

Effects of D-Cycloserine on Extinction of Conditioned Freezing

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The present study tested the prediction that D-cycloserine (DCS), a partial *N*-methyl-D-aspartate agonist, would facilitate extinction of conditioned freezing in male Sprague–Dawley rats. Rats received 5 light–shock pairings (conditioning). The following day, rats received 6 light-alone presentations (extinction training). Twenty-four hours later, rats received 1 light-alone presentation (test). Subcutaneous DCS injection before or after extinction training significantly enhanced extinction, and the dose–response curve for this effect was linear. Increasing the delay of DCS administration after extinction training led to a linear decrease in the facilitatory effect. The effect of systemic administration was replicated by intra-basolateral amygdala infusion. These results suggest that DCS facilitates extinction of conditioned freezing by acting on consolidation processes partly mediated by the basolateral amygdala.

The neural bases of learned fear are of major neuroscientific interest. Much of what we know about learned fear comes from studies of Pavlovian fear conditioning, which involves learning that certain environmental stimuli predict aversive events (Blanchard & Blanchard, 1972; Maren, 2001). In a typical study, an innocuous tone (the conditioned stimulus [CS]) is paired with a mild footshock (the unconditioned stimulus [US]). After a very few pairings (as few as one under certain conditions) long-lasting changes are established in the brain, such that the CS comes to elicit the behavioral, autonomic, and endocrine responses that are characteristically expressed in the presence of danger. These responses help prepare the animal for the ensuing aversive event (e.g., Bolles & Fanselow, 1980; Fanselow, 1994).

A large body of evidence suggests that the neural mechanisms involved in the acquisition and retention of conditioned fear depend on the action of the *N*-methyl-D-aspartate (NMDA) receptor, an excitatory amino acid receptor subtype (e.g., Collingridge & Bliss, 1987; LeDoux, 2000). NMDA receptor antagonists such as $+|-$ -10,11-dihydro-5-methyl-5H-dibenzo[ad]cycloheptene-5,10 imine (MK-801) and d,l-2-amino-5-phosphonovaleric acid (AP5) have been shown to block conditioned freezing (Maren, Aharonov, Stote, & Fanselow, 1996; Zhang, Bast, & Feldon, 2001), whereas NMDA agonists like glutamic acid and milacemide have been shown to facilitate shock avoidance learning (Flood, Baker, & Davis, 1990; Handelman, Nevins, Mueller, Arnolde, & Cordi, 1989). NMDA recognition sites are distributed throughout the central nervous system, with several specific high-density regions (see Monaghan & Cotman, 1985). One of those regions, which is also strongly implicated in Pavlovian fear conditioning, is the

lateral/basolateral amygdaloid nuclei (L/BLA). Infusion of AP5 into the BLA blocks the acquisition and expression of context-conditioned freezing (Fanselow & Kim, 1994; Maren et al., 1996) and cue-conditioned freezing (Lee & Kim, 1998), and the acquisition, but not the expression, of conditioned fear-potentiated startle (Campeau, Miserendino, & Davis, 1992; Miserendino, Sananes, Melia, & Davis, 1990).

One might expect that a neural site important for the acquisition and performance of conditioned fear would also be important for the extinction of conditioned fear. Although initially conceptualized as “unlearning,” extinction is now believed to involve the formation of new associations that compete with, or “mask,” the expression of the original conditioned-response-producing associations (Bouton, 1991; Konorski, 1948; Rescorla, 2001). If one assumes that extinction is a form of new learning, then it should be affected by pharmacological treatments that affect learning. To date, there has been little work undertaken on NMDA and extinction. However, the few studies focusing on the effects of NMDA antagonists on extinction support this view. For example, Falls, Miserendino, and Davis (1992) showed that infusion of AP5 into the BLA prior to extinction training dose-dependently blocked extinction of conditioned fear. Further, extinction of conditioned analgesia was blocked by systemic administration of MK-801 (Cox & Westbrook, 1994), and extinction of conditioned freezing was blocked by systemic administration of d(-)-3(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP; Quirk, Rosaly, Romero, Santini, & Muller, 1999). Both drugs were administered before extinction training. Only one recently published study has examined the effects of an NMDA agonist on extinction. Walker, Ressler, Lu, and Davis (2002) demonstrated the facilitation of conditioned fear extinction by D-cycloserine (DCS), a partial agonist that acts at the strychnine-insensitive glycine-recognition site of the NMDA receptor complex. In a series of six experiments, Walker et al. showed marked reductions in fear-potentiated startle (increased startle in the presence vs. absence of light previously paired with shock) following both systemic administration and BLA infusions of DCS prior to extinction training. DCS injections were also shown to enhance extinction dose dependently, and only in rats that also received extinction training.

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The present study was performed to answer a number of questions: (a) Can DCS administration prior to extinction training facilitate extinction of cue-conditioned freezing? (b) If so, does such facilitation also occur with post-extinction training administration, thus implicating consolidation mechanisms? and, (c) If so, is such an effect dose dependent, time dependent, and BLA mediated? The ability to pharmacologically enhance the extinction of intense fear memories has significant theoretical implications regarding the nature of extinction and memory (e.g., Rescorla, 2001), and significant clinical implications for disorders such as specific phobia, panic disorder, and posttraumatic stress disorder in which treatment often relies on the progressive extinction of fear memories (Dadds, Bovbjerg, Redd, & Cutmore, 1997; Foa, 2000).

