An Examination of the Extended Effects of D-Cycloserine Treatment on Fear Extinction in Rats

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An examination of the extended effects of d-cycloserine treatment on fear extinction in rats

By Rosa T. Kyek

This thesis submitted in partial fulfillment of the requirements for the degree of MS in Experimental Psychology with a concentration in Behavioral Neuroscience

Seton Hall University

May 2009
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Abstract

It has been suggested that post-traumatic stress disorder sufferers (PTSD) fail to extinguish the conditioned fear response to the traumatic stimuli which demonstrates the usefulness of an animal model for understanding human PTSD (Milad, 2006; Rauch, 2006). The N-methyl-D-aspartate (NMDA) system plays a critical role in the extinction of a fear response. In fact, NMDA antagonism has produced dose dependent blockages of extinction (Falls et al., 1992) while D-cycloserine (DCS), and NMDA agonist, has shown to be effective in facilitating the extinction process (Walker et al., 2002).

Experiment 1 did not find results similar to those found in the literature. Interestingly, DCS actually facilitated an augmented fear response with 24 hour, 7 day, and 30 delay periods between extinction sessions. Experiment 2 also did not find that DCS aids the extinction learning process but was able to eliminate the augmentation of the fear response found in experiment 1 by extinguishing animals to a criterion level before DCS manipulation. The lack of statistical power may have been the largest hindrance on the absence of effects seen in experiment 2. However, together these experiments show that an initial learning must take place in order for DCS to be effective. Ultimately, if there is certain sensitive population with which DCS can be efficacious, then an animal model of PTSD which utilizes DCS may be of limited value. In turn, DCS use in humans may only render a response in those that exhibit an initial reduction in fear. Otherwise, DCS may actually serve to enhance their fear response.
DCS and Fear Extinction

The extended effects of d-cycloserine treatment on fear extinction in rats.

Animal research investigating fear, anxiety, and disorders related to such involves animal models and typically uses Pavlovian fear conditioning paradigms in which a conditioned stimulus (CS), such as a light or tone, is paired with an aversive event (shock), or unconditioned stimulus (US). Throughout multiple trials of these pairings, the animal becomes conditioned to fear the CS usually causing increased anxiety and a conditioned response (CR). For example, rats generally exhibit this fear by freezing, a species specific behavior that is characterized by total lack of movement save for respiration. Later, in the process of extinction, the CS is presented to the animal in the absence of the US. The CR associated with the CS begins to diminish over trials, seemingly because this response is no longer adaptive. The conditioned response is thus used as an indirect measure of fear and the memory for the CS-US relationship. Although it is generally accepted that it is not that the fear has been forgotten, but rather the new association of the CS coupled with the absence of the US forms a new memory which then competes with the original association for fear expression (Milad et al., 2006).

Like experimental animals, humans may also employ the same processes to acquire and get rid of a fear response. It has been suggested that post-traumatic stress disorder sufferers (PTSD), however, fail to extinguish the conditioned fear response to the traumatic stimuli which demonstrates the usefulness of this exemplar for understanding human PTSD (Milad, 2006; Rauch, 2006).

Presently, therapies for PTSD and phobias include an exposure-based program, which closely resembles the extinction process used in animals (Foa, 2000; Rothbaum &
Patients are consistently presented with the stimuli once found to be traumatic without any negative or aversive consequences in hopes that their fear response will diminish over time. Nevertheless, this type of therapy has severe limitations because disordered patients typically do not respond normally over time to this form of extinction training in that the anxiety response habitually returns and does not usually transfer outside the experiment to real world anxiety inducing situations (Foa, 2000; van Minnen et al., 2002). Thus, PTSD is typically believed to support the idea of extinction failure.

Therefore, while the clinical applications of extinction training seems obvious, a clearer understanding of the neural mechanisms involved in extinction could generate improved biological approaches to treat fear and anxiety related disorders, like PTSD.

The Role of the Amygdala

The amygdala has long been implicated in the expression of fear and anxiety. The amygdala receives input from several sensory regions and mediates the expression of fear as well as anxiety, among other emotional responses (Fendt & Fanselow, 1999) and so is essential to formation and expression of aversive memories (Davis et al., 2005; Aggleton, 2000). Additionally, the central nucleus of the amygdala may mediate several different components of the fear response because it is this area that first projects to cortical and brain stem areas that control these responses (Kapp, Silvestri, & Guarraci, 1998). Barad (2006) also reviews literature indicating that the basolateral amygdala (BLA) is central to fear conditioning as well. The information from aversive stimuli is first processed in the BLA and is then forwarded to the central nucleus resulting in the fear response.
In animals, the extinction process can be disrupted by microinjections of NMDA receptor antagonists into the amygdala (Falls et al., 1992) and can be facilitated with microinjections of an NMDA agonist (Ledgerwood et al., 2003). Although it is believed that extinction is an inhibitory learning process, there is some evidence that suggests there is at least some reversal of an intact fear memory which occurs in the lateral amygdala. Lin et al. (2003) studied depotentiation, or a reversal of long-term potentiation (LTP), in the amygdala. Critical to learning and memory formation, LTP is the result of compounded neuronal firings, increasing the likelihood of those pathways to activate quicker in the future. Conversely, the phenomenon of depotentiation correlates with decreases in a conditioned fear response which is thought to be dependent on LTP in the amygdala. Thus, decreases in active amygdala pathways leads to a reduction in the overall behavioral response to fear. In demonstrating that this decrease (depotentiation) could be blocked by NMDA antagonists, Lin et al. (2003) showed that low-frequency stimulation of the lateral amygdala elicited this depotentiation (which required NMDA activation) as well as attenuated fear expression as measured by a fear-potentiated startle paradigm. Thus, they validated that the lateral amygdala is key to extinction memory processes and that neural deactivation is a central component to this process.

The extinction process in human fear conditioning also implicates the amygdala as a principal contributor to the process. Both positron-emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies have illustrated that amygdala activation is heightened during acquisition of conditioned fear (Fischer et al., 2000; Cheng et al., 2003 respectively) and in response to fearful faces in healthy adults (Fischer et al., 2003) suggesting there could be deficiencies in top-down control of the amygdala.
by structures involved in fear extinction for anxiety disordered people (Quirk et al., 2006).

