicle-injected snails, the number of pneumostome openings in MT was significantly lower than that in TS1 and not significantly greater than that in TS2. Thus, the criteria for LTM were met. Additionally, the number of attempted openings in TS2 was significantly lower than that in TS1; thus, learning occurred. For snails trained 2 days after the injection of WIN 55 (top right), neither learning nor LTM was found to be present; there were no significant differences between the number of attempted pneumostome openings in any of the sessions. For snails trained 4 days after the injection of WIN 55 (bottom left), there were no significant differences between the number of attempted pneumostome openings in any of the sessions. For snails trained 7 days after the injection of WIN 55 (bottom right), the number of attempted pneumostome openings in MT was significantly lower than that in TS1 and was not significantly greater than that in TS2. Moreover, the number of attempted openings in TS2 was significantly lower than that in TS1. Thus, we conclude that associative learning and LTM formation occurred. *P<0.05; **P<0.01.

and LTM formation (one-way ANOVA, P<0.001). A second cohort of snails experienced the traumatic stress (i.e. 50 pokes; see Materials and methods) followed 2 days later by training using the same two 0.5 h training sessions with a memory test 24 h later (Fig. 5B). This cohort of snails (n=20) exhibited neither learning nor LTM (one-way ANOVA followed by Tukey's *post hoc* test, P>0.05). In a third cohort of snails (n=32) trained 4 days after receiving the traumatic stress, learning and memory formation were also not observed (Fig. 5B; one-way ANOVA followed by Tukey's *post hoc* test, P>0.05). Finally, a fourth cohort of snails (n=34) that had received the traumatic stimulus 1 week previously showed learning and memory (Fig. 5B; one-way ANOVA followed by Tukey's *post hoc* test, P<0.001).

We next injected snails with a CBr antagonist (AM 251) before we applied the traumatic stimulus and then trained snails 2 days later (Fig. 6). In this cohort (Fig. 6B), learning and memory were still obstructed (one-way ANOVA followed by Tukey's *post hoc* test, P>0.05). In a second cohort, we injected the antagonist 4 h before the snails (n=14) received the traumatic stress. Again, when we trained these snails 2 days later, both learning and memory formation were obstructed (Fig. 6B; one-way ANOVA followed by Tukey's *post hoc* test, P>0.05). Finally, snails in a third cohort (n=20) were injected with the antagonist 1 h before receiving the severe traumatic stimulus, and were trained 4 days later. In this cohort of snails, learning and memory formation occurred (Fig. 6B; one-way ANOVA followed by Tukey's *post hoc* test, P<0.01). We performed control experiments with vehicle injection rather than AM 251, and found that in these control snails, learning and memory were still obstructed. Thus, AM 251 reduced the duration of the obstruction of memory formation by the traumatic stimulus.

We then determined the effect of the CBr antagonist on learning and memory formation in snails that were not subjected to the traumatic stress (Fig. 7). We first trained a cohort of snails (n=20) with the single 0.5 h training procedure, and LTM was not present