Method

Subjects

Adult male Sprague–Dawley rats (Gore Hill, Sydney, Australia) weighing between 300 and 400 g were used. The rats were housed in groups of 8 in plastic boxes (67 cm long \times 40 cm wide \times 22 cm high) in a colony room maintained on a 12-hr light–dark cycle. Food and water were continuously available. All procedures were approved by the Animal Care and Ethics Committee at The University of New South Wales. A total of 152 rats were used.

Apparatus

Rats were preexposed and conditioned in four standard conditioning chambers (20 cm long \times 12 cm wide \times 12 cm high), which were designated as Context A. Each chamber consisted of a Perspex ceiling, stainless steel rear wall, stainless steel mesh sides, and a hinged Perspex front door that locked magnetically. The floor consisted of stainless steel rods, 2 mm in diameter, spaced 13 mm apart (center to center). Each floor was located 8 cm above a stainless steel tray that served to collect boli and urine. Unscrambled 50-Hz AC shock from a constant current generator (constructed at The University of New South Wales) was delivered to the floor of each chamber. The chambers were contained in pairs in two sound-attenuating wooden cabinets, and each chamber was separated from its partner by a solid timber partition within each cabinet. Each of the chambers could be viewed through a Perspex window in the front door of each cabinet. In order to prevent the rats from being distracted by extraneous visual stimulation, each chamber was illuminated by a 15-W red light bulb, and the experimental room was also illuminated by red light. Prior to each session, two of the chambers were wiped with 0.5% acetic acid (in tap water) and the alternate two with 0.1% vanilla (in tap water). All programming, timing, and shock stimulus presentations were computer controlled.

For extinction training and extinction test sessions, the context (Context B) consisted of a single Perspex chamber (30 cm long \times 30 cm wide \times 35 cm high) placed inside a white-painted wooden sound-attenuating cabinet (52 cm long \times 58 cm wide \times 63 cm high). A Perspex window in the front door of the cabinet enabled viewing of the chamber. Vertical black and white stripes (2 cm wide) were applied to the outer side and rear walls of the chamber, and an additional vertically striped panel was inserted into the rear right-hand side corner of the chamber to create a chamber with five walls. Walls were wiped with 0.5% eucalyptus solution (in tap water) before each rat was placed into the chamber. The floor consisted of a cardboard tray filled with 4 cups of bedding and 2 ml of eucalyptus solution. An extractor fan mounted on the ceiling of the cabinet provided a masking noise. A 15-W red light bulb located on the inside left wall of the cabinet provided illumination.

In Experiments 2 and 3, an alternative apparatus (Context C) was used for extinction training and test. Context C consisted of two white-painted wooden sound-attenuating cabinets (52 cm long \times 58 cm wide \times 63 cm high), both of which contained a single chamber (30 cm long \times 23 cm wide \times 35 cm high). The front walls of these chambers were made of clear plastic, the hinged lids were made of wood, and the side and rear walls were constructed of steel and painted with 2-cm wide black and white vertical stripes. The floors of the chambers consisted of stainless steel rods, 2 mm in diameter, spaced 13 mm apart (center to center). These floors were fitted with cardboard inserts and covered with 2.5 cups of bedding and 1 ml of eucalyptus solution. A Perspex window in the front door of each cabinet enabled viewing of the chambers. An extractor fan was mounted on the back wall of each of the cabinets to provide a masking noise. Illumination was provided by a 15-W red light bulb located inside the door of each cabinet.

The CS was a white light globe positioned inside the door of each cabinet. Light intensity of the CS across contexts was equivalent (approximately 16–17 lx). During the experiments, the rats were observed, and their behavior recorded with a video camera and recorder positioned in front of the chambers.

Surgery and Histology

Rats that were to receive a unilateral cannula aimed at the right BLA (Experiment 5) were handled for 3 days prior to surgery. They were then injected intraperitoneally with 1.0 ml/kg (100.0 mg/ml) of the anesthetic ketamine (Ketapex) and 0.5 ml/kg (20.0 mg/ml) of the muscle relaxant xylazine (Rompun). Each rat also received a prophylactic intraperitoneal injection of 0.3 ml penicillin (Benicillin: 150 mg/ml procaine penicillin, 150 mg/ml benzathine penicillin, 20 mg/ml procaine hydrochloride). After its head had been shaved, each rat was placed into a stereotaxic instrument, with the incisor bar maintained at approximately 3.3 mm below horizontal zero in order to achieve a flat skull position. A 22-gauge guide cannula (Plastics One, Roanoke, Virginia) was implanted into the right hemisphere of the brain, aimed at the BLA. The tip of the guide cannula was positioned at 2.4 mm caudal to bregma, 5.1 mm lateral to the midline, and 8.5 mm ventral to the dura (Paxinos & Watson, 1998). The guide cannula was fixed in position with dental cement, anchored by three jeweler's screws. A dummy cannula (extending 2 mm beyond the guide) was kept in the guide at all times, except during infusion.

Behavioral procedures began approximately 4–7 days after surgery. Cannulated rats subsequently received an overdose of sodium pentobarbital and had their brains removed and frozen. Unfixed brains were cut into 40- μ m coronal sections on a cryostat, and the sections were stained with Cresyl violet to determine the location of the cannulas. The coordinates of the cannula placements were then identified at the microscope with reference to the atlas of Paxinos and Watson (1998).