Aside from work with healthy participants, clinical evidence has also shown that combat veterans with PTSD display an increase in amygdala activation when shown masked-fearful faces (Rauch et al., 2000) or reminders of the traumatic event (Liberzon et al., 1999) compared to combat-exposed controls as well as healthy participants.

Thus, the amygdala, and perhaps especially the lateral amygdala, has a profound influence on fear acquisition as well as fear expression and these results have been replicated in both animal and human experimental settings. However, because the literature generally describes the amygdala as a forerunner in fear processing, it is perhaps its interplay with other brain regions that are most important in fear extinction and ultimately fear expression. The infralimbic cortex (IL), a cortical sub region of the prefrontal cortex (PFC), sends an extensive number of connections to the amygdala and seems to intercede on amygdala controlled expression of fear as well (Floyd et al., 2001). Quirk et al. (2003) demonstrated that IL activation could inhibit the normal amygdala mediated behaviors involved in extinction. Consequently, when considering abnormal fear acquisition, such as in anxiety disorders, it is perhaps the pathway between the amygdala to the prefrontal cortex that could be compromised.

The Role of the Prefrontal Cortex

Considering the PFC more carefully, the ventral medial prefrontal cortex (vmPFC) may also play a large role in extinction learning and expression. It is this region that is thought to control the extinction process of long-term memories (Quirk et al., 2000). LeDoux et al. (1988) demonstrated that lesions to this area in rats did not have
an effect on acquisition of conditioned fear responses but did impair extinction. While some laboratories have failed to replicate this finding (Gewirtz et al., 1997), it is important to note that the medial prefrontal cortex (mPFC) extends a number of inputs to the amygdala and so may modulate the expression of conditioned fear during extinction (Milad et al., 2006; Morgan et al., 1993). Others have illustrated that vmPFC lesions, specifically those at the IL, do not inhibit extinction learning but may hinder subsequent recall at least one day later (Quirk et al., 2000) or impair the process such that it requires more initial training for equal results compared to control animals (Lebron et al., 2004).

In the study performed by Lebron and colleagues (2004), rats with lesions of the vmPFC extinguished normally but were impaired in extinction recall 24 hours later. Additionally, lesioned rats needed twice as many days to initiate extinction compared to control animals. This implicates the vmPFC as a storage site for extinction memories. However, lesioned animals responded to subsequent extinction sessions faster than lesioned counterpart rats without prior extinction experience. This ultimately proposes that vmPFC is not the sole site of extinction memories but it is a necessary component for adequate recall and access to extinction memories. Therefore, the vmPFC must be vital for the consolidation of extinction memories so that it is available for recall (Milad et al., 2006) and the collection of animal studies supports this notion.

Aside from lesion studies, recording investigations have also demonstrated that long-term potentiation (LTP) can result from the stimulation of glutamatergic pathways connecting to the mPFC (see Quirk, 2006 for review). Focus on such prefrontal pathways indicate that the mediodorsal thalamus (MD) pathway shows definite LTP-related changes with extinction training and that inactivation of the PFC by the thalamus
is associated with extinction recall (Herry & Garcia, 2002). However, disabling the MD-PFC pathway leads to a full recovery of the initial fear after extinction trials (Herry & Garcia, 2002).

In a recent review of prefrontal mechanisms involved in extinction, Barrett and colleagues (2003) concluded that the mPFC showed the most metabolic activity after extinction, specifically in the IL sub region. Furthermore, one such study reported positive correlations existing with the mPFC and areas associated with inhibition and extinction behavior and inverse correlations existing between the mPFC and areas associated with the expression of conditioned fear (Quirk, 2006). Together, these analyses support the idea that the prefrontal cortex, and perhaps especially its infralimbic region, is critical to consolidating extinction sessions into long-term storage and recalling those memories so that fear expression can be inhibited.

As mentioned, the PFC may be of most importance to fear extinction because its role may be to countermand or supersede the amygdala response to fear and thus hinder its expression. Quirk et al. (2006) describes this process as a feed-forward inhibition of the amygdala output neurons. In support of this idea, Quirk et al. (2003) and Milad et al. (2004) stimulated the IL which lead to decreased activity in pathways from the amygdala to the brainstem and also lead to decreases in common measures of fear expression. In turn, this directed interest towards ways to improve the extinction process by enhancing prefrontal mechanisms. Herry and Garcia (2002) stimulated the medial dorsal (MD) inputs before extinction training and reported no significant differences during extinction sessions. However, when tested 1 week later, the recollection for the extinction training was distinctly improved. They concluded that their LTP-evoking stimulation of mPFC
pathways prevented the normal decay of extinction memory that occurs over time. Indeed, electrical stimulation of the mPFC strengthens extinction learning and decreases fear behavior (Quirk, 2006). Thus, LTP of extinction, which is creating the new memory for extinction training, is ultimately related to synaptic strength in the PFC.

Similar to animal studies, research into the human fear experience parallels the findings often seen in laboratory settings. In such cases, prefrontal activation was seen during the recall of extinction (Phelps et al., 2004) and specifically during the extinction of eye blink conditioning suggesting that even in humans, the prefrontal cortex is necessary for aversive conditioning and fear learning (Rauch et al., 2006). PTSD sufferers also seem to show augmented perseveration in object-alternation tasks which involve the prefrontal cortex (Koenen et al., 2001). Thus, it seems that the animal literature parallels human findings of prefrontal deficiencies during the extinction process of fear conditioning and learning. One hypothesis involves the connection between the prefrontal regions and the amygdala. That is, a top-down failure of inhibition of the amygdala by the vmPFC (Rauch et al., 2000). However, some have also suggested a bottom-up malfunction of pre-existing amygdala dysfunction which later results in prefrontal interruption (Milad et al., 2006). These hypotheses could be further tested with longitudinal examinations of irregularities in either the PFC or amygdala, or both, from a developmental perspective.

It is thoroughly evident, however, that the prefrontal cortex does seem to play a role in the inhibition of general cognition (Bremner et al., 2000). This may explain the intrusive thoughts, or flashbacks, often experienced by sufferers of PTSD, specifically with combat related stress. One form of treatment that is common is the use of
benzodiazepines. This has shown to be effective in therapy but does not seem to prevent PTSD (Bremner et al., 2000) suggesting that there are certain alterations in benzodiazepine receptors that occur during the stress provoking period. These alterations may be most prevalent in the prefrontal cortex. Bremner et al. (2000) found that Vietnam War veterans with PTSD had prefrontal benzodiazepine distribution volumes 41% lower than that of healthy comparison subjects. One explanation which was offered suggested that perhaps a preexisting low level of benzodiazepine receptors in the prefrontal cortex may be a risk factor for developing PTSD.

