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## Explicit disassociation of a conditioned stimulus and unconditioned stimulus during extinction training reduces both time to asymptotic extinction and spontaneous recovery of a conditioned taste aversion

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### Abstract

Conditioned taste aversions (CTAs) may be acquired when an animal consumes a novel taste (CS) and then experiences the symptoms of poisoning (US). This aversion may be extinguished by repeated exposure to the CS alone. However, following a latency period in which the CS is not presented, the CTA will spontaneously recover (SR). In the current study we employed an explicitly unpaired extinction procedure (EU-EXT) to determine if it could thwart SR of a CTA. Sprague-Dawley rats acquired a strong CTA after 3 pairings of saccharin (SAC the CS) and Lithium Chloride (LiCl the US). CTA acquisition was followed by extinction (EXT) training consisting of either (a) CS-only exposure (CSO) or, (b) exposure to saccharin and Lithium Chloride on alternate days (i.e., explicitly unpaired: EU). Both extinction procedures resulted in  $\geq 90\%$  reacceptance of SAC, although the EU extinction procedure (EU-EXT) significantly decreased the time necessary for rats to reach this criterion (compared to CSO controls). Rats were subsequently tested for SR of the CTA upon re-exposure to SAC following a 30-day latency period of water drinking. Rats that acquired a CTA and then underwent the CSO extinction procedure exhibited a significant suppression of SAC drinking during the SR test (as compared to their SAC drinking at the end of extinction). However, animals in the EU-EXT group did not show such suppression in drinking compared to CSO controls. These data suggest that the EU-EXT procedure may be useful in reducing both time to extinction and the spontaneous recovery of fears.

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There are clear adaptive advantages to establishing and retaining fears. Fears evoke defensive reactions that protect individuals against future threats and help ensure survival. However, fears become maladaptive when they persist in contexts where threats are no longer present. Crippling clinical conditions such as panic attacks, phobias and the anxiety-laden flashbacks of Post Traumatic Stress Disorder (PTSD) are examples of pathological fear (for review, see Maren, 2005). The symptomatology of PTSD includes unsuccessful termination of fear responses (Yehuda, 2001) and resistance to extinction (EXT; Chorot & Sandin, 1993; Van der Kolk, 1994) as evidenced by spontaneous recovery (SR) of the fear (Rescorla, 2004). Thus, learning plays an important part in the expression of psychiatric symptoms that follow trauma,

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and the study of EXT and SR has important implications for mental illness, therapy and relapse (for review, see Bouton & Swartzentruber, 1991).

A growing literature is now addressing not only how fears are acquired, but also how they may be reduced (extinguished) and how SR of these fears may be attenuated or eliminated (Maren, 2005; Quirk, 2006; Quirk, Martinez, & Nazario-Rodríguez, 2007; Myers & Davis, 2002; Mickley et al., 2007). Much of the pre-clinical literature has employed Pavlovian fear conditioning paradigms such as the conditioned emotional response (CER). Here, a conditioned stimulus (CS), such as a tone, is paired with an aversive unconditioned stimulus (US), such as electric shock. After several such pairings, the tone elicits autonomic and behavioral fear responses, such as freezing, in anticipation of the shock (Blanchard and Blanchard, 1972; Fanselow, 1980; Quirk & Mueller, 2008; Quirk, 2006; Thomas, Longo, & Ayres, 2005).

In most studies aimed at reducing fearful responding, reduction of defensive behaviors is accomplished through the use of simple EXT - a form of learning that disassociates the CS and US by repeatedly presenting only the CS without the US (CS-only extinction: CSO) (Quirk & Mueller, 2008; Thomas, Longo & Ayres, 2005). Using these methods, rats that once froze at the sound of a tone paired with shock now move more freely in the presence of the tone.

Because EXT reduces or eliminates the avoidance behavior, it is tempting to assume that it erases the learned fear itself. However, allowing time to pass following EXT frequently evokes the re-emergence, or SR, of the conditioned response (Pavlov, 1927). The phenomenon of SR indicates that, even after many EXT trials, an animal retains a memory of conditioning that can provide a powerful basis for relapse (Quirk, 2002; Bouton, 2002).

The explicitly unpaired extinction procedure (EU-EXT) has been suggested as an alternative to simple CSO extinction methods. Essentially, following an original association between CS and US (CS+US), this procedure then extinguishes the original association by now providing a new negative CS-US contingency in which the CS *never* predicts the US. This methodology has been explored for over 40 years (Rescorla, 1969a,b; Baker, 1977; Kalat & Rozin, 1973) but most recently by Thomas and his associates (Rauhut, Thomas, & Ayres, 2001; Thomas & Ayres, 2004; Thomas, Longo, & Ayres, 2005). The EU procedure resulted in less fear (after 24 days) than did conventional CSO EXT (Thomas & Ayres). Moreover, the conditioned response extinguished and did not reappear between sessions (spontaneously recover) following the passage of time. Further, EU treatments thwarted both renewal and reacquisition of the CER (Rauhut, Thomas, & Ayres, 2001).

Our laboratory has extended the extinction and SR literature to the conditioned taste aversion paradigm (CTA) (Mickley et al., 2004; 2005; 2007). CTA is a defensive reaction to a learned fear (Parker, 2003) and may be acquired when an animal consumes a novel taste (CS) and then experiences the symptoms of poisoning (the US) (Garcia, Kimeldorf, & Hunt, 1961; Garcia, Kimeldorf, & Knelling, 1955). Later, the animal will avoid the taste previously associated with feelings of illness. CTA extinction results in a resumption of eating/drinking the once-avoided tastant (Rosas & Bouton, 1996; Mickley et al., 2004), but the taste aversion spontaneously recovers following a latency period of water drinking (Mickley et al., 2007).

