

Methamphetamine enhances memory of operantly conditioned respiratory behavior in the snail *Lymnaea stagnalis*

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SUMMARY

Amphetamines have been used as cognitive enhancers to promote learning and memory. Amphetamines are also drugs of abuse that may promote the initiation of strong memories that ultimately lead to addiction. To understand how methamphetamine (Meth) may be augmenting learning and memory, we chose a relatively simple system, the pond snail, *Lymnaea stagnalis*. We studied the effects of Meth exposure on the long-term memory (LTM), extinction and reinstatement of operantly conditioned aerial respiratory behavior in *Lymnaea*. We first determined doses of Meth that would acutely alter respiratory behavior. Next, we measured the impact of training snails in Meth solution or water (control group) using a training procedure that produces LTM (>6 h) in control conditions. Meth exposure impaired the expression of LTM 21 h after two training sessions, but this appeared to be a context-dependent effect only. However, snails exposed to 3.3 $\mu\text{mol l}^{-1}$ Meth during training had a decreased rate of extinction of the operantly conditioned memory. We then tested whether this decreased ability of snails to extinguish memory was due to enhanced LTM or impaired extinction of that memory. Snails were operantly conditioned in water and exposed to Meth 16 h after their last trial but 4–5 h prior to extinction. Meth produced an increase rather than a decrease in extinction rate. Thus, Meth impaired extinction only when snails were exposed to Meth during training. Last, we tested the effect of Meth on the ability to form LTM using a single training procedure that is suboptimal for LTM formation. Control snails did not demonstrate LTM, as expected, but pre-exposure of snails to 3.3 $\mu\text{mol l}^{-1}$ Meth 24 h prior to the single training session produced LTM 24 h later, indicating that Meth pre-exposure primed snails for LTM formation. Taken together, our studies suggest that LTM is strengthened by Meth such that extinction training is less effective. *Lymnaea* provides a simple and useful model system to dissect the cellular and/or molecular mechanisms of how Meth may initiate the formation of stronger memories.

Key words: methamphetamine, consolidation, extinction, memory, reinstatement, snail.

INTRODUCTION

Psychostimulants have been shown to enhance learning and memory (Carmack et al., 2010; Martinez et al., 1980; Soetens et al., 1995; Wood and Anagnostaras, 2009). An understanding of how these drugs initiate enhanced learning and memory is valuable from two perspectives: first, such drugs could be useful as cognitive enhancers in humans with memory impairments, and second, amphetamines as drugs of abuse are believed to produce persistent memory by inducing a type of pathological memory (Berke and Hyman, 2000; Hamilton and Kolb, 2005; Huang et al., 2009; Liu et al., 2005; O'Brien et al., 1992; Robinson and Kolb, 2004; Thomas et al., 2008; Wise, 2000; Wolf et al., 2004). Most of the focus on psychostimulants has been on the ability of amphetamines to enhance formation of several different forms of memory (Blais and Janak, 2006; Blais and Janak, 2007; Fenu and Di Chiara, 2003; Janak and Martinez, 1992; McGaugh, 2000; O'Carroll et al., 1988; Packard and Teather, 1998; Simon and Setlow, 2006; Wiig et al., 2009).

However, one of the obstacles to understanding detailed mechanisms by which basic learning and memory processes are enhanced after exposure to amphetamines is the complexity of the mammalian brain. In the present study we used the freshwater pond snail, *Lymnaea stagnalis*, which provides a relatively simple model

system suitable for studying learning, memory and reinstatement behavior after extinction (Lukowiak et al., 2006; Lukowiak et al., 1996; Sangha et al., 2003b). *Lymnaea* are bimodal breathers using both cutaneous and aerial systems. Aerial respiration occurs through a breathing tube called the pneumostome, and snails can be operantly conditioned to reduce opening of their pneumostome despite low oxygen levels that drive them to the surface to open this structure. In the operant conditioning procedure, snails are placed into a hypoxic environment, and every time they begin to open their pneumostome, they receive a tactile stimulus to this structure. The stimulus causes the snail to close the pneumostome, and after training, snails learn not to open their pneumostome.

Aerial respiratory behavior is driven by a central pattern generator (CPG) consisting of three neurons whose sufficiency and necessity have been experimentally determined: right pedal dorsal 1 (RPeD1), ventral dorsal 4 (VD4) and input 3 (IP3) interneurons (Syed et al., 1990; Syed et al., 1992). Learning, memory and extinction have all been shown to be dependent on RPeD1, which is dopaminergic (Cottrell et al., 1979; Sangha et al., 2004; Spencer et al., 2002). Dopaminergic neurons have been shown to be involved in learning both appetitive (Everitt and Robbins, 2005; Nestler, 2002; Wise et al., 1978) and aversive (Ader and Clink, 1957; Salamone, 1994; White et al., 1992) behaviors. Thus, the circuitry involving RPeD1

may be susceptible to alteration by psychostimulant treatment, and these changes may in turn be assessed by examining basic mechanisms of learning and memory underlying operant conditioning of aerial respiration as well as extinction and reinstatement of this learned response.

We previously reported that repeated cocaine exposure produced greater reinstatement of operantly conditioned respiratory behavior in *Lymnaea* (Carter et al., 2006). These studies suggested that initial molecular processes underlying long-term memory (LTM) formation were enhanced or that extinction was impaired by prior repeated cocaine treatment. In the present studies, we followed up this finding using another psychostimulant, methamphetamine (Meth). Because of its relative simplicity, *Lymnaea* provides an excellent model system to dissect the mechanisms by which Meth promotes learning and/or memory. As a first step toward understanding these mechanisms, we assessed the impact of Meth on LTM formation and extinction processes. We tested the hypothesis that Meth promotes learning and memory and/or impairs extinction learning such that the original memory formed under the influence of Meth is strengthened and more persistent. An understanding of these mechanisms in this relatively simple system is expected to lead to delineation of cellular pathways that produce the persistent memories established in the presence of psychostimulants.

MATERIALS AND METHODS

Animals

Laboratory-reared stocks of *Lymnaea stagnalis* L. were obtained from stocks at the University of Calgary, Canada, which were originally derived from snails established at *Vrije Universiteit* Amsterdam. A total of 311 snails were used for these experiments. Animals were kept in aerated dechlorinated water at 22–24°C. The snails had intermittent access to food (green lettuce supplied three times per week). All animals had a shell length of 2.3–3.0 cm before experimental use.

Drugs

(+)-Methamphetamine hydrochloride (Meth) was obtained from Sigma Chemical Company (St Louis, MO, USA). Concentrations of Meth (ranging from 0.3 to 3.3 $\mu\text{mol l}^{-1}$) are reported as weight/volume of the salt.

Drug exposure

For Experiments 1, 6 and 7, Meth was dissolved in 1 litre of normoxic water [Pullman City water equilibrated (de-chlorinated) for a minimum of 1 week; referred to as 'Water']. The various concentrations of Meth were dissolved in Water. For experiments 2–5, Meth exposure was given in hypoxic Water during training sessions (see details of each experiment below). Snails were exposed to Meth for 30 min in Experiment 1 (dose–response) and 45 min for all other experiments. For all experiments, animals were taken from a supply aquarium and acclimated to a smaller aquarium (referred to as the home aquarium) for a minimum of 24 h prior to beginning experimentation.

Operant conditioning procedure

A hypoxic environment [approximately 1.8 mg l^{-1} dissolved oxygen (prior to bubbling N_2 , dissolved oxygen was 8.0 mg l^{-1})] was created by bubbling N_2 through 800 ml of Water for 20 min. The rate of N_2 flow was reduced and snails were given a 10 min acclimation period in the 800 ml Water [dissolved oxygen was stable during the entire session, similar to that reported by Rosenecker et al. (Rosenecker et al., 2004)]. All sessions in hypoxia were conducted in this fashion

for either 30 min (Experiment 1) or 45 min (all other experiments). 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 initial training session were no longer used in the experiment.