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

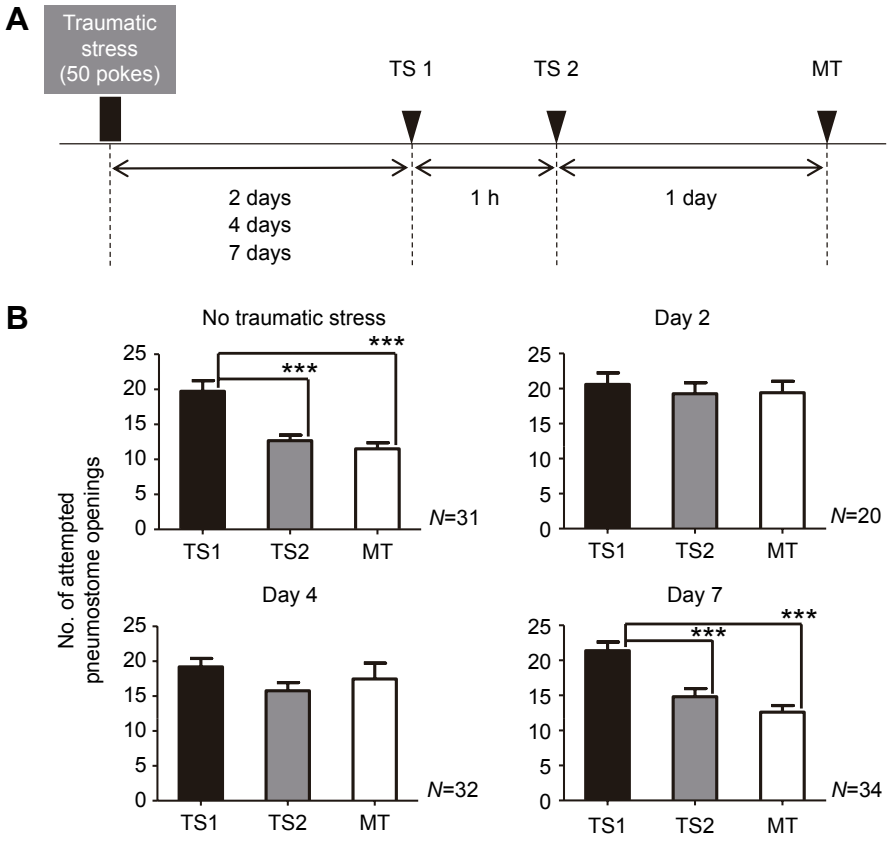

Fig. 5. Experiencing a severe stressor impedes learning and memory formation for at least 4 days. (A) Time line of the experimental procedure, showing the interval between when snails experienced the severe traumatic stressor and the initiation of operant conditioning. Four different cohorts of snails were used: one cohort did not receive the traumatic stressor, the other three cohorts did. Snails were trained (i.e. TS1 and TS2, separated by a 1 h interval) 2, 4 and 7 days after experiencing the severe traumatic stressor; LTM was tested 24 h after TS2. (B) The number of attempted pneumostome openings in each 0.5 h session. The data were analysed using a two-way rmANOVA ($F_{6,202}$=4.752, P=0.0001) followed by Tukey's *post hoc* test for multiple comparisons. For snails not exposed to the traumatic stressor (top left), the number of attempted pneumostome openings in MT was significantly lower than that in TS1 and was not significantly greater than that in TS2. Thus, the criteria for LTM were met. The number of attempted openings in TS2 was also significantly lower than that in TS1; thus, learning occurred. For snails trained 2 days (top right) or 4 days (bottom left) after experiencing the traumatic stressor, neither learning nor LTM was found to be present, as there were no significant differences in the number of attempted pneumostome openings. For snails trained 7 days after experiencing the traumatic stressor (bottom right), the number of attempted pneumostome openings in MT was significantly lower than that in TS1 and was not significantly greater than that in TS2. Moreover, the number of attempted openings in TS2 was significantly lower than that in TS1. Thus, we conclude that associative learning and LTM formation occurred. ***P<0.001.

when memory was tested 1 day later (two-tailed paired *t*-test, P>0.05; Fig. 7B, naive snails). In a second cohort of snails (n=20), we injected vehicle 1 h before training. In these snails, there was no enhancing effect on LTM formation, and these snails did not exhibit LTM (two-tailed paired *t*-test, P>0.05; Fig. 7B). Finally, in a third cohort of snails (n=24) injected with AM 251 1 h before training, LTM formation occurred 24 h (one-way ANOVA followed a Tukey's *post hoc* test, P<0.01) but not 48 h later (one-way ANOVA followed by Tukey's *post hoc* test, P>0.05; Fig. 7B).

Finally, we reanalysed all the data obtained in the first training procedures (i.e. TS1) in the three cohorts of snails shown in Fig. 7. There were no significant differences in the number of attempted pneumostome openings in TS1 of the three different cohorts, which each received a different treatment (one-way ANOVA followed by a Tukey's *post hoc* test, P>0.05). This shows that while AM 251 enhanced memory formation, it did not alter the sensitivity of the snails to the tactile stimulus used in the training procedure or the response to the hypoxic environment. Three further points need to be made here. The first is that this specific procedure and the operant conditioning procedures were performed by two different individuals, and this accounts in part for the difference in the number of attempted pneumostome openings. The second is that we also used a different batch of snails, as these experiments were performed some months after the other experiments. Finally, a different concentration of AM 251 was used in this particular set of experiments (18 µmol l^{-1} rather than 10 µmol l^{-1} used in the other experiments). Again, the concentration used here did not alter the responsiveness of snails to the tactile stimulus used.

DISCUSSION

Here, we showed that *Lymnaea* possess two G-protein-coupled receptor genes, which encode proteins closely related to well-characterized vertebrate CBrs, and these mRNAs are expressed in the *Lymnaea* CNS. The results obtained with the use of mammalian CBr agonists and antagonists suggest that cannabinoids play key roles in modulating learning and memory formation in *Lymnaea*. Further, we showed that subjecting snails to a severe traumatic tactile stressor blocks the ability of the snails to learn and form memory for at least 4 days. This obstruction of learning and memory was mimicked by application of the CBr agonist WIN 55. However, pre-treatment of snails with the CBr antagonist AM 251 decreased the effect of the severe traumatic stressor on the snails' ability to