While investigating another clinical population, Bremner (2002) reported that PTSD sufferers showed reduced overall activity of the PFC, while Rauch et al. (2003) added that recorded volumes of the perigenual PFC were significantly lower in PTSD populations. It is perhaps the genetic integrity of the prefrontal cortex which could pose as a risk factor in developing anxiety disorders, especially PTSD. Milad et al. (2005) suggested that the preservation of fear extinction memory is correlated with prefrontal cortical thickness. Therefore, if the prefrontal cortex is compromised, it is likely that the extinction process will be as well.

The Role of the NMDA System

Yet another system that may influence fear learning, at least in the amygdala, is the glutamate receptor of the N-methyl-D-aspartate (NMDA) type (Davis, 2005). Some forms of long-term potentiation (LTP) have been known to be dependent on NMDA receptor activity which point to this system's role in memory consolidation (Quartermain et al., 1994). Lanthorn (1993) states that since LTP is a necessary component of learning and memory and that the NMDA system seems to play an integral role, its specific
modulation may also underlie disorders of learning and memory and thus their treatment. According to Lanthorn (1993), the pattern of neural activation favorable to LTP matches that which is also favorable to NMDA activation. This pattern consists of one stimulus which disinhibits neural responses via the GABAergic system. A second stimulus, not more than 250 milliseconds later, produces a cumulative depolarization which in total is sufficient to activate NMDA channels. Essentially, the stimulation of the NMDA receptor complex increases the probability of inducing LTP (Lanthorn, 1993).

Interestingly, acquisition of fear has not been shown to be affected by NMDA agonism so it may be that only extinction, or learning to inhibit a response that is no longer adaptive, is affected by the NMDA system (Davis, 2005). In fact, NMDA antagonism has produced dose dependent blockages of extinction (Falls et al., 1992). At least one explanation for the discrepant findings comparing NMDA’s role in acquisition and extinction is that NMDA and glycine share similar chemically structural components and therefore bind to the same site on the NMDA receptor. In this case, it may be possible that glycine is already be inundating these sites during acquisition, thus hindering any attempts made by NMDA to bind there (Davis, 2005).

What is perhaps the most interesting to note is that NMDA blockers need only to be administered around the time of extinction, rather than only before or after an extinction session. While numerous studies have illustrated that d-cycloserine (DCS), an NMDA agonist, given prior to training can enhance performance and memory for extinction (see Lanthorn, 1993 for review), it has also been demonstrated that administration 3 hours post-extinction was sufficient to impair extinction recall in rats up to 24 hours later (Ledgerwood et al., 2003). Flood et al. (1992) also validated that DCS
administered post-training could aid retention of a T-maze (with foot shock) paradigm. These authors claim that DCS enhanced neural changes that typically occur shortly after learning sessions.

Although NMDA agonism may not be dependent on the order of drug administration with a learning task, activation's beneficial effects may be constrained by administration frequency. Quartermain et al. (1994) reported mice treated with DCS for 15 days performed significantly poorer in a spatial memory test for retention compared to mice that only received single injections. Their study demonstrated that chronic NMDA activation could lead to receptor desensitization. Thus, the NMDA system appears to be important to both the consolidation and maintenance of extinction learning, a system that could very well be malfunctioning in PTSD sufferers. On the other hand, chronic NMDA activation could prove to be an ineffective form of treatment. Therefore, perhaps the sensitivity of these related systems provides only a narrow range of treatment courses that do not result in impairments to extinction learning.

One clear advantage of investigating the role of NMDA in PTSD is the clinical relevance and usefulness of such approaches. DCS is a clinically safe compound that has been used to treat tuberculosis and has shown antibacterial effects at high doses (Davis, 2005) due to its ability to inhibit the metabolism of the amino acid, l-alanine (Baran et al., 1995). At high doses, DCS has been known to produce antidepressant and anxiolytic effects (Crane, 1959). Thus, exposure based programs used in conjunction with DCS could provide the best option for treatment and would subsequently add to the validity of animal models of PTSD. Indeed, some double-blind, placebo controlled studies have shown that DCS can improve associative learning in people with phobias (Ressler, 2004).
While there may be physiological abnormalities that distinguish clinically anxious people from those who are not, the evidence with animal extinction learning discerns that perhaps there is an emotional learning component which allows for the malformation of fear memories. It is thusly believed that pharmacological treatment, to combat the physiological dysfunction, and behavioral therapy, to resist the maladaptive learning, is the best options for treatment. This combination has been tested in both pre-clinical and situations that mimic those of animal extinction training.

Ressler et al. (2004) examined 27 patients with DSM-IV criteria for acrophobia in a virtual reality exposure paradigm (VRE) where participants controlled a virtual elevator upwards and stopped at the highest floor they could manage. The trial was double-blind and placebo controlled. Initially the authors found that DCS does not affect the level of fear or of avoidance during the first exposure (extinction/therapy day 1). Therefore, any promising results could not be attributed to any anxiolytic effects of DCS. However, during the second exposure, DCS patients demonstrated significantly lower subjective ratings of discomfort and elevated to significantly higher floors. This established that the facilitory effects of DCS occur during the intersession retention interval.

These positive outcomes were maintained after a 3 month follow-up period. Additionally, patients who had received DCS reported significantly more comfortability and less anxiousness in real-world height situations. In fact, they reported twice as many such encounters during the 3 month period. DCS also aided in reducing objective measures of anxiety. Those patients receiving DCS had significantly fewer spontaneous skin conductance fluctuations, a measure that is negatively correlated with anxiety, compared to those patients that did not receive DCS treatment.
As robust and lasting the results from this study are it is uncertain if these long lasting reductions in fear were the result of more extinction sessions in the DCS groups. In other words, it is presumed that the initial learning was sufficient to empower the DCS patients to face more real-world heights, and then these people are expected to face more response evoking situations without aversive consequences, in essence more extinction sessions. It is certainly plausible to consider that untreated groups would have had the same outcome had they confronted more real-world heights. Therefore, one cannot assume that DCS provided this reduction in overall fear level. Rather, its conjunction with additional extinction learning experiences may have contributed to the effect seen.

