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RESEARCH ARTICLE

Enhanced memory persistence is blocked by a DNA methyltransferase inhibitor in the snail *Lymnaea stagnalis*

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ABSTRACT

Lymnaea stagnalis provides an excellent model system for studying memory because these snails have a well-described set of neurons, a single one of which controls expression of long-term memory of operantly conditioned respiratory behavior. We have shown that several different manipulations, including pre-training exposure to serotonin (5-HT) or methamphetamine, submersion of snails after training to prevent memory interference, and exposure to effluent from predatory crayfish (CE), enhance memory persistence. Changes in DNA methylation underlie formation of strong memories in mammals and 5-HT-enhanced long-term facilitation in *Aplysia*. Here we determined the impact of the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-AZA; 87 µmol l⁻¹), on enhanced memory persistence by all four manipulations. We found that 5-HT (100 µmol l⁻¹) enhanced memory persistence, which was blocked by 5-AZA pretreatment. Snails pre-exposed to 3.3 µmol l⁻¹ Meth 4 h prior to training demonstrated memory 72 h later, which was not present in controls. This memory-enhancing effect was blocked by pre-treatment with 87 µmol l⁻¹ 5-AZA. Similarly, submersion to prevent interference learning as well as training in CE produced memory that was not present in controls, and these effects were blocked by pre-treatment with 87 µmol l⁻¹ 5-AZA. In contrast, 5-AZA injection did not alter expression of normal (non-enhanced) memory, suggesting that these four stimuli enhance memory persistence by increasing DNA methyltransferase activity, which, in turn, increases expression of memory-enhancing genes and/or inhibits memory suppressor genes. These studies lay important groundwork for delineating gene methylation changes that are common to persistent memory produced by different stimuli.

KEY WORDS: Snail, Long-term memory, Epigenetics, DNA methylation, Forgetting, Methamphetamine

INTRODUCTION

Persistent, unwanted memories are key contributors to post-traumatic stress disorder (PTSD) (American Psychiatric Association, 2013) and drug addiction (Robbins et al., 2008; Torregrossa et al., 2011; Volkow et al., 2012). However, a major obstacle to understanding detailed mechanisms by which memories are made more persistent after exposure to stimuli such as stress, drugs of abuse, or other stimuli that enhance memory persistence is the complexity of the mammalian brain. In the present study, we used

the freshwater pond snail, *Lymnaea stagnalis* (Linnaeus 1758), which provides a relatively simple, well-defined model system that is excellent for studying enhanced memory persistence (Lukowiak et al., 2006; Lukowiak et al., 1996; Sangha et al., 2003c). *Lymnaea stagnalis* are bimodal breathers via both cutaneous and aerial systems. Aerial respiration occurs through a breathing tube called the pneumostome, and snails can be operantly conditioned to reduce the frequency of opening of their pneumostome despite low oxygen levels that drive them to the surface to open this structure. In the operant conditioning procedure, the snail is placed into a hypoxic environment by bubbling nitrogen into the water, and every time it opens its pneumostome, it immediately receives a tactile stimulus to this structure. The stimulus causes the snail to close its pneumostome, and it consequently learns not to open its pneumostome (but will receive sufficient oxygen via cutaneous breathing for several hours).

Aerial respiratory behavior is driven by a central pattern generator consisting of three neurons whose sufficiency and necessity have been experimentally determined (Syed et al., 1990; Syed et al., 1992). Learning, memory, extinction and forgetting have all been shown to be dependent on the dopamine neuron right pedal dorsal 1 (RPeD1) in the central pattern generator (Cottrell et al., 1979; Sangha et al., 2003a; Sangha et al., 2004; Scheibenstock et al., 2002; Spencer et al., 2002). Thus, RPeD1 plays a crucial role in memory, and analysis of this simple model system presents the opportunity to identify the molecular events leading to persistent memory formation.

The goal of the present study was to determine whether there is a common mechanism underlying enhanced memory persistence induced by a variety of stimuli. Previous studies have demonstrated that a single 30 min training period normally produces memory that lasts for 3 h but not for 24 h (Lukowiak et al., 2000), and that two 30 min operant training sessions normally produce memory lasting 24 h but not 72 h (Parvez et al., 2005). Therefore, we used either a single training session and tested for memory 24 h later [serotonin (5-HT) and crayfish effluent (CE) experiments] or two training sessions and tested for memory persistence 72 h later [methamphetamine (Meth) and submersion experiments]. Four types of manipulations were chosen to enhance memory persistence. First, we tested the impact of 5-HT because of previous work describing enhanced memory in *Aplysia* via changes in DNA methylation status (Rajasethupathy et al., 2012). Second, we previously reported that the psychostimulant drugs cocaine (Carter et al., 2006) and Meth (Kennedy et al., 2010) enhanced long-term memory (LTM) in snails. We also demonstrated that pre-exposure to Meth prior to weak training produced LTM of respiratory behavior that was not found in controls. In the present study, we optimized the pre-exposure time to produce memory 72 h later in Meth-pre-exposed snails but not in controls. A third manipulation was the submersion of snails immediately after training, which enhances memory persistence

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List of abbreviations

5-AZA	5-aza-2′-deoxycytidine
5-HT	serotonin
CE	crayfish effluent
DNMT	DNA methyltransferase
LTM	long-term memory
Meth	methamphetamine
MT	memory test
PTSD	post-traumatic stress disorder
PW	artificial pond water
RPeD1	right pedal dorsal 1
TS1	training session 1
TS2	training session 2

(Lukowiak et al., 2014; Sangha et al., 2003a; Sangha et al., 2005), most likely by reducing interference learning. Interference learning is thought to prevent 72 h memory formation after the two-trial learning procedure described above. This is because, during training, snails are given the stimulus contingent upon pneumostome opening, but between training sessions and the test for memory 72 h later, they are allowed to surface breathe in their home aquarium without stimulation to the pneumostome, producing interference learning that results in forgetting (Sangha et al., 2003a; Sangha et al., 2005). Submersion by placing a grating to keep snails below the surface of eumoxic water after training prevents this surface breathing and thus prevents the interference, producing enhanced memory persistence. Fourth, snails were trained in CE, which we have previously shown enhances memory persistence for up to 8 days (Orr and Lukowiak, 2008).

The second step in determining whether there was a common mechanism for memory persistence was to test whether epigenetic changes would mediate this persistence. We focused on epigenetic changes because several recent studies have demonstrated that epigenetic changes accompany memory formation (for reviews, see Gräff and Mansuy, 2008; Roth and Sweatt, 2009; Zovkic et al., 2013a). In particular, strong memories such as those found in individuals with PTSD and drug addiction may be resistant to forgetting because these memories appear to be stabilized by epigenetic changes (Franklin et al., 2012; Nestler, 2014; Nielsen et al., 2012; Schmidt et al., 2013; Zovkic and Sweatt, 2013). The two major types of epigenetic modifications occur at the level of histone proteins that associate with DNA and at the level of the DNA itself via methylation of cytosine bases. In the present study, we focused on the role of DNA methylation by pretreating snails with a DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-AZA), because of earlier work demonstrating enhanced memory via DNA methylation after 5-HT treatment in *Aplysia* (Rajasethupathy et al., 2012). We tested whether this same inhibitor might serve as a common mechanism for blocking the memory-enhancing effects of the four different manipulations described.