Drug Administration

Systemic administration. DCS (Sigma-Aldrich, Castle Hill, New South Wales, Australia) in various doses (2.5, 5.0, 10.0, and 15.0 mg/kg) was freshly dissolved in sterile isotonic saline (0.9% wt/vol) and injected subcutaneously in a volume of 1.0 ml/kg. Control rats were subcutaneously injected with saline (0.9% wt/vol) in a volume of 1.0 ml/kg. Drug doses were chosen on the basis of the results of other behavioral studies (Land & Riccio, 1999; Pussinen et al., 1997; Walker et al., 2002), estimates of brain concentration after systemic administration (extrapolated from Loscher, Wlaz, Rundfeldt, Baran, & Honack, 1994), and findings relating in vitro drug concentrations to DCS effects on NMDA receptor function measured electrophysiologically (Priestley & Kemp, 1994; Watson, Bolanowski, Baganoff, Deppeler, & Lanthorn, 1990) and by stimulated ligand binding (Hamelin & Lehmann, 1995; Hood, Compton, & Monahan, 1989).

Intra-amygdala infusion. DCS (10 μ g dissolved in 0.5 μ l saline) or 0.5 μ l saline was infused over 1 min immediately after extinction training. The

infusion cannula was left in place for an additional minute before being withdrawn.

General Behavioral Procedures

Rats were handled for 2 min a day for at least 3 consecutive days and assigned to weight-matched groups prior to the start of each experiment. Behavioral procedures for Experiments 3–5 consisted of a context preexposure phase, a fear conditioning phase, an extinction training session, and a postextinction test. For Experiments 1 and 2, there was no preexposure phase.

On Day 1 (preexposure), each rat was transported to the laboratory and placed into the conditioning chamber for 30 min. On Day 2 (fear-conditioning), each rat was returned to the conditioning chamber and trained with shock (US) signaled by a visual (light) cue (CS). Two minutes after being placed in the dark chamber, the CS was presented for 10 s and coterminated with a single 0.8-mA, 0.8-s shock delivered through the grid floor of the chamber. There were a total of five light–shock presentations, with an intertrial interval of 60 s. After the fifth light–shock presentation, each rat remained in the chamber for an additional 50 s before being put back in its home cage and returned to the colony room. Between each rat, the chambers were wiped clean with either 0.5% acetic acid or 1% vanilla. On Day 3 (extinction training), rats were placed into a second context (Context B or C) for a total of 24 min. Two minutes after the rat was placed in the dark chamber, the CS was presented for 2 min. The CS was presented a total of six times during the 24 min session, with a 4-min intertrial interval. No shock was delivered during this session. Between each rat, the chamber was wiped with 0.5% eucalyptus solution, and the bedding was removed and changed (including addition of eucalyptus solution to bedding). On Day 4 (postextinction test), each rat was returned to the second context and, after 2 min, the light CS was presented once for 2 min. Between each rat, the chamber was wiped with 0.5% eucalyptus solution, and the bedding was changed.

Scoring

Each rat was scored for freezing during both the 24 min of the extinction training session and the 4 min of the extinction test. Freezing was scored as the absence of all movement, except that related to respiration (Fanselow, 1994). The behavior of each rat was videotaped, and freezing was rated with a time-sampling procedure in which each rat was observed once every 2 s. A percentage score was calculated for the proportion of the total observation period. A proportion of the sessions was rated by an additional observer who was unaware of the rats' group designations. There was a high degree of agreement between the two observers: The Pearson product–moment correlation between their ratings was $>.96$.

Statistical Analyses

Analysis of variance (ANOVA) of the percentage of conditioned freezing was the primary statistical approach. Simple main effects were assessed with two-tailed t tests for independent samples. The criterion for significance for all comparisons was $p < .05$.

Results

Experiment 1: Effect of DCS on Extinction of Conditioned Freezing

This experiment assessed the effect of DCS on extinction of conditioned freezing. A 2 (extinction, no extinction) \times 2 (saline, DCS) factorial design was employed. A total of 32 rats were handled and allocated to four weight-matched groups ($n = 8$ per group). For this experiment only, DCS or saline was administered

subcutaneously 15 min prior to extinction training in an attempt to replicate the work of Walker et al. (2002). According to Walker and colleagues (Experiment 2), 15 mg/kg DCS produced the maximal enhancing effect, so we used this dose. A single extinction training session was used, as pilot work indicated that this produced a moderate amount of extinction, against which a facilitatory effect of DCS could be detected. Rats in the extinction condition were conditioned, extinction trained, and postextinction tested as previously described. Rats in the no-extinction condition received the same treatment, except that instead of extinction training, they were merely transported to a location outside the testing room, injected with either saline or DCS, handled briefly 15 min later, and then returned to the colony room.

During the first 2 min of extinction training, prior to the first light CS (i.e., precue), there was a statistically significant difference in levels of freezing between rats injected with DCS and those given vehicle, $t(14) = 7.61$ (see Figure 1A). During the first presentation of the cue, the level of freezing for the DCS group rose quite sharply (for the saline group, the level of freezing remained approximately the same as it had been during the precue period). However, freezing fell gradually for both groups across successive light CS presentations, $F(5, 70) = 3.26$. The DCS group displayed marginally less freezing than the saline group, $F(1, 14) = 4.21, p = .06$.

On the postextinction test (see Figure 1B) the following day, rats that had been injected with DCS before extinction training displayed significantly less freezing to the light CS than rats injected with saline, $t(14) = 2.42$. Moreover, the two extinction groups froze significantly less than did the two no-extinction groups, $F(1, 28) = 27.02$. There was no significant difference between the DCS and saline no-extinction groups, $t(14) < 1$. Thus, DCS appears to act specifically to facilitate extinction mechanisms and does not have a more general effect on conditioned freezing measured 24 hr later in the absence of the drug.