Hofmann (2007) also reported similar results pairing DCS dosing with an exposure-based therapy program. In his clinical study, social anxiety patients made public speeches of increasing difficulty. Those who had been administered the DCS treatment exhibited significantly greater post-treatment improvements on clinical and self-report assessments of anxiety compared to patients receiving placebo treatment. Moreover, these results were maintained at a one month follow up evaluation.

Other clinical investigations have employed DCS as treatment for anxiety based disorders. Kushner et al. (2007) is currently focusing on acute administration of DCS and effectiveness of exposure therapy and response prevention in obsessive-compulsive disorder. Preliminary results thus far have shown that there are significant reductions in obsession-related distress compared to a placebo control group.

Furthermore, Tolin et al. (2006) explored the use of DCS with panic disorder. In their exposure-based program, they also presented significantly greater reductions in both clinical and self-report measures of panic disorder severity compared to placebo
counterparts. More impressively though, their findings indicated that 62% of patients receiving DCS were considered to be in remission compared to 21% of the placebo patients. After the follow-up interval, 75% of DCS patients were considered to be in remission compared to only 31% of placebo patients.

Noticeably advantageous results have sprung from clinical and animal studies utilizing DCS in exposure-based programs and extinction paradigms. Yet many patients still fail with such treatments. It is possible with perhaps better animal and sub-clinical models of anxiety disorders to observe larger and longer lasting effects of DCS.

Animal models have characteristically employed acute administrations of DCS and there is only one known study of sub-chronic or multiple administrations of DCS given during the extinction process (Parnas et al., 2005). Additionally, these single doses of DCS do not seem to have lasting effects on subsequent extinction trials (Silvestri, Kyek, & LeBlanc, 2007), yet long term treatment was not assessed. Presumably, treatment for phobias, panic disorder and PTSD would include multiple sessions of therapeutic exposure so it is important to assess whether an inhibited fear response can be sustained over longer periods of time and if drug administration could facilitate fear inhibition retention and reduce overall spontaneous recovery. At least two human studies showed that DCS has long lasting effects, yet no one has investigated the usefulness of an animal model. Parnas and colleagues (2005) reported that a 28 day period of pre-exposure of animals to DCS before acquisition training facilitated the extinction process but that this result was not found with pre-exposure just prior to acquisition training. Accordingly, it may be advantageous to test the duration of extinction facilitation while manipulating the inter-session interval and thus memory consolidation periods.
Pilot Study

Methods

Subjects

The subjects were 13 experimentally naïve male Sprague-Dawley rats. All animals weighed between 250 and 350 grams throughout the duration of the experiment. Food and water were available ad libitum. Rats were kept on a 12:12 hour light-dark cycle (lights on at 8am) and housed in pairs (one rat housed singly).

Apparatus

Fear conditioning was conducted in standard conditioning chambers using a light and noise conditioned stimuli presentation as well as a floor grid equipped to deliver shock. The shock was transferred via an ENV-414 shock/distributor (MED Associates, Inc., Georgia, VT) and the light and noise were conveyed by means of a custom written computer program using MED-PC (MED Associates Inc., Georgia, VT).

Procedure

Rats were handled for at least twice in 10 minute sessions prior to experimentation beginning up to 2 weeks after arrival. To habituate animals to the context, one habituation session was given prior to fear conditioning. For this, rats were placed into the conditioning chamber for 45 minutes with the same white noise that was present during conditioning.

For acquisition training, rats were again placed into the conditioning chamber and presented with 10 light/noise (CS) plus shock (US) pairings. The intensity of the shock and number of training sessions was manipulated to investigate which condition would provide the maximum amount of freezing that could be sustained over longer periods of
time. Rats were subjected to either a .5mA or 1.0mA shock in either one 10 trial session or two 10 trail sessions separated by 24 hours (Table 1). The rationale for such an investigation developed because an earlier study was unable to detect long term differences between groups seemingly because freezing had reached a minimal value (in essence a floor effect) too quickly (Silvestri, Kyek & LeBlanc, 2007). If extended effects of DCS are of particular interest, it is necessary that animals are trained under conditions that would maximize freezing during extinction so that if a difference does exist it can be recognized in multiple extinction sessions beyond just the first. One way to combat this issue may be to create a stronger initial conditioned fear association by increasing shock intensity and/or increasing the number of CS-US pairings.

Two initial extinction sessions were given after acquisition training concluded and were each separated by 24 hours. A third identical extinction session was given 1 week following the second. For each of these sessions, rats were placed into the conditioning chamber and presented with 10 CS alone trials. The percentage of time spent freezing during the 30 second CS presentation was recorded by trained observers.

<table>
<thead>
<tr>
<th>SHOCK INTENSITY</th>
<th>10 TRIALS</th>
<th>20 TRIALS OVER 2 DAYS</th>
<th>TOTAL</th>
</tr>
</thead>
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<tr>
<td>.75 mA</td>
<td>N= 3</td>
<td>N= 3</td>
<td>N= 6</td>
</tr>
<tr>
<td>1.0 mA</td>
<td>N= 3</td>
<td>N= 4</td>
<td>N= 7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>N= 6</td>
<td>N= 7</td>
<td>N= 13</td>
</tr>
</tbody>
</table>

Table 1. Pilot study manipulation of shock intensity and duration of acquisition
Data Analysis

Each rat was scored for freezing during each of the acquisition and extinction sessions. Freezing was considered to be a lack of all bodily movement except that required for breathing and was measured using a time sampling method. An observation was made every 3 seconds and a positive or negative judgment was made. A percentage score was then calculated for the proportion of the total observation time period spent freezing (Weber et al., 2007). Because only qualitative measures of freezing levels were necessary to determine the best methods to employ for the study’s experiments, no quantitative data analyses were conducted. A close visual inspection of the graphic representations of the results was performed and logically informed judgments were made based upon these.

