

Repeated cocaine effects on learning, memory and extinction in the pond snail *Lymnaea stagnalis*

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Summary

The persistence of drug addiction suggests that drugs of abuse enhance learning and/or impair extinction of the drug memory. We studied the effects of repeated cocaine on learning, memory and reinstatement in the pond snail, *Lymnaea stagnalis*. Respiratory behavior can be operantly conditioned and extinguished in *Lymnaea*, and this behavior is dependent on a critical dopamine neuron. We tested the hypothesis that repeated cocaine exposure promotes learning and memory or attenuates the ability to extinguish the memory of respiratory behavior that relies on this dopaminergic neuron. Rotating disk electrode voltammetry revealed a K_m and V_{max} of dopamine uptake in snail brain of $0.9 \mu\text{mol l}^{-1}$ and $558 \text{ pmol s}^{-1} \text{ g}^{-1}$ respectively, and the IC_{50} of cocaine for dopamine was approximately $0.03 \mu\text{mol l}^{-1}$. For operant conditioning, snails were given 5 days of 1 h day^{-1} immersion in water

(control) or $0.1 \mu\text{mol l}^{-1}$ cocaine, which was the lowest dose that maximally inhibited dopamine uptake, and snails were trained 3 days later. No changes were found between the two groups for learning or memory of the operant behavior. However, snails treated with $0.1 \mu\text{mol l}^{-1}$ cocaine demonstrated impairment of extinction memory during reinstatement of the behavior compared with controls. Our findings suggest that repeated exposure to cocaine modifies the interaction between the original memory trace and active inhibition of this trace through extinction training. An understanding of these basic processes in a simple model system may have important implications for treatment strategies in cocaine addiction.

Key words: cocaine, dopamine, reinstatement, *Lymnaea stagnalis*, long-term memory, snail.

Introduction

Addiction is characterized by persistent and compulsive drug craving that is present even after years of abstinence. Drug abuse studies have focused on learning and memory processes (O'Brien et al., 1992; Berke and Hyman, 2000; Wise, 2000; Robinson and Kolb, 2004; Wolf et al., 2004; Hamilton and Kolb, 2005; Liu et al., 2005). Changes occur within dopaminergic brain regions that are similar to those found in learning and memory studies, such as long-term potentiation and depression (LTP and LTD, respectively) (Bonci and Malenka, 1999; Thomas et al., 2000; Liu et al., 2005; Wolf et al., 2004). Morphological alterations are observed in brain areas such as the prefrontal cortex and nucleus accumbens after repeated psychostimulant exposure (Ferrario et al., 2005; Robinson and Kolb, 2004). Additionally, repeated exposure to these drugs produces changes in intracellular signaling that influence learning and memory (for a review, see Hyman, 2005).

Extinction training attenuates or reverses neuronal changes associated with the memory of drug-related cues (Sutton et al., 2003; Self et al., 2004). Extinction training consists of exposure to the conditioned stimulus, such as a drug cue, without the unconditioned stimulus, the drug. After repeated extinction sessions, the original behavior can return with no further stimulus [spontaneous recovery (Pavlov, 1927)] or with presentation of the unconditioned stimulus [reinstatement (Myers and Davis, 2002)]. Although extinction appears to be forgetting, it is an active learning process that occludes previously learned behavior but does not erase it (Bouton, 1994; Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004). Thus, the memory for extinction may critically impact the return, or reinstatement, of behavior such as drug-seeking behavior. It is not known whether repeated drug exposure produces a stronger initial memory of the trained behavior or if it impairs extinction learning so that reinstatement occurs more readily or easily, but distinguishing

which pathways have been altered is especially difficult in the complex mammalian brain. A first step toward addressing this issue is to measure how drugs of abuse impact learning, memory and reinstatement after extinction in a simpler neuronal circuit.

The pond snail, *Lymnaea stagnalis*, provides a relatively simple model suitable for studying learning, memory and reinstatement behavior after extinction (Lukowiak et al., 1996) (for a review, see Lukowiak et al., 2006). *Lymnaea* are bimodal breathers *via* both cutaneous and aerial systems. Aerial respiratory behavior is driven by a central pattern generator consisting of three neurons: right pedal dorsal 1 (RPeD1), ventral dorsal 4 (VD4) and input 3 interneuron (Syed et al., 1990; Syed et al., 1992). Learning, memory and extinction have all been shown to be dependent on RPeD1, which is dopaminergic (Scheibenstock et al., 2002; Spencer et al., 2002; Sangha et al., 2004). Thus, the circuitry involving RPeD1 may be susceptible to alteration after repeated cocaine treatment. These changes can be measured by examining learning and memory for operant conditioning of aerial respiration, and testing for the memory of extinction during reinstatement of this conditioned behavior, which appear to be RPeD1 dependent.

We tested the hypothesis that repeated cocaine exposure *enhances* learning and memory or *impairs* the memory for extinction during reinstatement of respiratory behavior. We first characterized dopamine uptake in *Lymnaea stagnalis* and measured the ability of cocaine to block this process using rotating disk electrode voltammetry and HPLC techniques. Second, we assessed whether repeated cocaine exposure enhanced learning and/or memory of operant conditioning of respiratory behavior. Third, we determined whether repeated cocaine exposure attenuated extinction memory during reinstatement of this conditioned behavior.

