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RESEARCH ARTICLE

Aversive operant conditioning alters the phototactic orientation of the marbled crayfish

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ABSTRACT

Aversive learning was applied to affect the phototactic behaviour of the marbled crayfish. Animals initially showed negative phototaxis to white light and positive taxis to blue light. Using an aversive learning paradigm, we investigated the plasticity of innate behaviour following operant conditioning. The initial rate of choosing a blue-lit exit was analysed by a dual choice experiment between blue-lit and white-lit exits in pre-test conditions. During training, electrical shocks were applied to the animals when they oriented to the blue-lit exit. Memory tests were given to analyse the orientation rate to the blue-lit exit in trials 1 and 24 h after training and these rates were compared with the pre-test. In general, animals avoided the blue-lit exit in the memory tests. When training was carried out three times, the long-term memory was retained for at least 48 h, although a single bout of training was also enough to form a long-term memory. Cooling animals at 4°C or injection of cycloheximide immediately after training altered the formation of long-term memory, but had no effect on short-term memory formation. Administration of the adenylate cyclase inhibitor SQ22536, the PKA inhibitor H89 or the CREB inhibitor KG-501 immediately after training also blocked the formation of long-term memory, but had no effect on short-term memory formation. Thus, our pharmacological behavioural analyses showed that new protein synthesis was necessary to form long-term memories and that the cAMP/PKA/CREB pathway is the main signal cascade for long-term memory formation in the marbled crayfish.

KEY WORDS: CREB, PKA, Learning, Long-term memory, Taxis

INTRODUCTION

As with vertebrates, many invertebrate animals have been recognised as having the ability to learn. Habituation is a well-known form of non-associative learning; the *Aplysia* siphon withdrawal reflex (Kandel, 2001, 2009), the proboscis extension response of honeybees (Hammer and Menzel, 1998) and the lateral giant (LG) mediated tail flip of the crayfish (Krasne and Woodsmall, 1969; Nagayama and Araki, 2015) have been well studied both behaviourally and physiologically. The learning process by which animals associate external predictors with important outcomes is called classical conditioning, while the learning process that arises from the consequences of their own behaviour is called operant conditioning. Studies of both types of associated learning have been carried out in *Aplysia* (Walters et al., 1979; Hawkins et al., 1983; Buonomano and Byrne, 1990; Brembs et al., 2002; Baxter and Byrne, 2018), pond snail (Lukowiak et al., 1996, 2003; Kemenes et al., 2011; Dong and Feng, 2017), *Drosophila* (Putz and Heisenberg, 2002; Guven-Ozkan and Davis, 2018), honeybees (Giurfa and Sandoz, 2020), crickets (Matsumoto et al., 2017), crabs (Abramson and Feinman, 1987, 1990; Kaczer and Maldonado, 2009; Magee and Elwood, 2013; Klappenbach et al., 2017), lobsters (Tomina and Takahata, 2010) and crayfish (Tierney and Andrews, 2013; Takahashi and Takahata, 2017; Johnson and Crane, 2018). Invertebrate animals have a simpler central nervous system than vertebrates, with fewer neurones. Many of the neurones are uniquely identifiable in invertebrates; these make the search for neural mechanisms more accessible and can suggest approaches for elucidating the activity associated with learning (Brembs, 2003).

Invertebrates, especially arthropods, frequently show stereotyped behaviours generated by innate motor programmes. For example, taxis is a basic form of orientation and an essential element of innate behaviour. Various arthropods show either positive or negative phototactic behaviours depending on their internal states, the environmental signals or both (Menzel and Greggers, 1985; Shirley and Shirley, 1988; Julian and Gronenberg, 2002; Ziegler et al., 2010; Lone et al., 2012). The marbled crayfish, *Procambarus virginalis* Lyko 2017 (Vogt et al., 2015), is a parthenogenetic crayfish (Scholtz et al., 2003; Martin et al., 2007). Marbled crayfish show negative phototaxis to white light during the day (Shiratori et al., 2017). When using a T-maze with a white-lit exit on one side and an unlit exit on the other, animals prefer the unlit exit. Furthermore, the marbled crayfish shows a positive phototaxis to blue light (C. Shiratori, unpublished data), similar to *Argulus japonicus*, a fish ectoparasite crustacean (Yoshizawa and Nogami, 2008). Thus, it could be readily assumed that the marbled crayfish would prefer a blue-lit exit if a dual choice experiment offers a choice between a blue-lit or white-lit exit. Does this phototactic behaviour reverse when aversive stimuli such as electrical shocks are applied to animals when they orient themselves to the blue-lit exit? If animals choose the white-lit exit after receiving electrical shocks, then this would confirm that innate phototactic behaviour can be modified by aversive operant conditioning. We investigated this possibility using an aversive learning paradigm with the application of electrical shocks. Because marbled crayfish produce genetically uniform clones, they are suitable animals for studying this learning process.

Learning processes can be stored as memories, and both short- and long-term memory have been described as storage mechanisms. It is generally accepted that the short- and long-term memory are defined as follows: short-term memory lasts minutes and involves covalent modifications of pre-existing proteins, whereas long-term memory lasts days, weeks or even longer, and requires gene expression, new mRNA and protein synthesis (Hawkins et al., 2006). After acquisition, the memory consolidation phase and the

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cascade of intracellular events subserving consolidation are highly conserved across animals. Cyclic AMP/PKA/CREB pathways are thought to be essential for long-term memory formation in *Aplysia* (Hawkins et al., 2006; Kandel, 2012), *Drosophila* (Davis, 2011) and honeybees (Eisenhardt, 2018). Here, we show that the marbled crayfish can form both short-term and long-term memories and examine the signal cascades required for long-term memory formation by studying pharmacological behaviour.

MATERIALS AND METHODS

Animals

Marbled crayfish were obtained from a laboratory population in our department. All individuals were the offspring of three mothers, which we acquired in 2010 from Prof. Tochinai's laboratory at Hokkaido University, Japan.