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

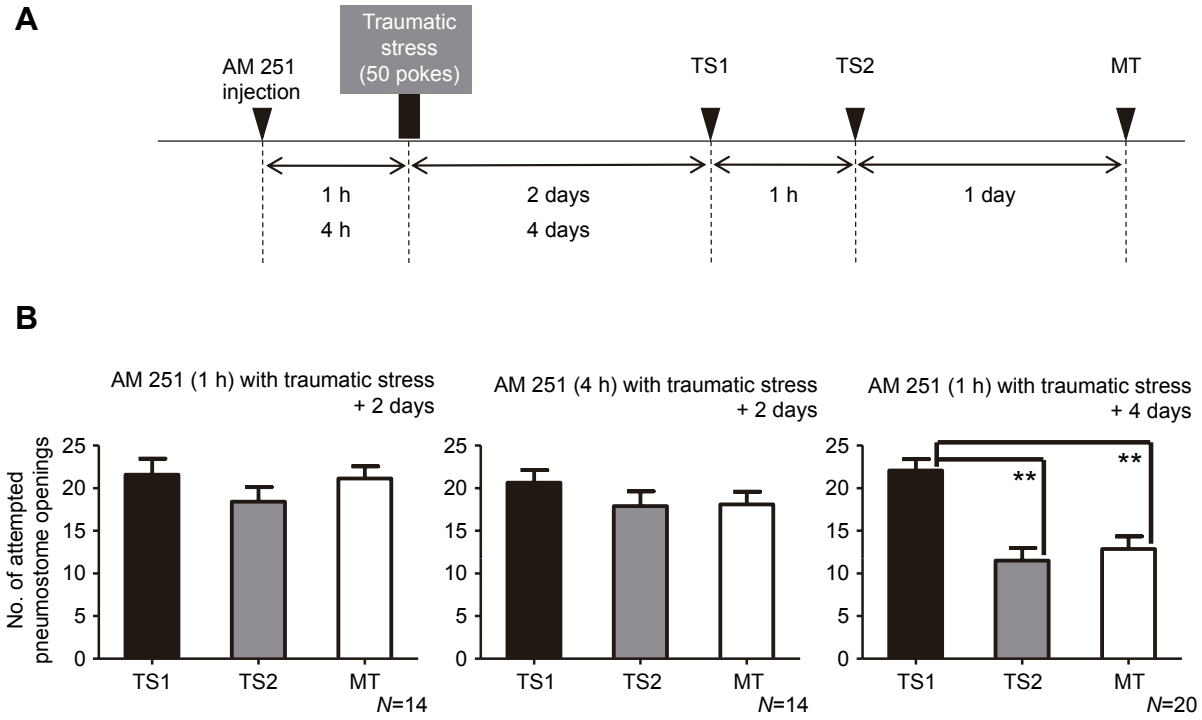

Fig. 6. AM 251 mitigates the severity of the effect of the traumatic stressor on learning and LTM formation. (A) Time line of the experimental procedure for three cohorts of snails, showing the interval (1 or 4 h) between when snails were injected with AM 251 (estimated final concentration in body, 2 µmol l^{-1}) and the onset of the severe traumatic stressor. Snails were then trained (i.e. TS1 and TS2, separated by a 1 h interval) either 2 or 4 days later; LTM was tested 24 h after TS2. (B) The number of attempted pneumostome openings in each 0.5 h session. Data were analysed using a two-way rmANOVA ($F_{4,90}$=3.262, P=0.0130) followed by Tukey's *post hoc* test for multiple comparisons. For snails injected with AM 251 1 h (left) or 4 h (middle) before experiencing the severe traumatic stressor and then trained 2 days later, neither learning nor LTM was found to be present, as there were no significant differences in the number of attempted pneumostome openings between any of the sessions. For snails injected with AM 251 1 h before experiencing the severe traumatic stressor and then trained 4 days later (right), the number of attempted pneumostome openings in MT was significantly lower than that in TS1 and was not significantly greater than that in TS2. Moreover, the number of attempted openings in TS2 was significantly lower than that in TS1. Thus, we conclude that associative learning and LTM formation occurred. **P<0.01.

learn and remember. That is, the duration of the impact of the applied stressor was shortened. Finally, injection of AM 251 into snails led to enhancement of LTM formation, suggesting that there is an ongoing tonic suppression on the neuronal circuits that mediate LTM formation.

In the present study, we first identified two *Lymnaea* cDNAs encoding CBr-like G-protein-coupled receptors with structurally distinct third cytoplasmic loops. To our knowledge, this is the first report of G-protein-coupled receptor genes closely related to the mammalian CBrs in molluscs. Interestingly, there were tissue-specific differences of the expression patterns of *Lymnaea* CBr-like 1 and 2 mRNA. *Lymnaea* CBr-like 1 mRNA was ubiquitously transcribed, whereas *Lymnaea* CBr-like 2 mRNA was specifically expressed in the CNS (Fig. 2). As shown in Fig. 1B, two subtypes of CBrs, termed CB_1 and CB_2, have been identified in mammals, and the tissue specificities of these receptors have already been reported. CB_1 is expressed mainly in the brain, but also in the lung, liver and kidney (Gérard et al., 1991); and CB_2 is expressed mainly in the immune system and haematopoietic cells (Pacher and Mechoulam, 2011). The CBr-like genes of *Lymnaea* were widely expressed in the whole body, suggesting that the cannabinoid system functions in the various tissues in *Lymnaea* as it does in mammals. Although we did not examine the pharmacological properties of the gene products of *Lymnaea* CBr-like genes and their fine distribution in the *Lymnaea* CNS, we believe that they are strong candidates for targets of WIN 55 and AM 251 in the *Lymnaea* CNS. In addition to *Lymnaea*, our *in silico* analyses also indicated that similar CBr-like genes exist in three other molluscan species (*A. californica*, *B. glabrata* and *L. gigantea*) and a brachiopod species (*L. anatine*). A previous report in *Aplysia* showing that Δ^9-tetrahydrocannabinol caused a depression in nerve cell excitability is consistent with the CBr-like genes being present in *Aplysia* (Acosta-Urquidi and Chase, 1975). The existence of the CBrs in another lophotrochozoan species, the leech (*H. medicinalis*), has previously been both physiologically and pharmacologically demonstrated (Li and Burrell, 2009).

Application of both the CBr antagonist AM 251 and 2-AG, the inhibitor of diacylglycerol lipase (which is necessary for the synthesis of the cannabinoid transmitter) blocked a change in synaptic plasticity in the leech (Li and Burrell, 2009). However, bath application of 2-AG and the CBr agonist CP55 940 induced a change in synaptic plasticity (Li and Burrell, 2009). It is unclear how the behaviour of the leech was altered by the induced changes in synaptic plasticity via CBr. In addition, Stefano et al. (1997) reported a partial cDNA sequence of the leech CBr (Stefano et al., 1997) but its deduced protein sequence partially resembles the mammalian CBr and adrenocorticotropic hormone receptor, and it was thought to be an artifact of PCR contamination (Elphick, 1998). Therefore, it is still unclear whether the leech has a complete set of genes involved in endocannabinoid signalling. CBr-like genes were also found in genomes of the tunicate chordates and lancelets (*C. intestinalis* and *B. floridae*; Elphick et al., 2003; Elphick, 2007), but their pharmacological properties have not yet been investigated. Further investigations involving identification and pharmacological characterization of invertebrate CBr-like proteins are required to