Materials and methods

Animals

Laboratory-reared stocks of *Lymnaea stagnalis* L. were originally obtained from stocks at the University of Calgary, Canada. Animals were kept in aerated dechlorinated tap water at 20°C on a 12 h:12 h light:dark cycle. The snails had constant access to food (lettuce and fish food supplied *ad libitum*). All animals were allowed to age at least four months and reach a shell length of 2.5–3.0 cm before experimental use.

Drugs

Cocaine hydrochloride and dopamine were obtained from Sigma Chemical Company (St Louis, MO, USA). Concentrations of cocaine (ranging from 0.03 to 10 $\mu\text{mol l}^{-1}$ for HPLC studies) are reported as weight/volume of the salt.

Voltammetry

To determine doses of cocaine that would effectively block dopamine uptake from the extracellular space, we first characterized dopamine uptake using rotating disk electrode (RDE) voltammetry to measure the kinetics of transport and

map the time course of dopamine uptake in isolated snail brain suspensions. We note here that dopamine uptake is described as the clearance of exogenously added dopamine from the extracellular space. As mentioned in the results for Fig. 2 below, we found a minor component of dopamine clearance that was not inhibited by cocaine and, we attributed this effect to either an uptake mechanism that is not sensitive to cocaine or to another mechanism unrelated to uptake, such as monoamine oxidase-mediated metabolism of dopamine.

A total of 40 snails was used. For each sample, five snail brains were rapidly removed, pooled and weighed. The tissue was then finely chopped on an ice-cold plate and transferred into 500 μl of buffer saturated with 95% O_2 /5% CO_2 in a glass chamber. The composition of the buffer was as follows: 51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} CaCl_2 , 1.5 mmol l^{-1} MgCl_2 , 5.0 mmol l^{-1} Hepes, adjusted to pH 7.9. The tissue was disrupted by pipetting, allowed to settle for 18 min, and washed by removing and replacing 250 μl of saline seven times (McElvain and Schenk, 1992). Voltammetry was conducted at 22°C. The RDE (Pine Instruments, Grove City, PA, USA) was lowered into the chamber and rotated at 2000 r.p.m. A potential of 450 mV relative to a Ag/AgCl reference electrode was applied with a potentiostat (LC-4B, Bioanalytical Systems Inc., Indianapolis, IN, USA). After collecting a baseline signal, various amounts of dopamine were administered using a constant-flow rate microsyringe. Increasing concentrations of dopamine were added (in $\mu\text{mol l}^{-1}$: 0.10, 0.15, 0.25, 0.5, 1.0). After each addition, the disappearance of dopamine was monitored with a Nicolet 310 digital oscilloscope. The initial rate of dopamine disappearance was estimated as described previously (Meiergerd et al., 1997). The velocity of uptake was expressed as $\text{pmol s}^{-1} \text{g}^{-1}$ wet mass.

High-pressure liquid chromatography

Use of the RDE technique allowed us to determine the time window over which dopamine disappearance occurred. Because dopamine uptake occurred over a matter of minutes rather than seconds, we could take advantage of measuring dopamine uptake with high-pressure liquid chromatography (HPLC) to unequivocally identify dopamine levels after cocaine addition to the tissue. Brain tissue (five pooled brains per sample) was prepared in the same manner as described for voltammetry, placed in the glass chamber and washed as described above, and rotated at 2000 r.p.m. using the RDE as a stirring rod only (i.e. not detecting changes in dopamine levels with this electrode). Cocaine (0.03–10 $\mu\text{mol l}^{-1}$) was added with a microsyringe and allowed to incubate for 30 s before adding 0.5 $\mu\text{mol l}^{-1}$ dopamine. After 2 min, the contents of the chamber were removed and centrifuged at 10 000 g at 4°C for 1 min. The supernatant was transferred to a tube containing 100 μl of 0.1 mol l^{-1} perchloric acid. Dopamine was measured by HPLC as described (Wayment et al., 2001).

Drug exposure

All operant behavior experiments involving drug pretreatment were done in a blinded fashion. Animals were randomly assigned

to one of two treatment groups: control or 0.1 $\mu\text{mol l}^{-1}$ cocaine. Control animals that were given training were conducted throughout the entire study, and the same cumulative data for controls are shown in Fig. 4C and Fig. 5A. The treatments were made by dissolving the appropriate amount of drug in 1 l of eumoxic pond water ($P_{\text{O}_2} > 75$ mmHg). Animals were placed into 1 l of the given treatment for 1 h daily. Immediately after the 1 h exposure, all animals were returned to their home aquaria. Exposures were repeated once each day for 5 days. The exposure time was based on several studies examining the ability of cocaine to produce locomotor sensitization in rats (Kalivas and Stewart, 1991; Robinson et al., 1998). At the end of this exposure time, animals were given 2 days in their home aquaria with no treatment to allow for complete wash-out of cocaine before beginning the training sessions.

Total breathing time procedure

To determine whether there were changes in breathing behavior due to drug exposure, we observed the total breathing time of animals immediately before the first drug exposure and 2 days after the last drug exposure, which corresponded with what would be the first day of training. All observations were performed in a hypoxic environment over a 45 min period. 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.

Operant conditioning procedure

The protocol for sessions for Training, Memory Test, Extinction and Test for Savings is shown in Table 1. A hypoxic environment ($P_{\text{O}_2} < 7$ mmHg) was created by bubbling N_2 through 800 mm of pond water at a rate of 2 ml min^{-1} for 20 min. Animals were given a 10 min acclimation period, which ended by gently pushing each snail below the surface to signify the beginning of the training period. Training consisted of gently touching 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 time at which the pneumostome opened was recorded, and total pneumostome openings over the training period were tabulated for individual snails.