These findings parallel those reported by Walker et al. (2002) with fear-potentiated startle. A new finding in the current experiment is that DCS appears to have a short-term depressive effect on contextual freezing (precue during training). This effect cannot be explained wholly in terms of a simple drug or performance effect, as a significant difference between the levels of precue freezing at the postextinction test for the DCS ($M = 14.62, SD = 21.97$) and saline ($M = 44.99, SD = 29.06$) extinction groups was also found, $t(14) = 2.36$. This latter finding suggests a longer lasting DCS effect on learning.

Experiment 2: Effect of DCS on Memory Consolidation

In Experiment 1, we demonstrated that pre-extinction training injections of DCS facilitate extinction in a conditioned freezing paradigm. Experiment 2 assessed the effects of post-extinction training DCS injections on conditioned freezing. A 2 (extinction, no extinction) \times 2 (saline, DCS) factorial design was used. A total of 32 rats were handled and allocated to four weight-matched groups ($n = 8$ per group). Rats were conditioned and postextinction tested as previously described. Two groups (one group of saline-injected rats and one group of DCS-injected rats) underwent extinction training on Day 2. Two groups (one group of saline-injected rats and one group of DCS-injected rats) did not receive extinction training on Day 2 and were handled only. Rats were

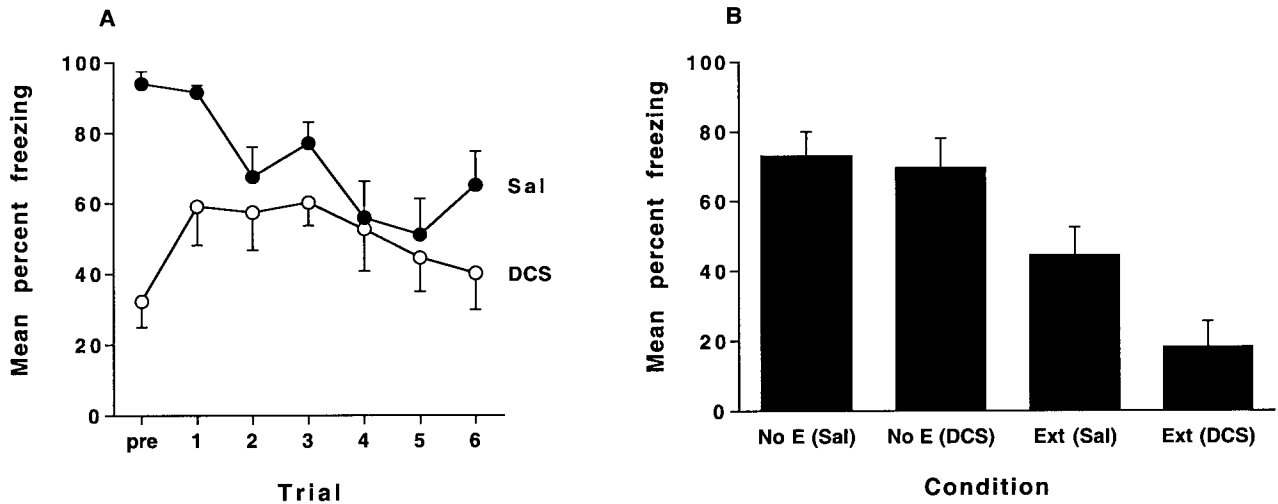


Figure 1. A: Effect of D-cycloserine (DCS) on conditioned freezing during the extinction training session. Mean (\pm SEM) percentage of time rats in Experiment 1 spent freezing during the 2-min period prior to the first presentation of the light conditioned stimulus (CS; pre) and during each of the six 2-min presentations of the light CS, after being injected with either saline (Sal) or DCS. B: Effect of DCS on conditioned freezing during the postextinction test. Mean (\pm SEM) percentage of time rats spent freezing during one 2-min presentation of the light CS in Experiment 1. No E = no extinction training; Ext = extinction training.

injected subcutaneously with either DCS (15 mg/kg) or saline immediately after extinction training or handling.

During the first 2 min of extinction training, prior to the first light CS presentation (precue), there was no apparent difference in levels of freezing between rats to be injected with DCS and rats to be injected with saline immediately after the training session (see Figure 2A). Indeed, in all subsequent precue group comparisons in

the remaining experiments, there were no differences between DCS and saline in levels of precue freezing during extinction training. During the first presentation of the light CS, the levels of freezing rose sharply in both groups but then fell gradually across successive cue presentations, demonstrating that extinction of fear to the light CS occurred in the short term, $F(5, 70) = 15.81$. There were no significant group differences across CS presentations.