RESULTS
Experiments 1 and 2: effect of 5-HT and 5-AZA on extended memory (24 h after single training session)
For all studies conducted throughout, memory was considered to be present if the number of attempted pneumostome openings in the memory test (MT) session was significantly lower than that of the first training session (TS1) and was not significantly higher than that of the last training session (TS2, when two training sessions were conducted). The first manipulation to extend memory was accomplished by administering 5-HT prior to training. Previous work demonstrated that the 5-HT antagonist, mianserin, blocked the

memory-extending effects of CE (Il-Han et al., 2010), so we hypothesized that prior treatment with 5-HT would enhance memory persistence in *L. stagnalis*, as was found for long-term facilitation in *Aplysia* (Bartsch et al., 1995). As with the CE experiment (see below), here we tested the effect of either 5-HT or 5-HT combined with 87 µmol l^{-1} 5-AZA on 24 h memory after a single training session. Control snails given two saline injections 2 h apart followed by a single training session (TS1) 1 h later did not demonstrate 24 h memory, as expected ($F_{1,6}$=0.32, P<0.593; Fig. 1A). However, 5-HT pre-treatment followed by saline extended memory to 24 h, and this 24 h memory was not present in yoked controls (trained: $F_{1,6}$=50, P<0.0004; yoked $F_{1,6}$=1.62, P<0.251; Fig. 1B). In contrast, 5-HT pre-treatment followed by 5-AZA blocked this extended memory 24 h later ($F_{1,6}$=3.57, P<0.108; Fig. 1C).

To test for non-specific effects on respiratory behavior, total breathing time was examined prior to treatment and 24 h later after the same three treatments (and 1 h after the second injection). Fig. 1D–F shows that there were no effects of any of the treatment combinations on total breathing time (saline/saline $F_{1,10}$=0.24, P<0.634; 5-HT/saline $F_{1,9}$=1.08, P<0.326; 5-HT/5-AZA $F_{1,9}$=0.31, P<0.594). We also did not observe any effects on the overall pattern of breathing, as measured by the average breathing time per pneumostome opening. Average breathing time per opening (in seconds) is as follows for the three treatment groups: saline/saline group: pre-obs=14.8±1.6, post-obs=13.4±2.6; $F_{1,10}$=0.21, P<0.658; 5-HT/saline group: pre-obs=17.0±3.4, post-obs=12.8±3.1; $F_{1,9}$=1.13, P<0.316; and 5-HT/5-AZA group: pre-obs=14.5±1.7, post-obs=15.0±1.9; $F_{1,9}$=0.11, P<0.749). These findings indicate that the effects we observed were most likely due to learning and memory events and not to non-specific effects on respiratory behavior.

Experiment 3: effect of 5-AZA on non-extended memory (24 h after two training sessions)
Because 87 µmol l^{-1} 5-AZA prevented the expression of extended memory, we determined whether treatment with this concentration of 5-AZA would impact 24 h LTM that is normally present after two training sessions, TS1 and TS2. Fig. 2 demonstrates that TS1 and TS2 produced 24 h memory that was not affected by 5-AZA (saline: $F_{2,40}$=6.92, P<0.0026; 5-AZA: $F_{2,72}$=8.72, P<0.0004). These findings indicate that memory that is normally present after sufficient training (non-enhanced) is not affected by 5-AZA.

Experiments 4–6: effect of Meth pre-exposure on extended memory (72 h)
Our previous study demonstrated that pre-exposure to Meth 24 h prior to training enhanced the ability to form 24 h LTM after a single training session (Kennedy et al., 2010). We conducted additional pilot studies (not shown) to assess the time interval during which Meth pre-exposure produced maximal effects on memory persistence, which was 4 h. Fig. 3A shows control snails that were pre-exposed to artificial pond water (PW) 4 h before training. As expected in controls, when given two training sessions 1 h apart, snails demonstrated learning on TS2, but no memory was apparent on the MT given 72 h later ($F_{2,23}$=8.60, P<0.0009). This finding is consistent with a previous study (Parvez et al., 2005). Pre-injection of snails with 87 µmol l^{-1} 5-AZA did not alter behavior compared with saline controls ($F_{2,24}$=6.46, P<0.0036; Fig. 3B). In contrast to the lack of memory expression at 72 h in PW controls, Meth pre-exposure 4 h prior to the first training session enhanced memory persistence, producing expression of memory 72 h later ($F_{2,46}$=17.39, P<0.0001; Fig. 3C). This 72 h memory was not present after 5-AZA treatment (Fig. 3D), suggesting that enhanced memory persistence
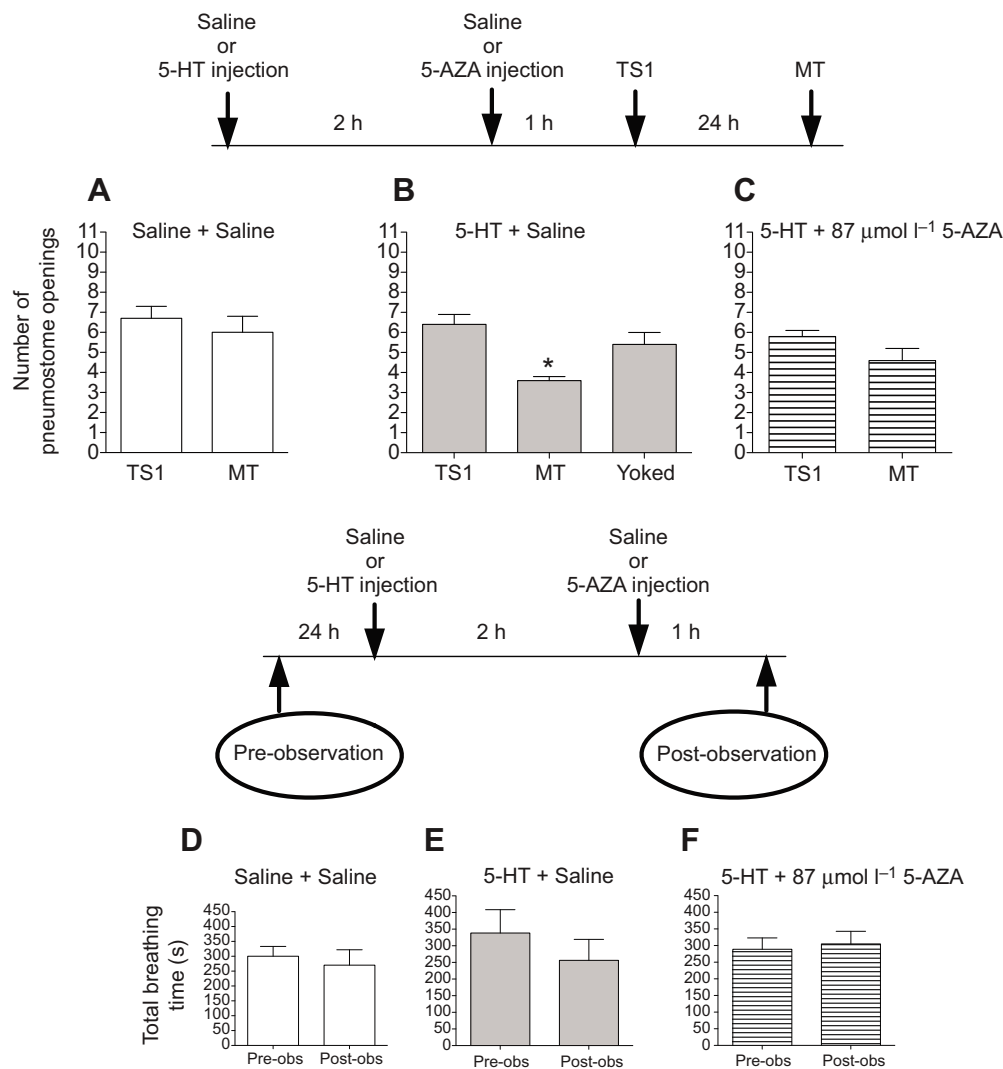
Fig. 1. Treatment of *Lymnaea stagnalis* with serotonin (5-HT) extends memory that is prevented by 5-aza-2'-deoxycytidine (5-AZA) treatment. (A–C) Mean ± s.e.m. number of pneumostome openings for the following treatments: (A) saline injection 2 h prior to a second saline injection 1 h before training session 1 (TS1; $N=7$); (B) 100 µmol l^{-1} 5-HT injection 2 h prior to a saline injection 1 h before TS1 ($N=7$); (C) 100 µmol l^{-1} 5-HT injection 2 h prior to an 87 µmol l^{-1} 5-AZA injection 1 h before TS1 ($N=7$). Snails were given a single training session and tested 24 h later. Treatment with 5-HT produced 24 h memory after a single training session that was not present in yoked controls. Expression of this extended memory was prevented by 5-AZA treatment after 5-HT injection. Timeline for treatment is shown at the top. MT, memory test. (D–F) Mean ± s.e.m. total breathing time over 30 min sessions given before (pre-obs) or after (post-obs) the following treatments: (D) saline injection 2 h prior to a second saline injection 1 h before post-obs ($N=11$); (E) 100 µmol l^{-1} 5-HT injection 2 h prior to a saline injection 1 h before post-obs ($N=10$); (F) 100 µmol l^{-1} 5-HT injection 2 h prior to an 87 µmol l^{-1} 5-AZA injection 1 h before post-obs ($N=10$). During breathing observations, snails were not given stimulation to the pneumostome upon opening. Injection of 5-HT alone or in combination with 5-AZA did not alter general respiratory behavior. Timeline for treatment is shown at the top. *$P<0.05$, comparing with TS1.