Journal of Experimental Biology (2017) 220, 3026-3038 doi:10.1242/jeb.159038

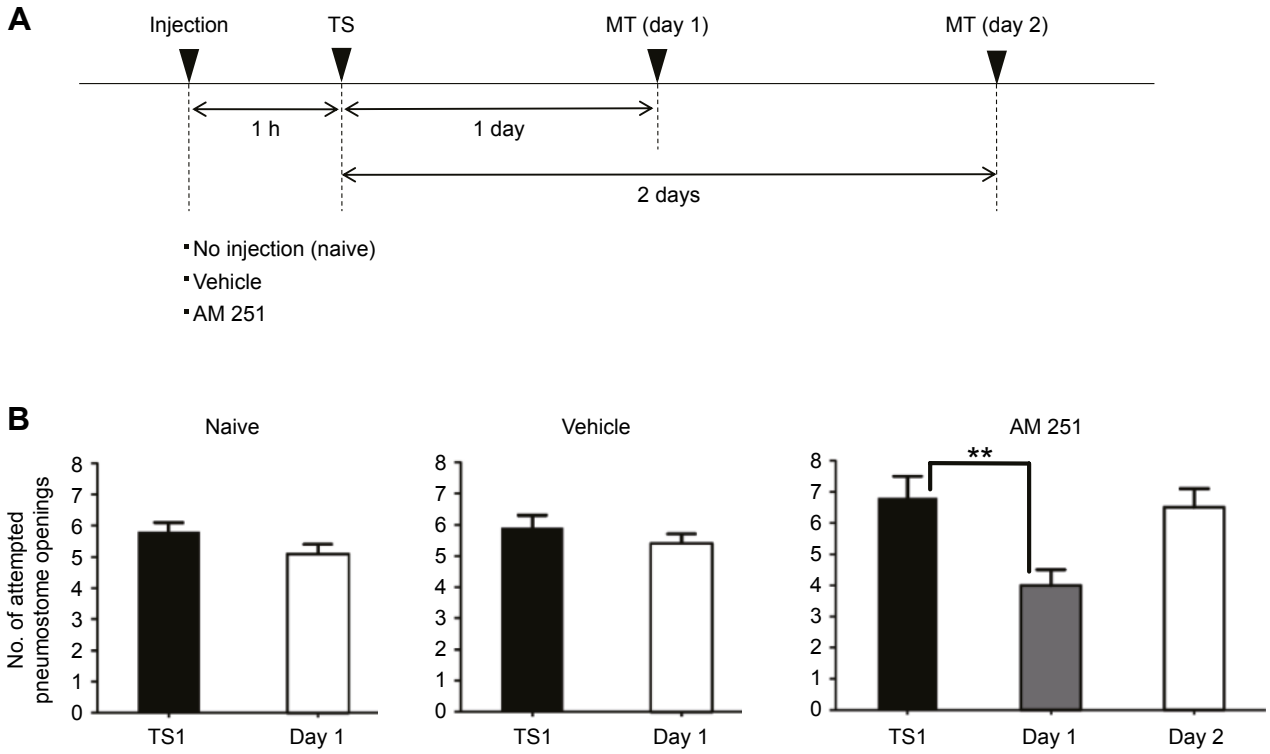

Fig. 7. AM 251 enhances LTM formation. (A) Time line of the experimental procedure, showing the interval between when snails were injected with AM 251 and operant conditioning (single 0.5 h training session). Three different cohorts of snails were used. (B) The number of attempted pneumostome openings. Data were analysed using a two-way rmANOVA ($F_{2,48}$=5.789, P=0.056) followed by Tukey's *post hoc* test for multiple comparisons. For snails (n=20) that received no injection (naive), the number of attempted pneumostome openings 1 day after training (MT) was not significantly lower than that in TS1 (left). Thus, memory was not formed. For snails that were injected with vehicle (0.1% DMSO/0.1% Tween 80) 1 h before training, and then tested 1 day later, MT was not significantly lower than that in TS1 (middle). Thus, memory was not formed. For snails (n=24) that were injected with AM 251 (18 µmol l^{-1}) 1 h before training, followed by a memory test either 1 or 2 days later (n=12 each), LTM was present after 24 h, as the number of attempted pneumostome openings for day 1 MT was significantly lower than that for TS1 (right). However, there was no significant difference in the number of attempted openings between the day 2 response and TS1; thus, memory was not present in the cohort tested 2 days following TS1. **P<0.01.

understand molecular evolution and diversification of the endocannabinoid system in invertebrates.

In the mammalian nervous system, CBrs are mainly found on the presynaptic terminals of central and peripheral neurons, where they modulate the release of different excitatory and inhibitory neurotransmitters, which include glutamate and gamma-aminobutyric acid (GABA), respectively, as well as many other transmitter types (Slanina and Schweitzer, 2005; Roberto et al., 2010; Katona and Freund, 2012; Wamsteeker-Cusulin et al., 2014). In *Lymnaea*, whether the putative CBrs are also primarily localized to pre-synaptic sites remains to be determined, and is the subject of ongoing experimentation. However, the data to date from numerous studies show that CBrs are indispensable for various forms of synaptic plasticity that are thought to play key roles in learning and memory formation (Castillo et al., 2012; Lee et al., 2015).

In mammals, the activation of the endocannabinoid system in many cases suppresses the formation of both working memory and LTM, whereas both forms of the memory are enhanced when the endocannabinoid system is inhibited (Puighermanal et al., 2012). It must be kept in mind, however, that often contradictory results are obtained when CBr agonists or antagonists are used. This may be dependent on the emotional state of the animal when the stressor is applied at the time of training. The arousal level (think of the Yerkes–Dodson/Hebb curve) of the animal is a key factor in determining the effects of the endocannabinoid system on memory. For example, in rodents, WIN 55 administration enhanced long-term object recognition memory when they were trained under a high arousal condition, but WIN 55 was ineffective with low-arousal training (Campolongo et al., 2013; Basavarajappa et al., 2014). Thus, WIN 55 enhanced long-term object recognition memory only when trained under high arousal conditions (O'Shea et al., 2004; Morena and Campolongo, 2014; Wamsteeker-Cusulin et al., 2014). Whether a similar arousal-dependent situation exists in *Lymnaea* will be tested in the future.