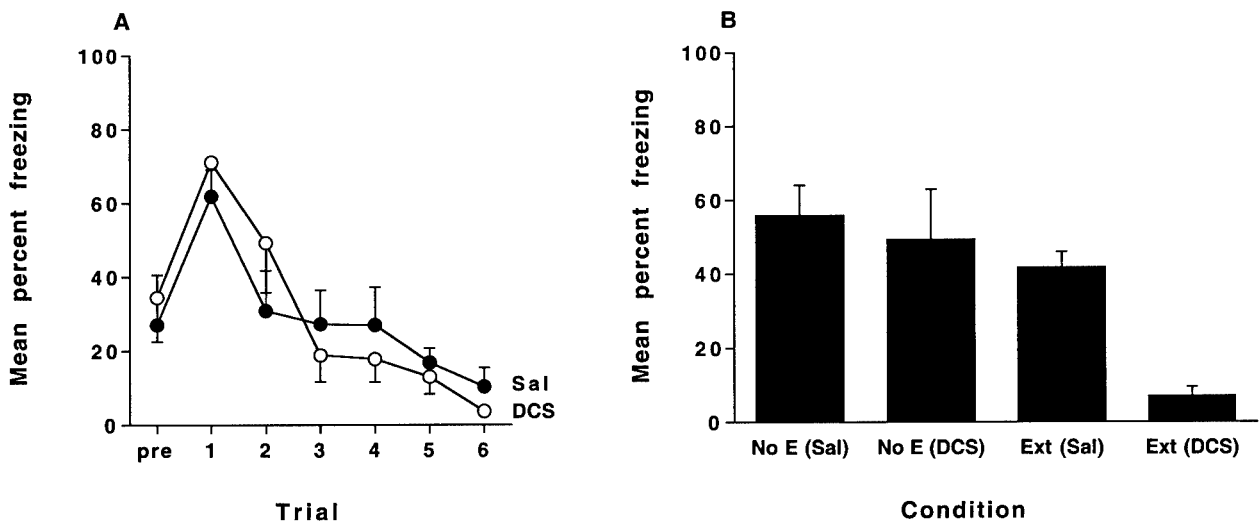


Figure 2. A: Conditioned freezing during the extinction training session. Mean (\pm SEM) percentage of time rats in Experiment 2 spent freezing during the 2-min period prior to the first presentation of the light conditioned stimulus (CS; pre) and during each of the six 2-min presentations of the light CS. Rats were injected with either saline (Sal) or D-cycloserine (DCS) immediately after the extinction training session. B: Effect of DCS on conditioned freezing during the postextinction test. Mean (\pm SEM) percentage of time rats spent freezing during one 2-min presentation of the light CS in Experiment 2. No E = no extinction training; Ext = extinction training.

On the postextinction test (Figure 2B) the following day, rats that had been injected with DCS immediately after extinction training displayed significantly less freezing than rats injected with saline, $t(14) = 6.99$. Moreover, the two extinction groups froze significantly less than the two no-extinction groups, $F(1, 28) = 11.50$. There was no significant difference in levels of freezing between DCS- or saline-injected rats in the no-extinction groups, $t(14) < 1$. These results suggest that DCS facilitates consolidation of new extinction memories.

Experiment 3: Dose-Response Function for the Effect of DCS on Extinction

Walker et al. (2002, Experiment 2) found that DCS facilitated extinction of fear-potentiated startle in a dose-dependent manner. It was therefore expected that there would be a DCS dose-response function for post-extinction training administration and conditioned freezing. A total of 31 rats were handled and allocated to four weight-matched groups ($n_s = 8$ except for the group receiving saline, for which $n = 7$). Because of the relatively high levels of precue freezing in the first two experiments, rats were subsequently preexposed to Context A for 30 min, 24 hr prior to conditioning. This effectively reduced the levels of precue freezing at test by an average of 20.22% across this and subsequent experiments. Rats were then conditioned as previously described. Immediately after extinction training on Day 3, each rat was injected with either saline or DCS (2.5, 5.0, or 10.0 mg/kg sc). Twenty-four hours later, all groups were postextinction tested.

During extinction training prior to saline/DCS injection, there were no group differences in freezing. Freezing gradually decreased over successive CS presentations, $F(5, 135) = 59.90$.

Figure 3 presents the percentage of freezing to the cue during the postextinction test, and suggests that DCS facilitated extinction in a dose-dependent manner. This was supported by the finding of a significant linear trend, $F(1, 27) = 7.54$. Conditioned freezing was significantly lower in rats injected with 10.0 (but not 2.5 or 5.0) mg/kg DCS after extinction training than in rats injected with saline, $t(13) = 2.76$. Although 10 mg/kg produced significant

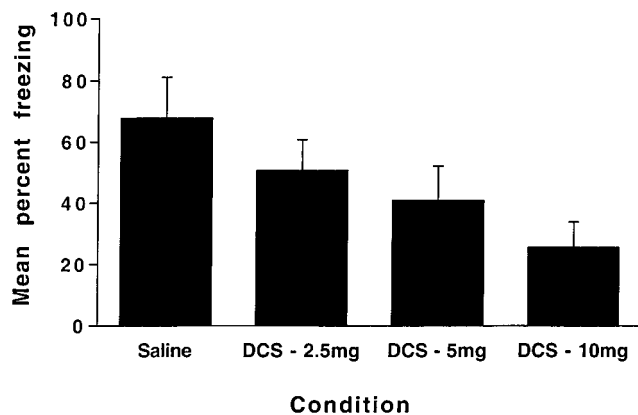


Figure 3. Dose-response function for the effect of D-cycloserine (DCS) on extinction. Mean (\pm SEM) percentage of time rats in Experiment 3 spent freezing during one 2-min presentation of the light conditioned stimulus (CS). Rats were injected with saline or DCS (2.5, 5.0, or 10.0 mg/kg sc) immediately after extinction training.

facilitatory effects (i.e., 27.77% freezing), 15 mg/kg appeared to be the more effective dose (i.e., 7.01% freezing in Experiment 2).