Freely behaving and yoked controls

We performed both freely behaving and yoked control experiments to confirm that the changes we observed in respiratory behavior were contingent upon application of the stimulus immediately after the snail opened its pneumostome. For analysis of the freely behaving control group, snails were placed into the hypoxic environment as described above, but no stimuli were given to the pneumostome. The number of pneumostome openings was recorded over each session. For analysis of the yoked control groups, yoked snails were 'paired' with partners that were operantly trained as described above. Snails were placed into the same hypoxic conditions used in the operant conditioning procedure. Then, yoked animals received a gentle tactile stimulus to their pneumostome area whenever their yoked partner's pneumostome opened and was subsequently stimulated. Thus, stimulus of the pneumostome was not contingent upon opening the pneumostome as it was in trained animals. This training was repeated using the same training sequence as used in the operant conditioning procedure. Pneumostome openings were also tabulated as in the operant conditioning procedure. Freely behaving and yoked controls were given the same number and timing of sessions as described under experiment 2–4 and shown in Table 1.

Extinction training and reinstatement

In some experiments described below, we conducted extinction sessions. Three extinction sessions of 45 min each were performed as described by McComb et al. (McComb et al., 2002); see Table 1. The first extinction session was begun 1 h after Trial 3, and the second extinction session was given 1 h after the first session. The third extinction session was given 24 h later. However, during extinction, no stimulus to the pneumostome was given. Animals were allowed to freely perform aerial respiration. The time of each pneumostome opening was recorded. To test for the memory of

Table 1. Training protocol for operant conditioning in Experiments 2–4

Trial 1 (45 min with tactile stimulation to pneumostome)
Home (1 h)
Trial 2 (45 min with tactile stimulation to pneumostome)
Home (21 h)
Trial 3 (45 min with tactile stimulation to pneumostome)
Home (1 h)
Extinction 1 (45 min with no stimulation)
Home (1 h)
Extinction 2 (45 min with no stimulation)
Home (21 h)
Extinction 3 (45 min with no stimulation)
Home (2 h)
Reinstatement (45 min with tactile stimulation to pneumostome)
All sessions except 'Home' were performed under hypoxic conditions.

extinction, tactile stimulation was given during a fourth session, the Reinstatement session, starting 2 h after the last extinction session.

Experiment 1: Meth dose–response of respiratory behavior

To determine whether there were changes in breathing behavior after acute Meth exposure and to determine the range of doses to use for subsequent experiments, we recorded the total breathing time of animals for a 30 min period 1 h *prior* to Meth exposure, for a 30 min period *during* Meth exposure, and for a 30 min period 1 h *after* Meth exposure. All observations were performed in a hypoxic environment. During the 1-h intervals, snails were returned to their home tanks in normoxic water. To determine total breathing time, the time of each pneumostome opening and its subsequent closing were recorded so that the duration of breathing time could be determined for each pneumostome opening. Duration for each opening was summed over the entire session to obtain total breathing time.

Experiments 2–4: effect of Meth during training on LTM, extinction and reinstatement

We next tested whether training in Meth would alter LTM 21 h later, and, subsequently, whether it would alter extinction (Experiment 3) and/or reinstatement (Experiment 4; see Table 1 for full procedure). Based on the dose–response curve results, we chose to use both the $1.0\ \mu\text{mol l}^{-1}$ and $3.3\ \mu\text{mol l}^{-1}$ dose of Meth. The basic training module consisted of three 45-min sessions, all under hypoxic conditions: Trial 1, Trial 2, and Trial 3. During these sessions, the conditioning procedure was applied and the number of pneumostome openings was recorded. Trial 1 and Trial 2 were separated by a 1 h inter-trial interval during which the animals were housed in their home aquaria. Trial 3 was given 21 h after Trial 2. This training protocol has been shown to create a memory that lasts for up to 5 days (McComb et al., 2002). Extinction 1 was given 1 h after Trial 3, and Extinction 2 was given 1 h after Extinction 1. After a 21 h period, Extinction 3 was given, followed by Reinstatement 2 h later.

Previous research indicates that the ability of snails to recall learned behavior is dependent on the context in which the memory is tested. Haney and Lukowiak (Haney and Lukowiak, 2001) found that if snails were trained in the presence of a food odorant (carrot), they could exhibit recall only if tested in the food-odorant context in which they were initially trained. Thus, immersion of snails in $1.0\ \mu\text{mol l}^{-1}$ and $3.3\ \mu\text{mol l}^{-1}$ Meth during the initial two trials in Experiment 2 may have been perceived as a different context and thus given the appearance of impaired memory on Trial 3, which is given in the *absence* of Meth. To test for this possibility, an additional group of snails was tested using the standard protocol, but snails were instead submerged in $3.3\ \mu\text{mol l}^{-1}$ Meth solution during Trial 3 in addition to during Trials 1 and 2. The number of pneumostome openings was recorded for Trials 1–3.

Experiment 5: effect of Meth in freely behaving and yoked control groups

To determine whether the changes in the number of pneumostome openings observed after training for LTM, extinction and reinstatement were due to stimulation of the pneumostome contingent upon its opening, we also conducted six additional control groups: Water freely behaving, Water yoked, $1.0\ \mu\text{mol l}^{-1}$ Meth freely behaving, $1.0\ \mu\text{mol l}^{-1}$ Meth yoked, $3.3\ \mu\text{mol l}^{-1}$ Meth freely behaving, and $3.3\ \mu\text{mol l}^{-1}$ Meth yoked. Snails were given the exact same protocol as described in Table 1 but were not given any pneumostome stimulation (freely behaving) or were given a stimulus near the pneumostome area independently of the yoked snail's

position in the beaker whenever the partner they were yoked to opened its pneumostome and was stimulated.

Experiment 6: effect of Meth after training and before extinction on extinction and reinstatement

In this experiment, we sought to determine whether the changes we observed in extinction in Experiment 3 were due to the impact of Meth exposure on initial learning (consolidation) processes or on extinction learning processes. Snails were operantly trained as in Experiment 3 (Trials 1–3), and then given a 16 h period in the home aquaria prior to a 45 min exposure to $3.3\ \mu\text{mol l}^{-1}$ Meth in normoxic Water. This 16 h period of time off was given because we wished to avoid any possible impact of Meth exposure on the consolidation of memory from the Trial 3 session, which is another training session. After Meth exposure, snails were placed back into their home aquaria for 4–5 h and then were given Extinction 1, Extinction 2, Extinction 3, and Reinstatement exactly as described above for Experiments 2–4.

Experiment 7: effect of Meth pre-exposure on LTM formation

These studies were conducted to determine whether *prior* exposure to Meth would enhance the formation of LTM. We based our modified training protocol on that of Lukowiak et al. (Lukowiak et al., 2000) in which a single 30-min training session did not produce LTM when tested 24 h later. Our pilot experiments indicated that a single 45-min training session also did not produce the expression of LTM 24 h later, so we used the 45-min training session, since this was the time used for all other experiments in which snails were trained. Based on the results of Experiment 1, we chose a dose of $3.3\ \mu\text{mol l}^{-1}$ Meth for pre-exposure. Snails were exposed to $3.3\ \mu\text{mol l}^{-1}$ Meth in normoxic Water for a 45 min period. After a 24 h period in their home aquaria, snails were then given a single 45 min Trial 1 in hypoxic Water as described above. After another 24 h period in their home aquaria, snails were given a 45 min Trial 2, and the number of pneumostome openings for each of the two sessions was recorded.

Definition of learning, memory, extinction memory and reinstatement

We defined learning as a significant decrease in pneumostome openings compared with the previous session (McComb et al., 2002). Demonstration of LTM in Trial 3, given 21 h after Trial 2, was determined by two criteria: (1) the number of pneumostome openings during a session must be significantly less than observed during Trial 1, and (2) the number of pneumostome openings must not be significantly more than in Trial 2.