Marbled crayfish with a body length of 4–6 cm from rostrum to telson were used for all experiments. They were each maintained in separate opaque containers of 23×18×8.5 cm (length×width×height) with a 5 cm water depth under a 12 h:12 h (06:00 h to 18:00 h) light:dark cycle at a room temperature of approximately 23°C. Each animal was fed equal amounts of small food pellets (Kyorinn, Japan) once a week. Animals that moulted in the week before experiments or those in the process of egg-laying were not used for this study.

Plus-maze and electrical shocks

Experimental trials were carried out in dimly red-lit (LED light: LEE-S2026R, KHK Co., Japan) dark rooms during the day, from 09:00 to 16:00 h. The plus-maze was constructed from a transparent acrylic cube (Fig. 1). The plus-maze consisted of two entrance arms and two side arms of 250 mm in length, 50 mm in width and 50 mm in height. A hole with a diameter of 25 mm was made near the exit of each side arm to position either blue or white LED lights (underwater fishing lamps: ISARIBI L-60, ING Co., Japan). The blue light intensity was 3.2×10^{18} photons m^{-2} s^{-1} and the white light intensity was 1.87×10^{18} photons m^{-2} s^{-1}, measured using a photonic multichannel analyser (HSU-100 Asahi Spectra Co., Japan). The marbled crayfish were allowed to settle underwater at one of the entrance gates of the plus-maze for at least 5 min. Marbled crayfish were released by opening the gate and they were allowed to choose an exit. When an animal settled at entrance 1, barrier 1 was opened but barrier 2 remained closed. For animals released from entrance 1, the white LED light was on the right exit and the blue light was on the left. When animals settled at entrance 2, only barrier 2 opened, resulting in the white LED light being on the left exit and the blue light on the right. Animals were randomly placed in either entrance 1 or 2 to prevent spatial learning.

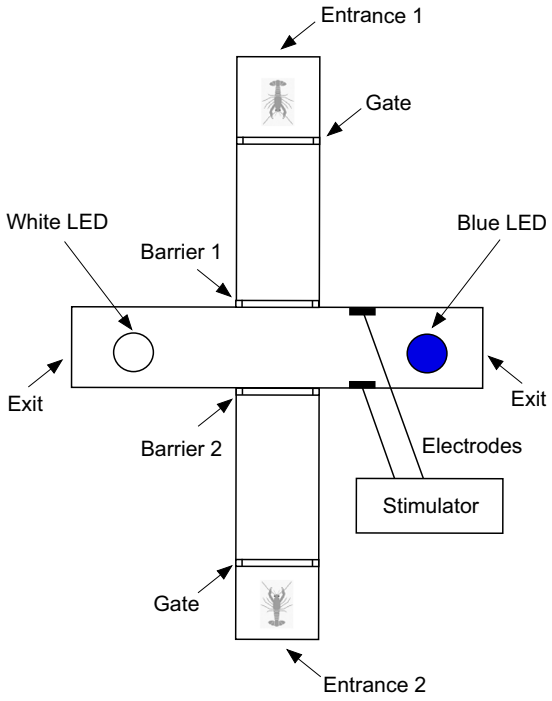

Fig. 1. Plus maze. Marbled crayfish were allowed to settle in entrance 1 or 2 for a minimum of 5 min. Animals were then released by opening the gate and the blue and white LED lights on the side arms were switched on. When gate 1 was released, barrier 1 was opened but barrier 2 remained closed. When gate 2 was released, only barrier 2 was opened. In the training session, when animals accessed the blue-lit exit, electrical shocks were applied from silver electrodes placed on the side arm of the blue-lit exit.

Experimental procedure

Experiments consisted of three sessions: pre-test, training and memory test.

Pre-tests

For each animal, the pre-test was repeated three times with a minimum interval of 10 min. Crayfish usually chose an exit within 3 min of being released from the entrance gate. Crayfish that returned to the entrance gate or that did not choose an exit within 5 min were excluded from analysis. Ninety-seven animals out of 145 (67%) chose the blue-lit exit two or more times in the three trials. These animals were consecutively used in the training and memory test sessions.

Training

Two silver plates were placed inside the side arm near the blue LED light (Fig. 1) and connected to a stimulator (SEN-5201; Nihon Kohden, Japan). When animals turned toward the blue-lit exit and their head crossed between the electrodes, square pulses of 20 V with 3 ms duration at 50 Hz were delivered to the stimulating electrodes for 1 s. A stimulus of 20 V is the minimum intensity required to evoke animals' backward escape. Training was repeated three times for each animal with minimum intervals of 10 min. In one series of experiments, the training trial with the electrical shock was only performed once.

Memory tests

After the third training trial, animals were isolated for 1 h in a stock chamber to rest and then a memory test was executed three times at 10 min intervals. No electrical shocks were applied in memory tests even if the animals selected the blue-lit exit. After the 1 h memory tests, tested animals were isolated for 24 h in the stock chamber, and then retested three times for the 24 h memory tests. For some animals, memory tests were performed just once at 48 or 96 h after the training session.

Icing

Immediately after training, some animals were placed in a new tank filled with water at 23°C for 30 min, while other animals were placed in water at 4°C for 30 min either immediately, 2 h or 4 h after training.

Drug injection

The following pharmacological agents were acquired from Sigma-Aldrich (St Louis, MO, USA): SQ22536 as an adenylate cyclase

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inhibitor, H89 dihydrochloride hydrate (H89) as a protein kinase A (PKA) inhibitor, cycloheximide (CHX) as a protein synthesis inhibitor, and naphthol AS-E phosphate (KG-501) as a cAMP-response element-binding protein (CREB) inhibitor (Best et al., 2004). SQ22536 was dissolved in physiological saline (van Harreveld, 1936) and prepared at a 50 µmol l^{-1} concentration as described by Mita et al. (2014). Other drugs were dissolved as 1000× stock solutions with a maximum final concentration of 0.1% dimethylsulphoxide (DMSO), and then diluted in saline to their final concentration. The 10 µmol l^{-1} of H89 used was in accordance with the protocol described by Momohara et al. (2016). Concentrations of KG-501 and CHX were determined by our preliminary observations, in which 10 µmol l^{-1} KG-501 and 100 µmol l^{-1} CHX were the maximum concentrations that produced no postural or behavioural changes in the animals. Each drug was prepared to the required concentration just prior to the experiments. After the third training trial was complete, 0.5 ml of each drug was injected into the pericardial sinus of the animals. Injections took place through the dorsal carapace within the caudal third of the pericardia to avoid damaging the underlying heart with a 27-3/4 gauge needle. The injection site was determined based on descriptions in Alcaro et al. (2011). After drug injection, the animals were isolated in stock containers until memory tests were conducted.