CTA has a number of unusual properties that challenge the basic tenets of traditional learning and memory theory (Domjan, 1993; Bures, Bermudez-Rattoni, & Yamamoto, 1998). Therefore we sought to determine if the EU extinction procedure would alter a CTA in a way that is similar to that already reported in studies using the CER paradigm. Specifically, in the studies reported here, we tested the generalizability of the EU-EXT procedure by comparing its ability to reduce a conditioned taste aversion as compared to a procedure in which only the CS is presented (CSO). Further, we assessed the efficacy of the EU-EXT procedure in reducing or eliminating SR of a CTA. We hypothesized that EU-EXT would be an effective means of

achieving re-acceptance of a once-avoided taste and that it would reduce the SR of a taste aversion. Our data were consistent with these predictions.

## Methods

### Subjects

A total of 47 naive male Sprague-Dawley rats (Mean  $\pm$  SEM weight = 420.82  $\pm$  12.17g), supplied by Zivic Laboratories (Zelienople, PA) were used in this experiment. Animals were housed in individual plastic cages (44.45 cm long  $\times$  21.59 cm wide  $\times$  20.32 cm deep) with corncob bedding (Bed o'cobbs, Andersons Industrial Products, Maumee, OH). A 12-hr light-dark cycle (lights on at 0600 hr) was maintained, and temperature was kept within 23–26°C. Rats also had free access to Purina Rat Chow (no. 5001, PMI Nutrition International, Brentwood, MO) for the duration of the study. All animals were handled briefly during daily weighings.

Procedures were approved by the Baldwin-Wallace College Institutional Animal Care and Use Committee. Animals were procured and cared for according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and in compliance with the Animal Welfare Act.

### Experimental Design and Group Assignment

At the start of the study, rats were randomly assigned to one of 4 treatment groups (**CTA+CSO-EXT**, **CTA+EU-EXT**, **NO CTA+“CSO-EXT”**; **NO CTA+“EU-EXT”** – see descriptions, below and in Table 1). **CTA** rats received CS+US pairings while the CS/US presentations for the **NO CTA** rats were available non-contingently on different days in order to avoid formation of the CTA. Once assigned, each individual **NO CTA** control rat was matched, as closely as possible, to an individual **CTA** rat of similar weight. The **NO CTA** rats served as yoked controls and received the same number of CS/US presentations as their experimental pairs. As part of a related study, we sacrificed about half of the rats from each of our treatment groups for immunohistochemical analysis of the brains following the extinction stage of our experiment (data to be available in a future report) (see Table 1).

### Materials

All chemicals were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). Lithium chloride (LiCl) was dissolved in physiological saline to produce a final concentration of 81mg/ml and was administered at a dose of 81mg/kg. Saccharin salt was dissolved in deionized water to a final concentration of 0.3%, by mass, saccharin solution (SAC). All consummatory tests (SAC or water) involved a single bottle.

### Conditioning Procedure

Rats were conditioned and tested in their home cages. All contextual cues were kept constant throughout each phase of the study. Animals were habituated to a 23 hr water deprivation schedule beginning 2 days prior to the first conditioning trial and maintained on this schedule throughout the study. This relatively brief period of water deprivation acclimation ensured that animals were motivated to drink SAC (the CS) when it was first presented. Fluid consumption was recorded daily to the nearest tenth of a gram.

On the first conditioning day, the water-deprived rats were given 30 min access to 0.3% SAC. Following SAC exposure, SAC bottles were removed and animals assigned to a **CTA** group (refer to Table 1 for group nomenclature) received an intraperitoneal injection of LiCl (81mg/kg; 81mg/ml; i.p.). Fifteen minutes post-injection, the animals were given 30 min access to tap water to prevent dehydration. **CTA** animals (Mean weight  $\pm$  SEM = 429.28  $\pm$  25.60g) received

the CS-US pairings on conditioning days 1, 3, and 5. Interim days 2, 4, and 6 served as rest periods during which the *CTA* animals received two 30 min presentations of water separated by a 15 min interval (replacing the LiCl injection period experienced on days 1, 3, and 5). At the end of the *CTA* training, the 23 animals were designated as *CTA* and continued into the extinction phase of the experiment as either *CTA+EU-EXT* or *CTA+CSO-EXT* animals (extinction condition randomly assigned prior to the start of the experiment).

An additional control group (*NO CTA*; N=24; Mean weight  $\pm$  SEM = 381.58  $\pm$  6.88g) that did not receive CS-US pairings, but instead, received explicitly unpaired presentations of both the CS and US during the conditioning phase, were included in this study to account for any residual effects of LiCl and SAC exposures. These *NO CTA* animals received 30 min access to SAC and then, 15 min later, 30 min access to water on days 1, 3, and 5 of conditioning. On alternate days 2, 4, and 6 of conditioning, *NO CTA* animals were injected with LiCl during the 15 min interval between the two water presentations. This explicitly unpaired conditioning procedure allowed the *NO CTA* animals to receive both the CS and US throughout conditioning without forming an association or subsequent aversion to SAC (Mickley et al., 2004; 2007).

### Extinction Procedure

After day 6 of the conditioning phase, the extinction phase of our study began. Beginning with day 1 of extinction training, animals received 30-min exposure to SAC every-other day (odd-numbered days). During the initial stages of extinction, *CTA* rats drink very little of the CS. Therefore, fifteen minutes after SAC exposure, the animals received 30 min access to water in order to prevent dehydration.