For extinction sessions, since animals are not stimulated when they open their pneumostome, the number of pneumostome openings is no longer necessarily related to the amount of breathing time allowed after surfacing, since snails can surface breathe through the pneumostome as long as necessary without being stimulated. Thus, we compared the number of pneumostome openings during Extinction sessions 1–3 with the number of openings in freely behaving and in yoked controls, since the latter groups were also not stimulated (freely behaving) or were not stimulated in a manner that was contingent upon pneumostome opening (yoked). We therefore defined extinction as a significant increase in the number of pneumostome openings compared with Extinction 1.

For the Reinstatement test, snails were again given the pneumostome stimulus upon opening. Snails were considered to have reinstated their previous behavior if the number of pneumostome openings was significantly less than in Trial 1 and not significantly higher than Trial 3.

Data analyses

For all data that included more than one session or treatment, a two-way ANOVA with a repeated measure over session was conducted followed by a Fisher's least significant difference (LSD) test in the case of a significant interaction. For data that included only a single session or single treatment, a one-way ANOVA was conducted. The level of significance was set at $P < 0.05$.

RESULTS

Experiment 1: Meth dose–response effects on respiratory behavior

We first determined the dose of Meth that would have noticeable effects on respiratory behavior without producing long-lasting changes in this behavior after removing snails from Meth. These studies were conducted to guide the remaining studies in which operant conditioning was done. We chose a method similar to that used by Browning and Lukowiak (Browning and Lukowiak, 2008). For this procedure, total breathing time and the number of pneumostome openings were measured initially (pre-observation period) in snails that were placed into hypoxic Water and 1 h later were immersed in Water (‘0’ dose) or one of three concentrations of Meth (0.3, 1.0, 3.3 $\mu\text{mol l}^{-1}$) for 30 min. For total breathing time (Fig. 1A) a two-way ANOVA showed a significant effect of session

($F_{2,126}=7.0$, $P < 0.0013$). Immersion in 1.0 $\mu\text{mol l}^{-1}$ Meth suppressed total breathing time when compared with the pre-observation period (session $F_{2,47}=8.0$, $P < 0.0016$). In animals receiving the highest dose of Meth (3.3 $\mu\text{mol l}^{-1}$), total breathing time was not suppressed during Meth immersion compared with their pre-observation period. During post-observation, total breathing time in all groups was not different from that during pre-observation. The data in Fig. 1B show the number of pneumostome openings. We give these data here because this is the measure we report throughout. A two-way ANOVA showed a significant effect of session ($F_{2,126}=6.6$, $P < 0.0018$). A one-way ANOVA across sessions within each treatment revealed that snails immersed in either 1.0 or 3.3 $\mu\text{mol l}^{-1}$ Meth suppressed the number of pneumostome openings compared with the pre-observation period (1.0 $\mu\text{mol l}^{-1}$ Meth: session $F_{2,47}=9.5$, $P < 0.0006$; 3.3 $\mu\text{mol l}^{-1}$ Meth: session $F_{2,50}=4.1$, $P < 0.026$). During post-observation, the number of pneumostome openings did not completely return to pre-observation baseline for the 1.0 $\mu\text{mol l}^{-1}$ Meth group but did rebound to pre-observation baseline levels for the 3.3 $\mu\text{mol l}^{-1}$ Meth group. For all subsequent experiments, we chose to examine the impact of either 1.0 $\mu\text{mol l}^{-1}$ and/or 3.3 $\mu\text{mol l}^{-1}$ Meth on operant conditioning, extinction and reinstatement because these doses were apparently perceived by the snail and yet had only relatively mild effects on respiratory behavior under basal (non-stimulated) conditions.

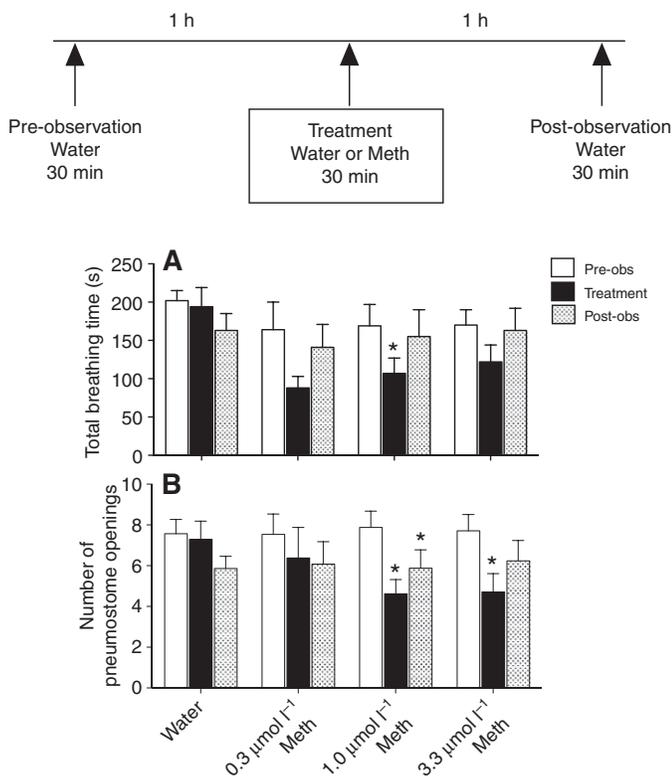


Fig. 1. Dose–response effects of acute methamphetamine (Meth) exposure on respiratory behavior. (Top) Experimental protocol. Snails were observed for 30 min in Water, then 1 h later were observed for 30 min while immersed in Water (control) or Meth, and 1 h later were observed for an additional 30 min in Water. (A) Total breathing time and (B) the number of pneumostome openings before, during and after Meth exposure. Data are expressed as mean \pm s.e.m. of total breathing time (sec; A) and number of pneumostome openings before (B). All observations were made under hypoxic conditions. * $P < 0.05$, compared with the pre-observation period within the same treatment group. Number of snails tested: 21 (Water); 13 (0.3 $\mu\text{mol l}^{-1}$ Meth); 16 (1.0 $\mu\text{mol l}^{-1}$ Meth); 17 (3.3 $\mu\text{mol l}^{-1}$ Meth).

Experiment 2: effect of Meth during training on LTM

We next determined whether training in Meth would promote learning and/or memory such that LTM is expressed to a greater extent. To determine this, we chose a training procedure (see Materials and methods and Table 1) in which snails were trained in the presence of Meth for two trials and then tested for LTM 21 h later in the absence of Meth. This procedure was used to assess whether memory in the absence of Meth would be altered after training in the presence of Meth. The training procedure we used has been shown to last for 5 days in control snails (McComb et al., 2002). Fig. 2 shows that training in Meth altered respiratory behavior across sessions. There was a main effect of treatment (treatment $F_{2,88}=4.92$; $P < 0.0094$), session (session $F_{2,176}=43.79$; $P < 0.0001$), and a treatment \times session interaction (interaction $F_{1,176}=3.66$; $P < 0.0069$). In Water controls, two trials given 1 h apart resulted in learning in Trial 2 compared with Trial 1, and also resulted in LTM when tested the following day in Trial 3. When snails were trained in 1.0 $\mu\text{mol l}^{-1}$ Meth, a different pattern emerged. Snails showed learning on Trial 2 compared with Trial 1, but on Trial 3, although the number of pneumostome openings was still less than in Trial 1, it was higher than in Trial 2; thus, these animals did not fulfill the criteria for LTM. Snails trained in 3.3 $\mu\text{mol l}^{-1}$ Meth had a higher number of pneumostome openings in Trial 1 compared with Water controls in Trial 1 and demonstrated significant learning during Trial 2. However, no LTM was expressed during Trial 3 in this group. Thus, training in Meth during Trials 1 and 2 did not alter learning expressed in Trial 2, but training in Meth suppressed the expression of LTM during Trial 3.