Statistical analyses

Statistical analyses to compare the difference between choosing the blue-lit exit in the pre-tests and memory tests were carried out using a generalized linear mixed model (GLMM) with a binomial distribution and a logit-link function using R 3.4.3. The model used exit choice (i.e. blue-lit exit or white-lit exit) as a response variable and individual ID as a random effect. The model also included drug treatment (e.g. saline, SQ22536, etc.), number of training trials (i.e. one shock or three shocks given), ice treatment (e.g. 23°C or 4°C) and trial number as fixed factors.

RESULTS

Aversive learning paradigm

In all three pre-test trials, released marbled crayfish showed a preference for the blue-lit exit. A total of 86%, 84% and 83% animals chose the blue-lit exit in each trial, respectively (Fig. 2A). During the training session, the rates of the animals choosing the blue-lit exit decreased in all three trials (Fig. 2B). Typically, the animal entered the side path to the blue-lit exit, but responded to the electrical shock with a tail flip. Even though a few animals forced their way through the electrical shock to reach the blue-lit exit, most ultimately chose the white-lit exit after several unsuccessful attempts to access the blue-lit exit. In the three training sessions, 12%, 2% and 7% of animals chose the blue-lit exit. Because no significant differences were found in the exit choice among the first, second and third trials in the pre-test or training sessions, the rates for individual animals choosing the blue-lit exit were summed from all three trials of the session.

Using 18 marbled crayfish, we examined whether aversive learning could be established (Fig. 2C). In the pre-tests, the animals preferred the blue-lit exit (binomial test, $P<0.001$). The rate of

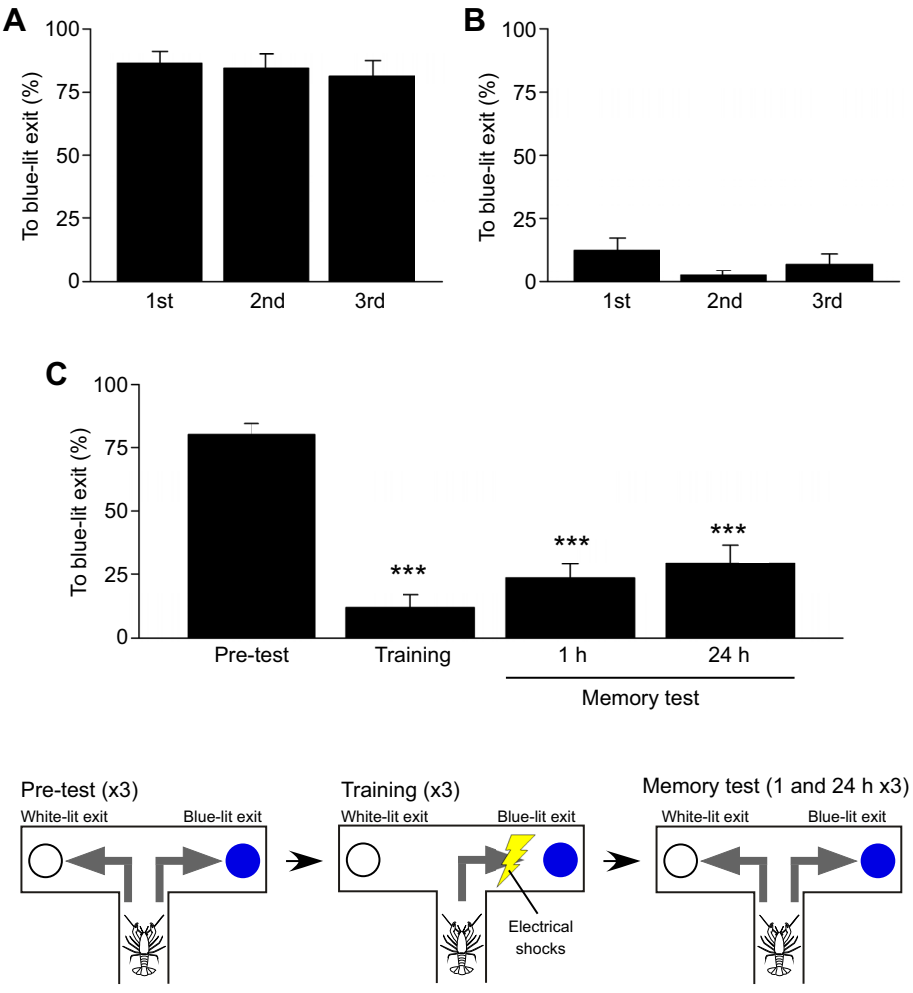

Fig. 2. Dual choice experiment to select between blue-lit and white-lit exits. (A) The rate at which marbled crayfish chose the blue-lit exit during three pre-test trials with a minimum interval of 10 min between trials. (B) The orientation rate to the blue-lit exit during three training session trials. (C) Aversive conditioning and memory formation. The rate of choosing the blue-lit exit was plotted for the pre-test, training and memory tests. Memory tests were performed at 1 and 24 h after the training session. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from pre-test (GLMM, ***$P<0.001$).