Experiment 4: Post-Extinction Training DCS Administration Delay Function

If DCS influences extinction through the facilitation of memory consolidation, then the basic DCS facilitation effect should decrease as a function of the length of time between extinction training termination and DCS administration. To test this, a total of 36 rats were preexposed, fear conditioned, and extinction trained, as previously described. Rats were then injected with either DCS or saline at varying intervals (0, 30, 120, or 240 min) after completion of the extinction training session (DCS: $n_s = 5, 5, 7,$ and 8 ; and saline: $n_s = 3, 3, 3,$ and 2 , respectively). Twenty-four hours later, all groups were postextinction tested.

There were no group differences in freezing during extinction training prior to saline-DCS injection. Freezing gradually decreased over successive CS presentations, $F(5, 155) = 51.54$. As expected, a one-way ANOVA of the levels of freezing to the light CS by saline rats during the postextinction test revealed that the delay of saline administration had no impact. Thus, the data from the saline-injected rats were collapsed into one condition, the saline control.

Figure 4 presents post-extinction test freezing and suggests that DCS facilitated extinction in a manner dependent on the interval between the termination of extinction training and DCS administration. A significant linear trend indicated that with increasing DCS administration delay, the amount of freezing increased, $F(1, 21) = 6.74$. Direct comparisons revealed that the 240-min DCS group did not freeze significantly less than the saline control group, $t(17) = 1.80, p = .09$, although the other three DCS groups did: 120-min, $t(16) = 2.96$; 30-min, $t(14) = 4.88$; 0-min, $t(14) = 5.12$. The last result represents a replication of the basic DCS facilitating effect reported in Experiments 2 and 3. Overall, it appeared that increasing the delay of DCS administration after the extinction session led to a decrease in the facilitating effect of DCS, with there being no advantage by 240 min.

Experiment 5: Intra-Amygdala DCS Infusions

Previous studies have indicated that NMDA receptors in the amygdala, and more specifically the basolateral amygdala (BLA), play a critical role in the extinction of conditioned fear (Falls et al., 1992; Lee & Kim, 1998; Walker et al., 2002). It is possible that the effect of systemically administered DCS reported in the above experiments was mediated by actions at BLA NMDA receptors; this experiment tested this notion. Twenty-one rats with intra-BLA cannulations received preexposure, fear conditioning, extinction training, and testing for conditioned freezing, as described previously. Immediately after being removed from the chamber for extinction training, rats were infused with either saline or 10 μ g DCS (in 0.5 μ l saline). Rats in both groups were postextinction tested 24 hr later.

The cannula placements for all rats are presented in Figure 5A. The brains of 2 saline rats and 4 DCS rats had damage to the area dorsal to the BLA, notably the caudate putamen (CPu), and were therefore excluded. Figures 5B (extinction training) and 5C (post-extinction test) present the behavioral data of the remaining 8

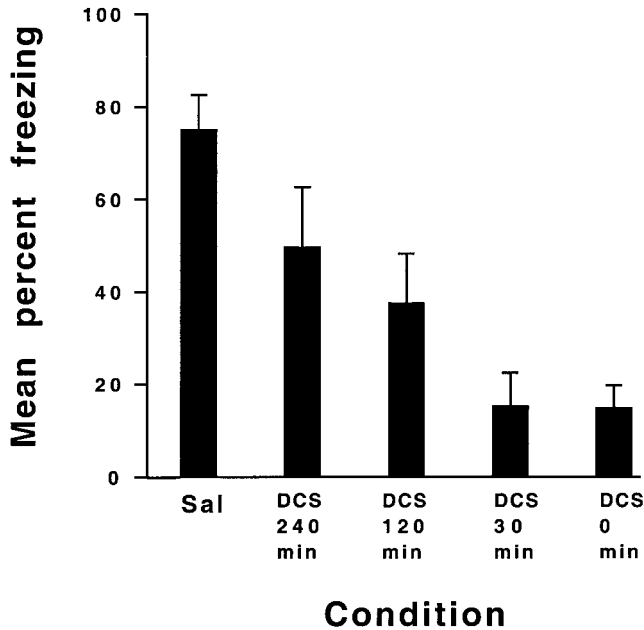


Figure 4. Effect of varying the delay of D-cycloserine (DCS) administration following extinction training. Mean (\pm SEM) percentage of time rats in Experiment 4 spent freezing during one 2-min presentation of the light conditioned stimulus (CS). Saline (Sal) or DCS was administered 240 min, 120 min, 30 min, or immediately after extinction training.

saline and 7 DCS rats. Statistical analyses confirmed that there were no differences between the groups during extinction training (prior to infusion) and that freezing decreased over trials, $F(5, 65) = 30.79$. As suggested in Figure 5C, conditioned freezing to the light CS was significantly lower in rats that received BLA DCS infusions immediately after extinction training compared with rats that received BLA saline infusions immediately after extinction training, $t(13) = 2.43$. There was no significant difference between the pre-cue (contextual) test freezing for saline ($M = 17.93$, $SD = 18.65$) and DCS ($M = 11.28$, $SD = 12.98$), $t(13) < 1$. It should be noted that the average level of freezing to the light CS of the excluded rats (saline = 42.02, DCS = 0.00) was not statistically different from the included rats: saline, excluded versus included, $t(8) < 1$; DCS, excluded versus included, $t(9) = 1.48$, $p = .17$, thus it is likely that the damage to the brains of the excluded rats was sustained during decapitation, that is, after testing.