Since snails trained in Meth did not express LTM in Trial 3 as shown in Fig. 2 above, we reasoned that Meth-treated snails may not have demonstrated memory in Trial 3 because this session was done in the absence of Meth, whereas the first two trials were done in the presence of Meth. This reasoning was based on work by Haney and Lukowiak (Haney and Lukowiak, 2001) who demonstrated that snails display context-dependent memory. That is, training in one context does not produce apparent LTM when snails are tested in a context different from that training context. Thus, in the present study, snails may have perceived Meth as a context different from Water. To test

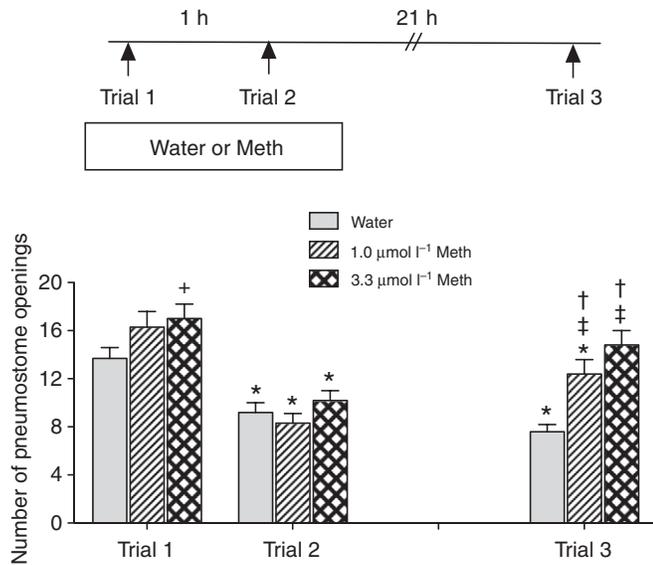


Fig. 2. Meth exposure during training does not allow for expression of LTM when tested in the absence of Meth. (Top) Experimental protocol. (Bottom) The number of pneumostome openings was measured over Trials 1–3. Data are mean \pm s.e.m. for number of pneumostome openings. Animals were trained in Water ($N=24$), $1.0\ \mu\text{mol l}^{-1}$ Meth ($N=38$) or $3.3\ \mu\text{mol l}^{-1}$ Meth ($N=29$) during Trial 1 and Trial 2. All treatment groups demonstrated learning in Trial 2 compared with Trial 1. Water controls showed LTM in Trial 3, whereas Meth-exposed snails did not express LTM in Trial 3. * $P<0.05$, compared with Trial 1 within the same treatment group; † $P<0.05$, compared with Trial 2 within the same treatment group; ‡ $P<0.05$, compared with Water controls during the same session.

for this possibility, snails were trained *and* tested for LTM in the presence of Meth. For comparison, Water controls and snails trained in $3.3\ \mu\text{mol l}^{-1}$ Meth but tested in Water are taken from Fig. 2, and the results of this comparison are shown in Fig. 3. A two-way ANOVA revealed a main treatment, session, and treatment \times session interaction (treatment $F_{2,65}=15.4$, $P<0.0001$; session $F_{2,130}=36.9$, $P<0.0001$; interaction $F_{4,130}=4.9$, $P<0.0011$). The main finding was that snails immersed in $3.3\ \mu\text{mol l}^{-1}$ Meth during Trials 1–3 exhibited LTM in Trial 3 comparable to Water controls. These findings suggest that $3.3\ \mu\text{mol l}^{-1}$ Meth (and likely $1.0\ \mu\text{mol l}^{-1}$ Meth, but this remains untested) provided a contextual change between the first two trials (in which Meth was present) and Trial 3 (in which Meth was absent). This suggests that snails exposed to Meth during Trials 1 and 2 form LTM but do not express it during Trial 3 unless Meth is also present.

Experiment 3: effect of Meth during training on extinction

We next asked the question: would training in Meth promote LTM such that the memory is more persistent, as evidenced by a resistance to extinction? This question was examined because there is evidence that extinction learning may be impaired in mammals given repeated psychostimulants (Borowski and Kokkinidis, 1998). We subjected the same snails as used in the tests in Fig. 2 to extinction training (i.e. a tactile stimulus was not delivered to the pneumostome when the snail opened it in each of three 45 min extinction training sessions; see Materials and methods, and results from Experiment 5 below). Fig. 4A–D shows the response to extinction sessions among the three treatment groups. It is important to note that during extinction sessions, snails are not stimulated upon opening their pneumostome, so the appropriate comparisons are between operantly conditioned snails during extinction and snails that were given all the same treatments and sessions but not

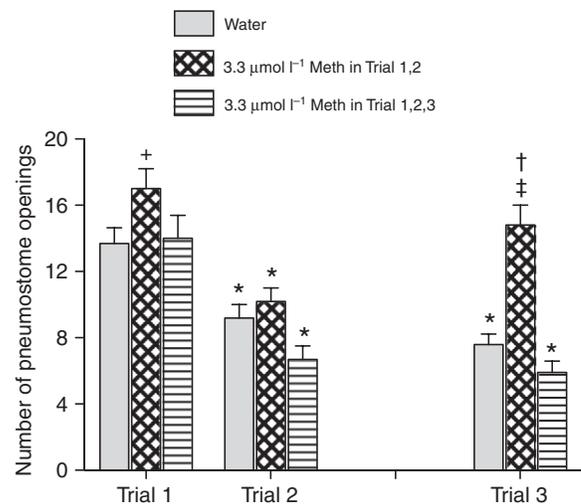
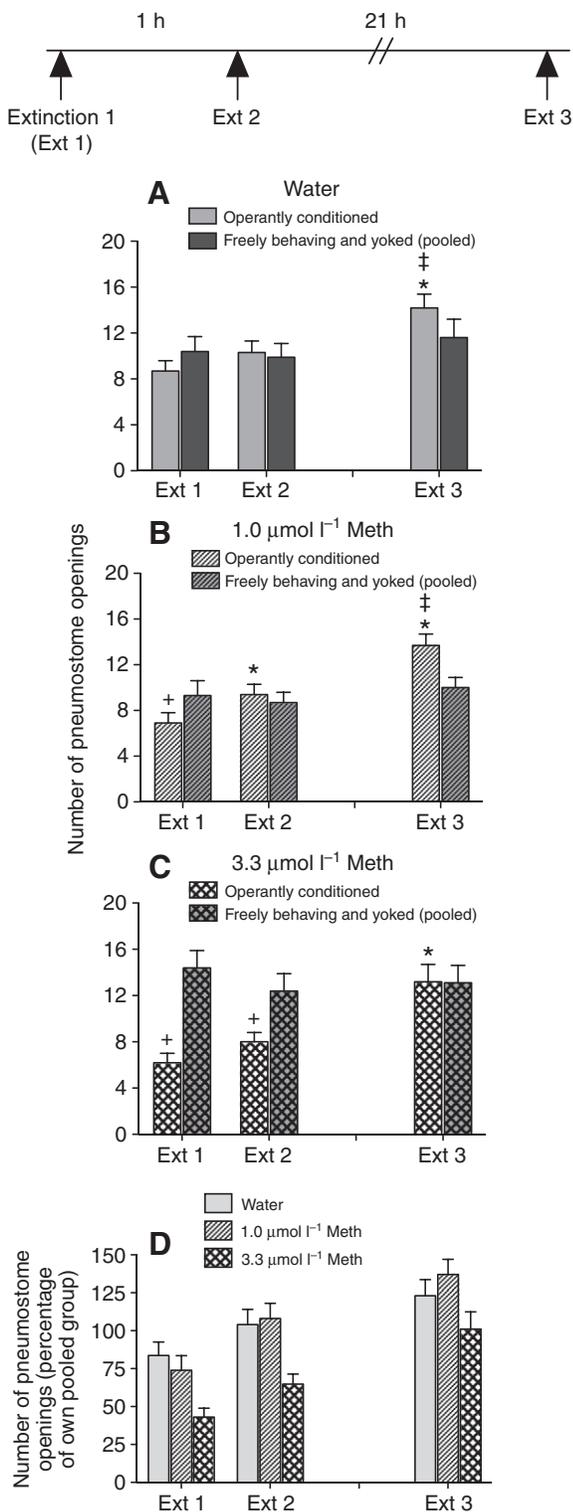