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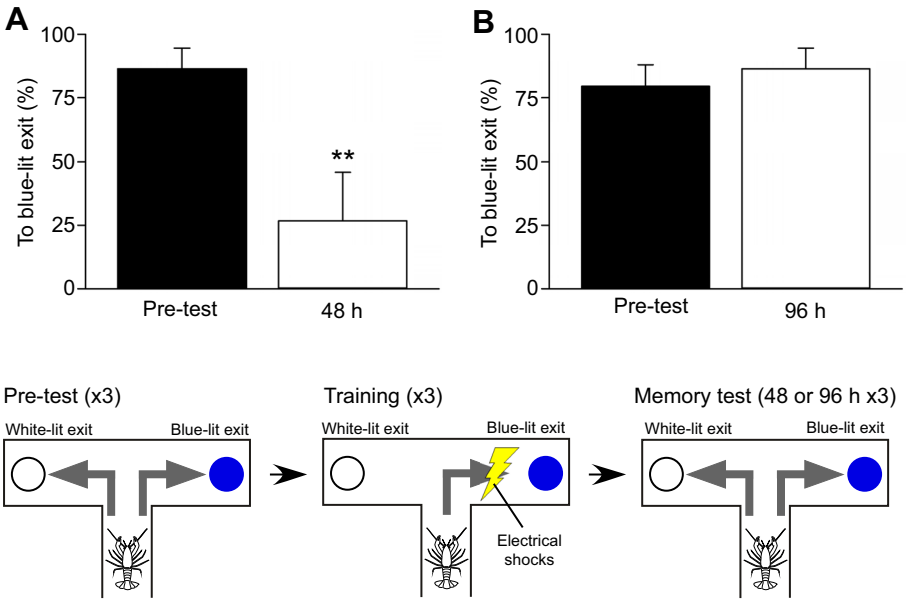

Fig. 3. Memory tests at 48 or 96 h after the training session. (A) 48 h; (B) 96 h. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, $^{**}P<0.01$).

orientation towards the blue-lit exit was 80.4±4.1% (mean±s.e.m.). During the training session, the rate of choosing the blue-lit exit was reduced to 11.8±4.9%. The orientation rate to the blue-lit exit was 23.5±5.5% in the 1 h memory test and 29.4±6.9% in the 24 h memory test. The rate of choosing the blue-lit exit in both training and memory tests was statistically significantly lower than in the pre-tests (GLMM, $P<0.001$ pre-test versus training, pre-test versus 1 h memory test, and pre-test versus 24 h memory test). Thus, the marbled crayfish associated the blue light with the aversive electrical shocks and learned to avoid that exit.

Using the aversive learning paradigm, memory tests were performed twice, at 1 and 24 h following the training session. To exclude the influence of the 1 h memory test in the 24 h memory test, for some animals, the memory test was performed only once, at either 48 h ($n=5$) or 96 h ($n=5$) after the training session (Fig. 3). The orientation rate to the blue-lit exit was 86.7±8.2% in the pre-tests but decreased significantly to 26.7±19.4% in the 48 h memory test (GLMM, $P=0.0097$; Fig. 3A). In contrast, the rate of choosing the blue-lit exit was 86.7±8.2% in the 96 h memory test (Fig. 3B) with no difference from the pre-test at 80.0±8.2% (GLMM, $P=0.3348$).

We examined whether a single electrical shock as training would be enough to establish aversive learning (Fig. 4). In five animals, one training trial was performed after the pre-test and then memory tests were performed at 1 and 24 h following the single training trial. The rate of choosing the blue-lit exit was 80.0±8.2% in the pre-test, and it decreased to 33.3±10.5% in the 1 h memory test and to 26.7±12.5% in the 24 h memory test, which was significantly lower (GLMM, $P=0.0277$ pre-test versus 1 h memory test and $P=0.0082$ pre-test versus 24 h memory test).

Marbled crayfish show a negative phototaxis; they avoid white light illumination (Shiratori et al., 2017). When marbled crayfish (five animals, 10 trials each) were released from the gate and chose either the white-lit exit or the unlit exit, the orientation rate to the white-lit exit was only 15.0±3.4% (Fig. 5). Animals were more likely to choose the unlit exit (binomial test, $P<0.001$). We

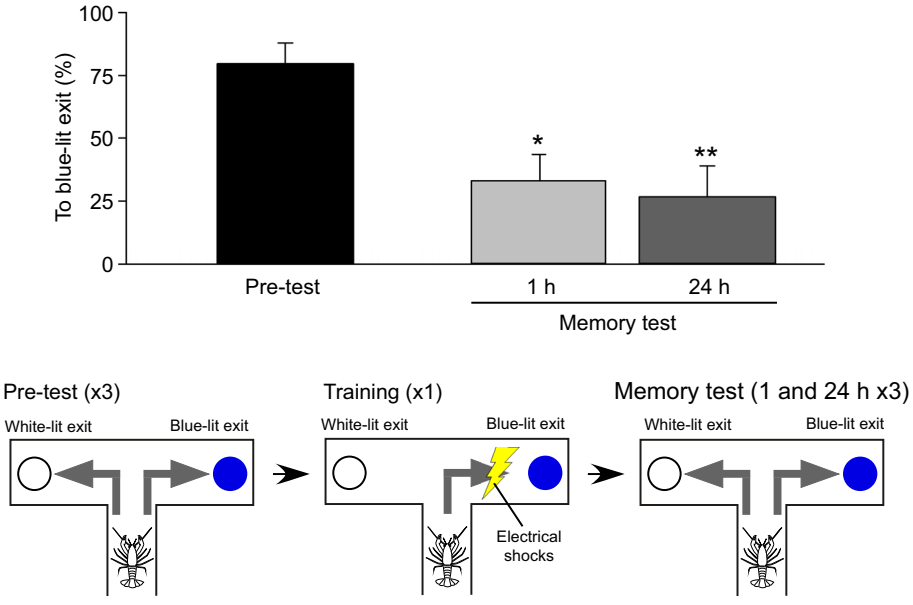

Fig. 4. Aversive conditioning in marbled crayfish with a single training session. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, $^{*}P<0.05$, $^{**}P<0.01$).