The basic training module consisted of three 45 min sessions: Training 1, Training 2, and the Memory Test. During these sessions, the conditioning procedure was applied and the time of all pneumostome openings recorded. Training 1 and Training 2 were separated by a 1 h inter-training interval, during which the animals were housed in their home aquaria. The Memory Test followed Training 2, 24 h later. This training protocol has been shown to create a memory that lasts for up to 5 days (McComb et al., 2002).

Yoked controls

We performed yoked control experiments to confirm that the decrease in respiratory behavior was contingent upon

Table 1. *Training protocol for operant conditioning*

Training 1 (45 min with tactile stimulation to pneumostome)
Home (1 h)
Training 2 (45 min with tactile stimulation to pneumostome)
Home (24 h)
Memory Test, MT (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 (24 h)
Extinction 3 (45 min with NO stimulation)
Home (2 h)
Test for Savings, TS (45 min with tactile stimulation to pneumostome)

All sessions were performed under hypoxic conditions.

application of the stimulus to the open pneumostome. Yoked animals and their freely behaving partners were placed into the same hypoxic conditions used in the operant conditioning procedure. Yoked animals received a gentle tactile stimulus to their pneumostome area whenever their yoked partner's pneumostome was open. 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.

Extinction training

Three extinction sessions of 45 min each were performed with the same temporal sequence as the operant conditioning procedure and were done according to the method of McComb et al. (McComb et al., 2002). The first extinction session was begun 1 h after the Memory Test, 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 extinction, tactile stimulation was given during a fourth session, the Test for Savings, starting 2 h after the last extinction session.

Definition of learning, memory, extinction memory/reinstatement

We defined learning as a significant decrease in pneumostome openings compared with the previous session (McComb et al., 2002). Demonstration of memory was determined by two criteria: (1) the number of pneumostome openings during a session must be significantly less than observed during Training 1 (T1), and (2) the number of pneumostome openings must not be significantly more than on Training 2 (T2), or the last training session. We defined

extinction memory during the reinstatement test as a violation of either criterion for memory after the Test for Savings. Thus, for extinction memory to be present during the test for reinstatement, (1) the number of pneumostome openings on the Test for Savings must not be significantly less than on Training 1, or (2) the number of pneumostome openings on the Test for Savings must be significantly more than on the Memory Test.

Data analyses and statistical testing

Data obtained from RDE voltammetry experiments were plotted and fitted to the Michaelis–Menten equation using non-linear regression (GraphPad Prism, San Diego, CA, USA). The K_m and V_{max} values were obtained from the Michaelis–Menten equation. Data obtained from HPLC studies were plotted and the IC_{50} estimated by examining the resultant line to determine the cocaine concentration at which dopamine uptake was half-maximal. To determine the effect of training on respiratory behavior for each treatment group, data from each set of cohorts was subjected to a one-way analysis of variance (ANOVA) followed by a *post-hoc* Fisher's least significant difference (LSD) test to compare sessions within groups. In the case where two treatments were compared across days (Figs 3 and 8), a two-way ANOVA was conducted followed by a Fisher's LSD test in the case of a significant interaction. The level of significance was set at $P < 0.05$.

Results

Fig. 1 shows the results of dopamine uptake kinetics in isolated snail brains using RDE voltammetry. Concentrations of dopamine from $0.1 \mu\text{mol l}^{-1}$ to $1.0 \mu\text{mol l}^{-1}$ were added sequentially to each preparation to determine the maximum velocity at which dopamine was cleared from the extracellular solution, presumably due to uptake into the tissue. This procedure has been performed by our laboratory for rat brain tissue (e.g. Wayment et al., 2001). This characterization revealed a K_m of $0.9 \mu\text{mol l}^{-1}$ and a V_{max} of $558 \text{ pmol s}^{-1} \text{ g}^{-1}$ wet mass for dopamine.

The ability of cocaine to inhibit dopamine uptake in isolated snail brains was assessed using HPLC (Fig. 2). Initial pilot studies were done using RDE voltammetry, but were confirmed for the presence of dopamine using HPLC analysis. When cocaine was applied to preparations *without* exogenous dopamine addition, it did not alter extracellular dopamine levels at any dose tested (0.1 – $10 \mu\text{mol l}^{-1}$; data not shown). The IC_{50} for cocaine to inhibit dopamine uptake in isolated snail brains was approximately $0.03 \mu\text{mol l}^{-1}$.

For behavioral studies, we chose a dose of $0.1 \mu\text{mol l}^{-1}$ cocaine because this was the lowest dose that maximally inhibited dopamine uptake. Previous work showed that respiration was significantly increased when *Lymnaea* were acutely exposed to hydrogen sulfide (Rossenegger et al., 2004). To determine whether repeated cocaine exposure may have altered basal respiration levels, we measured the behavior of snails before and after exposure to cocaine. After measuring