The *NO CTA* control group was continued into the extinction phase of the study, but since they had no aversion to extinguish, these control animals were yoked to animals in experimental groups (*CTA+CSO-EXT* or *CTA+EU-EXT*) based on initial weights and were considered to have “extinguished” on the day that their yoked *CTA* counterpart reached the asymptotic extinction criterion (see below).

On days when there was no SAC exposure (even-numbered days), animals received two 30 min presentations of tap water. Within 15 min of the first water exposure, all animals designated as *CTA+EU-EXT* or *NO CTA+“EU-EXT”* were injected with LiCl (81mg/kg, i.p.) and all animals designated as *CTA+CSO-EXT* or *NO CTA “CSO-EXT”* were injected with a comparable volume of physiological saline (i.p.; refer to Table 1). Fifteen minutes post-injection, animals were given a second, 30-min opportunity to drink to tap water.

The extinction phase of the study was complete once rats reached a 90% SAC re-acceptance level (see extinction criteria discussed, below). *Note:* approximately half the rats were sacrificed for brain assays on the day they achieved asymptotic extinction (histology data not reported here). See Table 1 and the *Experimental Design and Group Assignment* section for details regarding N.

### Spontaneous Recovery Test

Upon reaching the extinction criterion (90% SAC reacceptance, see below), animals were given access to water only for the next 29 days (refer to Table 1 for group Ns). During this period, rats received two 30-min presentations of water (spaced 15 min apart) each day. This procedure corresponded to the temporal characteristics of the previous *CTA* and *EXT* training regimens. Thirty days following the end of extinction training, rats were exposed to SAC for 30 min as a test of *CTA SR*.

## Statistical Analysis

We wished to estimate levels of normal, familiar baseline SAC drinking as a means to evaluate the degree to which the rats in this study had extinguished their CTA. However, recording several days of baseline SAC pre-exposure in our animals would have impeded future CTA training, due to latent inhibition effects. Moreover, we also wished to avoid the bias associated with the rat's initial hesitation to consume novel substances (neophobia; Domjan & Gillan, 1977). Therefore, normal, familiar SAC consumption was determined by averaging SAC consumption on the third day of exposure from a separate group ( $N = 10$ ) of similarly-sized rats not used in the current study.

We adopted the naming convention originally established by Nolan et al. (1997) to describe the phases of CTA extinction. Saccharin drinking levels to enter each of the three phases of extinction were defined as a percentage of baseline saccharin consumption: Static (less than 10% of baseline), Dynamic (10%–80% of baseline) and Asymptotic (greater than 80% of baseline). In this experiment, the end point criterion for asymptotic extinction was defined as SAC consumption greater than or equal to 90% of the baseline (Mickley et al., 2004).

SPSS software (Chicago, IL) was used for all statistical analyses. A repeated measures analysis of variance (RM-ANOVA; Kirk, 1982) was used to evaluate SAC consumption within and between groups that received either *NO CTA* or *CTA* training during the conditioning phase of the experiment. An independent samples t-test was used to compare the mean total liquid consumption (SAC+H<sub>2</sub>O, i.e., total volume of these liquids consumed during their sequential, single-bottle, presentations each day) of these two groups of animals during the conditioning phase of the study. An independent samples t-test was also performed to assess: (1) differences in the mean total liquid consumption (SAC+H<sub>2</sub>O) by the *CTA+CSO-EXT* and *CTA+EU-EXT* rats during the first 15 days of EXT training, (2) between-group differences in the total days to asymptotic extinction as well as the durations of the static, dynamic and asymptotic phases, and (3) SAC drinking of these two groups on the asymptotic extinction and SR test days. Finally, paired samples t-tests evaluated group differences in SAC consumption on the day of asymptotic extinction compared to the SR test day. SR of a CTA was operationally defined as significant suppression of SAC drinking as compared to the level of SAC consumption at the point of asymptotic extinction. Statistical significance was evaluated using an  $\alpha = 0.05$ .

## Results

### Conditioning

The amount of SAC consumed over the three day conditioning period indicated that all the *CTA* animals had acquired a strong taste aversion, whereas the *NO CTA* rats did not acquire a CTA (refer to Figure 1). On the first day of conditioning, the animals showed a neophobic response, indicated by the low consumption of SAC. A repeated measures ANOVA [Treatment (*CTA* or *NO CTA*)  $\times$  Trial] revealed a significant treatment effect [ $F(1, 45) = 334.761, p < 0.001$ ], a significant change in SAC drinking over trials [ $F(2, 90) = 39.055, p < 0.001$ ], and a significant interaction [ $F(2, 90) = 164.834, p < 0.001$ ]. In addition, two separate repeated measures ANOVAs were run to analyze SAC consumption over the three days of conditioning for the *CTA* and *NO CTA* groups. This analysis showed that the *CTA* groups had a significant decline in SAC drinking over the three days [ $F(1, 23) = 56.894, p < 0.001$ ], and also that the *NO CTA* groups had a steady rise in SAC consumption [ $F(1, 22) = 161.234, p < 0.001$ ].

In order to confirm that the thirst and general consummatory behaviors were not different between the rats that formed a CTA and those that did not, we compared the mean total liquid consumption (SAC+H<sub>2</sub>O; presented sequentially, as described above, in single-bottle tests) of

the two groups of animals. The mean daily total liquid consumption of rats in the *CTA* group [Mean volume (ml) consumed  $\pm$  SEM = 21.34  $\pm$  0.74] was not significantly different from rats in the *NO CTA* group [Mean volume (ml) consumed  $\pm$  SEM = 21.54  $\pm$  0.95].