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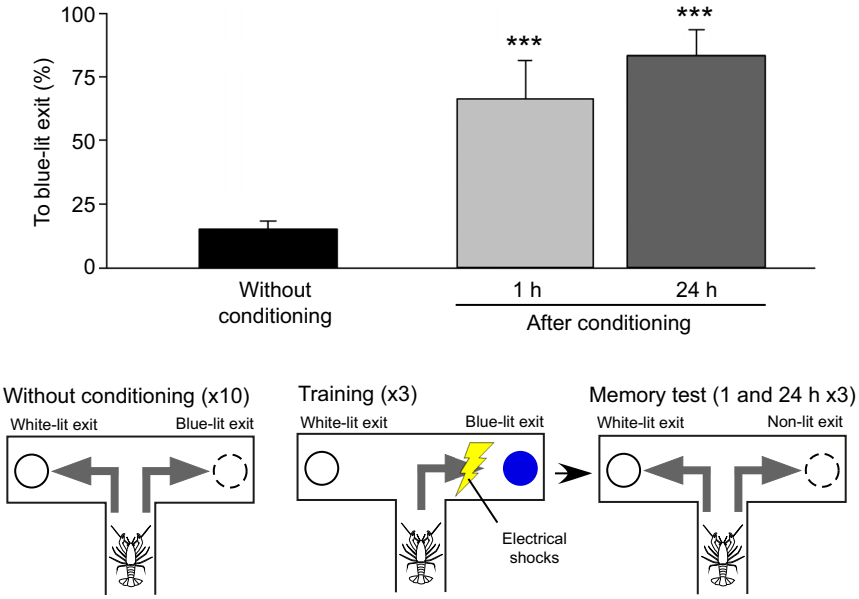

Fig. 5. The reversal from a negative to a positive phototactic response to white light after training in marbled crayfish. Dual choice experiments to select between white-lit and unlit exits. Marbled crayfish avoided orienting to the white-lit exit prior to training but preferred the white-lit exit following training. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, ***P<0.001).

examined whether negative phototaxis in response to the white light could change following the establishment of aversive learning. Initially, animals were tested to determine their choice between either a white-lit exit or an unlit exit, these animals were then placed in a training session and given a choice between either the blue-lit exit (with associated shocks) or the white-lit exit three times. After this training, the animals were tested again to determine whether they would choose either the white-lit or the unlit exit three times at 1 and 24 h following the training session. The orientation rate to the white-lit exit was 66.7±14.9% in the 1 h memory test and 83.3±10.5% in the 24 h memory test. Both rates were significantly higher than the first test prior to conditioning (GLMM, P<0.001 for both pre-test versus 1 h memory test and pre-test versus 24 h memory test). Animals were more likely to choose the white-lit exit 24 h after training (binomial test, P=0.0129).

Short-term and long-term memory formation

Memory tests indicated the establishment of both short-term and long-term memory after aversive learning. To confirm the functional pathway for memory formation, we examined the effect of cooling after the training session. Fulton et al. (2008) reported that cooling inhibits protein synthesis. When the animals (n=6) were placed in the chamber filled with water at 23°C for 30 min immediately after the training session, the orientation rate to the blue-lit exit was 11.1±5.0% in the 1 h memory test and 5.6±5.6% in the 24 h memory test (Fig. 6A). Both rates were significantly lower than the pre-test at 88.9±7.0% (GLMM, P<0.001 pre-test versus 1 h memory test and pre-test versus 24 h memory test). In contrast, when the animals (n=6) were placed in the chamber filled with water at 4°C for 30 min immediately after the training session, the rate at which the animals chose the blue-lit exit was 5.6±5.6% in the 1 h

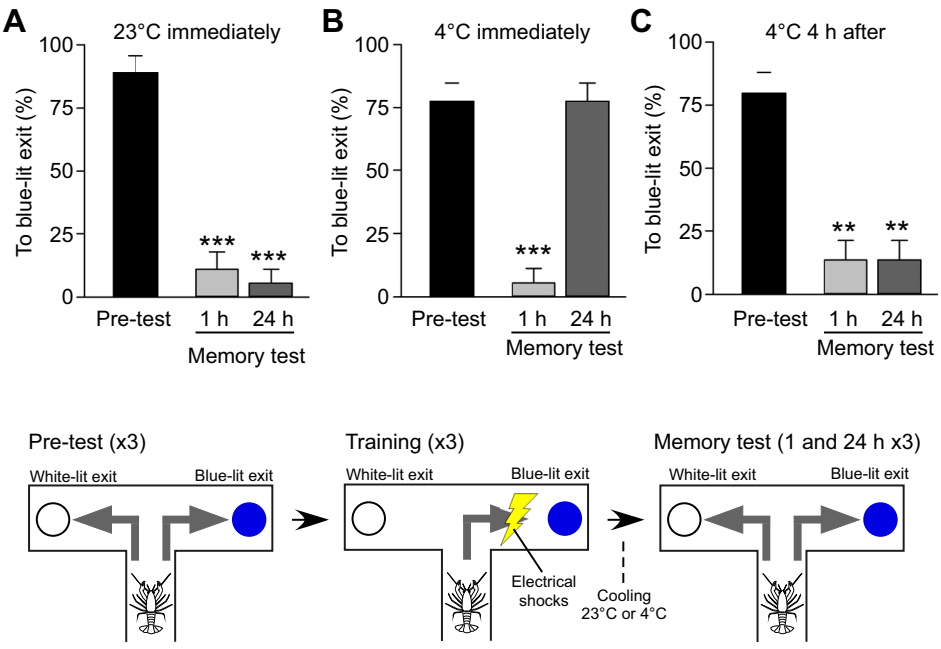

Fig. 6. Effect of cooling on memory formation in marbled crayfish. (A) The rate of orientation to the blue-lit exit during pre-tests and memory tests for animals kept at 23°C immediately after training. (B) The rate of orientation to the blue-lit exit during pre-tests and memory tests for animals kept at 4°C immediately after training. (C) The rate of orientation to the blue-lit exit during pre-tests and memory tests for animals kept at 4°C 4 h after training. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, **P<0.01, ***P<0.001).