Results

The results of the pilot experiment illustrated that shock intensity did not factor into the strength of the conditioned fear association during the initial extinction sessions (Figure 2-3 left). However, rats who had received the larger intensity shock did sustain a more pronounced conditioned response one week later during a third extinction session (Figure 4 left). This is presumably because there is not a difference between a .75mA shock and a 1.0mA shock in terms of initial aversiveness yet the salience of a larger shock may have been large enough to sustain a greater fear response over an extended period of time. When rats were given 20 training trials over two days, their freezing during both extinction trials remained heightened compared to the freezing behavior exhibited by the rats only receiving 10 training trials on one day (Figures 2-3 right). Thus, it was concluded that the condition which induced the strongest initial association
for the fear related stimuli as well as the strongest sustained fear response was that which received 1.0mA shocks and 20 acquisition trials which increased the duration of training as well as the intensity of the shock (Figure 5).

**Figure 1.** First extinction session (24 hrs. after acquisition training). Left side shows groups collapsed across shock intensity. Right side shows groups collapsed across number of training trials (20 trials is over 2 consecutive days).

**Figure 2.** Second extinction session (24 hrs. after extinction 1). Left side shows groups collapsed across shock intensity. Right side shows groups collapsed across number of training trials (20 trials is over 2 consecutive days).
Figure 3. Third extinction session (7 days after the extinction 2). Left side shows groups collapsed across shock intensity. Right side shows groups collapsed across number of training trials (20 trials is over 2 consecutive days).

Figure 4. The group receiving both the greater 1.0mA shock and increased training trials seemed to be the only group sustaining a more enhanced fear response indicated as the only group that remained above 50% freezing throughout the session.
Experiment 1

Methods

Subjects

The subjects were 21 experimentally naïve male Sprague-Dawley rats. All animals were between 250 and 350 grams throughout the duration of the experiments. Food and water were available ad libitum. Rats were kept on a 12:12 hour light-dark cycle (lights on at 8am) and housed in pairs. All protocols were approved by the Seton Hall Animal Use and Care Committee and all guidelines for the care and use of animals set by the United States Public Health were strictly followed.

Apparatus

The apparatus used was identical to that used in the pilot study.

Procedure

Handling and habituation procedures were identical to that used in the pilot study. For acquisition training rats were again placed into the conditioning chamber and presented with 10 light/noise plus shock pairings (light/noise: 5 seconds; shock: 0.5 seconds, 1.0mA; 120 second variable intertrial interval). After this training session, rats were returned to their home cages. Twenty-four hours later, another conditioning session was administered using the same conditioning procedure described above.

The first extinction session took place 24 hours after this initial conditioning session. For this, rats were placed into the conditioning chamber and presented with 10 light/noise alone trials. An identical second extinction session was administered to all rats 24 hours following the first session. There were four experimental groups consisting of rats that received subsequent extinction sessions beginning 24 hrs., 7 days, or 30 days
after the initial two extinction sessions to assess both memory for extinction training and analyze the extended effects of DCS treatment (Figure 6). Such time intervals were chosen because Parnas et al. (2005) had demonstrated that DCS can have long-lasting effects on extinction but their animals were pre-exposed to DCS prior to acquisition. The extended effects of drug administration given after initial extinction sessions were not assessed. Therefore, a delay of at least 4 weeks was necessary; however it seemed appropriate to evaluate the developing time-course effects of DCS at shorter intervals as well. The 24 hr. delayed group was the only group to receive extinction sessions each day until fully extinguished or until the drug group is no longer significantly different than the control group. All other delay groups received extinction sessions spaced apart by their respective time delays.

![Figure 5. Schematic representation of experiment 1](image)
**Drug**

D-cycloserine (DCS) was obtained from Sigma Chemical Company. Rats were first trained and given the first extinction session as described in the procedure above. Immediately after this first extinction session, rats were given a systemic injection of either 30 mg/kg DCS or vehicle. The same injection was given after the second extinction session only. No rats were given injections after any subsequent extinction sessions.

The rationale for administering the drug post-extinction came from previous studies that showed no behavioral differences from drug administrations given prior to or after extinction sessions (see introduction). Therefore it seemed advantageous to minimize the effects of state dependent learning which could result from an anxiety provoking state due to injections given before extinction session.

**Data Analysis**

The methods used to analyze the amount of freezing were identical to that used in the pilot study. Mixed factorial ANOVA was the primary statistical approach for all acquisition and extinction sessions. The between-subject factors were drug treatment and delay while the within-subjects factor was blocks. In each analysis, the dependent measure was percentage of time spent freezing and the independent variables were trial block and delay group. Each trial block consisted of 2 trials (Woods & Bouton, 2006). A rejection criterion of $p < .05$ was used for all analyses.
Results

Acquisition

The first and second acquisition sessions were combined to have an overall indication of acquisition behavior. The analysis was conducted as a Groups (2) x Blocks (10) x Delay group (3) design with blocks as a within-subject variable and delay and group as between subjects variable. And, since the analyses of these sessions were only used to have assurance that animals acquired an observable amount of fear, no analyses between acquisition sessions was necessary. A main effect of blocks was observed indicating that animals’ freezing behavior significantly increased over trials \[F(9, 135) = 14.312, p = .000\] (Figure 7). There was also a main effect of delay group \[F(2, 15) = 5.262, p = .019\]. A Tukey’s HSD post-hoc analysis revealed a difference between the 7 day and 30 day delay groups \[F(2, 15) = 5.262, p = .015\]. The difference is largely due to slightly less freezing overall in the 30 day group compared to the 7 day group. Figure 7). There was no main effect of drug group which signifies that these two groups did not differ in terms of acquisition behavior before the drug manipulation took place. There were also no interaction effects between any of these variables.
Acquisition

![Graph showing acquisition data for DCS and Vehicle groups over different blocks and time points.](image)

Figure 6. Acquisition 1 and 2. Blocks 1-5 reflect first acquisition; blocks 6-10 reflect the second acquisition session.

Initial Extinction

Extinctions 1 and 2 were combined to have an overall indication of initial extinction behavior. Injections of DCS or vehicle were given only after these sessions which were separated by 24 hours. They were also unique in that the time delay manipulation did not take place until after these initial extinction sessions.