Fig. 3. Meth exposure during training produces context-dependent effects. Data are mean \pm s.e.m. for the number of pneumostome openings across Trials 1–3. Data from the first two groups (Water and $3.3\ \mu\text{mol l}^{-1}$ Meth in Trials 1 and 2) are taken from Fig. 2. Snails submerged in $3.3\ \mu\text{mol l}^{-1}$ Meth during all three trials demonstrated LTM in Trial 3, similar to Water controls, suggesting that impairment of LTM expression when snails are initially trained in the presence of Meth and tested for LTM in the absence of Meth demonstrates context specificity. For $3.3\ \mu\text{mol l}^{-1}$ Meth in Trials 1, 2 and 3, $N=15$. * $P<0.05$, compared with Trial 1; † $P<0.05$, compared with Trial 2 within the same treatment group; ‡ $P<0.05$, compared with Water controls during the same session.

stimulated (naïve, freely behaving group) or given non-contingent stimulation of the pneumostome area (yoked group). Since a two-way, repeated measures ANOVA did not show any differences between the freely behaving snails and yoked snails within any of the three treatment groups, we pooled these two groups within each treatment group (see Experiment 5). In Water operantly conditioned snails (Fig. 4A), the number of pneumostome openings across extinction sessions was not different from their pooled group except in Extinction 3, in which they opened their pneumostome to a significantly greater extent than their pooled group (session $F_{2,92}=9.8$, $P<0.0001$; interaction $F_{2,92}=3.6$, $P<0.030$). By contrast, the $1.0\ \mu\text{mol l}^{-1}$ Meth-treated group (Fig. 4B) demonstrated a significant reduction in the number of pneumostome openings in Extinction 1, and, as in Water controls, significantly elevated the number of openings in Extinction 3 compared with their pooled group (session $F_{2,120}=19.5$, $P<0.0001$; interaction $F_{2,120}=8.2$, $P<0.0004$). In the $3.3\ \mu\text{mol l}^{-1}$ Meth-treated group (Fig. 4C), the number of pneumostome openings in both Extinction 1 and Extinction 2 was significantly suppressed compared with their pooled group, and never surpassed this pooled group in Extinction 3, as the other two treatment groups had (treatment $F_{1,43}=6.9$, $P<0.012$; session $F_{2,86}=14.4$, $P<0.0001$; interaction $F_{2,86}=11.7$, $P<0.0001$). Fig. 4D shows the number of pneumostome openings when normalizing for their own pooled group and comparing across all three treatment groups. A two-way ANOVA showed a significant effect of treatment (treatment $F_{2,88}=6.91$; $P<0.0016$) and session (session $F_{2,176}=45.98$; $P<0.0001$). The treatment effect appeared to be due to the diminished rate of extinction in snails trained in $3.3\ \mu\text{mol l}^{-1}$ Meth compared with Water controls, whereas the lower dose of Meth did not produce differences in response compared with Water controls. Thus, exposure to $3.3\ \mu\text{mol l}^{-1}$ Meth during



Trial 1 and Trial 2 produced an LTM that was more resistant to extinction. Importantly, we also delivered the first two extinction sessions to all animals shown in Fig. 3 that were exposed to $3.3 \mu\text{mol l}^{-1}$ Meth during all three trials (and expressed memory on trial 3). We found that they also were resistant to extinction, similar to those snails shown in Fig. 4C (pneumostome openings = 5.9 ± 0.7 for Ext 1; 6.7 ± 0.7 for Ext 2). This indicates that the change of context from Meth during Trial 3 to no Meth during Ext 1 did not alter the resistance to extinction in Meth-exposed snails.

Fig. 4. Meth exposure during training diminishes the rate of extinction. (Top) Experimental protocol. Data are mean \pm s.e.m. for number of pneumostome openings. (A) Water: operantly conditioned and pooled Water controls (freely behaving + yoked; $N=24$). (B) Exposure to $1.0 \mu\text{mol l}^{-1}$ Meth during Trial 1 and Trial 2: operantly conditioned and pooled Meth controls ($N=24$). (C) Exposure to $3.3 \mu\text{mol l}^{-1}$ Meth during Trial 1 and Trial 2: operantly conditioned and pooled Meth controls ($N=16$). Data for operantly conditioned snails are taken from Fig. 2 (see Fig. 2 for the number of snails in these groups). (D) Number of pneumostome openings in all three operantly conditioned treatment groups was normalized to the percentage of their own pooled responses. Operantly conditioned snails in all three treatment groups demonstrated extinction from Extinction 1 to Extinction 3. By contrast, yoked and freely behaving snails did not alter the number of pneumostome openings across sessions. A main treatment effect in D is due to a decrease in the rate of extinction in the $3.3 \mu\text{mol l}^{-1}$ Meth group. * $P < 0.05$, compared with Extinction 1 session within the same treatment group; † $P < 0.05$, compared with operantly conditioned group during the same session.

Experiment 4: effect of Meth during training on reinstatement

In the last phase of this set of experiments, we tested in the same snails that were used in the experiments in Figs 2 and 4 to see whether suppressed pneumostome-opening behavior could be reinstated once it was extinguished. If Meth treatment enhanced reinstatement when snails were again given pneumostome stimulation upon opening, then this would provide evidence that a stronger memory was formed and/or that extinction processes were impaired. Fig. 5A shows the number of pneumostome openings during reinstatement compared with openings during Trials 1–3 in the three treatment groups. There was a significant effect of treatment (treatment $F_{2,88}=3.43$; $P < 0.0366$), session (session $F_{3,264}=51.60$; $P < 0.0001$), and treatment \times session interaction (interaction $F_{6,264}=4.29$; $P < 0.0004$). In all three groups, there was significant reinstatement (number of openings lower than Trial 1 but not higher than Trial 3). A remaining question, however, was whether snails trained in $3.3 \mu\text{mol l}^{-1}$ Meth were actually demonstrating greater reinstatement because, while their response on reinstatement was similar to those of Water controls, their Trial 1 (baseline) openings were higher than those of Water controls. We therefore normalized the reinstatement response to their Trial 1 behavior, and this is shown in Fig. 5B. A one-way ANOVA did not reveal a significant difference among groups.

Experiment 5: effect of Meth in freely behaving and yoked control groups

For each of the three treatment groups we also ran two additional controls: freely behaving and yoked snails (see Materials and methods). This was done to determine whether exposure to the drug alone in the absence of any pneumostome stimulation (referred to as ‘freely behaving’) or exposure to drug plus non-contingent pneumostome stimulation (referred to as ‘yoked’) would alter behavior across sessions. Data from the extinction sessions of the freely behaving and yoked animals are shown in Fig. 4A–D, but here we show all the data from Trial 1 to reinstatement. First, we compared whether there were differences across all sessions in each treatment group. Since there were no significant differences, the freely behaving and yoked groups were combined for each treatment group. Fig. 6 shows the response across all sessions for each treatment group. A one-way, repeated measures ANOVA indicated no significant differences across session for any of the three groups, demonstrating that snails require stimulation contingent upon pneumostome opening in order to express learning, memory, extinction and reinstatement.