Lastly, to verify that the *CTA+CSO-EXT* and *CTA+EU-EXT* animals had acquired the same level of aversion to SAC, the animals' SAC consumption levels on the first day of extinction (after all conditioning trials) were compared. The mean SAC consumption of the *CTA+CSO-EXT* group [Mean volume (ml) consumed  $\pm$  SEM = 0.08  $\pm$  0.027] was not significantly different from that of the *CTA+EU-EXT* group [Mean volume (ml) consumed  $\pm$  SEM = 0.01  $\pm$  0.06].

## Extinction

Rats in the *CTA+CSO-EXT* and *CTA+EU-EXT* groups achieved the same levels of asymptotic extinction (% of baseline). Rats in the *CTA+EU-EXT* group drank amounts of SAC on the day of asymptotic extinction that were not reliably different [ $t(15.91) = 1.74, p = 0.10$ ; unequal variances] from the volumes consumed by rats assigned to the *CTA+CSO-EXT* group. However, the mean times to reach asymptotic extinction for these groups were significantly different (see Figure 2). The *CTA+EU-EXT* animals extinguished the CTA more rapidly than did the *CTA+CSO-EXT* group [ $t(21) = 3.00, p = 0.007$ ]. To further explore this difference, the lengths of each of the phases of extinction (as described by Nolan et al., 1997) were determined and compared. The data revealed a significant *CTA+CSO-EXT* vs. *CTA+EU-EXT* group difference in the days to complete the static phase of extinction (i.e., to return to 10% of baseline SAC drinking) (see Figure 3) [ $t(21) = 2.52, p = 0.02$ ]. However there were no differences between these groups in the length of time to reach the dynamic and asymptotic phases of extinction.

An additional analysis was performed to determine if the *CTA+EU-EXT* animals had altered hydration during the extinction phase. The total daily liquid consumed (SAC + H<sub>2</sub>O) during the first 15 days of the extinction process was compared between the *CTA+EU-EXT* and *CTA+CSO-EXT* groups [Mean volume (ml) consumed/day  $\pm$  SEM = 20.71  $\pm$  1.06 and 21.97  $\pm$  1.06, respectively]. An independent samples *t*-test revealed that there was not a significant difference in liquid consumption between these groups.

## Spontaneous Recovery (SR) Test

The SR test data revealed that the *CTA+EU-EXT* group drank significantly more SAC than the *CTA+CSO-EXT* group [ $t(9) = 2.47, p = 0.04$ ] when rats had an opportunity to drink the sweet liquid following a 29-day water-only latency period. A paired sample *t*-test determined that the volumes of SAC consumed by the rats in the *CTA+EU-EXT* group on the day of asymptotic extinction and on the SR test day were not significantly different, indicating that the animals did not experience SR of the CTA [ $t(4) = -0.55, p = 0.66$ ]. However, the *CTA+CSO-EXT* group drank significantly less SAC on the SR test day than on the day of asymptotic extinction [ $t(5) = 2.72, p = 0.04$ ], which indicates SR of the CTA (see Figure 4).

As expected, the *NO CTA* animals drank large amounts of SAC throughout the study once the animals overcame the initial neophobia. The average volume of SAC consumed at a time parallel to when their yoked CTA pairs were reaching asymptotic extinction was 28.92  $\pm$  2.65 ml [Mean SAC consumption  $\pm$  SEM]. This average daily consumption did not change significantly (following 30 days of water drinking) during the final "SR" test: Mean SAC consumption (ml)  $\pm$  SEM = 27.620  $\pm$  1.27. Additionally, the *NO CTA+EU-EXT* [Mean SAC consumption (ml)  $\pm$  SEM = 28.42  $\pm$  1.82] and the *NO CTA+CSO-EXT* [Mean SAC consumption (ml)  $\pm$  SEM = 27.420  $\pm$  1.67] did not show any differences in SAC consumption at the time of the "SR" test. In these non-conditioned animals, repeated non-contingent

exposures to the CS and/or US did not lead to a suppression of SAC drinking over the course of this study.

## Discussion

In this study, we compared the effectiveness of two CTA extinction procedures: (1) presentation of the CS only (*CSO-EXT*), and (2) presentation of the CS explicitly unpaired with the US (*EU-EXT*). Our data suggest that both methods of extinction produced the same level of asymptotic reacceptance of the once-avoided taste. However, the *CTA+EU-EXT* procedure produced more rapid extinction of a CTA than did exposure to the CS only. Further, *CTA+EU-EXT* significantly inhibited spontaneous recovery of the CTA when the CS was re-introduced 30-days after asymptotic extinction was achieved.