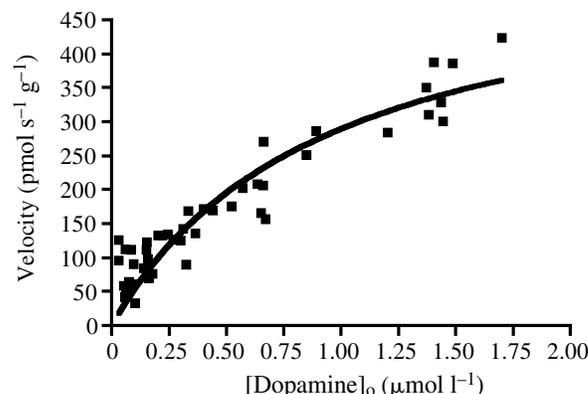


Fig. 1. Michaelis–Menten analysis of dopamine uptake in isolated snail brains measured by rotating disk electrode voltammetry. Sequential addition of dopamine to the preparation showed a K_m of $0.9 \mu\text{mol l}^{-1}$ dopamine and a V_{max} of $558 \text{ pmol dopamine s}^{-1} \text{ g}^{-1}$ wet mass. A total of 40 animals was used for this experiment ($N=8$; 5 pooled brains used for each experiment). Because the concentration of outside dopamine ($[\text{dopamine}]_o$) varied slightly as a function of the rate of uptake when each sequential addition of exogenous dopamine was made, individual data points are shown rather than averaged data. The data were fitted to the Michaelis–Menten equation using non-linear regression. The curve does not significantly deviate from the model ($P=0.36$ via the runs test).

total breathing time of snails in hypoxic water for 45 min, animals were divided into two cohorts and received either water only (control) or $0.1 \mu\text{mol l}^{-1}$ cocaine. Two days after the last exposure, total breathing time was again recorded. Fig. 3A indicates that snails exposed repeatedly to $0.1 \mu\text{mol l}^{-1}$ cocaine significantly increased the amount of time spent performing aerial respiration compared with the pre-cocaine exposure session. The number of pneumostome openings is shown in Fig. 3B. The number of openings before and after cocaine exposure was not different across time when performing a two-way ANOVA, but there was a trend toward an increase in pneumostome openings in snails given repeated exposure to $0.1 \mu\text{mol l}^{-1}$ cocaine.

We also confirmed that contingent application of a stimulus to the pneumostome area during aerial respiration was necessary for operant conditioning of the respiratory behavior. Three groups of snails were tested under hypoxic conditions. Control animals were allowed to freely perform aerial respiration. Yoked animals received a gentle tactile stimulus to the pneumostome area when the animal they were yoked to opened its pneumostome. Fig. 4A,B shows that animals that did not receive the tactile stimulus or those yoked animals given the stimulus but not paired with pneumostome openings did not alter their respiratory behavior over the three sessions. However, contingently trained animals that received a tactile stimulus to the pneumostome area each time they opened their pneumostome significantly decreased aerial respiration per session compared with Training Session 1 (Fig. 4C). Thus, the training protocol produced associative learning in these

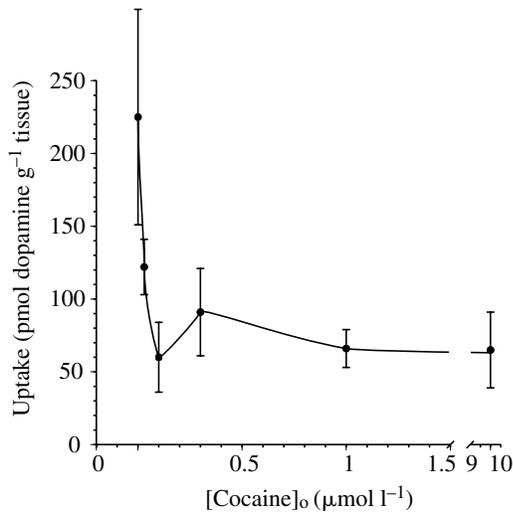


Fig. 2. Inhibition of dopamine uptake by cocaine in isolated snail brains measured by HPLC. Cocaine doses ranging from 0.03–10.0 $\mu\text{mol l}^{-1}$ were used, and dopamine uptake is reported as mean \pm s.e.m. of dopamine levels remaining outside the tissue ($N=6$ for controls; $N=3$ for 0.03 $\mu\text{mol l}^{-1}$; $N=4$ for all other doses). The IC_{50} for cocaine was estimated to be approximately 0.03 $\mu\text{mol l}^{-1}$.

animals, consistent with the findings first reported by Lukowiak et al. (Lukowiak et al., 1996).

To determine if cocaine affected learning and memory in *Lymnaea*, we exposed two cohorts of animals to pond water (controls) or 0.1 $\mu\text{mol l}^{-1}$ cocaine. Animals were then given the same training regimen as shown in Fig. 4. Both groups showed significant learning in Training 2 (Fig. 5) because there were fewer pneumostome openings than during Training 1. Both groups also showed evidence of memory in the Memory Test 24 h later (significantly less than Training 1 but not significantly more than Training 2). A *t*-test conducted on Training 1 between the control and cocaine-exposed groups indicated that the total number of pneumostome openings on this day was higher in the cocaine-pretreated group compared with controls ($P<0.013$). Because of our previous findings when measuring total breathing time (Fig. 3A), we speculated that this was due to increased motivation or need for respiration in the 0.1 $\mu\text{mol l}^{-1}$ cocaine pretreated snails compared with controls.

Each group of snails was then given extinction training, and performance was measured during the Test for Savings (Fig. 6). Control animals showed memory for the previous extinction sessions during the test for reinstatement (Test for Savings significantly higher than Memory Test). However, snails treated with 0.1 $\mu\text{mol l}^{-1}$ cocaine did *not* demonstrate a memory for extinction sessions because their performance during the test for reinstatement was not significantly higher than that on the Memory Test, and in fact, they demonstrated a significantly *lower* number of pneumostome openings than on the Memory Test. Thus, reinstatement was greater in cocaine-pretreated snails than in controls.