induced by Meth pre-exposure is dependent on DNA methylation state.

To demonstrate that the effects of 87 µmol l^{-1} 5-AZA and Meth pre-exposure were specific to learning and memory expressed at 72 h rather to an effect of drug treatments on general respiratory behavior, we measured the impact of the same treatment in non-contingent controls. Snails were given all treatments exactly as before, but they were given the tactile stimulus independent of opening their pneumostome, with the stimulus given near their pneumostome whenever their 'partner' opened their pneumostome to breath. There were no significant effects of either 5-AZA or Meth on the number of pneumostome openings across TS1, TS2 or MT,
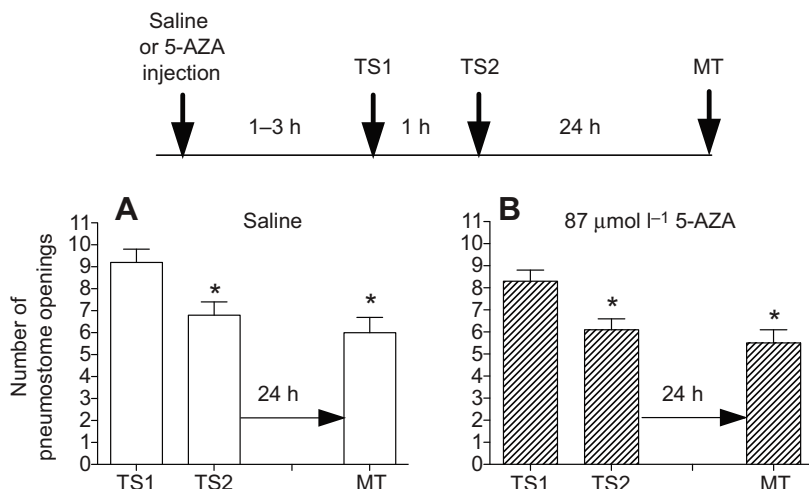