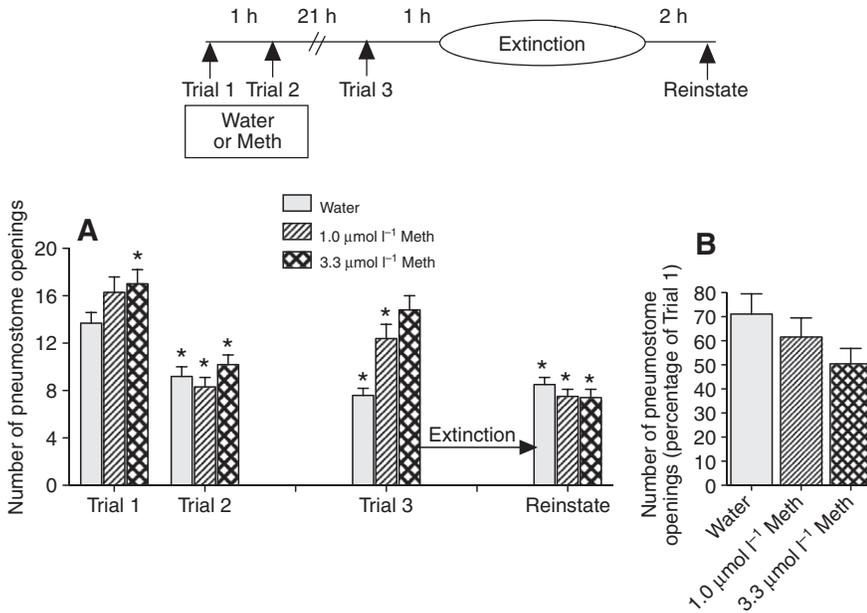


Fig. 5. Snails demonstrate reinstatement of extinguished respiratory behavior. (Top) Experimental protocol. The number of pneumostome openings are shown for Trials 1–3 and reinstatement. Data are taken from Fig. 2 for Trials 1–3 and are shown here for statistical comparison with data from the reinstatement session. Data are mean \pm s.e.m. for number of pneumostome openings. (A) Number of pneumostome openings during training and reinstatement. (B) Reinstatement in the three treatment groups normalized to their Trial 1 response. All three treatment groups demonstrated significant reinstatement after extinction. * $P < 0.05$, compared with Trial 1.

Experiment 6: effect of Meth after training and before extinction on extinction and reinstatement

Since the results obtained thus far showed a resistance to extinction when snails were trained in Meth during Trial 1 and Trial 2 (Fig. 4), we next tested whether Meth would alter extinction if snails were instead trained in Water and immersed in Meth just hours prior to

extinction sessions. After Trial 3, snails were given a 45 min immersion in Meth 16 h later to avoid any possible effects of Meth on consolidation of memory that may occur during Trial 3. After an interval of 4–5 h, snails were then given Extinction sessions 1–3 as before, followed by a test for reinstatement. Fig. 7 shows that Meth exposure prior to extinction did not significantly suppress

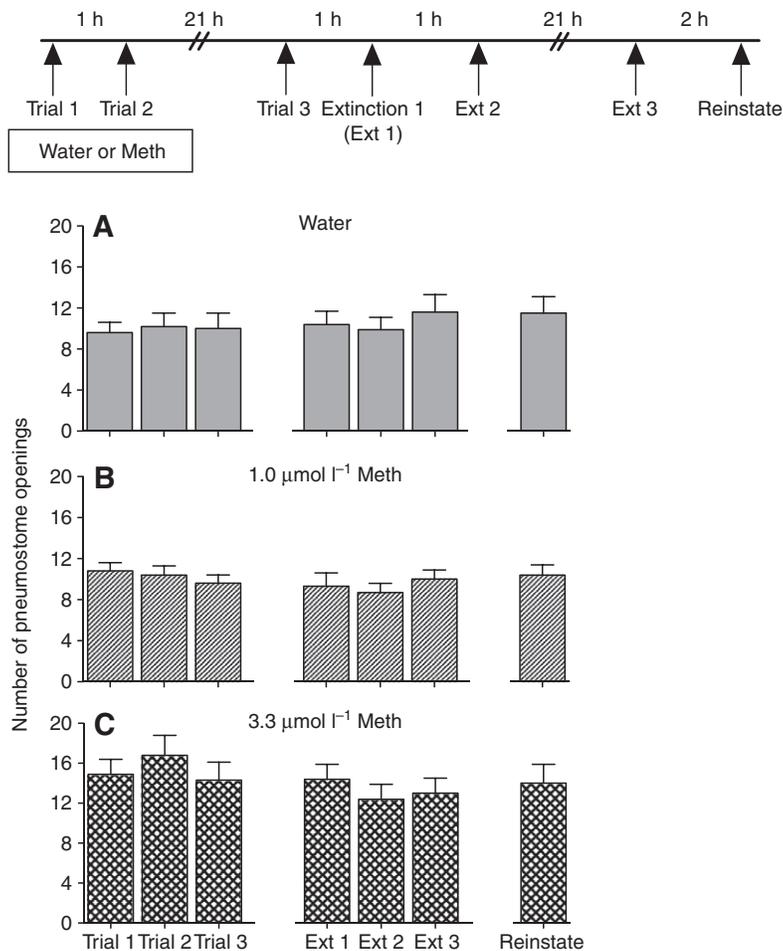


Fig. 6. Formation of LTM, extinction and reinstatement requires pneumostome stimulation contingent upon pneumostome opening. (Top) Experimental protocol. Data are mean \pm s.e.m. for the number of pneumostome openings during all phases of training, extinction and reinstatement in control groups of snails exposed to (A) Water, (B) 1.0 $\mu\text{mol l}^{-1}$ Meth or (C) 3.3 $\mu\text{mol l}^{-1}$ Meth and given either no pneumostome stimulation (freely behaving) or stimulation independent of opening their pneumostome (yoked). Freely behaving and yoked groups were pooled. No differences in pneumostome openings across sessions were found in any of the three treatment groups. For Water controls, $N = 24$; 1.0 $\mu\text{mol l}^{-1}$ Meth, $N = 24$; 3.3 $\mu\text{mol l}^{-1}$ Meth, $N = 16$.

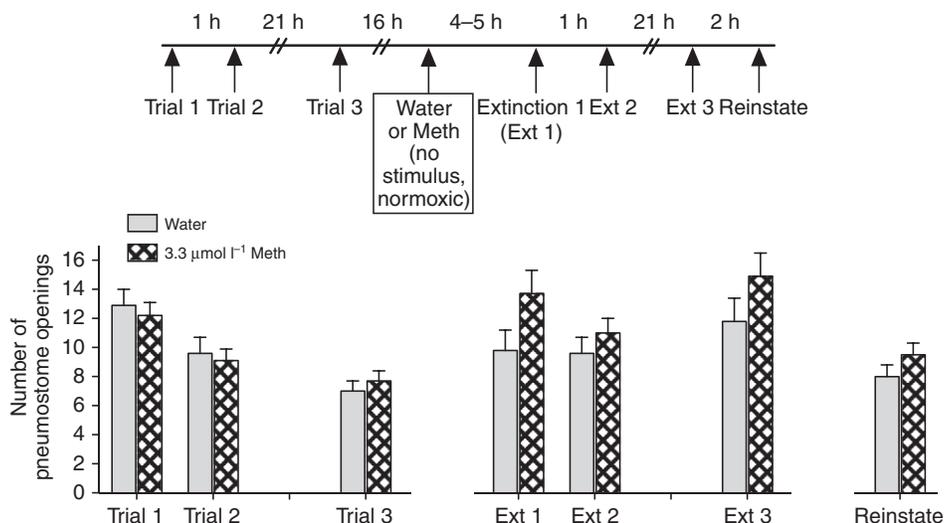


Fig. 7. Exposure to Meth after training but prior to extinction enhances extinction learning. (Top) Experimental protocol. Data are mean \pm s.e.m. for the number of pneumostome openings during all phases of training, extinction and reinstatement in snails exposed to either Water or $3.3 \mu\text{mol l}^{-1}$ Meth after training but prior to extinction. $3.3 \mu\text{mol l}^{-1}$ Meth given 4–5 h prior to Extinction 1 produced greater extinction learning. For Water controls, $N=20$; $3.3 \mu\text{mol l}^{-1}$ Meth, $N=22$.

extinction but instead slightly but significantly enhanced extinction. A two-way ANOVA conducted for the extinction and reinstatement sessions revealed a significant treatment effect (treatment $F_{1,40}=5.18$, $P<0.028$) and session effect (session $F_{3,120}=5.71$, $P<0.0011$). We conclude that exposure to $3.3 \mu\text{mol l}^{-1}$ Meth just hours before extinction training did not suppress the rate of extinction as was observed when snails were trained in this dose of Meth, but instead slightly enhanced extinction memory, which occluded the initial learning that occurred during operant conditioning in Trials 1–3.