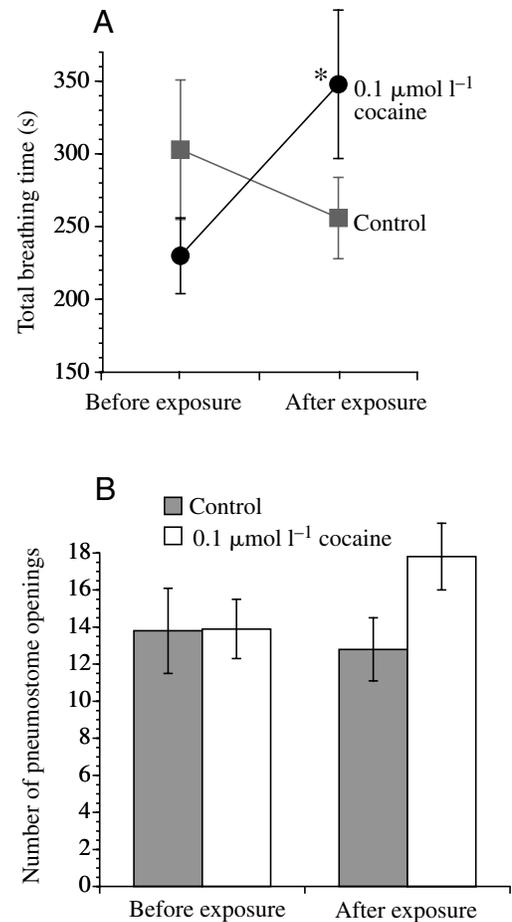


Fig. 3. Basal respiration in control and cocaine-exposed snails. To determine changes in respiratory behavior due to cocaine exposure, total breathing time was measured in snails placed in hypoxic pond water over a 45 min session before exposure to cocaine and 2 days after the last (fifth) exposure. Values are means \pm s.e.m. (A) Animals exposed to 0.1 $\mu\text{mol l}^{-1}$ cocaine ($N=13$) showed significantly higher total breathing time after exposure to cocaine than before exposure. No significant change in total breathing time was observed in controls ($N=12$). (B) Total number of pneumostome openings was also tabulated for the same snails as in A. Animals receiving 0.1 $\mu\text{mol l}^{-1}$ cocaine showed a non-significant trend toward more pneumostome openings after exposure. * $P<0.05$, compared within the treatment group to the before exposure values.

Our study showing total breathing time before and after exposure to cocaine indicated that animals treated with 0.1 $\mu\text{mol l}^{-1}$ cocaine had an increased respiratory drive. Therefore, the decrease in respiratory behavior during the Test for Savings may have been due to a return to pre-exposure respiratory levels due to the passage of time over which training sessions were given. To test for this possibility, we repeated the previous experiment in which snails were repeatedly exposed to 0.1 $\mu\text{mol l}^{-1}$ cocaine as before but no tactile stimulus was applied, and animals were allowed to behave freely under the hypoxic condition. There was no difference in the number of pneumostome openings across sessions for the

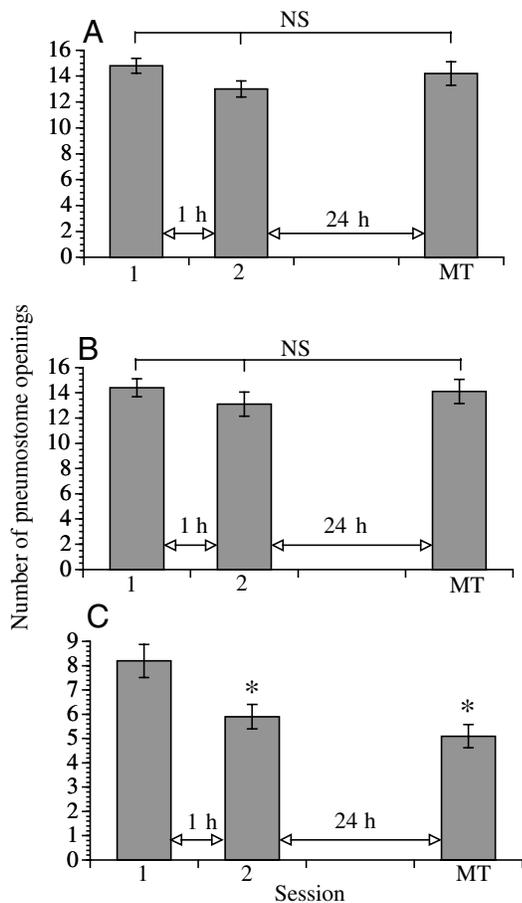


Fig. 4. Respiratory behavior in freely behaving, yoked and operantly conditioned snails. Pneumostome activity was measured over three 45 min training sessions for (A) freely behaving animals ($N=18$), (B) yoked animals ($N=17$) and (C) operantly conditioned animals ($N=31$). Values are means \pm s.e.m. for number of pneumostome openings. Animals operantly conditioned show a significant decrease in pneumostome openings across sessions. The other two treatment groups show no difference in pneumostome openings over the training time course. 1, Training session 1; 2, Training session 2; MT, Memory Test; NS, not significant. * $P<0.05$ compared with performance during Training session 1 within each treatment group.

freely behaving animals (Fig. 7). This result suggests that the effect of prior cocaine exposure on respiratory drive did not diminish over the 3-day testing period.