Fig. 2. Treatment of *L. stagnalis* with 5-AZA does not alter non-extended (24 h) memory. Mean ± s.e.m. number of pneumostome openings for the following treatments: (A) saline injection 1–3 h prior to TS1 ($N=21$); (B) 87 µmol l^{-1} 5-AZA injection 1–3 h prior to TS1 ($N=37$). Two training sessions produced memory that lasted for 24 h, and this was not altered by 5-AZA treatment. Timeline for treatment is shown at the top. *$P<0.05$, comparing with TS1.
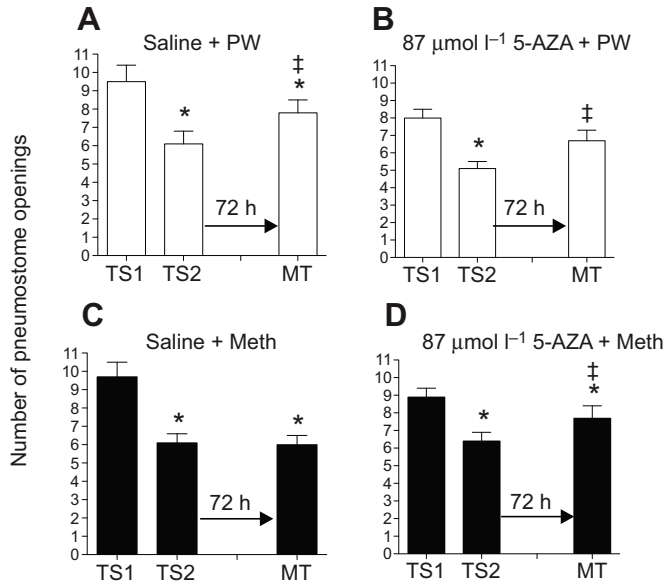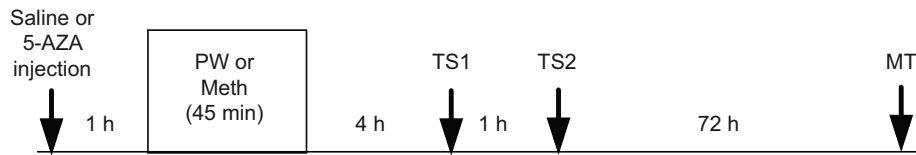
Fig. 3. Treatment of *L. stagnalis* with 5-AZA prevents methamphetamine (Meth)-induced extended memory. (A–D) Mean ± s.e.m. number of pneumostome openings for the following treatments: (A) saline injection and immersion in artificial pond water (PW) 4 h prior to TS1; (B) 87 µmol l⁻¹ 5-AZA injection and immersion in PW 4 h prior to TS1 (*N*=22); (C) saline injection and immersion in 3.3 µmol l⁻¹ Meth 4 h prior to TS1; (D) 87 µmol l⁻¹ 5-AZA injection and immersion in 3.3 µmol l⁻¹ Meth 4 h prior to TS1 (*N*=22). Injection of 87 µmol l⁻¹ 5-AZA prevented Meth-induced extension of memory but had no effect in PW controls. Timeline for treatment is shown at the top. (E–H) Mean ± s.e.m. total breathing time over 30 min sessions given before (pre-obs) or after (post-obs) the following treatments: (E) saline injection and immersion in PW 4 h prior to TS1 (*N*=16); (F) 87 µmol l⁻¹ 5-AZA injection and immersion in PW 4 h prior to TS1 (*N*=14); (G) saline injection and immersion in 3.3 µmol l⁻¹ Meth 4 h prior to TS1 (*N*=16); (H) 87 µmol l⁻¹ 5-AZA injection and immersion in 3.3 µmol l⁻¹ Meth 4 h prior to TS1 (*N*=16). In all sessions, snails were not given stimulation to the pneumostome upon opening. Injection of 5-AZA or immersion in Meth did not alter general respiratory behavior. *$P<0.05$, comparing with TS1; ‡$P<0.05$, comparing MT with TS2.
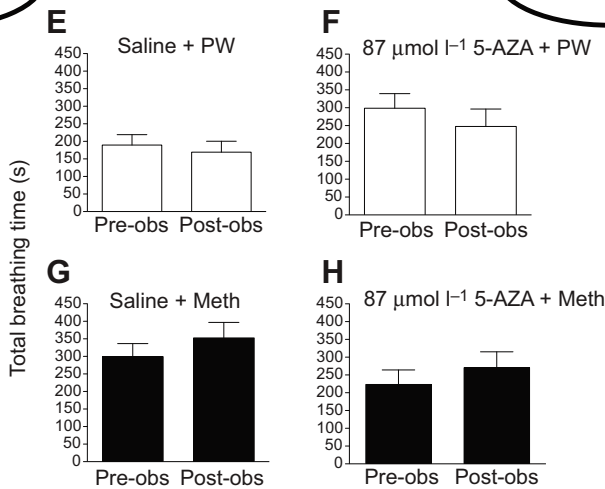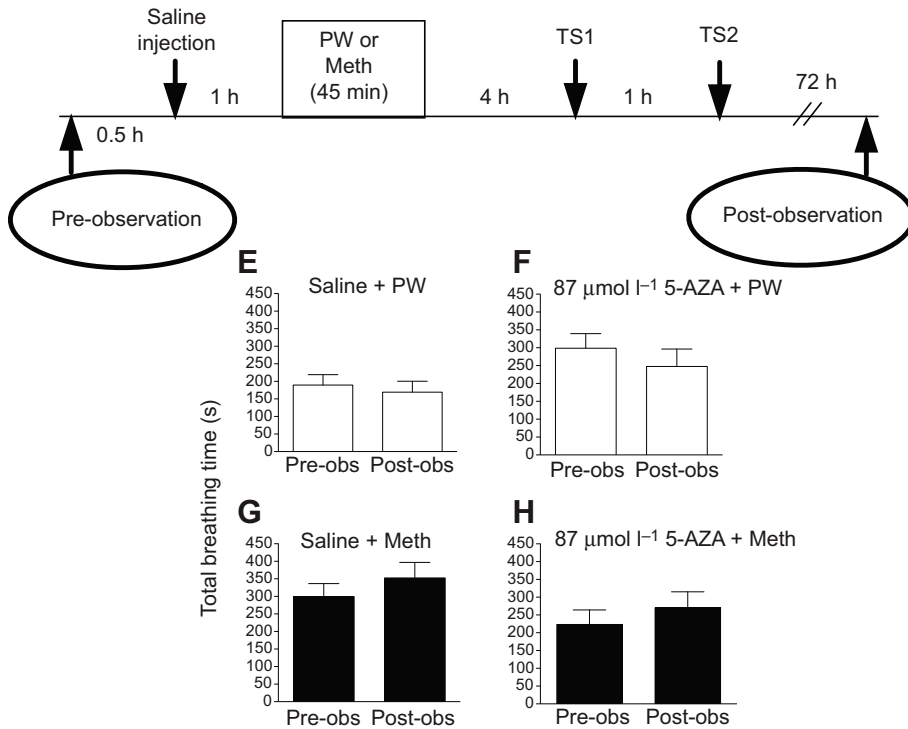
when the stimulus was not contingent upon opening (saline/PW: $F_{2,14}=1.96$, $P<0.178$; 5-AZA/PW: $F_{2,10}=0.34$, $P<0.720$; saline/Meth: $F_{2,30}=0.36$, $P<0.697$; 5-AZA/Meth: $F_{2,42}=1.49$, $P<0.236$; Table 1). These findings indicate that the impact of 5-AZA and Meth pre-exposure was dependent on learning and memory-associated events.

A further test for non-specific effects of drug treatment was conducted to assess general respiratory behavior. Snails were given a pre-observation period to assess the total breathing time before any drug manipulations were carried out. Training and testing were

conducted as before, and total breathing time was again measured when the MT would be given. As shown in Fig. 3E–H, there were no significant effects on total breathing time in any of the treatment groups (saline/PW: $F_{1,15}=0.83$, $P<0.376$; 5-AZA/PW: $F_{1,13}=0.93$, $P<0.352$; saline/Meth: $F_{1,15}=1.74$, $P<0.206$; 5-AZA/Meth $F_{1,15}=1.55$, $P<0.232$). The average breathing time (in seconds) per pneumostome opening was not different in the saline/PW group (pre-obs=30.9±5.1, post-obs=21.4±1.7; $F_{1,15}=2.69$, $P<0.122$). However, we did observe a decrease in the saline/Meth group (pre-

Table 1. Pneumostome openings in *Lymnaea stagnalis* snails given non-contingent stimulation and treated with 5-aza-2′-deoxycytidine (5-AZA) and methamphetamine (Meth)

Treatment (N)	TS1	TS2	MT
Saline + PW (8)	8.4±1.5	8.1±0.6	6.2±0.8
87 µmol l^{-1} 5-AZA + PW (6)	7.7±1.4	7.0±0.8	8.3±1.6
Saline + Meth (16)	6.1±0.9	6.9±1.0	6.9±0.8
87 µmol l^{-1} 5-AZA + Meth (22)	5.0±0.5	5.0±0.6	6.0±0.6

MT, memory test; PW, artificial pond water; TS1, training session 1; TS2, training session 2.

obs=31.0 ± 3.0, post-obs=22.8 ± 2.6; $F_{1,15}=4.63$, $P<0.048$). The reduction in average breathing time per pneumostome opening in snails treated with saline/Meth indicates that Meth increased the number of openings per breathing session, at least when allowed to freely breathe without stimulation to the pneumostome, which was reversed when treated with 5-AZA, as we did not observe any differences in snails treated with the combination of 5-AZA/Meth (pre-obs=30.5 ± 3.3, post-obs=31.1 ± 2.8; $F_{1,15}=0.03$, $P<0.871$). However, this effect is difficult to reconcile with that observed in Fig. 3C, because snails allowed to freely breathe without stimulation increased pneumostome openings post-observation, while Fig. 3C results indicate that snails treated with Meth decreased the number of pneumostome openings over the same passage of time when contingently stimulated. In snails treated with 5-AZA/PW, we found a decrease in average breathing time per pneumostome opening (pre-obs=41.2 ± 5.0, post-obs=24.7 ± 2.6; $F_{1,13}=9.10$, $P<0.0099$). However, this decrease did not reflect the pattern of changes found for 5-AZA alone in the PW controls contingently stimulated shown in Fig. 3A.