We know in *Lymnaea* that different stressors tweak the ability to learn and form LTM (Lukowiak et al., 2014a). Some stressors (e.g. predator detection) lead to enhanced memory formation (Orr and Lukowiak, 2008), whereas other stressors (e.g. crowding) lead to suppression of memory formation (de Caigny and Lukowiak, 2008). In addition, a combination of stressors (e.g. crowding and low environmental calcium) leads to complete obstruction of learning and all forms of memory (i.e. short, intermediate and long-term memory) (Dalesman et al., 2011). It is unclear at present how the stressors actually cause memory formation to be enhanced or suppressed. However, at least for enhanced memory formation following exposure to certain stressors (e.g. predator detection) and bioactive agents (e.g. methamphetamine), epigenetic changes (i.e. DNA methylation) have been shown to play a necessary role (Lukowiak et al., 2014b). From the data obtained here, we are confident that CBrs play a key role in both modulating learning and the subsequent formation of memory as the result of snails experiencing different stressors.

Here, we showed that snails lose their ability to learn and form memory following exposure to the severe traumatic stressor. The inability to learn and form memory persists for at least 4 days and we found that snails regained their ability to learn and form LTM 1 week after the traumatic event. We mimicked this traumatic effect by injecting the CBr agonist WIN 55 into the snails, and we saw a similar time course for the snails' inability to learn and form memory. Consistent with our hypothesis that putative CBrs play a key role in the inability to learn and form memory after a traumatic event, we found that if we injected a CBr antagonist (i.e. AM 251) before the snails received the traumatic stimulus, the effect of the trauma on learning and memory was reduced. Finally, again consistent with our hypothesis that putative CBrs play a key role in memory formation, we found that injection of AM 251 into snails (i.e. untrained and not traumatized) enhanced their ability to form LTM.

In our previous investigations on the effect of different stressors on LTM formation, we have not seen such long-lasting effects as we saw here following either presentation of the severe traumatic stressor or injection of WIN 55. Typically, the induced modification persists for only a few hours. For example, experiencing a low calcium environment blocks LTM formation (Dalesman et al., 2011). However, even after snails experienced the low calcium environment for 1 week, it only took 1 h in the normal calcium environment to relieve the suppressive effect on LTM formation. We also showed that predator detection enhances LTM formation (Orr and Lukowiak, 2008). Again, however, snails had to be trained immediately after predator detection; a 1 h interval between detection of the predator and training in a predator-free environment was sufficient to negate the enhancing effects of predator detection on LTM formation. In contrast, here the effects of the traumatic experience were long lasting (4 days) as were the effects of WIN 55 injection. A working hypothesis to explain these findings is that the traumatic experience and/or the injection of WIN 55 causes an extreme fear state that is incompatible with learning and memory, as Ruehle et al. (2012) pointed out the endocannabinoid system regulates the establishment and maintenance of the fear state.

We conclude that the endocannabinoid system normally plays a role in learning and memory formation when some level of stress is involved in the snails. Neither WIN 55 nor AM 251 injection had a significant effect on homeostatic aerial respiratory behaviour. This suggests to us that the central terminations of the peripheral sensory neurons that drive this behaviour (Lukowiak et al., 1996; Karnik et al., 2012) via the 3-neuron central pattern generator (Syed et al., 1990, 1992) are not affected by the endocannabinoid agonists or antagonists. Our data also suggest that the tactile sensory neurons (Steffensen et al., 1995; Inoue et al., 1996) that convey mechanosensory information are also not affected by the agonists or antagonists. If these sensory neurons were affected, we would expect to see differences in the number of pokes delivered to the pneumostome during the operant conditioning procedure, when snails received either the agonist or antagonist.

We did not expect that injection of AM 251 into snails would cause enhancement of memory formation. This finding suggests that there may be a tonic inhibitory effect of the putative CBrs on the ability of snails to form LTM. Suppression of memory formation may occur to 'keep down the cost' of making memories that are of too little consequence for the snail. Data complementary to this idea have been obtained in rodent work previously (Takahashi et al., 2005; Terranova et al., 1996; Robinson et al., 2008). In those studies, blockade of the CBrs produces memory-enhancing effects on spatial and associative memory tasks. In addition, in birds, similar data were obtained with a slight twist (Shiflett et al., 2004), in that there was a 'cost' to the improved memory as a result of blocking the CBrs. This cost is that newly formed memories may be blocked from forming as a result of proactive interference. It may be that this notion will be more easily elucidated at the neuronal level in the *Lymnaea* model system. Further experimentation will be necessary to establish whether this is the correct hypothesis. It could be that stressors or bioactive agents that cause enhancement of LTM formation in *Lymnaea* do so via the putative CBrs. The present results will enable us to better understand how the endocannabinoid system is involved in learning acquisition and memory formation, and these results will also reveal how to control the traumatic stress effect.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Investigation: H.S., T.W., D.H., S.L., J.F.; Data curation: H.S., M.S., E.I., K.L.; Writing - original draft: H.S., T.W., D.H.; Writing - review & editing: M.S., E.I., K.L.; Funding acquisition: E.I., K.L.

Funding

This work was supported by a grant from Canadian Institutes of Health Research (MOP 64339) and Natural Sciences and Engineering Research Council of Canada (227993-2013) to K.L. and KAKENHI from Japan Society for the Promotion of Science (24657055 and 25291074) and Waseda University grants for Specific Research Projects in Japan (2016B-068, 2016B-069 and 2016S-037) to E.I.

Data availability

Transcriptome shotgun assembly contigs for *Lymnaea* CBr-like 1 (FX183817) and CBr-like 2 (FX186161) and full-length cDNAs for *Lymnaea* CBr-like 1 (LC093511) and CBr-like 2 (LC093512) have been deposited in GenBank.