We also normalized the performance on the Test for Savings as a percentage of the performance on Trial 1 the first day, and as a percentage of performance on the last extinction test. The reason for normalization was that the initial number of pneumostome openings was significantly higher in the cocaine-pretreated group compared with controls. Fig. 8 shows the comparison of normalized performance between groups on the Test for Savings, which is a demonstration of how well animals activate extinction memory during reinstatement when they are again given stimulation to the pneumostome. The cocaine-pretreated group demonstrated a significant decrease in

extinction memory compared with controls, suggesting an increased reinstatement of the originally learned behavior after cocaine.

Discussion

To our knowledge, this is the first report on the effects of repeated cocaine exposure on operant conditioning behavior in a snail. Characterization of dopamine uptake and cocaine-mediated inhibition of dopamine uptake are important first steps toward elucidating the effects of cocaine on learning, memory and extinction in this simple model system. Our main finding is that repeated cocaine exposure impaired extinction memory, producing enhanced reinstatement of operantly conditioned respiratory behavior in *Lymnaea*.

Dopamine uptake in *Lymnaea*

The RDE studies indicate that dopamine uptake had a K_m value similar to the range of K_m values for the rat prefrontal cortex, nucleus accumbens and striatum (range $0.47\text{--}1.3\ \mu\text{mol l}^{-1}$) (McElvain and Schenk, 1992; Povlock and Schenk, 1997; Meiergerd et al., 1997). The V_{max} for dopamine uptake in the snail was much closer to that of the nucleus accumbens or striatum ($375\text{--}582\ \text{s}^{-1}\ \text{g}^{-1}$ wet mass) than that reported for the prefrontal cortex [$31.7\ \text{pmol s}^{-1}\ \text{g}^{-1}$ wet mass]. The IC_{50} for cocaine inhibition of dopamine uptake was lower ($0.03\ \mu\text{mol l}^{-1}$) than the K_i or IC_{50} reported for cocaine in brain tissue or heterologous expression systems for mammalian dopamine transporter (range $0.14\text{--}2.0\ \mu\text{mol l}^{-1}$) (Kilty et al., 1991; Jones et al., 1995; Povlock and Schenk, 1997; Wu and Gu, 1999). It is also possible that the effects of cocaine on dopamine disappearance in the snail could occur through uptake by the norepinephrine transporter, as in mammals (Carboni et al., 1990; Moron et al., 2002). Maximal inhibition of dopamine uptake occurred in the presence of $0.1\ \mu\text{mol l}^{-1}$ cocaine, but a somewhat diminished inhibition was observed in the presence of $0.3\ \mu\text{mol l}^{-1}$ cocaine. The mechanism for this reduced inhibition at intermediate cocaine concentrations is unclear, but the effect may be related to a combined effect of cocaine on all three monoamine transporters. Cocaine in the range of $1\ \text{nmol l}^{-1}$ to $1\ \mu\text{mol l}^{-1}$ could alter the dynamics of dopamine and other neurotransmitters because it significantly increases calcium influx in *Lymnaea* neurons (Vislobokov et al., 1993). However, at all doses tested, cocaine only partially inhibited dopamine uptake (about 70%), even in the presence of $10\ \mu\text{mol l}^{-1}$ cocaine. This finding suggests the existence of a mechanism for clearance of dopamine from the extracellular space that is not affected by cocaine, similar to that reported elsewhere (Wayment et al., 2001).

Cocaine effects on *Lymnaea* learning, memory and extinction/reinstatement

Snails receiving $0.1\ \mu\text{mol l}^{-1}$ cocaine demonstrated learning and memory similar to controls; however, extinction memory was less active or impaired in cocaine-pretreated snails because

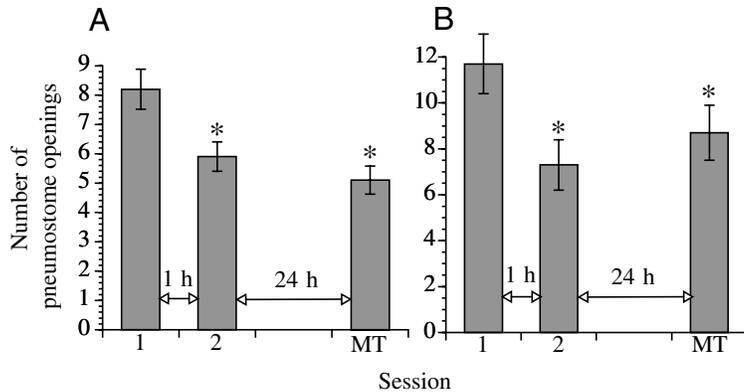


Fig. 5. Learning and memory in control and cocaine-exposed snails. (A) Control ($N=31$; from Fig. 4C) and (B) $0.1 \mu\text{mol l}^{-1}$ cocaine-treated ($N=15$). Values are mean \pm s.e.m. for number of pneumostome openings. Both groups demonstrated learning and memory. 1, Training session 1; 2, Training session 2; MT, Memory Test; $*P < 0.05$ compared with performance during Training session 1 within each treatment group.

they reinstated to a greater extent than did controls. Three possibilities to explain this finding are considered.

First, repeated exposure to $0.1 \mu\text{mol l}^{-1}$ cocaine may have altered metabolism, producing increased motivation for respiration early after 'withdrawal', but at the time they were given the Test for Savings, their metabolic need was back to control levels. This would give the appearance of better reinstatement on the Test for Savings, because there would no longer be an increased need to surface breath. However, the respiratory behavior of snails exposed to $0.1 \mu\text{mol l}^{-1}$ cocaine and not operantly conditioned, did not alter pneumostome openings over a similar time course, so this is an unlikely explanation.