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memory test and 77.8±7.0% in the 24 h memory test (Fig. 6B). The orientation rate of the 1 h memory test was significantly lower than the 77.8±7.0% rate of the pre-test (GLMM; $P<0.001$) but no significant difference was found between the pre-test and the 24 h memory test (GLMM, $P=1$). Thus, cooling only prevented the formation of long-term memory. When cooling was done 4 h after the training session ($n=5$), the orientation rate to the blue-lit exit decreased to 13.3±8.2% in both the 1 and 24 h memory tests (Fig. 6C), significantly lower than the pre-test (GLMM, $P=0.0025$ pre-test versus 1 h memory test and pre-test versus 24 h memory test). Cooling 2 h after training did not prevent long-term memory formation (not shown), suggesting that long-term memories are formed within an hour.

Effect of cycloheximide (CHX)

CHX is a known protein synthesis inhibitor. Because cooling immediately after training prevented long-term memory formation, we examined the effect of CHX injection on memory formation. When DMSO as a vehicle control was injected alone following the training session ($n=7$), the orientation rate to the blue-lit exit was 75.0±8.3% in the pre-test, 29.2±9.8% in the 1 h memory test and 20.8±10.8% in the 24 h memory test (Fig. 8A). The rate of the animals choosing the blue-lit exit in both the 1 and 24 h memory tests was significantly lower than the pre-test (GLMM; $P=0.0037$ pre-test versus 1 h memory test, and $P<0.001$ pre-test versus 24 h memory test). As shown in Fig. 7B, injection of 100 µmol l^{-1} CHX ($n=6$) immediately after the training session decreased the orientation rate to the blue-lit exit to 11.1±7.0% in the 1 h memory test, making it significantly lower than the pre-test (GLMM, $P<0.001$). However, the rate at which the animals chose the blue-lit exit was 83.3±7.5% in the 24 h memory test, which was similar to the 88.9±7.0% in the pre-test (GLMM, $P=0.6318$).

Effect of SQ22536

SQ22536 is an adenylate cyclase inhibitor that suppresses cAMP synthesis. When physiological saline as a vehicle control was injected alone after the training session ($n=6$), the orientation rate to the blue-lit exit was 83.3±7.5% in the pre-test, 11.1±7.0% in the 1 h memory test and 27.8±10.2% in the 24 h memory test (Fig. 8A). The rate at which the animals chose the blue-lit exit in both the 1 and 24 h memory tests was significantly lower than the pre-test (GLMM, $P<0.001$ pre-test versus 1 h memory test, and pre-test versus 24 h memory test). Before injection of 50 µmol l^{-1} SQ22536, the orientation rate to the blue-lit exit was 83.3±7.5% ($n=6$) in the pre-test (Fig. 8B); this decreased to 16.7±7.5% in the 1 h memory test after SQ22536 injection, which was significantly lower (GLMM, $P<0.001$). In the 24 h memory test, the rate of choosing the blue-lit exit was 77.8±7.0%. No statistical difference was found between the pre-test and the 24 h memory test (GLMM, $P=0.6745$).

Effect of H89 and KG501

H89 acts as a PKA inhibitor and KG-501 acts as a CREB inhibitor. In the pre-test prior to injection of either 10 µmol l^{-1} H89 ($n=6$) or 10 µmol l^{-1} KG-501 ($n=6$), the orientation rates to the blue-lit exit were 83.3±7.5% (Fig. 9A) and 77.8±7.0% (Fig. 9B), respectively. After H89 injection immediately after the training session, the orientation rate to the blue-lit exit was 5.6±5.6% in the 1 h memory test and 88.9±7.0% in the 24 h memory test (Fig. 9A). The rate in the 1 h memory test was significantly lower than that in the pre-tests (GLMM, $P<0.001$), but no significant difference between the pre-test and 24 h memory test was observed (GLMM, $P=0.6318$). Similarly, the orientation rate to the blue-lit exit at the 1 h memory test following the KG-501 injection was 11.1±7.0%, significantly lower than the pre-test (GLMM, $P<0.001$), while the 24 h memory test was not significantly different from the pre-test (GLMM, $P=0.7007$).

DISCUSSION

In this paper, we demonstrated for the first time the effectiveness of aversive operant conditioning in the marbled crayfish. The acquired memory was retained for at least 2 days and long-term memories were formed through the cAMP/PKA/CREB signal cascade.

Establishment of aversive learning

Marbled crayfish show negative phototaxis to white light illumination (Shiratori et al., 2017). They also show positive phototaxis to blue and green light illumination, but no phototaxis in response to red light illumination (C. Shiratori, personal

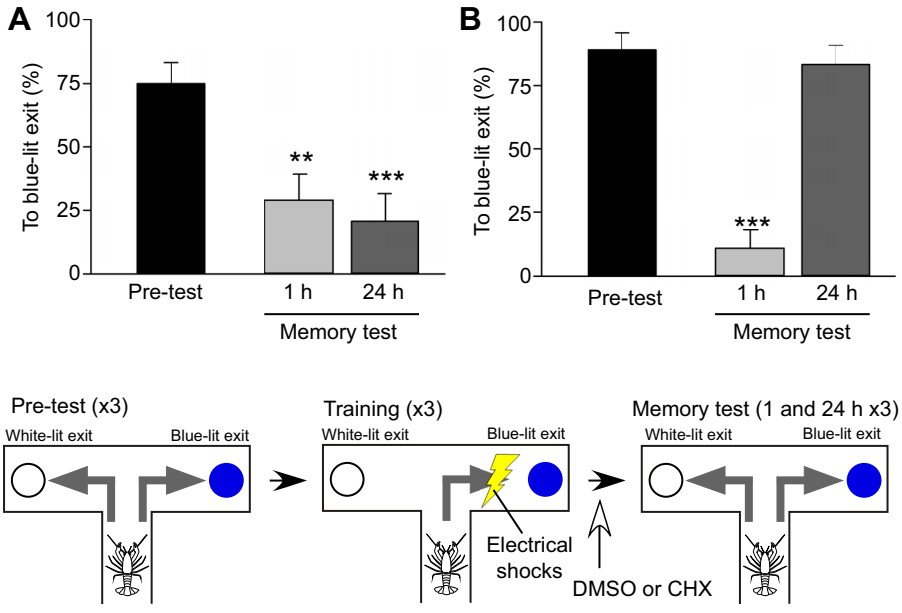

Fig. 7. Effect of CHX on memory formation in marbled crayfish. The rate of orientation to the blue-lit exit of animals during pre-tests and memory tests. (A) DMSO alone or (B) 100 µmol l^{-1} CHX with DMSO was injected immediately after training. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, $^{**}P<0.01$, $^{***}P<0.001$).