References

Acosta-Urquidi, J. and Chase, R. (1975). The effects of delta9-tetrahydrocannabinol on action potentials in the mollusc *Aplysia*. *Can. J. Physiol. Pharmacol.* **53**, 793-798.

Aonuma, H., Kaneda, M., Hatakeyama, D., Watanabe, T., Lukowiak, K. and Ito, E. (2016). Relationship between the grades of a learned aversive-feeding response and the dopamine contents in *Lymnaea*. *Biol. Open* **5**, 1869-1873.

Aonuma, H., Kaneda, M., Hatakeyama, D., Watanabe, T., Lukowiak, K. and Ito, E. (2017). Weak involvement of octopamine in aversive taste learning in a snail. *Neurobiol. Learn. Mem.* **141**, 189-198.

Atsak, P., Roozendaal, B. and Campolongo, P. (2012). Role of the endocannabinoid system in regulating glucocorticoid effects on memory for emotional experiences. *Neuroscience* **204**, 104-116.

Bacon, F. (1620). The New Organon (Bobbs-Merrill)./Bacon, F. (1960). *The New Organon and Related Writings* (ed. F. H. Anderson). Indianapolis, IN: Bobbs-Merrill.

Basavarajappa, B. S., Nagre, N. N., Xie, S. and Subbanna, S. (2014). Elevation of endogenous anandamide impairs LTP, learning and memory through CB1 receptor signaling in mice. *Hippocampus* **24**, 808-818.

Campolongo, P., Roozendaal, B., Trezza, V., Hauer, D., Schelling, G., McGaugh, J. L. and Cuomo, V. (2009). Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. *Proc. Natl. Acad. Sci. USA* **106**, 4888-4893.

Campolongo, P., Morena, M., Scaccianoce, S., Trezza, V., Chiarotti, F., Schelling, G., Cuomo, V. and Roozendaal, B. (2013). Novelty-induced emotional arousal modulates cannabinoid effects on recognition memory and adrenocortical activity. *Neuropsychopharmacology* **38**, 1276-1286.

Castillo, P. E., Younts, T. J., Chávez, A. E. and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron* **76**, 70-81.

Dalesman, S. and Lukowiak, K. (2010). Effect of acute exposure to low environmental calcium on respiration and locomotion in *Lymnaea stagnalis* (L.). *J. Exp. Biol.* **213**, 1471-1476.

Dalesman, S. and Lukowiak, K. (2012). How stress alters memory in 'smart' snails. *PLoS ONE* **7**, e32334.

Dalesman, S., Braun, M. H. and Lukowiak, K. (2011). Low environmental calcium blocks long-term memory formation in a freshwater pulmonate snail. *Neurobiol. Learn. Mem.* **95**, 393-403.