Experiments 7–9: effect of submersion and 5-AZA on extended memory (72 h)

Because 87 µmol l^{-1} 5-AZA reduced the expression of extended memory while leaving intact non-extended memory, we next employed a different manipulation that has previously been shown to extend memory to determine whether 5-AZA would also prevent its expression. In this set of experiments, snails were given underwater submersion after training and before testing for memory. In respiratory operant training experiments, snails learn that, in the interval after training and before testing 72 h later, they are allowed to surface breathe without their pneumostome being stimulated. This is expected to produce interference of the recently learned information that stimulation of the pneumostome is contingent upon its opening. Thus, this interference leads to forgetting, while this interference is prevented in submerged snails (Sangha et al., 2003a). Fig. 4A shows that saline controls not submerged do not show 72 h memory, as expected ($F_{2,34}=5.54$, $P<0.0083$). In contrast, Fig. 4B shows that saline controls given submersion during the 72 h interval between training and the MT demonstrated memory 72 h later ($F_{2,58}=15.2$, $P<0.0002$), replicating a previous study (Sangha et al., 2003a). Injection of 87 µmol l^{-1} 5-AZA 1–3 h prior to training did not alter non-submerged snail respiratory behavior ($F_{2,32}=4.80$, $P<0.015$; Fig. 4C), but it prevented the expression of extended memory 72 h later in the submerged group ($F_{2,44}=7.71$, $P<0.0013$; Fig. 4D). These results are similar to the findings from Meth-enhanced memory persistence, indicating that 5-AZA may promote forgetting.

Non-contingent controls given saline or 87 µmol l^{-1} 5-AZA and submersion did not alter their number of pneumostome openings across the three sessions (TS1, TS2, MT) in which they were given non-contingent pneumostome stimulation (saline/submerge: $F_{2,20}=0.33$, $P<0.721$; 5-AZA/submerge: $F_{2,28}=2.32$, $P<0.117$; Table 2).

An additional set of controls to assess total breathing time was also conducted to determine whether 87 µmol l^{-1} 5-AZA and/or submersion produced non-specific effects on respiratory behavior. Total breathing time in the post-observation session was not different from that in the pre-observation session saline ($F_{1,8}=0.15$, $P<0.711$; 5-AZA: $F_{1,7}=1.26$, $P<0.299$; Fig. 4E,F). The average breathing time per pneumostome opening (in seconds) was not different in either group (saline group: pre-obs=28.9 ± 7.0, post-obs=40.9 ± 3.4; $F_{1,7}=2.38$, $P<0.167$; 5-AZA group: pre-obs=47.8 ± 17.8, post-obs=33.6 ± 6.5; $F_{1,7}=0.73$, $P<0.421$). Collectively, these data indicate that the effects of 5-AZA combined with submersion are dependent on learning and memory events rather than on non-specific effects of drug and submersion treatment on respiratory behavior.

Experiment 10: effect of CE and 5-AZA on extended memory (24 h after single training session)

An additional manipulation to extend memory in snails was to train snails for respiratory behavior in water taken from their natural predator, crayfish (Orr and Lukowiak, 2008). This water, referred to as CE, was used to examine 24 h memory that normally is not present after a single training session. CE produces robust 24 h memory after a single training session in saline controls ($F_{1,20}=239$, $P<0.0001$), but this 24 h memory is blocked by pre-treatment with 87 µmol l^{-1} 5-AZA ($F_{1,19}=2.98$, $P<0.101$; Fig. 5A,B). Several control studies have previously shown that CE does not alter non-specific effects of memory, such as behavior in yoked controls and total breathing time (Orr et al., 2009; Orr et al., 2007). The present study shows that 5-AZA effects are specific to enhanced memory and not to memory tested only 72 h later.

DISCUSSION

We have previously shown that three of the four memory manipulations used here, including Meth exposure prior to training, underwater submersion after training and exposure to CE during training, enhance memory persistence. Here we replicated these results and, in addition, determined that memory persistence is also enhanced by pre-treatment with 5-HT. The present study extends these findings to demonstrate that the persistent memory induced by all four manipulations in *L. stagnalis* is blocked by the DNMT inhibitor, 5-AZA, given prior to training. In contrast, 5-AZA treatment had no effect on the expression of learning, as assessed by TS2 performance, or on non-extended memory, whether measured 24 or 72 h later, suggesting that changes in DNA methylation state at least partially mediate the enhanced memory persistence. These findings are the first to implicate a role for DNMTs in augmented memory in *L. stagnalis*.

Methylation of DNA is accomplished by DNMTs (DNMT1, DNMT3a and DNMT3b in mammals). In general, DNA methylation silences the expression of genes via recruitment of other proteins, including histone deacetylases and methyl-CpG binding domain proteins (Bird, 2002; Gräff and Mansuy, 2008; Miranda and Jones, 2007). In some cases, however, DNA methylation has also been found to activate gene expression (Kotini et al., 2011). Recently, studies in mammalian systems have demonstrated that DNA methylation state is dynamic and influenced by various environmental manipulations, including stress exposure (Chertkow-Deutsher et al., 2010; Kinnally et al., 2011; Uchida et al., 2011), fear conditioning (Lubin et al., 2008; Miller et al., 2010; Miller and
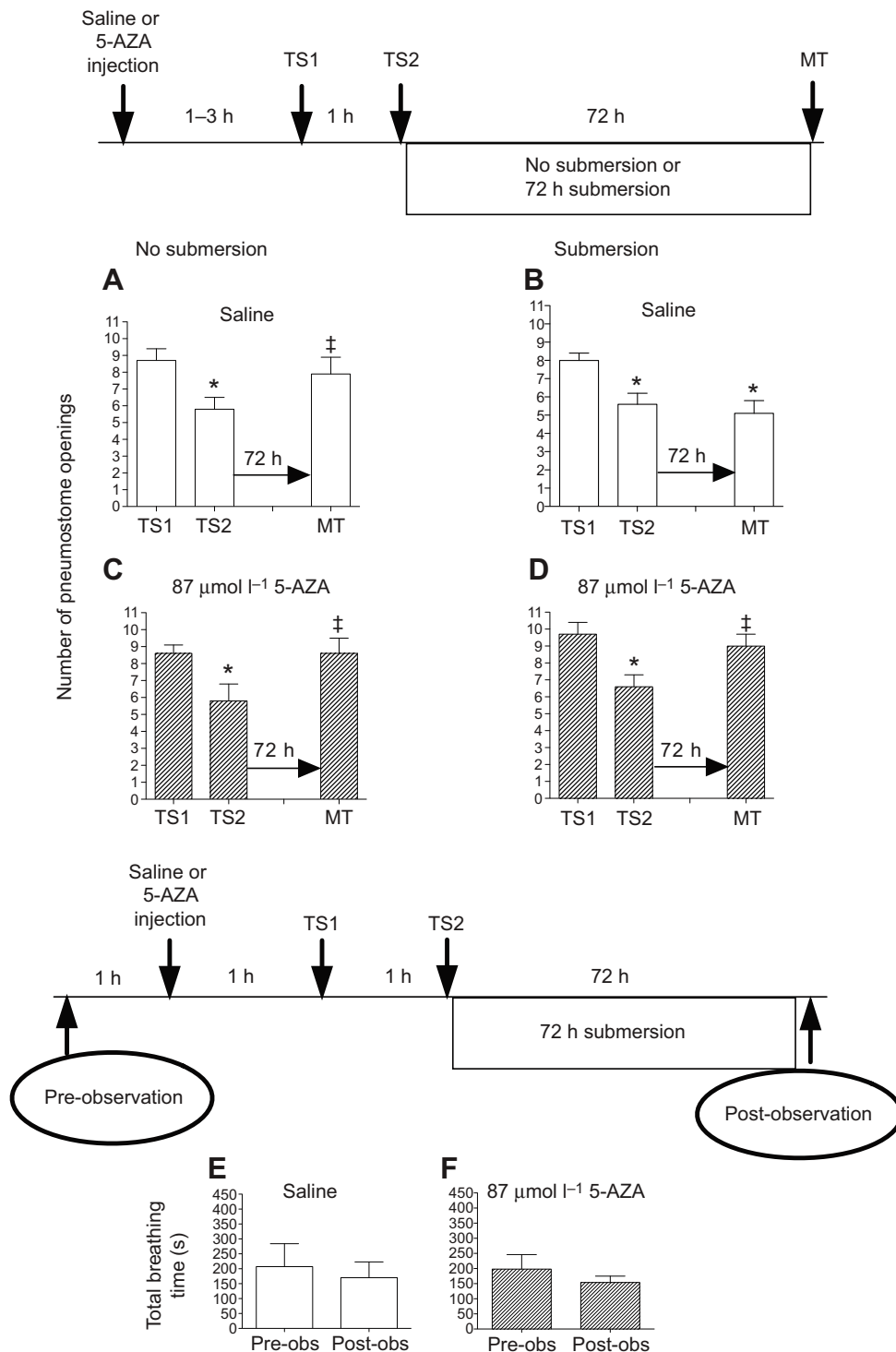
Fig. 4. Submersion of *L. stagnalis* extends memory that is prevented by 5-AZA treatment. (A–D) Mean ± s.e.m. number of pneumostome openings for the following treatments: (A) saline injection 1–3 h prior to TS1, no submersion (*N*=18); (B) saline injection 1–3 h prior to TS1, 72 h submersion (*N*=30); (C) 87 µmol l^{-1} 5-AZA injection 1–3 h prior to TS1, no submersion (*N*=17); (D) 87 µmol l^{-1} 5-AZA injection 1–3 h prior to TS1, 72 h submersion (*N*=23). Injection of 5-AZA prevented the memory-enhancing effects of submersion on memory measured 72 h later. Timeline for treatment is shown at the top. (E,F) Mean ± s.e.m. total breathing time over 30 min sessions given before (pre-obs) or after (post-obs) the following treatments: (E) saline injection and 72 h submersion (*N*=9); (F) 87 µmol l^{-1} 5-AZA injection and 72 h submersion (*N*=8). In all sessions, snails were not given stimulation to the pneumostome upon opening. Injection of 5-AZA or 72 h submersion did not alter general respiratory behavior. Timeline for treatment is shown at the top. *$P<0.05$, comparing with TS1; ‡$P<0.05$, comparing MT with TS2.