Experiment 7: effect of Meth pre-exposure on LTM formation

Because of potential conflicts in the contextual effects of Meth given during training (see Fig. 3 above) and to test whether Meth exposure would increase memory using a different protocol, we determined whether pre-exposure to Meth would enhance LTM formation. Meth was therefore given prior to a single 45-min training session and then LTM was tested 24 h later. Control snails, given only a single training session will not demonstrate memory 24 h later because the training is suboptimal for forming LTM (Lukowiak et al., 2000). Therefore, if Meth enhances LTM formation, we would expect to observe the expression of LTM 24 h later in this group. The results (Fig. 8) show the response in Water controls and snails exposed to $3.3 \mu\text{mol l}^{-1}$ Meth (45 min) 24 h prior to a single session training. A two-way ANOVA revealed a significant treatment \times session interaction (interaction $F_{1,30}=10.28$; $P<0.0032$). This result shows that snails exposed to Water 24 h prior to Trial 1 did not demonstrate a decrease in the number of pneumostome openings during Trial 2, suggesting that no LTM was formed, as expected. However, pre-exposure to $3.3 \mu\text{mol l}^{-1}$ Meth 24 h prior to Trial 1 resulted in LTM formation, as evidenced by a significant decrease in the number of pneumostome openings in Trial 2 given 24 h after Trial 1. We also tested the effect of $3.3 \mu\text{mol l}^{-1}$ Meth on total breathing time and the number of pneumostome openings in freely behaving animals. We did this to determine whether the suppression in pneumostome openings that we attributed to LTM formation in snails exposed to Meth 24 h prior to Trial 1 was not due to non-specific effects of Meth. Snails were observed for total breathing time and number of

pneumostome openings for a 45 min session in hypoxic Water, then 1 h later were exposed to $3.3 \mu\text{mol l}^{-1}$ Meth. Either 1 or 4 h later (not shown), snails were tested in hypoxic Water for total breathing time and number of pneumostome openings and then both groups were again tested 24 h later. No changes were observed across any of the time points whether they were tested 1 or 4 h later, and so the data for the 24 h time point were pooled ($N=16$ total). Thus, across pre-exposure and post-exposure 24 h later, there were no differences in

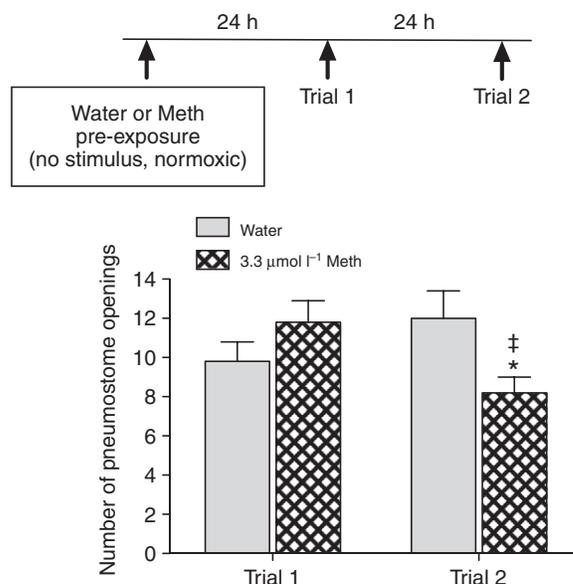


Fig. 8. Pre-exposure to Meth enhances formation of LTM in a single session. (Top) Experimental protocol. Data are mean \pm s.e.m. for the number of pneumostome openings during Trial 1 and Trial 2. ($N=16$ /group). Snails pre-exposed to $3.3 \mu\text{mol l}^{-1}$ Meth 24 h prior to Trial 1 demonstrated LTM 24 h after this single training session, whereas snails exposed to Water did not express LTM 24 h later. * $P<0.05$, compared with Trial 1; † $P<0.05$, compared to Water controls during the same session.

total breathing time (268 ± 39 s pre-exposure; 238 ± 40 s 24 h post-exposure; $P < 0.54$) or the number of pneumostome openings (8.9 ± 1.1 pre-exposure; 7.8 ± 1.2 24 h post-exposure; $P < 0.43$). Thus, the decrease in pneumostome openings in Trial 2 in snails exposed to Meth 24 h prior to training in Trial 1 appeared to be due to the formation of LTM.

DISCUSSION

The primary findings from these studies are the following: (1) Meth exposure appeared to produce contextual effects distinctive from the control (Water) environment such that training in Meth and testing in Water produced an inability to express LTM; (2) Meth exposure during the first two training sessions rendered test snails more resistant to extinction than control snails; (3) Meth exposure *after* training and *prior to* extinction did not impair but instead slightly enhanced the effect of extinction training (i.e. better occlusion of the originally formed LTM); and (4) pre-exposure to Meth 24 h before a single operant conditioning training session enhanced the ability of snails to form LTM.

Effect of training in Meth on extinction and reinstatement

Based on our previous results with cocaine pre-treatment of snails (Carter et al., 2006) and previous work showing that psychostimulants enhance memory (see Introduction), we predicted that Meth used during initial training would enhance LTM such that extinction would be impaired and/or reinstatement would be greater than that in the Water control snails. Meth exposure during training diminished the extinction rate of the trained behavior. Extinction is an active learning process that suppresses previously learned behavior but does not erase it (Bouton, 1994; Eisenberg et al., 2003; Lattal et al., 2006; Pedreira and Maldonado, 2003; Suzuki et al., 2004). Thus, training in Meth for the first two sessions during Trial 1 and Trial 2 appears to have enhanced the processes of memory formation such that extinction learning was less effective. Alternatively, training in Meth may have activated downstream pathways within RPeD1 or other neurons that actively inhibit subsequent extinction learning. This result is similar to what we previously found for the effects of cocaine pre-exposure, in which reinstatement of operantly conditioned breathing behavior was enhanced compared with controls (Carter et al., 2006). In that study, however, animals were exposed for 5 days to cocaine and then trained 3 days after the last cocaine exposure. It remains to be tested whether repeated Meth pre-exposure would have similar effects.

Any inhibitory action of Meth on RPeD1 during extinction is specific to whether snails were initially trained in Meth, because when animals were trained in Water only and exposed to Meth *after* training but before extinction, we observed the opposite effect: Meth enhanced extinction learning. These findings are opposite to those described in previous reports of rodents, which described an inhibitory effect (Borowski and Kokkinidis, 1998) or no effect (Blais and Janak, 2007; Carmack et al., 2010; Mueller et al., 2009) of amphetamine on extinction. The differences may be due to the drug that was used (Meth *versus* amphetamine), the type of task, or the time the drug was given relative to the extinction session (several hours prior to, immediately prior to, or after the session). These observations lead us to speculate that the ability of Meth to enhance or impair extinction depends on when snails are exposed to Meth relative to learning the original task. Meth may interfere with extinction when it is present during training, but if present after training and either before or during extinction, Meth may enhance the ability of extinction to occlude an already present memory. It should be noted that Meth may also promote the competing process

of reconsolidation, in which a specific memory is thought to become labile after retrieval (Misanin et al., 1968; Nader et al., 2000) and subsequently strengthened by a reconsolidation process. Recent observations in our laboratory suggest that snails trained in Meth form a memory for operant conditioning that is not amenable to disruption during the reconsolidation period, unlike what is found in Water-exposed controls (Sorg et al., 2009). Thus, retrieval of memory that occurred during Trial 3 could have strengthened that memory because reconsolidation processes that compete with extinction may have been augmented by Meth given during training.