Discussion

The principal finding of this study is that DCS, a partial agonist at the strychnine-insensitive glycine-recognition site on the NMDA receptor complex, facilitates extinction of conditioned fear after either systemic injections (Experiments 1–4) or intra-BLA infusions (Experiment 5). On a more functional level, DCS appears to influence both the acquisition (as demonstrated in Experiment 1 and by Walker et al., 2002) and consolidation (Experiments 2–5) of extinction memory. Because DCS reduced conditioned freezing only in rats that also received extinction training, the effects of DCS cannot be attributed either to DCS-

related neurotoxicity or to anxiolytic drug actions still present 24 hr after drug administration (i.e., during testing).

The findings of this study with conditioned freezing replicate those of Walker et al. (2002) with fear-potentiated startle. DCS, administered prior to extinction training (Experiment 1), significantly enhanced the extinction of conditioned fear. Further, when administered subcutaneously immediately after extinction training, a dose–response effect was demonstrated (Experiment 3; 2.5, 5.0, and 10.0 mg/kg). Across all experiments, the optimal dose appeared to be 15 mg/kg. A dose–response curve was also reported by Walker et al. (2002, Experiment 2; 3.25, 15.00, and 30.00 mg/kg ip), with there being little difference between 15 and 30 mg/kg. Moreover, post-extinction training DCS infusions directly into the BLA (unilaterally; Experiment 5) mimicked the systemic results. A similar finding was demonstrated by Walker et al. (Experiment 6) with pre-extinction training bilateral intra-amygdala infusions. Combined, these results add further support to the current belief that extinction is new learning, and that the formation of new associations can be enhanced by the action of a drug, namely DCS, that facilitates NMDA receptor activity. This action is dose dependent, that is, greater enhancing effects are obtained with higher DCS doses, but only to a point. Both this study and Walker et al. have found 15 mg/kg DCS to produce the optimal facilitatory effect. Doses of 30 mg/kg have led to only marginally greater DCS effects on the extinction of conditioned freezing (Ledgerwood & Cranney, 2002) and no greater effects on the extinction of fear-potentiated startle (Walker et al., 2002). The intra-BLA experiments strongly suggest that the actions of DCS are mediated by actions at the BLA NMDA receptors.

Extending the work of Walker et al. (2002), a number of novel findings are reported in the current study. First, the DCS facilitatory effect appears to generalize across measures of conditioned fear. Whereas Walker et al. used the extinction of conditioned fear-potentiated startle, the current study used the extinction of conditioned freezing, and similar results were obtained both systemically and with intra-amygdala infusions. Both sets of researchers however, used light as the CS, and work has yet to be undertaken using CSs of other sensory modalities (e.g., olfactory, auditory).

A second novel finding in this study is the suggested role of NMDA in the consolidation of extinction learning. Walker et al. (2002) focused on pre-extinction training administration of DCS and thus were able to manipulate the acquisition of extinction learning. To date, most studies on DCS and learning administer DCS prior to training (e.g., Flood, Morley, & Lanthorn, 1992; Land & Riccio, 1997, 1999; Matsuoka & Aigner, 1996; Monahan, Handelsmann, Hood, & Cordi, 1989). Although none of these studies focused specifically on extinction, all found DCS to result in superior learning and memory expression on a variety of appetitive and aversive tasks. In contrast, very few studies have examined post-training DCS administration (e.g., Steele, Dermon, & Stewart, 1996). The underlying assumption in the pre-extinction training DCS administration studies is that DCS affects initial acquisition processes by, for example, heightening attention to the stimuli. An alternative explanation is that DCS affects consolidation processes that begin to occur during extinction training (see Abel & Lattal, 2001). In the current study, DCS was found to significantly enhance the extinction of conditioned freezing when administered immediately after extinction training (Experiment 2).