These findings are, in many ways, consistent with those of other laboratories that have used CER paradigms to assess the effectiveness of *EU-EXT* to thwart renewal/relapse of conditioned fears (Rauhut, Thomas, & Ayres, 2001; Thomas, Longo, & Ayers, 2005). Although these investigators reported that the EU procedure retarded the rate of extinction in the classical fear conditioning paradigm, our data indicate that the *EU-EXT* procedure allows more-rapid extinction of a CTA. A comparison of the methodologies of the CER and CTA may provide some hints about why this is the case. The time courses of extinction are clearly very different in the 2 paradigms. Progress on CER extinction may be observed in a matter of minutes whereas movement into the dynamic phase of CTA extinction may take a month, or longer. This is due, in part, to the schedule of CS and US exposures necessary for each paradigm. Within the classical fear conditioning paradigm, multiple presentations of the CS and US are made available within an hour (Thomas, Longo, & Ayers). But the CSs and USs of CTA extinction are presented once every 24 or 48 hours (Mickley et al., 2004; 2005; 2007). These different time periods may very well affect the ability of the animal to consolidate, retrieve and/or reconsolidate contingencies associated with the CS or US. The duration of CS and US exposures during each presentation also differs between CERs and CTAs. Tone and shock presentations characteristic of CER studies are under precise control of the experimenter and are typically of short duration. In our studies, however, taste exposures were voluntary and therefore determined by our subjects. Gustatory sensations were of unknown duration, and the malaise associated with LiCl may have lasted an hour or more (Meachum & Bernstein, 1990). Perhaps these longer exposures to the CS and US in the CTA paradigm are differentiating factors that affect the relative rates of CTA vs. CER extinction. Finally, animals may perceive different salencies in the two types of extinction learning discussed here. Rejecting potential food sources can have devastating consequences for an animal (perhaps more so than the discomfort of transient foot pain) and engenders additional risk-taking that may facilitate the speed of CTA extinction learning.

The differences between the behavioral results produced by the *EU-EXT* and *CSO-EXT* procedures raise some important theoretical questions. Is it possible that fear of the CS has been somehow intensified by the *CSO-EXT* procedure but not the *EU-EXT* methods? Eysenck (1968; 1979) argued that, in patients with high anxiety, the CR (e.g., an internal state of fear) is uncomfortable and may serve as an aversive US substitute. Consistent with this proposition, “incubation of fear” would increase over successive non-reinforced presentations of the CS. Thus, reinforcement of the CS may continue during extinction if the CR is elicited by the CS during this process. The theory has been tested (although never in the context of CTA) and experimental findings have not always supported Eysenck’s original proposition (Richards & Martin, 1990). However, “incubation of fear” has been demonstrated under certain, well-defined but limited, experimental conditions in which CS presentations are spaced and the level of physiological arousal is high (Cain, Blouin, & Barad, 2004). In our study, the CS was presented at the same frequency (once, every-other day) in both the *EU-EXT* and *CSO-EXT*

conditions. Therefore the frequency of CS exposure does not allow us to differentiate the two extinction methods or explain the dissimilar behavioral results they produce. The interleaving of the US presentations every-other day during extinction training may have provided a heightened sense of arousal in the *EU-EXT* animals as compared with the *CSO-EXT* rats. If this is the case, then we would expect a greater “incubation of fear” in the rats undergoing the EU procedure when, in fact, their SAC avoidance reactions (as measured by a reduced SR of the CTA) were attenuated by this extinction method. Therefore, our data seem more consistent with the hypothesis that greater excitation during extinction leads to greater extinction (Rescorla, 2000) – a finding harmonious with the clinical observation that effective treatment depends on generating a sufficient level of anxiety and sympathetic activation to induce effective behavioral extinction (Stampfl & Levis, 1967; Holmes, Moulds, & Kavanaugh, 2007).

Could our *EU-EXT* procedure have caused habituation to the US or turned the CS into a conditioned inhibitor? According to Thomas, Longo and Ayres (2005), the EU procedure does neither of these things in the context of a CER paradigm. It should be noted, however, that our findings differ from those of Thomas, Longo and Ayres (2005), because rats exposed to the *EU-EXT* condition extinguished their CTAs more rapidly than the rats that went through the *CSO-EXT* treatments. It may be the case that the EU procedure converted SAC into a conditioned inhibitor (CI) and thus prevented the appearance of spontaneous recovery. It should be noted, however, that our paradigm was not a typical CI paradigm where a CTA is created and then later a “safe” solution is paired with the CS without consequential illness. This procedure creates a clear preference for the safe solution (Best, 1975). However, Calton, Mitchell and Schachtman (1996) showed that a CI can also be produced through a CSO CTA extinction process. Their CIs passed retardation and summation tests normally applied to evaluate CIs. In a retardation test, the putative CI should be slow to acquire (or re-acquire) excitatory responding relative to a novel stimulus. In a summation test, the CI is presented in compound with a known excitor and the conditioned responding to the stimulus compound is expected to be low relative to the excitor alone. Calton, Mitchell and Schachtman’s (1996) findings contrast with the results from other laboratories which failed to demonstrate that an extinguished CS acts as a CI when tested in compound with an excitatory CS (LoLordo & Rescorla, 1966; Rescorla, 1967; Reberg, 1972; Hendry, 1982). However, the authors attribute this to the earlier studies’ failure to provide sufficient extinction to produce an inhibitor and suggest that, in order for extinction to become a “net” CI, the level of inhibition must exceed that of excitation to the CS. Both the *EU-EXT* and *CSO-EXT* procedures employed in the current study produced the same levels of asymptotic extinction – suggesting equal levels of inhibition were achieved. However, it was only the *EU-EXT* procedure that suppressed SR of the CTA. Further, data from conditioned fear paradigms indicate that it takes substantially longer to produce CI than extinction (B.L. Thomas, personal communication, 2009), while our data indicate that the EU-EXT rats extinguished their CTA more rapidly than did rats that underwent the CSO procedure. While our data provide a necessary first step in describing the behavioral sequelae following the *EU-EXT*- vs. *CSO*-mediated extinction of a CTA, we did not employ summation or retardation tests to determine the extent to which our findings are attributable to conditioned inhibition. This is a logical and important next step that must be taken as we assess the underlying mechanisms that support the behavioral results we report here.