The initial extinction data are shown in Figure 8. In this groups x trials mixed factorial design, there was a main effect of blocks indicating that animals' freezing behavior significantly decreased over trials \(F(9, 135) = 20.035, p = .000\). Also, a main effect of drug group was detected \(F(1, 15) = 14.536, p = .002\), however contrary to the initial hypothesis, animals treated with DCS had significantly heightened levels of freezing compared to rats treated with vehicle. Additionally, a main effect of delay group \(F(2, 15) = 5.109, p = .020\) demonstrated that the groups may have differed before the time delay took place.
Similarly, significant blocks x delay group \( F(18, 135) = 3.672, p = .000 \) and blocks x drug group interactions \( F(9, 135) = 2.836, p = .004 \) were found which reflect the differences that diverge the 7 day delay group from the 24 hour and 30 day groups in terms of initial extinction behavior. Interestingly, there was a marginally significant blocks x drug group x delay group interaction \( F(18, 135) = 1.481, p = .106 \) which may indicate further that these groups not only differed in terms of their initial freezing behavior, but also in terms of their responsiveness to DCS (Figure 8).

**Initial Extinction**

![Initial Extinction Graph](image)

**Figure 7.** Initial Extinction. Blocks 1-5 reflects the first extinction session while blocks 6-10 reflect the second extinction session.

**Delayed Extinction**

In figure 9, extinctions 3 and 4 were combined to evaluate the overall indication of delayed extinction behavior. Injections of DCS or vehicle were not given only these sessions and so they reflect the behavior patterns of rats after acute injections of DCS or vehicle and a time delay.

A main effect of blocks was found \( F(9, 135) = 2.898, p = .004 \) indicating that decreases in freezing levels were still occurring after these time delays. There was also a
main effect of drug $[F(1, 15) = 18.234, p = .001]$ where animals treated with DCS displayed significantly greater amount of freezing during these later delayed extinction sessions. Additionally, a main effect of delay group $[F(2, 15) = 4.074, p = .039]$ indicates that the amount of initial extinction retained during these later sessions differed with respect to delay group. Tukey's HSD post-hoc analyses revealed a difference between the 24 hour and 7 day delay groups $[F(2, 15) = 4.074, p = .041]$ as well as between the 24 hour and 30 day delay groups, however this difference is only marginally significant $[F(2, 15) = 4.074, p = .083]$ (Figure 9). The graph suggests a significant three-way interaction with the greatest drug effect in the 30 day group and the least in the 7 day group, but the interaction was not significant, $F(13, 195) = 1.3, p > .05$. This lack of significance is most likely due to the high degree of variability in the data when all three groups are included in the analysis. For example, if separate ANOVAs are calculated on the three delay groups a significant drug x blocks interaction was observed in the 30 day group, $F(9, 36) = 2.30$, but not the 7 day group, $F(9, 63) < 1$, or the 24 hr group, $F(9, 36) = 1.40, p > .05$. 
**Figure 8.** Delayed Extinction. Blocks 1-5 reflect extinction 3 (24 hrs, 7 days, or 30 days after extinction 2). Blocks 6-10 reflect extinction 4 (48 hrs, 14 days, or 60 days after extinction 2).

**Discussion**

The experiment revealed that intraperitoneal administrations of DCS following initial extinction session serves more to augment the fear associated behavior rather than to reduce it. This is in contrast to a number of studies demonstrating that DCS enhances extinction (Lanthom, 1993; Flood et al., 1992; Parnas et al., 2005). There are several different reasons for the disparity in these results.

The seemingly beneficial effects of DCS stem from its neural underpinnings. By binding to the glycine receptor site on the NMDA receptor complex, DCS facilitates excitatory NMDA transmission which has been linked to long-term potentiation (LTP) and ultimately, learning (Quartermain et al., 1994). Moreover, NMDA neurotransmission is a possible mechanism by which experiences are translated from short-term to long-term memory (Sweatt, 1999). Assuming that short-term memories are necessary for long-term memory formation, it may be crucial to ascertain if short-term memories are in fact being formed during initial extinction sessions.
Only one other known study has formally addressed this issue. Weber, Hart, and Richardson (2007) investigated the effects of DCS on extinction of learned fear to an olfactory cue and determined that DCS is ineffective at facilitating extinction retention if no short-term extinction learning occurs prior to injection. The present study certainly supports this idea by demonstrating that when there was no effect of trials during extinction session 1 (prior to injections) or 2 (prior to 2nd injection), there was essentially no initial extinction learning that took place. Under these conditions, DCS would be predicted to be ineffective; this lack of effect is precisely what was observed in the present study.

Therefore, while DCS may facilitate NMDA transmission, its qualitative effects on fear related behavior may translate to its ability to convert short-term into long-term memories. Based on these findings, Weber et al. (2007) consequently divided animals into groups of “extinguishers” and “non-extinguishers” and found that when there was a significant amount of extinction in the short-term; DCS was facilitative in converting those memories into long-term memories as evidenced by retention tests. They concluded that this reflects a “quantitative” effect that DCS may have on memory, augmenting the amount of information that can be transferred into long-term storage. Such an argument could be used to explain the behavior found in the 7 day delay group, which never produced group differences at any time point in extinction training. Still, the performance in both the 24 hour and 30 day delay groups cannot be completely explained in the same manner. Their fear associated behavior was counter to both the original hypothesis and previous studies exploring DCS and extinction (Lanthorn, 1993; Flood et al., 1992; Parnas et al., 2005) in that DCS treated animals reliably displayed more fear.
related behavior than did control animals which seems to indicate a more complex nature of NMDA neurotransmission and its role in extinction learning.

DCS may serve better to strengthen initial extinction learning such that it is less susceptible to decay over time (Weber et al., 2007). This concept is borrowed from Rescorla (2004) who established that, in associative learning paradigms, what is learned first is the most resilient compared to what is learned later, elucidating why extinguished responses “spontaneously recover” after time. In other words, the acquisition training given could impair subsequent extinction learning retention and DCS may aid extinction learning by hindering spontaneous recovery. Presently, the 24 hour and 30 day delay groups illustrated that the amount of spontaneous recovery (block 1 in extinction sessions 2-4) was greater in the DCS treated animals. Thus, it is hypothesized here that not only was DCS ineffective at aiding extinction learning, but that it actually supported a more robust recovery after short (24 hours) and extended (30 days) but not intermediate (7 days) periods of time. Perhaps during these short and long retention intervals, DCS was able to enhance the last associative learning session, namely acquisition because the argument can be made that no learning took place during the extinction session prior to the DCS injection. Furthermore, if DCS serves to strengthen long-term memories, such that they are less susceptible to degradation over time (Weber et al., 2007), and if acquisition was the last long-term memory which was formed, then the present results could be indicating that DCS facilitated the strengthening of that memory which was obvious after both short (24 hours) and long (30 days) delays.
Experiment 2

This experiment was designed to test the previously stated hypothesis that the lack of effect of DCS in the first experiment was due to a failure to learn the initial extinction training. This objective will be accomplished by confirming all animals learn a criterion amount of extinction training before given injections of DCS or vehicle.