Sweatt, 2007), non-associative learning (Rahn et al., 2013), neuronal activity (Guo et al., 2011) and neural plasticity in normal learning and memory (Sultan et al., 2012). Related to the proposed studies, fear conditioning, a model for PTSD in rodents, and cocaine or chronic stress treatment increase DNA methylation, and inhibition of this methylation prevents the behavior (e.g. freezing behavior, responding for cocaine reward) (Anier et al., 2010; LaPlant et al., 2010; Miller et al., 2010), indicating that active DNA methylation and demethylation processes modify memory.

In contrast to studies in mammals, few studies have examined the role of DNA methylation in mollusks. DNMTs appear to be absent in *Drosophila melanogaster* (Zemach et al., 2010) and *Caenorhabditis elegans* (Simpson et al., 1986), although they are present in other invertebrates, and genes that are heavily methylated are among the most evolutionarily conserved (Sarda et al., 2012). Recently, Rajasethupathy and co-workers (Rajasethupathy et al., 2012) found that long-term facilitation within the sensorimotor synapse of the marine snail *Aplysia* produced by 5-HT application is prevented by the addition of the DNMT inhibitor RG108. This suppressive effect of RG108 was due to methylation of the transcriptional memory suppressor CREB2 via Piwi-interacting RNAs, leading to blockade of 5-HT-induced long-term facilitation.

Table 2. Pneumostome openings in snails given non-contingent stimulation and treated with 5-AZA and submersion

Treatment (N)	TS1	TS2	MT
Saline + Submerge (11)	4.4±0.8	3.6±0.7	4.3±0.9
87 µmol l⁻¹ 5-AZA + Submerge (15)	4.3±0.5	4.1±0.7	6.1±1.0

In agreement with this study, our current finding extends the role of 5-HT and DNMT inhibition on memory persistence from the non-associative conditioning procedure in *Aplysia* to an operant conditioning procedure in *L. stagnalis*.

The most parsimonious explanation for our findings is that the four manipulations we used to enhance memory persistence might increase memory formation via increased DNMT activity either to promote stronger memory that increases transcription of genes (Chahrour et al., 2008) that act as memory promoter genes (e.g. CREB1) or to inhibit active forgetting processes via decreased expression of memory suppressor genes [e.g. CREB2 (Landry et al., 2013)], as described above for *Aplysia*. Specific proteins have been implicated in forgetting. Studies in mammals have revealed a role for protein phosphatases in suppressing LTM formation (Genoux et al., 2002; Lee and Silva, 2009; Malleret et al., 2001; Mansuy et al., 1998); these enzymes are also important for memory in *Lymnaea* (Rosenegger et al., 2008). Such memory suppression may be due to a passive process of ongoing, basal phosphatase activity such that a decrease in phosphatase activity would ensue after stressful stimuli to extend memory. In addition, a more active suppression of protein phosphatase activity through hypermethylation of its genes combined with increased expression of memory-promoting molecules through hypomethylation of their genes occurs in mammals after fear conditioning (Miller et al., 2010; Miller and Sweatt, 2007) and cocaine treatment (Anier et al., 2010). Thus, it may be the combined action of hypomethylation of memory enhancer genes and hypermethylation of memory suppressor genes that promotes enhanced memory persistence.
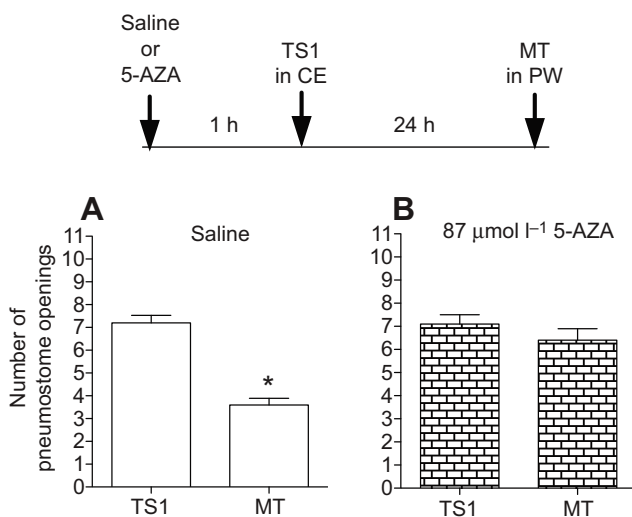

Fig. 5. Training of *L. stagnalis* in effluent from predatory crayfish (CE) extends memory that is prevented by 5-AZA treatment. Mean ± s.e.m. number of pneumostome openings in the following treatments: (A) saline injection 1 h prior to TS1 in CE (N=21); (B) 5-AZA injection 1 h prior to TS1 in CE (N=20). Snails were given a single training session and tested 24 h later. Snails given a single training session in CE demonstrated 24 h memory that was prevented by prior treatment with 5-AZA. Timeline for treatment is shown at the top. *P<0.05, comparing with TS1.