Although the current experiments do not speak directly to the underlying reasons why the *EU-EXT* process shortens extinction as compared to the *CSO-EXT* procedure, the shapes of the extinction curves may offer some clues (see Figure 3 to get a sense of the time rats from our two EXT groups spent in each phase of extinction). Rat CTA extinction curves often resemble a probit function (Mickley et al., 2004), with relatively long static phases followed by rapid re-acceptance of the once-poisoned CS as the animals quickly move to asymptotic levels of



consumption. In the current study, once rats start to re-accept the once-avoided SAC, the EU and CSO extinction curves are quite similar in their time course. However, the EU procedure seems to make the rats more likely to re-initiate CS sampling. Since animals in the *CTA+CSO-EXT* and *CTA+EU-EXT* groups drank liquids (SAC + water) in about the same volumes/day, this readiness to initiate CS exposure cannot be attributed to greater motivation (thirst). Future studies may shed more light on the neuro-behavioral mechanisms that mediate this propensity of rats to more quickly challenge their “bait shyness” during the course of the *EU-EXT* procedure.

Rats in the *CTA+EU-EXT* group moved through the static phase of extinction more rapidly than did rats in the *CTA+CSO-EXT* group and thereby also achieved asymptotic extinction more quickly. Thus the *CTA+EU-EXT* animals experienced comparatively few extinction trials. Given this information, one might expect that residual effects of the CTA would be more prominent in this group. However, if this was the case, there would have been some bias towards enhanced SR of the CTA. Instead, our *CTA+EU-EXT* animals exhibited a *reduced* SR, and their SAC drinking was not significantly less than their drinking at the end of the extinction procedure. Thus, the *EU-EXT* procedure was sufficiently powerful to overcome the potential tendency to retain aspects of the CTA memory in those animals that experience fewer extinction trials.

Our data provide additional information regarding the ongoing debate about whether extinction produces “new learning” (i.e., that the CTA memory is retained but there is a new understanding that it no longer applies in this new temporal context) (Bouton & Bolles, 1979; Bouton & Swartzentruber, 1991; Rescorla, 2001; Baeyens, Eelen, & Crombez, 1995; and review in Mickley et al., 2007) or “unlearning” (i.e., that the CTA memory is erased) (Bouton & Swartzentruber, 1991; Richards, Farley, & Alkon, 1984). Previous data from our laboratory suggest that the patterns of *c-Fos* expression (an indicator of neural activity) in the brains of rats that have undergone the *CSO-EXT* process following acquisition of a CTA do not resemble the brains of rats that have been exposed to the same CS and US but did not acquire the CTA (*NO CTA*; Mickley et al., 2004; Mickley et al., 2007). Thus, our *CTA+CSO-EXT* data have been consistent with a variety of other studies indicating that extinction induces new learning (for reviews, see Myers, Ressler, & Davis, 2008; Quirk & Mueller, 2008).

However, since the *CTA+EU-EXT* procedure seems to attenuate SR of a CTA, this raises the possibility that, under some circumstances, elimination or unlearning of the fear itself may be achieved. This implies that the CS re-enters a state that is functionally identical to the state of a neutral stimulus that was never involved in a CS+US contingency (Richards, Farley, & Alkon, 1984; Barad, 2006). At this stage, it is uncertain the extent to which *EU-EXT* procedures might be effective in producing a reversal of neurophysiological markers indicative of a CTA memory. However, our laboratory is currently studying patterns of *c-Fos* protein expression in the brains of rats exposed to *CTA+CSO-EXT* vs. *CTA+EU-EXT* procedures (Mickley et al., 2008).

In conclusion, our data indicate that the *EU-EXT* procedure is an effective way to attenuate SR of a CTA. These finding parallel similar results reported by other laboratories using the CER paradigm (Rauhut, Thomas, & Ayres, 2001; Thomas & Ayres, 2004; Thomas, Longo, & Ayres, 2005). Following additional pre-clinical testing, health care providers treating disorders where fear is prominent may wish to consider how the *EU-EXT* procedures described here may best be adapted in order to facilitate a variety of therapeutic approaches (Basoglu, Salcioglu, & Livanou, 2007).