Methods

Subjects

The subjects were 18 experimentally naïve male Sprague-Dawley rats. All animals were between 250 and 350 grams throughout the duration of the experiments. Food and water were available ad libitum. Rats were kept on a 12:12 hour light-dark cycle (lights on at 8am) and housed in pairs. All protocols were approved by the Seton Hall Animal Use and Care Committee and all guidelines for the care and use of animals set by the United States Public Health were strictly followed.

Apparatus

The apparatus used was identical to that used in the pilot study and in the first experiment.

Procedure

The procedures were identical to that of the first experiment with the exception of the number of initial (pre-drug) extinction trials. Rather than two extinction sessions followed by DCS or vehicle injections, animals now received as many extinction sessions necessary until extinction learning was demonstrated. For this, rats were given an extinction session every 24 hours after acquisition training until the average freezing during the final block of the session reached 50% of their original recalled fear magnitude.
(measured as the amount of freezing exhibited during the first block of the first extinction session). The time delay manipulation was not taken into consideration until the animal had reached criterion freezing levels. Therefore, once the animals reached criterion, they were given a single injection of DCS or vehicle and placed back in their home cages for the 24 hour, 7 day, or 30 day delay period as described in the first experiment.

\textit{Drug}

The drug used was identical to that used in the first experiment.

\textit{Data Analysis}

The methods used to analyze the amount of freezing were identical to that used in the pilot study and the first experiment. Mixed Factorial ANOVA was the primary statistical approach for all acquisition and extinction sessions. In each analysis, the dependent measure was percentage of time spent freezing and the independent variables were trial block as a within-subjects factor and delay and drug treatment as between-subjects factors. Each trial block consisted of 2 trials (Woods & Bouton, 2006). A rejection criterion of $p < .05$ was used for all analyses.

\textit{Results}

\textit{Acquisition}

The first and second acquisition sessions were combined to have an overall indication of acquisition behavior. Since the analyses of these sessions were only used to have assurance that animals acquired an observable amount of fear, no analyses between acquisition sessions was necessary. A main effect of blocks was observed indicating that animals' freezing behavior significantly increased over trials [$F (9, 153) = 29.90, p = .000$] (Figure 10). There was no main effect of delay group, nor was there a main effect of
drug group which signifies that these two groups did not differ in terms of acquisition behavior before the drug manipulation took place. There were also no interaction effects between any of these variables.

**Figure 9.** Acquisition. Blocks 1-5 reflect acquisition 1 while blocks 6-10 reflect acquisition session 2.

*Extinction*

The first and second extinction sessions were combined to have an overall indication of extinction behavior. A main effect of blocks was found indicating freezing behavior has significantly decreased over the course of 10 blocks (20 trials) of extinction \( [F (9, 117) = 11.226, p = .000] \). There was no main effect of drug group \( (\eta^2 = .018) \) but there was a significant main effect of delay group \( [F (2, 13) = 4.228, p = .039] \). Tukey’s HSD post-hoc analysis revealed that rats remained at more elevated levels of freezing when extinction took place 24 hours following drug/vehicle administration compared to when it took place 30 days following. There was also a marginally significant difference between the 24 hr. delay group and the 7 day delay group \( [F (2, 13) = 4.228, p = .090] \). Similar to the difference found between the 24 hr. and 30 day delay groups, this analysis indicated that overall freezing levels were higher in the 24 hr. delay group compared to
the 7 day delay group. While there was no interaction between drug group and delay group ($\eta^2 = .220$), or blocks ($\eta^2 = .042$), nor between blocks x delay group x drug group ($\eta^2 = .097$), there was an interaction effect observed between blocks and delay group [$F (18, 117) = 1.836, p = .029]$. More specifically this interaction is due to a greater decline in freezing over blocks in the 24 hr and the 30 day groups than in the 7 day group. (Figure 11).

![Extinction](image)

**Figure 10.** Extinction. Blocks 1-5 reflect extinction 1 while blocks 6-10 reflect extinction session 2.

**Discussion**

The results of the second experiment do not coincide with the animal literature demonstrating that NMDA agonism can aid the extinction process (Ledgerwood et al., 2003). Nor do the results corroborate with the more specific clinical literature that implicates DCS as an effective compound to use in extinction-like exposure therapy for anxiety disorder sufferers (Ressler et al., 2004; Tolin et al., 2006; Kushner et al., 2007; Hofmann et al., 2008). Again, the underlying causes of these results are unknown. There are perhaps methodological issues that can be addressed.
The amount of statistical power to detect group differences is a clear hindrance on supporting the original hypothesis. Based on visual observation alone, it appears as though DCS has a facilitory effect on extinction learning, especially for short (24-48 hours) and intermediate (7-14 days) time periods. During long (30-60 days) time periods, it seems as though this facilitory effect vanishes and there is no longer a difference between animals treated with DCS and those treated with vehicle. It is thus perceived that the lack of statistical power in this study, due to the small number of subjects, is possibly the largest contributor to the analyzed results found here.

It is also arguable that the dosage used was insufficient to reveal group differences. Parnas, Weber, and Richardson (2005) were able to document the facilitory effect of DCS on conditioned fear extinction in rats using a dosage of only 15 mg/kg. However, their design utilized multiple exposures to the drug. Weber et al. (2007) also used a dosage of 15 mg/kg and were able to observe the supportive effects of DCS when “extinguishers” were distinguished from “non-extinguishers”. If extinction memory processes can be facilitated using 15 mg/kg then the current use of 30 mg/kg is certainly substantiated. So conceivably, DCS may have a more narrow range of optimal dosages in terms of fear extinction. Flood, Morley, and Lanthorn (1992) established that in the range of dosages from 2.5 to 50 mg/kg, 10-39 mg/kg seemed to produce the greatest amount of memory retention aid during a T-maze foot shock avoidance task. Despite the fact that their study employed subcutaneous injections, it did not differ greatly in terms of experimental goals. In essence, 30 mg/kg administered intraperitoneally should promote extinction learning and foster a reduction in freezing behavior.
There is no known study that employed the same methods that are currently used. The idea of extinguishing animals to a criterion level spawned from the findings of Weber et al. (2007) who distinguished differences in DCS responsiveness when animals were identified as “extinguishers” and “non-extinguishers”. The present aim was not to validate this claim specifically but to study the extended effects of DCS on fear extinction. Thus, all animals were given the opportunity to become “extinguishers” and none were identified as “non-extinguishers”. In other words, although some animals did not reach a criterion freezing reduction in a short amount of time, these animals remained in the data analysis rather than being classified as “non-extinguishers”. Presently, most animals reached a 50% reduction in their freezing levels by the 2nd or 3rd extinction session. Nevertheless, those that did not reach similar levels until the 4th or 5th extinction session were all analyzed as equals. These seemingly small inter-animal differences may have actually played a larger role in the absence of an effect of DCS. Future investigations should examine the role DCS plays at low, moderate, and high amounts of extinction training as well as its role in fast, moderate, and slow extinguishers.