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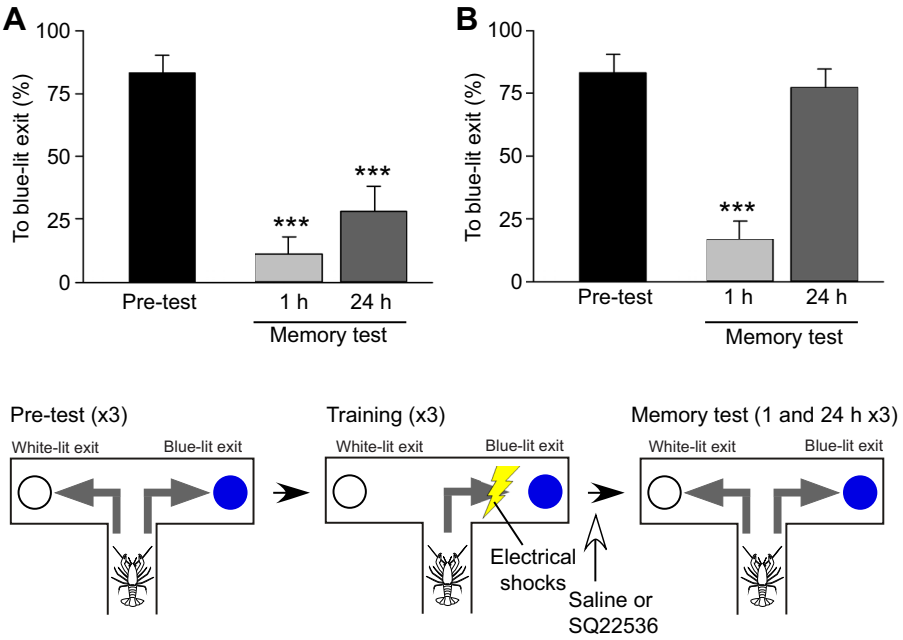

Fig. 8. Effect of SQ22536 on memory formation in marbled crayfish. The rate in which the animals chose the blue-lit exit of animals during pre-tests and memory tests. (A) Saline alone or (B) 50 μmol l^{-1} SQ22536 with saline was injected immediately after training. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, ***P<0.001).

observation). In the dual choice experiments between the blue-lit and the white-lit exits in this study, animals preferred the blue-lit exit prior to conditioning. The crayfish *P. clarkii* have been demonstrated to have both violet and yellow retinular cells in their compound eyes (Nosaki, 1969; Eguchi et al., 1973). The marbled crayfish central nervous system is similar to that of *P. clarkii*, and seems to be capable of colour vision (Faulkes, 2016). Colour vision is known to be present in many crustaceans. Mantis shrimp have 12 spectrally different photoreceptors (Marshall and Oberwinkler, 1999) and seem to be capable of colour vision (Marshall et al., 1996). Blue crabs also have colour vision and use colour in determining their choice of mates (Baldwin and Johnsen, 2009). The fish ectoparasite *Argulus japonicus* can distinguish between lights of different colours, and shows positive phototaxis in response to blue>yellow>green>red light (Yoshizawa and Nogami, 2008).

The aversive learning paradigm induced a reversed preference of phototactic behaviour in the marbled crayfish following training using electrical shocks. After three training trials, animals avoided the blue-lit exit for more than 48 h. Furthermore, a single training trial was enough to form long-term memory. Electrical shocks are a powerful motivating factor as an aversive sensory experience to the marbled crayfish, and they quickly learn to avoid the situation. Aversive conditioning using electrical shocks has also been performed in crabs (Denti et al., 1988; Magee and Elwood, 2013). In the crab *Chasmagnathus granulates*, however, passive avoidance learning has been detected at up to 3 h, but no retention was shown after 24 h (Denti et al., 1988).

After the training session, the marbled crayfish's preferential response to white light illumination reversed from negative to positive, as shown using dual choice experiments between white-lit and unlit exits. This indicates that the nature of taxis can change via

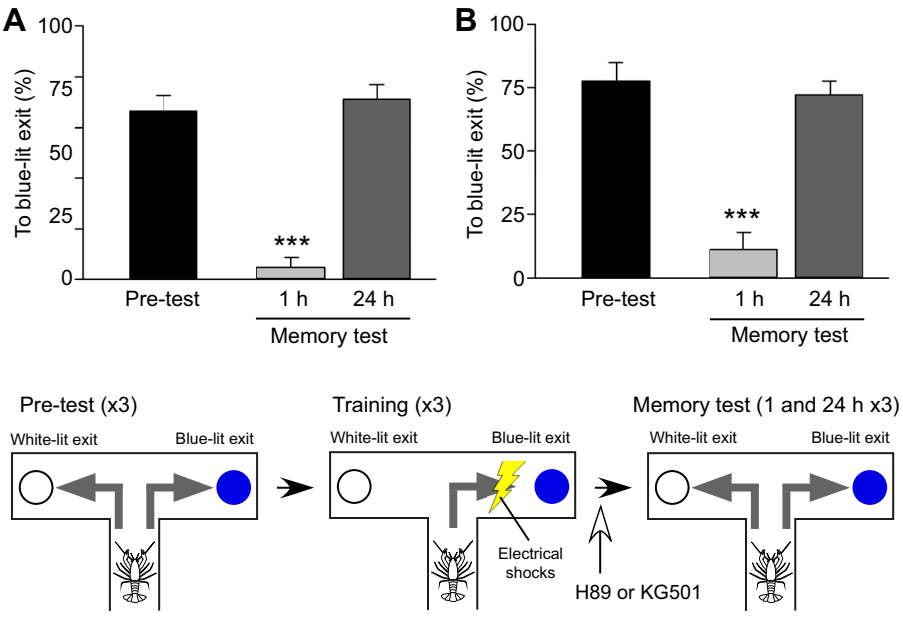

Fig. 9. Effects of H89 and KG-501 on memory formation in marbled crayfish. The rate of orientation to the blue-lit exit of animals during pre-tests and memory tests. (A) H89 or (B) KG-501 was injected immediately after training. Diagrams indicate the aversive learning paradigm. Asterisks indicate a significant difference from the pre-test (GLMM, ***P<0.001).