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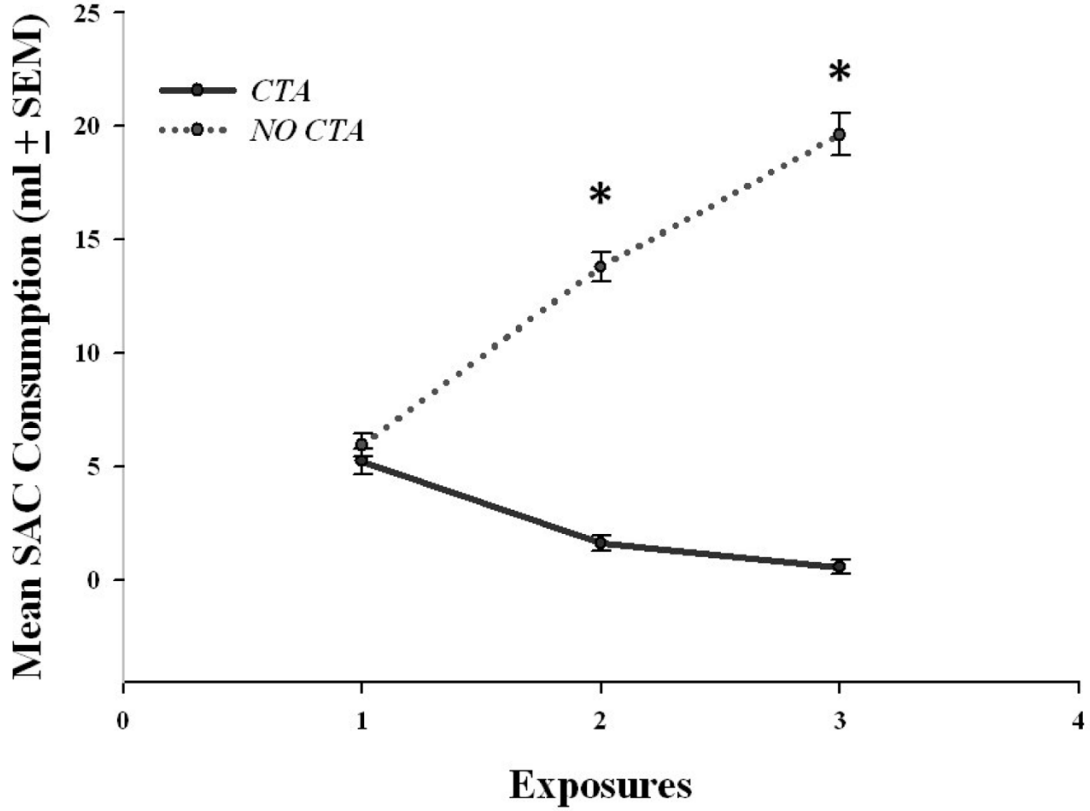
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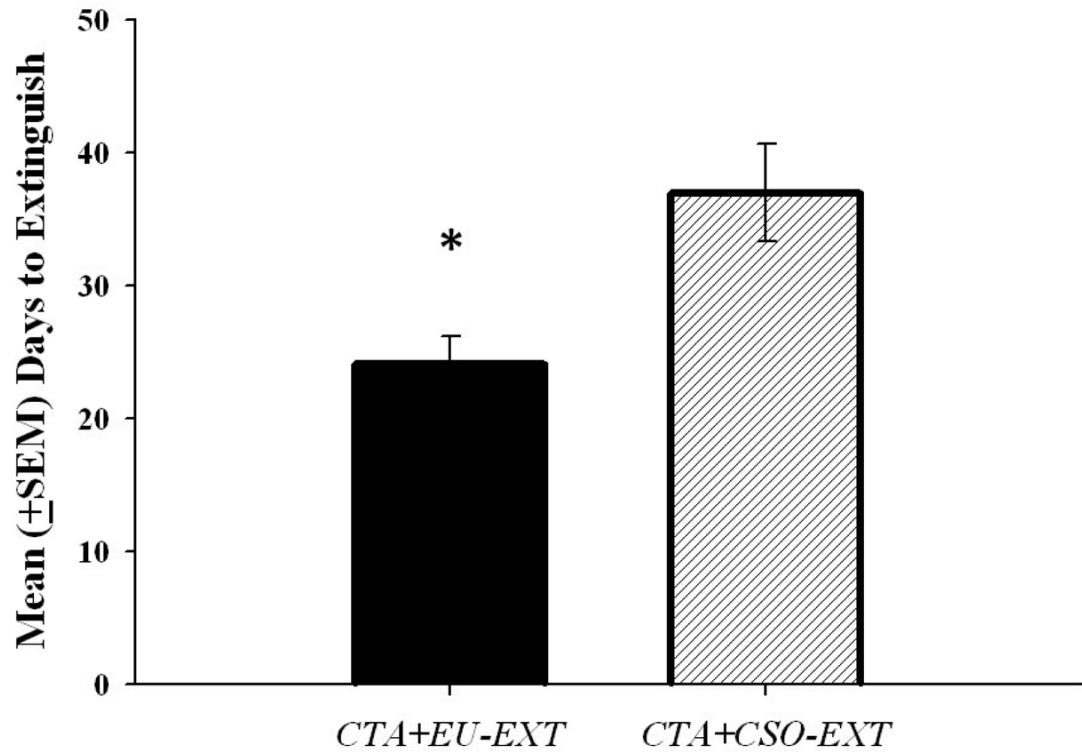
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## SAC Consumption after either CS+US Pairing or Explicitly Unpaired CS/US Exposure