The concept that forgetting is induced by interference events just after initial learning or by decay processes has been discussed for many years (Altmann, 2009; Jenkins and Dallenback, 1924; Jonides et al., 2008; Lewandowsky et al., 2009; Wixted, 2004). Several previous studies in *Lymnaea* support the idea that forgetting is an active process. First, as first described by Lukowiak and co-workers (Sangha et al., 2003a; Sangha et al., 2005) and replicated in the present study, retroactive interference of a competing behavior (breathing from the pneumostome without reinforcement upon opening after training) leads to LTM that does not last for 72 h after two short training sessions, while underwater submersion immediately after training prevents this interference, and memory is still observed 72 h later. This effect is context dependent (Sangha et al., 2003a) and also requires the dopamine neuron RPeD1 (Sangha et al., 2005). A second line of evidence supporting an active forgetting process in *Lymnaea* is that cooling snails just after training also extends memory, likely by preventing active forgetting (Sangha et al., 2003b). A third set of experiments manipulated the level of calcium, which, in *Lymnaea*, is required to be >20 mg l⁻¹ for LTM formation (Knezevic et al., 2011). A single training session produced memory for 24 h when snails were maintained in 80 mg l⁻¹ calcium. However, if they were maintained in low calcium (20 mg l⁻¹) immediately after training, memory persisted for up to 96 h. The conclusion was that the new, interfering memory could not be formed in the absence of calcium. An example of a molecular mechanism for an active forgetting process has been described in *Drosophila*. Shuai et al. (Shuai et al., 2010) examined the molecular mechanisms of early memory forgetting by interference events that involved the expression of Rac, a member of the Rho family of GTPases that ultimately alters the actin cytoskeleton. In this model system, the molecules involved in forgetting appeared to be separate from those involved in memory. Activation of Rac promoted memory decay, and inhibition of Rac activity decreased the rate of memory decay. Thus, these findings support the idea that there are active forgetting processes during early learning that could be inhibited to enhance memory persistence.

One possibility for enhanced memory persistence induced by all four manipulations is that these stimuli serve as stressors to produce changes in gene expression. Stressful stimuli alter DNA methylation state (see above) (e.g. Zovkic et al., 2013b) and this increased methylation may result from upstream events, including increased glutamate transmission, neuropeptide transmission and increased transcription factor activation (Stankiewicz et al., 2013). The presence of CE is clearly a stressor for *Lymnaea* (Lukowiak et al., 2010; Lukowiak et al., 2014). Drugs of abuse such as cocaine and Meth activate stress pathways in the hypothalamic-pituitary-adrenal axis (Moldow and Fischman, 1987) along with its downstream consequences, and these drugs also can increase DNMT activity in rodents, (Jayanthi et al., 2014; LaPlant et al., 2010; Numachi et al., 2007). Underwater submersion and 5-HT may also activate similar stress-related downstream events involving DNMT activation. In addition, UHRF1 binding protein is upregulated after LTM formation in *Lymnaea* (Rosenegger et al., 2010); UHRF1 plays a key role in DNMT1 activity (Avvakumov et al., 2008), and may influence DNA methylation to produce enhanced memory.

Conclusions

This study has laid the groundwork for identifying gene methylation changes that are common among all four stimuli to produce enhanced memory persistence of operantly trained behavior. A major advantage to using *Lymnaea* to determine the mechanisms underlying memory persistence is the simplicity of the central

pattern generator (three neurons) that drives respiratory behavior (Lukowiak et al., 2008; Lukowiak et al., 2010). A single neuron (RPeD1) is necessary for learning, memory and forgetting in *Lymnaea*. Therefore, the study of operantly trained behavior in *Lymnaea* presents a powerful opportunity to conduct single-cell methylation analysis (Kantlehner et al., 2011) for identifying the pattern of methylation changes that produce enhanced memory persistence.

MATERIALS AND METHODS
Animals
Laboratory-reared *L. stagnalis* were obtained from stocks at the University of Calgary, Canada, that were originally derived from snails established at Vrije Universiteit Amsterdam. Animals were kept in aerated dechlorinated water at 22–24°C at Washington State University or in artificial pond water (PW) at the University of Calgary. Artificial PW was made using the following formula: $0.26\,g\,l^{-1}$ Instant Ocean® (Spectrum Brands Inc., Madison, WI, USA) with additional calcium sulfate dihydrate or calcium chloride dihydrate to make our standard calcium of $80\,mg\,l^{-1}$ (Dalesman and Lukowiak, 2010). For all training sessions and home aquaria between training/memory sessions, snails were kept in PW. The snails had intermittent access to food (green leaf lettuce supplied three times per week). All snails had a shell length of 2.3–3.0 cm before experimental use. Snails were labeled and acclimated for 24 h to artificial PW in a smaller aquarium (referred to as the home aquarium) prior to beginning the experiment. Timelines for all experiments are provided in each figure.

Drugs
(+)-Methamphetamine hydrochloride (Meth), 5-AZA and 5-HT were obtained from Sigma Chemical Company (St Louis, MO, USA). The concentration of Meth ($3.3\,\mu mol\,l^{-1}$) is reported as weight/volume of the salt. Meth was dissolved in PW. The 5-AZA was dissolved in sterile saline, and 5-HT ($100\,\mu mol\,l^{-1}$) was dissolved in buffer (composition in $mmol\,l^{-1}$: 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, 5.0 HEPES, pH 7.9). Based on pilot studies in the Sorg and Lukowiak laboratories, we tested the effect of different doses of 5-AZA and found that the lowest dose tested, $8.7\,\mu mol\,l^{-1}$, did not significantly alter enhanced memory persistence and that the highest dose tested, $870\,\mu mol\,l^{-1}$, appeared to have non-specific suppression of respiratory behavior in PW controls. Therefore, the middle dose of $87\,\mu mol\,l^{-1}$ 5-AZA was chosen for all studies described here. Saline, 5-AZA or 5-HT (100 µl for all three drugs) was injected into the haemocoel through the foot of the snail. The concentrations and injection times were determined and optimized based on our pilot data from breathing observation studies.

Operant conditioning procedure
A hypoxic environment (P_{O2}<931 Pa) was created by bubbling N₂ through 800 ml of PW for 20 min (except for experiments with CE, which used 500 ml of CE; see individual experiments for details). The rate of N₂ flow was reduced and snails were given a 10 min acclimation period [dissolved oxygen was stable during the entire session, similar to that reported by Rosenegger et al. (Rosenegger et al., 2004)]. In a hypoxic environment, *Lymnaea* significantly increase their respiratory behavior (Lukowiak et al., 1996). All training and test sessions in hypoxia were conducted in this fashion for 30 min. The experiment was begun by gently pushing each snail below the surface to signify the beginning of the training period. Training consisted of gently poking the open pneumostome with a sharpened wooden probe. This stimulus caused immediate closure of the pneumostome but did not cause the snail to withdraw into its shell. The total number of pneumostome openings over the training period was tabulated for each snail. Because of possible floor effects after training, snails that opened their pneumostomes fewer than four times in the first training session were no longer used in the experiment. Between training and test sessions, snails were returned to their home eumoxic aquaria. For snails given underwater submersion, a mesh screen was placed into the home aquaria to prevent snails from surface breathing. For snails trained in CE, 500 ml of water was taken from a 3 l bucket where a crayfish was placed for 1 h.