acquired learning, though phototactic behaviour is generated by an innate motor programme embedded genetically. Many phototactic behaviours have been reported to reverse in sign (negative or positive) depending on the animal's internal state, such as developmental stage, growth or sex (Julian and Gronenberg, 2002; Lone et al., 2012; Miljeteig et al., 2014), or the environmental conditions, such as salinity or light intensity (Shirley and Shirley, 1988; Ziegler et al., 2010). Marbled crayfish show negative phototaxis in the daytime and positive phototaxis during the night (Shiratori et al., 2017). The present study is the first to report that phototaxis has plasticity and reverses according to associated learning experiences. Because serotonin mediates positive phototaxis while dopamine mediates negative phototaxis in the marbled crayfish (Shiratori et al., 2017), certain biogenic amines seem to intervene in the switching of phototaxis through aversive conditioning.

Long-term memory formation

It is widely accepted that in a variety of animals the form of memory changes from short-term to long-term in a time-dependent manner (Squire, 1987). In various invertebrates, cooling induces a blockade of long-term memory formation (Erber, 1976; Quinn and Dudai, 1976; Sangha et al., 2003; Fulton et al., 2008). Cooling is thought to induce the disruption of protein synthesis (Fulton et al., 2008). Consolidation of appetitive long-term memory in *Lymnaea* is disrupted by cooling applied immediately after conditioning, while no such effect is observed after delaying the treatment to 10 min after conditioning (Fulton et al., 2008). Our study was consistent with these findings, as cooling immediately after training disrupted the formation of long-term memory, but cooling 2 or 4 h after training had no effect. Injection of CHX immediately after training also altered long-term memory formation without any effect on short-term memory. CHX is a protein synthesis inhibitor and also impairs the formation of long-term aversive memories in *Drosophila* (Hirano et al., 2013) and the long-term habituation of the escape response in the crab (Pedreira et al., 1995). Thus, new protein synthesis that contributes to structural and functional changes is necessary in the formation of long-term memories in the marbled crayfish.

Cyclic AMP signalling is involved in various forms of learning across taxa of both vertebrates and invertebrates. Signal cascades for long-term memory formation are similar in many invertebrates, including *Aplysia*, *Drosophila* and honeybees, and share common principles with those underlying learning in vertebrates (Eisenhardt, 2018). Transcription for new protein synthesis is initiated when CREB binds to its target gene (Alberini, 2009). In vertebrates, CREB-dependent transcription is activated by several kinases, including PKA and CaM kinase II. With genetic sequencing techniques, signal cascades for operant conditions in *Drosophila* have been clarified. A receptor-coupled G-protein and Ca^{++} influx activating adenylyl cyclase produce cAMP and activate PKA. Another phosphorylation step from the activated PKA and MAPK activates CREB (Impey et al., 1999; Brembs, 2003). The cAMP/PKA/CREB signal cascade is also essential for long-term memory formation in *Aplysia* (Hawkins et al., 2006; Kandel, 2012). In the present study, either SQ22536, H89 or RG-501 injected immediately after training altered the formation of long-term memory, but did not affect the formation of short-term memory. SQ22536 decreases cAMP synthesis, H89 inhibits PKA activation and KG-501 blocks CREB from binding to its target gene. Thus, the cAMP/PKA/CREB signal pathway is also responsible for long-term memory formation in the marbled crayfish. This is the first report to describe a signal cascade for the formation of crustacean long-term memories. At present, however, it is still unclear whether CREB exists in the central nervous system of the marbled crayfish. Various isotypes of CREB have been already identified in many other animal species (e.g. Sadamoto et al., 2004). Cloning of CREB is thus indispensable to clarify this point.

Several studies have shown the roles biogenic amines have during learning and memory processes. For example, serotonin is necessary for aversive memory in *Drosophila* (Sitaraman et al., 2008). In many insects, dopamine has been reported to be related to aversive learning, while octopamine is related to appetitive processing (Schwaerzel et al., 2003; Unoki et al., 2005). At the moment, however, the idea that each amine is exclusively involved in one type of memory is under review, because opposing actions of dopamine or octopamine on both aversive and appetitive memory formation are found in *Drosophila* (Kim et al., 2007; Krashes et al., 2009; Liu et al., 2012) and crabs (Kaczer and Maldonado, 2009; Klappenbach et al., 2012). During aversive operant conditionings in the marbled crayfish, uncertainty remains as to which amine may trigger the G-protein and activate the downstream cAMP cascade. In the crayfish LG-mediated tail flip system, serotonin and octopamine affect social-status-dependent modulation of habituation by means of activation of the downstream second messenger systems of cAMP and IP_3 cascades, respectively (Araki and Nagayama, 2012; Araki et al., 2005). Serotonin increases the cAMP level and octopamine increases the IP_3 level, resulting in a slower rate of habituation. The serotonin 5HT1 receptor mediates the reversal of phototaxis from negative to positive with a decrement of cAMP level during the dark adaptation of the marbled crayfish, while the dopamine DA1 receptor mediates the reversal of phototaxis from positive to negative with an increase in cAMP level during light adaptation (Shiratori et al., 2017). Octopamine had no significant effect on phototaxis in their study. To this day, the role of biogenic amines in aversive learning is unclear and further research will be indispensable for clarification.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.O., N.H., T.N.; Methodology: S.O., N.H.; Formal analysis: T.A.; Investigation: S.O., N.H.; Data curation: N.H., T.A.; Writing - original draft: T.N.; Writing - review & editing: S.O., N.H., T.N.; Supervision: T.N.; Funding acquisition: T.N.

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