Dalesman, S., Sunada, H., Teskey, M. L. and Lukowiak, K. (2013). Combining stressors that individually impede long-term memory blocks all memory processes. *PLoS ONE* **8**, e79561.
de Caigny, P. and Lukowiak, K. (2008). Crowding, an environmental stressor, blocks long-term memory formation in *Lymnaea*. *J. Exp. Biol.* **211**, 2678-2688.
Elphick, M. R. (1998). An invertebrate G-protein coupled receptor is a chimeric cannabinoid/melanocortin receptor. *Brain Res.* **780**, 170-173.
Elphick, M. R. (2007). BfCBR: a cannabinoid receptor ortholog in the cephalochordate *Branchiostoma floridae* (Amphioxus). *Gene* **399**, 65-71.
Elphick, M. R. (2012). The evolution and comparative neurobiology of endocannabinoid signalling. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **367**, 3201-3215.
Elphick, M. R., Satou, Y. and Satoh, N. (2003). The invertebrate ancestry of endocannabinoid signalling: an orthologue of vertebrate cannabinoid receptors in the urochordate *Ciona intestinalis*. *Gene* **302**, 95-101.
Gérard, C. M., Mollereau, C., Vassart, G. and Parmentier, M. (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem. J.* **279**, 129-134.
Goodman, J. and Packard, M. G. (2015). The influence of cannabinoids on learning and memory processes of the dorsal striatum. *Neurobiol. Learn. Mem.* **125**, 1-14.
Hatakeyama, D., Okuta, A., Otsuka, E., Lukowiak, K. and Ito, E. (2013). Consolidation of long-term memory by insulin in *Lymnaea* is not brought about by changing the number of insulin receptors. *Commun. Integr. Biol.* **6**, e23955.
Hauer, D., Ratano, P., Morena, M., Scaccianoce, S., Briegel, I., Palmery, M., Cuomo, V., Roozendaal, B., Schelling, G. and Campolongo, P. (2011). Propofol enhances memory formation via an interaction with the endocannabinoid system. *Anesthesiology* **114**, 1380-1388.
Hebb, D. O. (1955). Drives and the C.N.S. (conceptual nervous system). *Psychol. Rev.* **62**, 243-254.
Inoue, T., Tasasaki, M., Lukowiak, K. and Syed, N. (1996). Identification of a putative mechanosensory neuron in *Lymnaea*: Characterization of its synaptic and functional connections with the whole-body withdrawal interneuron. *J. Neurophysiol.* **76**, 3230-3238.
Ito, E., Yamagishi, M., Takigami, S., Sakakibara, M., Fujito, Y. and Lukowiak, K. (2015). The Yerkes-Dodson law and appropriate stimuli for conditioned taste aversion in *Lymnaea*. *J. Exp. Biol.* **218**, 336-339.
Karnik, V., Braun, M., Dalesman, S. and Lukowiak, K. (2012). Sensory input from the osphradium modulates the response to memory-enhancing stressors in *Lymnaea stagnalis*. *J. Exp. Biol.* **215**, 536-542.
Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772-780.
Katona, I. and Freund, T. F. (2012). Multiple functions of endocannabinoid signaling in the brain. *Annu. Rev. Neurosci.* **35**, 529-558.
Lee, S.-H., Ledri, M., Tóth, B., Marchionni, I., Henstridge, C. M., Dudok, B., Kenesei, K., Barna, L., Szabó, S. I., Renkecz, T. et al. (2015). Multiple forms of endocannabinoid and endovanilloid signaling regulate the tonic control of GABA release. *J. Neurosci.* **35**, 10039-10057.
Lemak, M. S., Bravarenko, N. I., Bobrov, M. Y., Bezuglov, V. V., Ierusalimsky, V. N., Storozhuk, M. V., Malyshev, A. Y. Balaban, P. M. (2007). Cannabinoid regulation in identified synapse of terrestrial snail. *Eur. J. Neurosci.* **26**, 3207-3214.
Li, Q. and Burrell, B. D. (2009). Two forms of long-term depression in a polysynaptic pathway in the leech CNS: one NMDA receptor-dependent and the other cannabinoid-dependent. *J. Comp. Physiol. A* **195**, 831-841.
Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-$\Delta\Delta C_T$ method. *Methods* **25**, 402-408.
Lukowiak, K., Ringseis, E., Spencer, G., Wildering, W. and Syed, N. (1996). Operant conditioning of aerial respiratory behaviour in *Lymnaea*. *J. Exp. Biol.* **199**, 683-691.
Lukowiak, K., Adatia, N., Krygier, D. and Syed, N. (2000). Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. *Learn. Mem.* **7**, 140-150.
Lukowiak, K., Orr, M., de Caigny, P., Lukowiak, K. S., Rosenegger, D., Il-Han, J. and Dalesman, S. (2010). Ecologically relevant stressors modify long-term memory formation in a model system. *Behav. Brain Res.* **214**, 18-24.
Lukowiak, K., Sunada, H., Teskey, M. L., Lukowiak, K. and Dalesman, S. (2014a). Environmentally relevant stressors alter memory formation in the pond snail *Lymnaea*. *J. Exp. Biol.* **217**, 76-83.
Lukowiak, K., Heckler, B., Bennett, T. E., Schriner, E. K., Wyrick, K., Jewett, C., Todd, R. P. and Sorg, B. A. (2014b). Enhanced memory persistence is blocked by a DNA methyltransferase inhibitor in the snail *Lymnaea stagnalis*. *J. Exp. Biol.* **217**, 2920-2929.
Matias, I., Bisogno, T., Melck, D., Vandenbulcke, F., Verger-Bocquet, M., De Petrocellis, L., Sergheraert, C., Breton, C., Di Marzo, V. and Salzet, M. (2001). Evidence for an endocannabinoid system in the central nervous system of the leech *Hirudo medicinalis*. *Mol. Brain Res.* **87**, 145-159.
McPartland, J. M. (2004). Phylogenomic and chemotaxonomic analysis of the endocannabinoid system. *Brain Res. Brain Res. Rev.* **45**, 18-29.
McPartland, J. M. and Glass, M. (2003). Functional mapping of cannabinoid receptor homologs in mammals, other vertebrates, and invertebrates. *Gene* **312**, 297-303.
McPartland, J., Di Marzo, V., De Petrocellis, L., Mercer, A. and Glass, M. (2001). Cannabinoid receptors are absent in insects. *J. Comp. Neurol.* **436**, 423-429.
Mechoulam, R. and Parker, L. A. (2013). The endocannabinoid system and the brain. *Annu. Rev. Psychol.* **64**, 21-47.
Morena, M. and Campolongo, P. (2014). The endocannabinoid system: an emotional buffer in the modulation of memory function. *Neurobiol. Learn. Mem.* **112**, 30-43.
Oakes, M. D., Law, W. J., Clark, T., Bamber, B. A. and Komuniecki, R. (2017). Cannabinoids activate monoaminergic signaling to modulate key *C. elegans* behaviors. *J. Neurosci.* **37**, 2859-2869.
Orr, M. V. and Lukowiak, K. (2008). Electrophysiological and behavioral evidence demonstrating that predator detection alters adaptive behaviors in the snail *Lymnaea*. *J. Neurosci.* **28**, 2726-2734.
Orr, M., Hittel, K., Lukowiak, K. S., Han, J. and Lukowiak, K. (2009). Differences in LTM-forming capability between geographically different strains of Alberta *Lymnaea stagnalis* are maintained whether they are trained in the lab or in the wild. *J. Exp. Biol.* **212**, 3911-3918.
O'Shea, M., Singh, M. E., McGregor, I. S. and Mallet, P. E. (2004). Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. *J. Psychopharmacol.* **18**, 502-508.
Pacher, P. and Mechoulam, R. (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Prog. Lipid Res.* **50**, 193-211.
Puighermanal, E., Busquets-Garcia, A., Maldonado, R. and Ozaita, A. (2012). Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids. *Philos. Trans. R. Soc. B* **367**, 3254-3263.
Roberto, M., Cruz, M., Bajo, M., Siggins, G. R., Parsons, L. H. and Schweitzer, P. (2010). The endocannabinoid system tonically regulates inhibitory transmission and depresses the effect of ethanol in central amygdala. *Neuropsychopharmacology* **35**, 1962-1972.
Robinson, L., McKillop-Smith, S., Ross, N. L., Pertwee, R. G., Hampson, R. E., Platt, B. and Riedel, G. (2008). Hippocampal endocannabinoids inhibit spatial learning and limit spatial memory in rats. *Psychopharmacology (Berl.)* **198**, 551-563.
Ruehle, S., Rey, A. A., Remmers, F. and Lutz, B. (2012). The endocannabinoid system in anxiety, fear memory and habituation. *J. Psychopharmacol.* **26**, 23-39.
Sadamoto, H., Takahashi, H., Okada, T., Kenmoku, H., Toyota, M. and Asakawa, Y. (2012). De novo sequencing and transcriptome analysis of the central nervous system of mollusc *Lymnaea stagnalis* by deep RNA sequencing. *PLoS ONE* **7**, e42546.
Salzet, M. and Stefano, G. B. (2002). The endocannabinoid system in invertebrates. *Prostaglandins Leukot. Essent. Fatty Acids* **66**, 353-361.
Sangha, S., Scheibenstock, A. and Lukowiak, K. (2003a). Reconsolidation of a long-term memory in *Lymnaea* requires new protein and RNA synthesis and the soma of right pedal dorsal 1. *J. Neurosci.* **23**, 8034-8040.
Sangha, S., Scheibenstock, A., Morrow, R. and Lukowiak, K. (2003b). Extinction requires new RNA and protein synthesis and the soma of the cell RPeD1 in *Lymnaea stagnalis*. *J. Neurosci.* **23**, 9842-9851.
Sangha, S., Scheibenstock, A., Martens, K., Varshney, N., Cooke, R. and Lukowiak, K. (2005). Impairing forgetting by preventing new learning and memory. *Behav. Neurosci.* **119**, 787-796.
Scheibenstock, A., Krygier, D., Haque, Z., Syed, N. and Lukowiak, K. (2002). The soma of RPeD1 must be present for LTM formation of associative learning in *Lymnaea*. *J. Neurophysiol.* **88**, 1584-1591.
Shiflett, M. W., Rankin, A. Z., Tomaszycki, M. L. and DeVoogd, T. J. (2004). Cannabinoid inhibition improves memory in food-storing birds, but with a cost. *Proc. Biol. Sci.* **271**, 2043-2048.
Slanina, K. A. and Schweitzer, P. (2005). Inhibition of cyclooxygenase-2 elicits a CB1-mediated decrease of excitatory transmission in rat CA1 hippocampus. *Neuropharmacology* **49**, 653-659.
Stefano, G. B., Salzet, B. and Salzet, M. (1997). Identification and characterization of the leech CNS cannabinoid receptor: coupling to nitric oxide release. *Brain Res.* **753**, 219-224.
Steffensen, I., Syed, N. I., Lukowiak, K., Bulloch, A. G. M. and Morris, C. E. (1995). Sensorin-A immunocytochemistry reveals putative mechanosensory neurons in *Lymnaea* CNS. *Invert. Neurosci.* **1**, 207-213.
Sunada, H., Lukowiak, K. and Sakakibara, M. (2012). Repetitive noxious stimulus altered the shadow-induced withdrawal behavior in *Lymnaea*. *Acta Biol. Hung.* **63** Suppl. 2, 179-189.
Sunada, H., Lukowiak, K. and Ito, E. (2017). Cerebral giant cells are necessary for the formation and recall of memory of conditioned taste aversion in *Lymnaea*. *Zool. Sci.* **34**, 72-80.
Syed, N. I., Bulloch, A. G. M. and Lukowiak, K. (1990). In vitro reconstruction of the respiratory central pattern generator (CPG) of the pond snail *Lymnaea*. *Science* **250**, 282-285.
Syed, N. I., Ridgway, R. L., Lukowiak, K. and Bulloch, A. G. M. (1992). Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. *Neuron* **8**, 767-774.