For all studies, snails were subjected to one of the following training procedures: (1) a single 30 min training session in PW or CE; or (2) two 30 min training sessions separated by a 1 h interval. A memory test (MT) session was performed after 24 h or more (e.g. 72 h) to test whether LTM was present. All MT sessions were performed in PW.

Yoked controls, non-contingent stimulus controls and total breathing time analysis
For some experiments, we performed yoked control experiments. Yoked control animals underwent the same experimental treatment as operantly trained snails with one exception. For the yoked control training session, the snails received exactly the same number of stimuli using the same pattern of stimulation as those of the operant conditioning group. That is, they received the tactile stimulus near the pneumostome when the snail to which they were 'yoked' to opened its pneumostome in the operant conditioning procedure. Because snails are not withdrawn into their shells, the area near the pneumostome is accessible enough to provide a stimulus even when it remains closed and snails are submerged. Thus, the presentation of the tactile stimulus was not contingent on an attempted pneumostome opening of the yoked control snail. In the MT session, these snails received the tactile stimulus to the pneumostome when they attempted to open their pneumostomes. Between all sessions, snails were returned to their home eumoxic aquaria.

For other experiments, we performed additional non-contingent stimulus controls that received a non-contingent stimulus during all sessions (training and MT sessions) to confirm that the changes we observed in respiratory behavior in other experiments were dependent on application of the stimulus immediately after the snail opened its pneumostome. These were similar to yoked controls in that these snails were 'paired' with partners that were operantly trained as described above. In these snails, we tabulated the number of pneumostome openings even though they were not necessarily paired with a stimulus to the pneumostome. This training was repeated as for the operant conditioning procedure. Between all sessions, snails were returned to their home eumoxic aquaria.

To ensure that the drug (see below) or injections in these experiments did not cause any adverse effect on snails' baseline aerial respiratory behavior that might indicate changes in general metabolic state, breathing observation sessions were performed. In breathing observation sessions, snails were placed in a 1 l beaker containing 800 ml of hypoxic PW, given a 10 min acclimatization period, and their breathing behavior was observed in a 30 min session (the same amount of time as for the training sessions). The time at which each snail opened and closed its pneumostome was recorded. From these data, the total breathing time before and after drug treatments was calculated and compared to determine whether the drugs affected the snail's ability to perform aerial respiration. We also calculated the average breathing time per pneumostome opening. Between all sessions, snails were returned to their home eumoxic aquaria.

Criteria for memory
Memory was considered to be present if the number of attempted pneumostome openings in the MT session was significantly lower than that of the first training session (TS1) and was not significantly higher than that of the last training session (TS2 when two training sessions were conducted).

Experiments 1 and 2: effect of 5-HT and 5-AZA on extended memory (24 h after single training session)
Previous work demonstrated that the 5-HT antagonist, mianserin, blocked the memory-extending effects of CE (Il-Han et al., 2010). We therefore determined whether 5-HT would enhance memory persistence and also whether 5-AZA would prevent any memory-enhancing effects of 5-HT. For Experiment 1, snails were injected with 5-HT at 2 h prior to an injection of either saline or 5-AZA followed by a single 30 min training session 1 h later. Memory was tested 24 h later. For snails injected with saline instead of 5-AZA, we also conducted a yoked control test to demonstrate that 5-HT effects were dependent on contingent pneumostome stimulation. For Experiment 2, we conducted additional control tests for total breathing time and average breathing time per pneumostome opening after 5-HT and 5-

AZA injections. For testing whether total breathing time or average breathing time per pneumostome opening was altered, pre- and post-observation sessions (tactile stimuli were not presented in these sessions) were used. The time interval between the pre- and post-observation sessions was 24 h.

Experiment 3: effect of 5-AZA on non-extended memory (24 h after two training sessions)

This experiment was conducted to determine whether 5-AZA would affect non-extended memory, that is, memory normally present at 24 h after two training sessions (Parvez et al., 2005). We injected saline or 5-AZA (87 µmol l^{-1}) as before and assessed the effect on LTM 24 h later.

Experiments 4–6: effect of Meth pre-exposure on extended memory (72 h)

Meth exposure was carried out by placing snails in 1 l of normoxic 3.3 mmol l^{-1} Meth solution for 45 min. This dose and duration of Meth exposure was chosen based on a previous study in our laboratory (Kennedy et al., 2010). Pilot studies examined the effect of 1, 4, 8 or 24 h pre-exposure to Meth on memory (data not shown). Based on the results of this smaller study, we chose to further test the impact of Meth pre-exposure 4 h prior to operant training as described above. During the 4 h interval, snails were returned to their home tanks in normoxic water.

To determine the degree to which extended memory by Meth was dependent on DNA methylation, in Experiment 4, we injected either saline or the DNMT inhibitor 5-AZA (87 µmol l^{-1}) into snails 1 h prior to Meth pre-exposure. Snails were trained and tested as before. In Experiments 5 and 6, we conducted additional controls by testing the impact of 5-AZA injection followed by Meth-pre-exposure on pneumostome stimulation that was not contingent upon pneumostome opening, and on total breathing time and average breathing time per pneumostome opening in the absence of pneumostome stimulation.

Experiments 7–9: effect of submersion and 5-AZA on extended memory (72 h)

To determine whether 5-AZA injection prior to training would affect memory extension by underwater submersion during the 72 h period between the training sessions and the MT, snails were given either saline or a 5-AZA injection 1–3 h prior to the first training session. We had determined in pilot studies that the outcome of the MT was not different when snails were injected 1, 2 or 3 h prior to training. After the second training session, snails were returned to their eumoxic home aquaria and a mesh screen was placed in the tank such that snails could not surface breathe. Placing snails under the mesh in eumoxic PW did not in any apparent way harm the snails. In Experiments 8 and 9, we conducted additional submersion controls by testing the impact of 5-AZA injection followed by sessions during which pneumostome stimulation was not contingent upon pneumostome opening or by sessions during which total breathing time and average breathing time per pneumostome opening in the absence of pneumostome stimulation was measured.

Experiment 10: effect of CE and 5-AZA on extended memory (24 h after single training session)

Our previous study demonstrated that two training sessions in CE produced memory that would last for up to 8 days (Orr and Lukowiak, 2008). Here we used an abbreviated protocol, training snails with a single session and testing for memory 24 h later. We have shown that a single 30 min training session does not produce memory 24 h later in PW (Lukowiak et al., 2000). Snails trained in CE and tested in PW demonstrate memory 24 h later (Orr and Lukowiak, 2008). For Experiment 10, snails were injected with either saline or 5-AZA followed by a single 30 min training session 1 h later. Memory was tested 24 h later.

Data analyses

For all data where more than two time points were compared, a one-way ANOVA with a repeated measure over session was conducted followed by a Fisher's protected least significant difference test in the case of a significant effect. For data in which only two groups were compared, a two-tailed Student's paired t-test was used. The level of significance was set at $P<0.05$. The F- and P-values for all data are reported.

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