The CPG that drives aerial respiratory behavior consists of three interneurons: RPeD1, VD4 and IP3. Chemosensory information activates RPeD1, and this neuron initiates rhythmic activity in the CPG. RPeD1 activates the IP3 interneuron, which then activates motor neurons to open the pneumostome (Syed et al., 1990; Syed et al., 1992). After operant training, the activity of RPeD1 is suppressed in a greater percentage of isolated brain preparations (Spencer et al., 1999) and is also suppressed after a reinforcing stimulus in semi-intact preparations in which the brain–pneumostome innervation remains intact (Spencer et al., 2002). This latter effect was found only in animals that successfully learned the behavior, suggesting that RPeD1 at least partly underlies the behavioral changes observed during operant conditioning. Numerous studies provide strong evidence to support the idea that the RPeD1 dopaminergic neuron is a fundamental component of memory for operantly conditioned respiratory behavior. Ablation of the RPeD1 soma (which leaves intact the neurites and demonstrates a normal firing in response to stimuli) blocks the formation of LTM without altering the retrieval of that memory (Scheibenstock et al., 2002). In addition, ablation of the RPeD1 soma is necessary for both extinction (Sangha et al., 2003b; Sangha et al., 2004) and reconsolidation (Sangha et al., 2004) of operantly conditioned respiratory behavior. Either this dopaminergic neuron stores the memory itself, or it is a critical conduit for memory expression of conditioned behavior. Given that RPeD1 is a dopaminergic neuron, Meth may alter RPeD1 directly. Alternatively, Meth may influence the other two CPG neurons, or it may activate other neuronal pathways that prime the necessary molecular processes in a neuron such as RPeD1, which is a necessary site for LTM. Finally, it is also possible that Meth may alter peripheral neurons that send chemotactic information to the CPG or to motor neurons involved in pneumostome opening and closing. Regardless of the mechanism, there may be an interaction with Meth exposure and the expression of initial learning during Trial 1, because Meth exposure suppressed pneumostome openings compared with baseline behavior when no learning occurred (Fig. 1B), but it slightly enhanced pneumostome openings when learning occurred (Fig. 2, see Trial 1). Therefore, despite exposure to Meth producing more persistent learning, it resulted in less within-session learning during Trial 1.

Effect of Meth pre-exposure on LTM formation

The finding (Fig. 8) that Meth pre-exposure enhances LTM formation following a single training session coupled with the finding (Fig. 7) that pre-exposure of snails to Meth before extinction training slightly enhanced the occlusion of the original memory by the extinction trials, suggest to us that Meth initiates cellular and/or molecular changes that ultimately prime the neurons necessary for LTM. Whether this is a direct effect on neurons such as RPeD1 or an indirect effect *via* other afferent neurons remains to be determined. With regard to drug addiction in humans, our observation suggests that Meth effects on learning may last for several hours after the acute effects have dissipated, potentially pairing learning and memory events with contextual cues or other

discrete cues that are encountered well after Meth exposure is discontinued. We are currently evaluating various time intervals between the Meth exposure and training to determine the optimal window over which LTM formation is enhanced.

Our results from the single session training procedure cannot readily be explained by Meth-induced suppression of general metabolism, for two reasons. First, total breathing time in hypoxic water was not altered at 1, 4 or 24 h after Meth exposure to $3.3 \mu\text{mol l}^{-1}$ Meth (see Fig. 1 for 1 h results; see also text for Fig. 8). Second, even when snails were exposed to two 45-min sessions in Meth during Trial 1 and Trial 2, they did not decrease the number of pneumostome openings in Trial 3 given 24 h later (due to contextual effects; see Fig. 3) or in freely behaving or yoked controls. Thus, there does not appear to be a simple metabolic change underlying the decrease in pneumostome openings. Therefore, the most straightforward explanation is that Meth pre-exposure enhanced LTM formation.

The fact that Meth when given 24 h prior to a single training session (Fig. 8) strongly enhanced performance in Trial 2 suggests that exposure to this drug initiates a set of cellular and/or molecular events that primes the snail for enhanced LTM formation. Also supporting this finding are our observations from Experiments 2–4 in which snails received their last Meth exposure (during Trial 2) approximately 24 h prior to Extinction 1 and demonstrated during Extinction 1 an apparent better memory for Trial 3, where they were trained in Water. Several previous studies in mammalian systems have also demonstrated an augmentation by psychostimulants in the expression of memory for aversive stimuli (Blaiss and Janak, 2006; Blaiss and Janak, 2007; Carmack et al., 2010; Davies et al., 1974; Fenu and Di Chiara, 2003; Wood and Anagnostaras, 2009), spatial stimuli (McGaugh, 2000; Packard and Teather, 1998), and appetitive stimuli (Oscos et al., 1988). Typically, however, these drugs are administered just after the initial training during the consolidation period rather than prior to initial training. In snails, this procedure may not be feasible because the learned suppression of respiratory behavior after training is context dependent (Haney and Lukowiak, 2001). Indeed, in the present study, Meth appears to have produced context-dependent effects, because snails trained in Meth but tested in Water in Trial 3 the next day did not express memory, whereas snails trained in Meth and tested in Meth the next day did express memory.

The mechanism(s) by which Meth enhances LTM is unknown. One possibility is that Meth lowers the threshold necessary for the molecular processes that cause LTM formation and thus enables training procedures that usually lead only to intermediate-term memory (ITM) to now be sufficiently strong enough to elicit LTM. In this sense, our data are analogous to earlier findings (Parvez et al., 2006; Parvez et al., 2005) that 'ITM-training' leaves a residual memory trace such that even a single stimulus to the pneumostome is now sufficient to cause LTM formation. ITM has been described in snails after a single 30-min training session (Sangha et al., 2003a) and exists for approximately 3–4 h. ITM requires protein synthesis but does not require RNA transcription, whereas LTM requires both (Sangha et al., 2003a). It does not appear likely that the enhanced LTM was due to the acute and short-lasting effects of Meth itself, such as increased norepinephrine transmission, since animals were removed from Meth for a full 24 h. Possible candidates for promoting memory-enhancing effects are those involved in downstream pathways activated by drugs of abuse (Nestler, 2001). Such drugs of abuse, including Meth, increase dopaminergic neurotransmission, which activates PKA and the phosphoprotein, dopamine- and cyclic AMP-regulated phosphoprotein (DARPP-32; also known as

PPPR1RB), and this molecule in turn inhibits protein phosphatase-1 (Greengard et al., 1999; Lin et al., 2002; Svenningsson et al., 2005). Likely candidates involved in the cascade of events include protein kinase A (PKA), protein kinase C (PKC), and/or inhibition of phosphatase activity; the last two have been shown to be involved in boosting ITM into LTM in *Lymnaea* (Rosenegger et al., 2008). Meth may inhibit the active process of forgetting *via* effects on protein phosphatase activity (Lin et al., 2002; Snyder et al., 2000). Given the relatively long period of time between Meth exposure and the first training session (24 h), Meth may also influence the ability to alter synapse formation *via* factors such as the EGF-like peptide found in *Lymnaea* (Hamakawa et al., 1999; van Kesteren et al., 2008).

Conclusions

Our studies in *Lymnaea* concur with previous reports in mammals that psychostimulants enhance the ability to form LTM. The expression of LTM was not greater than controls in snails that were both trained and tested in Meth. Therefore, this enhanced memory may not be manifested as an increase in the *magnitude* of memory expression so much as an increase in the *persistence* of memory expression, as indexed by an inability to extinguish the memory as rapidly. Our studies show that not only did training in Meth produce a resistance to extinction and thus appear to enhance memory persistence, but that pre-exposure to Meth allowed for LTM formation in a sub-optimal training paradigm, implicating cellular and/or molecular mechanisms that prime the snail for enhanced memory formation. An understanding of these mechanisms in this relatively simple system is expected to lead to delineation of cellular pathways that produce the persistent memories established in the presence of psychostimulants.

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