A second possibility is that repeated cocaine exposure increased the consolidation time for memory formation by increasing the time to produce new RNA transcripts. If cocaine is acting to delay the mechanisms responsible for altered gene activity, performing the Test for Savings at a later time might reveal a memory for extinction. This explanation however,

seems unlikely because snails pretreated with cocaine demonstrated normal long-term memory on the Memory Test.

A third intriguing possibility is that repeated exposure to cocaine may have decreased the plasticity of the respiratory central pattern generator in *Lymnaea* by interfering with extinction memory. The lack of expression of extinction memory might be due to a loss in the ability to adapt to a changing environment after repeated cocaine. In rodents, the effects of repeated cocaine and other drugs of abuse have been shown to reduce plasticity at behavioral, morphological, electrochemical and neurochemical levels (Kolb et al., 2003; Robinson and Kolb, 2004; Briand et al., 2005; Hamilton and Kolb, 2005; Goto and Grace, 2005; Thompson et al., 2004). Briand et al. found that a complex environment enhanced fear-conditioned learning, but that prior repeated exposure to amphetamine prevented this enhancement by the complex environment (Briand et al., 2005). Similarly, Kolb and coworkers (Kolb et al., 2003; Hamilton and Kolb, 2005) showed that repeated drug exposure produced a resistance to

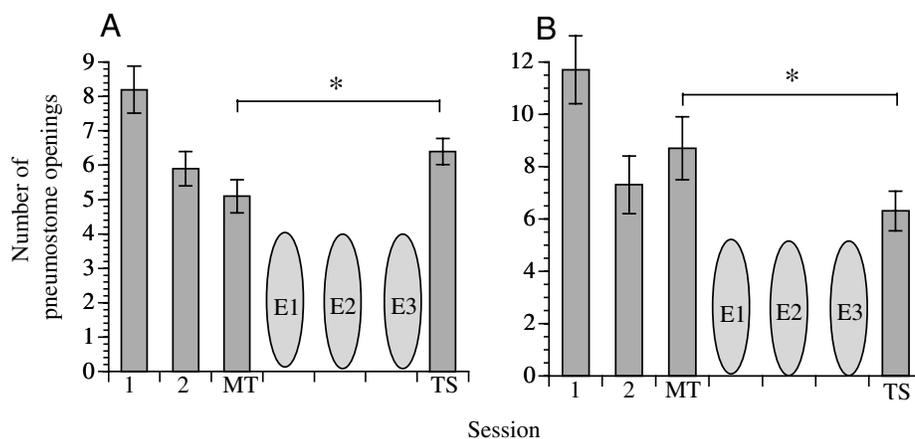


Fig. 6. Test for savings of extinction memory during reinstatement in control and cocaine-exposed snails. After operant conditioning, animals were exposed to extinction training but no pneumostome stimulus was administered (shown by ovals E1, 2 and 3; no data indicated). Testing for extinction memory was conducted during the Test for Savings 2 h after the last extinction training session, and this Test for Savings session is shown for the same animals as those used for the data in Fig. 5. (A) Control and (B) $0.1 \mu\text{mol l}^{-1}$ cocaine treated. Values are mean \pm s.e.m. for number of pneumostome openings. Animals treated with $0.1 \mu\text{mol l}^{-1}$ cocaine did not meet the requirements for extinction memory during reinstatement. Snails pretreated with $0.1 \mu\text{mol l}^{-1}$ cocaine showed a significant *reduction* in the number of pneumostome openings on the Test for Savings compared with the Memory Test. Training session 1; 2, Training session 2; MT, Memory Test; TS $*P < 0.05$ compared with MT within the same treatment group.

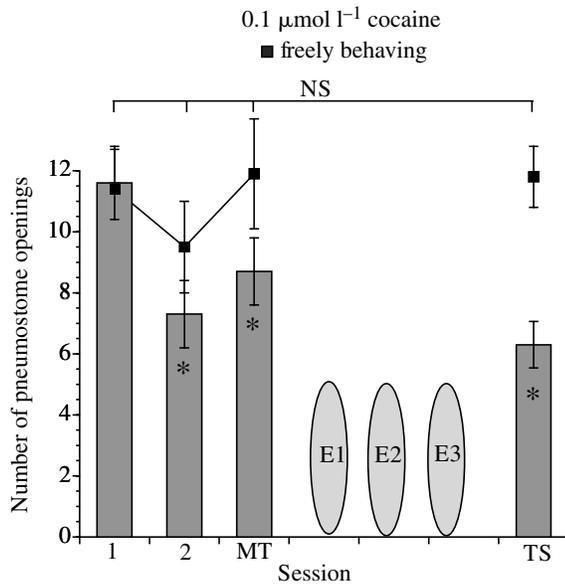


Fig. 7. Respiratory behavior after pretreatment with $0.1 \mu\text{mol l}^{-1}$ cocaine in freely behaving snails over time. Comparison of animals pretreated with $0.1 \mu\text{mol l}^{-1}$ cocaine and given operant training (shown in Fig. 6B) with freely behaving animals similarly pretreated but given *no* pneumostome stimulation ($N=15$) indicates that respiratory behavior does not change over the time frame used for trained animals. Values are mean \pm s.e.m. for number of pneumostome openings. Training session 1; 2, Training session 2; MT, Memory Test; N.S.=not significant; E1–3, extinction training sessions. * $P<0.05$ compared with Training session 1 within the same treatment group.

modification of dendritic branching in neurons that normally occurs in response to a complex environmental exposure. This loss of plasticity may be responsible for the long-lasting nature of addiction. Cocaine or other drugs of abuse may alter

inhibitory pathways activated during extinction such that reinstatement of the originally trained behavior occurs more readily after presentation of the original (unconditioned) stimulus.