Takahashi, R. N., Pamplona, F. A. and Fernandes, M. S. (2005). The cannabinoid antagonist SR141716A facilitates memory acquisition and consolidation in the mouse elevated T-maze. *Neurosci. Lett.* **380**, 270-275.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731-2739.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725-2729.

Tan, H., Ahmad, T., Loureiro, M., Zunder, J. and Laviolette, S. (2014). The role of cannabinoid transmission in emotional memory formation: implications for addiction and schizophrenia. *Front. Psychiatry* **5**, 1-12.

Terranova, J.-P., Storme, J.-J., Lafon, N., Pério, A., Rinaldi-Carmona, M., Le Fur, G. and Soubrié, P. (1996). Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. *Psychopharmacology (Berl.)* **126**, 165-172.

Teskey, M. L., Lukowiak, K. S., Riaz, H., Dalesman, S. and Lukowiak, K. (2012). What's hot: the enhancing effects of thermal stress on long-term memory formation in *Lymnaea stagnalis*. *J. Exp. Biol.* **215**, 4322-4329.

van Nierop, P., Bertrand, S., Munno, D. W., Gouwenberg, Y., van Minnen, J., Spafford, J. D., Syed, N. I., Bertrand, D. and Smit, A. B. (2006). Identification and functional expression of a family of nicotinic acetylcholine receptor subunits in the central nervous system of the mollusc *Lymnaea stagnalis*. *J. Biol. Chem.* **281**, 1680-1691.

Wamsteeker-Cusulin, J. I., Senst, L., Teskey, G. C. and Bains, J. S. (2014). Experience salience gates endocannabinoid signaling at hypothalamic synapses. *J. Neurosci.* **34**, 6177-6181.

Wistrand, M., Käll, L. and Sonnhammer, E. L. L. (2006). A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Protein Sci.* **15**, 509-521.

Wolf, S. and Grünewald, S. (2015). Sequence, structure and ligand binding evolution of rhodopsin-like G protein-coupled receptors: a crystal structure-based phylogenetic analysis. *PLoS ONE* **10**, e0123533.

Yamagishi, M., Watanabe, T., Hatakeyama, D. and Ito, E. (2015). Effects of serotonin on the heartbeat of pond snails in a hunger state. *Biophysics (Nagoya)* **11**, 1-5.

Yerkes, R. M. and Dodson, J. D. (1908). The relation of strength of stimulus to rapidity of habit-formation. *J. Comp. Neurol. Psychol.* **18**, 459-482.

Journal of Experimental Biology