Another methodological shortcoming may be that the 24 hour, 7 day, and 30 day delay groups were not run concurrently although all the animals began the initial handling phase at the same age. Because of this discrepancy, the 7 day rats were run at an age approximately 1-2 months older than those of the 24 hour group. The 30 day delay group rats, partially because of their long time delay periods, were also 1-2 months older than those of the 7 day delay group and 3-4 months older than those of the 24 hour delay group. The individual rat data results seem to be according to an age-dependent curve. That is, no animal in the 24 hour delay group took more than 4 extinction session
to reach the criterion level, and only 1 animal needed the 4th session. In the 7 day delay group, 1 animal took 4 extinction sessions, and 1 animal took 5 sessions. Finally, in the 30 day delay group, when the animals were substantially older, 3 animals took 4 extinction sessions to reach criterion and 2 needed the 5th session. Thus it seems as though older rats may become “non-extinguishers” or at least much slower extinguishers and in-turn may appear to be less responsive to DCS manipulation compared to much younger rats who were also given a much shorter time delay with which to recall extinction learning. In the present case, it is unknown whether such differences are due to memory impairments related with aging such that older rats may be unable to recall extinction learning after long delays or if the results stem from the greater amounts of time needed to learn the initial extinction training. Because these issue were not related to the current aims, further discussion is beyond the scope of this paper. What is known is that the co-variance of age and time delay group has created a possible problem in the data analysis and may have contributed to the results.

**General Discussion**

Although both experiments failed to validate previous research findings, there is useful information to take from them. For one, the effects of DCS may be small and not generalizable to all types of fear or anxiety related disorders, and more importantly, DCS may only be highly efficacious in a small population of experimental animals while many remain non-responsive. Such factors that could divide these groups are age, learning speed, and cognitive and memory load.

Based on the current findings, it appears as though animal models of PTSD that employ DCS and fear extinction are of limited value. The premise, the idea that PTSD
may have its underpinnings based on a primitive learned fear response, is certainly valid. However, there are likely more complex neural deficits that largely contribute to the overall presentation of the disorder. An advantage humans with psychological disorders have is the ability of conscious re-evaluation of psychotic symptoms which has shown to be effective in reducing psychotic symptomatology (Rauch et al., 2006). As of yet, it is not known whether cognizant reappraisal of aversive stimuli relies on the same neurocircuitry as does behavioral therapies involved in extinction training (Rauch et al., 2006). It has been said that emotional regulation activates the lateral prefrontal region whereas, as mentioned before, extinction training engages vmPFC (Rauch et al., 2006; Ochsner et al., 2002). And conversely, some studies have found similar activations for both of these processes (Rauch et al., 2006). Therefore, cognitive strategies may in fact appoint the same means of fear extinction (Rauch et al., 2006). Undoubtedly, if a correlation between these two processes is established, it will aid and guide the future of clinical treatment of PTSD. Conversely, since it assumed that experimental animals cannot engage evaluative cognition, if the capacity to do so is of considerable importance, then animal models of PTSD would be rather trivial.

What can be drawn from this investigation is that DCS is only effective in certain neural states. Such beneficial effects may also be constrained by the idea that DCS can, under the right circumstances, act similar to an NMDA antagonist (Lanthorn, 1993). If the NMDA complex is stimulated at an inappropriate time, it can block subsequent LTP. In essence, this allows NMDA and endogenous NMDA agonists to perform like antagonists. This also means that DCS can take on properties of an NMDA antagonist (Lanthorn, 1993).
Lanthorn (1993) states that DCS may only have learning and memory enhancing effects when glycine levels are low and that at high levels of glycine, DCS has only the efficacy of itself alone which is minimal since DCS is only a partial agonist of the glycine site. As mentioned earlier, if glycine is already inundating binding sites on the NMDA receptor complex, the additional DCS may actually be detrimental, in effect, to learning, even presumably, fear extinction learning. These ideas may partially explain the results seen in experiment 1 where DCS may have stimulated the NMDA complex prematurely, causing a subsequent blockage in LTP during initial extinction training.

Concluding Remarks

Despite the fact that Experiment 2 was unable to uncover any significant effects of DCS on extinction, it did reveal that the counter-intuitive findings of Experiment 1 can be eliminated by extinguishing animals to criterion. Therefore, the overall conclusions extracted from this study at least partially confirm the findings of Lanthorn (1993) as well as Weber, Hart, and Richardson (2007) by demonstrating the behavioral efficacy of DCS over extended periods of time.

Many animal models of PTSD have chosen to focus on specific brain regions to explore the behavioral value of DCS treatment (Silvestri, Kyek, & Leblanc, 2007). In truth, there are several brain locales and risk factors that may play roles of varying importance in PTSD and other anxiety related disorders. For example, parental PTSD, prenatal environments with a mother with PTSD (Yehuda et al., 1998), genetic background (Wakizono et al., 2007; Stein et al., 2002), and smaller hippocampal volumes (Gilbertson et al., 2002) all appear to be worthy of note. Animal models have recognized increased amygdala responses as well as hypofrontality, especially in the medial
prefrontal region, as important regions for understanding PTSD (Davis & Whalen, 2001; Davis et al., 2005; Fendt & Fanselow, 1999). Therefore, while future investigations should continue to explore NMDA agonism's contribution to extinction learning both on a focal and distributed level, perhaps DCS, only a partial agonist for the NMDA system is not the most effective way to build a beneficial animal model for human disorder.
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