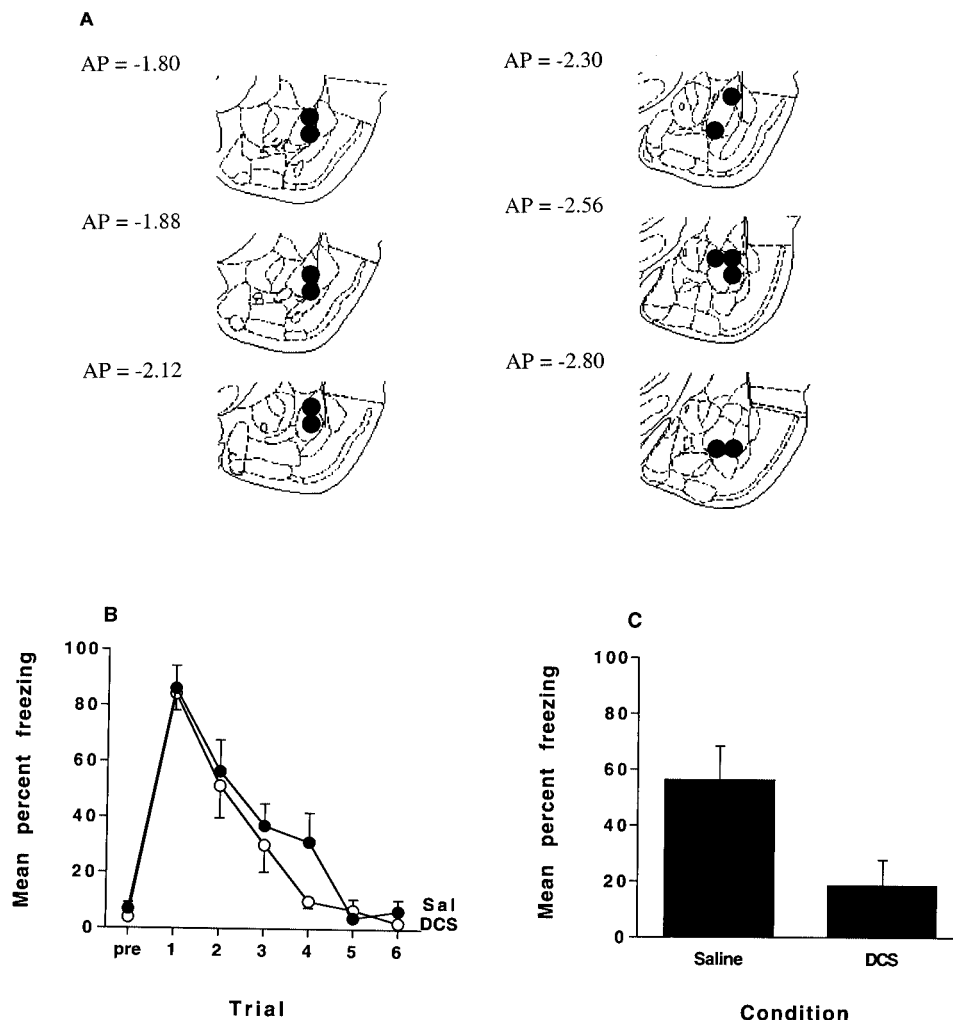


Figure 5. A: Cannula tip placements transcribed onto atlas plates adapted from Paxinos and Watson (1998). The distance from bregma is indicated to the left. All cannula tips were located in the basolateral amygdala (BLA). Each cannula tip is represented by one filled circle, with the exception of one circle in AP = -2.12 and one circle in AP = -2.56, each of which represents two cannula tips. Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figures 26–31, Copyright (1998), with permission from Elsevier Science. B: Effect of intra-BLA D-cycloserine (DCS) infusions on extinction. Mean (\pm SEM) percentage of time rats in Experiment 5 spent freezing during the 2-min period prior to the first presentation of the light conditioned stimulus (CS; pre) and during each of the six 2-min presentations of the light CS. Rats were infused with saline (Sal) or DCS immediately after extinction training. C: Effect of intra-BLA DCS infusions. Mean (\pm SEM) percentage of time rats in Experiment 5 spent freezing during one 2-min presentation of the light CS. Rats were infused with saline or DCS immediately after extinction training.

This finding, when combined with the other three post-extinction training administration experiments reported in this study, is strongly suggestive of a role of NMDA in the consolidation of extinction learning. This does not exclude the possibility of NMDA playing a role in acquisition processes in studies with pre-extinction training administration of DCS.

A third novel finding in this study relates to a point which was alluded to, but not examined, by Walker et al. (2002). That is, the effect of DCS on the short-term (or within-session) development of extinction. Port and Seybold (1998) examined the within-session effects of DCS during extinction of an appetitive instru-

mental response and actually found that DCS attenuated extinction. In Experiment 1, in which DCS was administered before extinction training, the difference between saline and DCS groups in the decrement of freezing over the six CS presentations during extinction training was only marginally significant, yet there was a clear difference at the 24-hr test. These findings are consistent with those found by Quirk et al. (1999) in which systemic injection of an NMDA antagonist (CPP) prior to extinction did not block extinction in the short-term but did reduce it substantially in the long-term. Our findings are also comparable to those of Maren et al. (1996) and Santini, Muller, and Quirk (2001) showing that

NMDA receptors appear to be relevant for consolidation processes leading to longer term, but not shorter term, new learning. Thus, NMDA receptors appear unnecessary for the initial formation and short-term stability of extinction memory but are required for long-term extinction memory.

Finally, Experiment 4 of the current series showed that increasing the delay of DCS administration after extinction training led to a linear decrease in the facilitatory effect, with there being no effect by 240 min. That is, DCS was only effective if administered within 120 min after extinction training. This finding would seem to suggest that NMDA receptors are active in the consolidation process for a short window of time (0 min to at least 120 min). There have been a number of relevant studies regarding windows, or phases, of consolidation and the role of NMDA mediation. For example, Bourtchouladze et al. (1998) suggested that there are two consolidation phases for contextual fear memories. They argued that the first consolidation phase, which occurs immediately after conditioning, is mediated by NMDA receptor activation, whereas the second phase, which occurs 240 min later, appears to be mediated by dopaminergic systems. Santini et al. (2001), however, argued that there may be several phases of NMDA-mediated memory consolidation, some occurring days after the training event. Clearly, further research is required to elucidate the neural and functional mechanisms underlying extinction memory consolidation.

Evidence that the extinction of conditioned fear memories is facilitated by the actions of a partial agonist, DCS, at NMDA receptor sites in the BLA, has considerable theoretical and clinical relevance. For example, several theorists have argued that there is considerable overlap between the functional and neural mechanisms involved in conditioned fear and clinical anxiety (Rosen & Schulkin, 1998). Thus, patients suffering from anxiety-related disorders could benefit from pharmacological treatments such as NMDA agonists during the course of therapeutic fear-extinction procedures such as systematic desensitization. Further research is required to determine clinically relevant characteristics of the DCS facilitating effect, such as its generalizability across time and context.

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