Lymnaea is expected to provide a valuable model for exploring the cellular, molecular and electrophysiological changes in neuronal pathways important for extinction. In semi-intact preparations of *Lymnaea*, central nervous system (CNS) neurons innervating the pneumostome and the pneumostome itself are left intact so that electrophysiological properties of the RPeD1 neuron can be compared before, during and after conditioning (McComb et al., 2005). Operant learning decreases rhythmic firing of the RPeD1 neuron and decreases the ability of RPeD1 neurons to elicit pneumostome opening movements. This decrease is directly correlated with the decreased pneumostome openings after operant conditioning. It is unknown if extinction training causes a return to pre-training levels of RPeD1 neuronal firing rate or the ability of this neuron to produce pneumostome openings. However, the RPeD1 neuron is required for extinction learning (Sangha et al., 2004) and extinction behavior is thus expected to be reflected in the firing properties of this neuron. Extinction of respiratory operant conditioning requires altered gene activity and new protein synthesis in RPeD1 neurons in *Lymnaea* (Sangha et al., 2003). Studies in rodents suggest that extinction involves a change in glutamate receptors (Sutton et al., 2003; Self et al., 2004), and similar mechanisms may be present in *Lymnaea*.

Increased respiratory behavior after cocaine

The increase in respiratory behavior observed in snails treated with $0.1 \mu\text{mol l}^{-1}$ cocaine could be a result of compensatory actions by neurons to the cocaine challenge, essentially a withdrawal response from cocaine. However, respiratory behavior did not change over the 5 days after

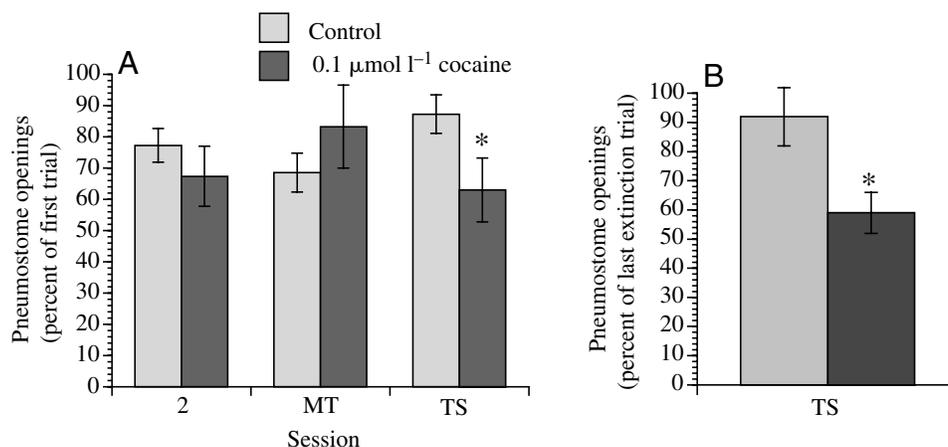


Fig. 8. Comparison of normalized performance between control and cocaine-exposed snails. Performance of control animals and animals treated with $0.1 \mu\text{mol l}^{-1}$ cocaine. Data are reported as mean \pm s.e.m. of pneumostome openings measured on the Test for Savings (TS) as a percent of openings on Training session 1 (A) and the last extinction day (B). Animals treated with $0.1 \mu\text{mol l}^{-1}$ cocaine showed a significant reduction in extinction memory during reinstatement compared with control snails. 2, Training session 2; MT, Memory Test. * $P<0.05$, compared with controls on the same day.

cocaine exposure in freely behaving animals. A similar effect of hydrogen sulfide on respiratory behavior in *Lymnaea* was reversible (Rossenegger et al., 2004), but it is not clear if the same is true for the effect of cocaine on respiratory behavior. The increased respiratory behavior may be a form of behavioral sensitization. Snails immersed for 30 min day⁻¹ for 5 days in 0.1–1.0 μmol l⁻¹ cocaine do not demonstrate increased locomotion on day 5 compared with day 1 (unpublished observations); therefore, behavioral sensitization by repeated cocaine does not appear in the form of increased locomotor activity as it does in rodents (Wise and Bozarth, 1987).

In summary, these studies show for the first time that repeated cocaine exposure alters extinction memory during reinstatement of operant conditioning in *Lymnaea*. Our work provides a basis to further explore the impact of repeated cocaine on extinction and reinstatement processes occurring in a simplified and well-delineated neural circuitry. *Lymnaea* should provide an excellent model to further examine the fundamental processes underlying extinction learning, as well as extinction forgetting that may occur during spontaneous recovery and reinstatement of the originally learned behavior. Our findings suggest that repeated exposure to cocaine modifies the interaction between the original memory trace and active inhibition of this trace through extinction training. An understanding of these processes may have important implications for treatment strategies in cocaine addiction.

List of abbreviations

DAT	dopamine transporter
HPLC	high-performance liquid chromatography
LSD	least significant difference
LTD	long-term depression
LTP	long-term potentiation
RDE	rotating disk electrode
RPed1	right pedal dorsal 1
VD4	ventral dorsal 4

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