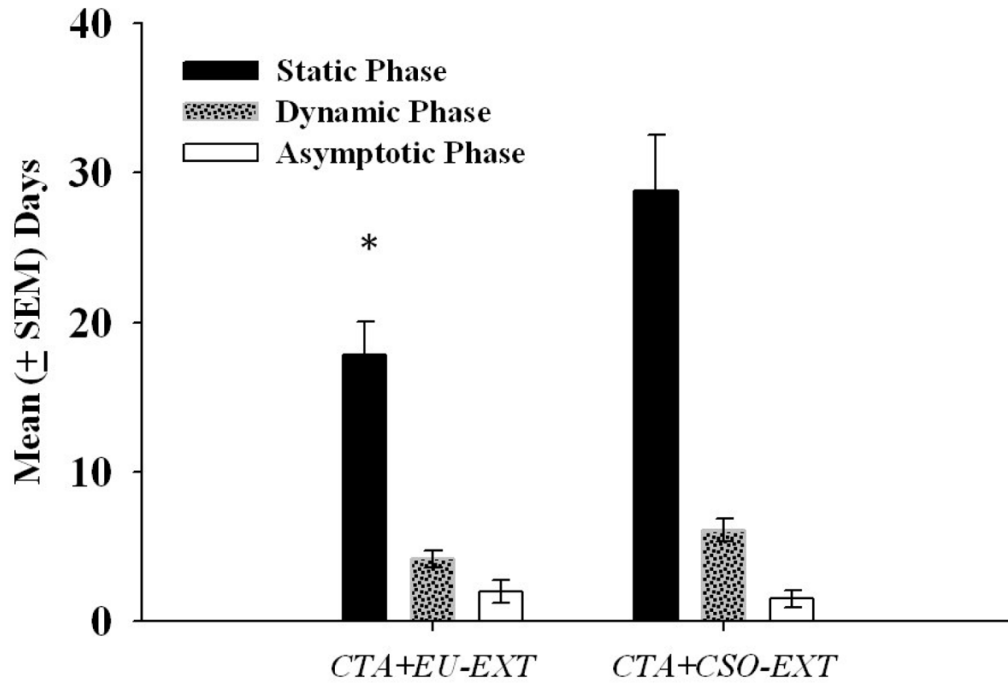
**Figure 1.** Mean volume of SAC consumption ( $\pm$  SEM) after either three CS+US pairings (*CTA*) or three explicitly unpaired CS/US exposures (*NO CTA*). The *CTA* group showed a significant decrease in the amount of SAC consumed over the three exposures. The *NO CTA* group showed a significant increase in SAC consumption over the same three periods. This indicates that the *CTA* groups had acquired the CTA, whereas the *NO CTA* group did not. \* = significantly different from the *CTA* animals ( $\alpha = 0.05$ ).

## Mean Days to Asymptotic Extinction



**Figure 2.** Mean days ( $\pm$  SEM) for animals to reach asymptotic extinction. Animals either underwent the *CTA+EU-EXT* or *CTA+CSO-EXT* procedure. The *CTA+EU-EXT* group took significantly fewer days to extinguish the learned fear than the *CTA+CSO-EXT* group. \* = significantly different from the *CTA+CSO-EXT* animals ( $\alpha = 0.05$ ).

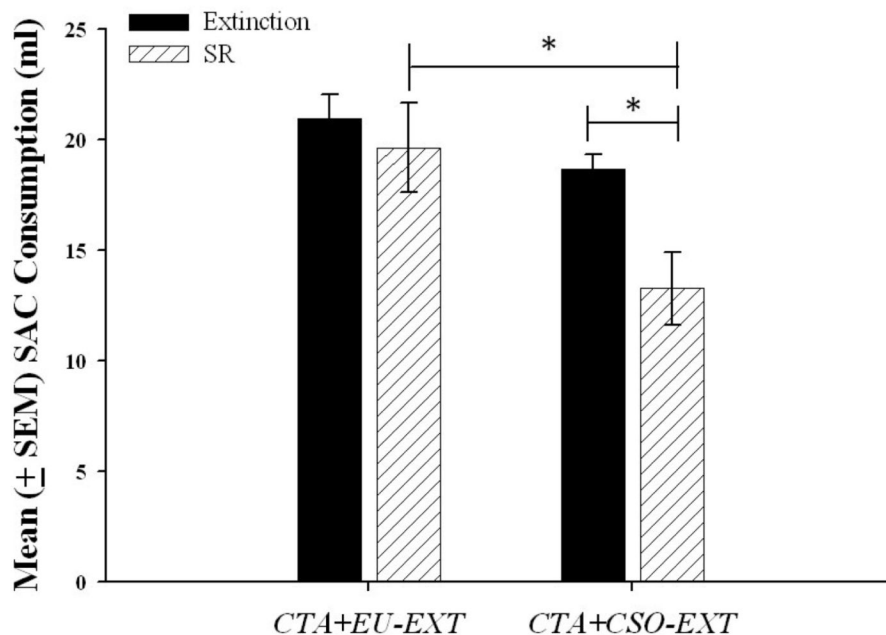
## Mean Days Spent In Each Phase Of Extinction



**Figure 3.**

Mean days ( $\pm$  SEM) spent in each phase of extinction (Nolan et al., 1997) for animals that underwent either *CTA+EU-EXT* or *CTA+CSO-EXT* procedures. Rats that experienced the *CTA+EU-EXT* procedure spent significantly fewer days in the static phase (SAC reacceptance of less than 10% of baseline) than the *CTA+CSO-EXT* group [\* = significantly different from the *CTA+CSO-EXT* animals ( $\alpha = 0.05$ )]. However, the *CTA+EU-EXT* and *CTA+CSO-EXT* groups spent comparable number of days in both the dynamic (SAC reacceptance greater than 10 % but less than 80% from baseline) and asymptotic phases (SAC reacceptance of greater than 80% from baseline).

## Comparison of SAC Consumption at Extinction and SAC Consumption on SR Test Day



**Figure 4.**

Mean volume of SAC consumption ( $\pm$  SEM) on the day of asymptotic extinction and on the subsequent SR test day for both the *CTA+EU-EXT* and *CTA+CSO-EXT* animals. On the day of the final extinction test, the *CTA+EU-EXT* and *CTA+CSO-EXT* groups drank comparable amounts of SAC ( $p > 0.05$ ; see text). The *CTA+CSO-EXT* group drank significantly more SAC on the last day of extinction than on the day of the SR test, indicating a spontaneous recovery of the CTA. However, the *CTA+EU-EXT* animals drank nearly the same amount of SAC on the day of extinction as they did on the SR test day, suggesting that the EU-EXT procedure may be effective in blocking SR. \* = groups indicated are significantly different,  $\alpha = 0.05$ .



Table 1

## Group Nomenclature and Treatments

Conditioning Phase - Group Designation	Group N	CTA Conditioning		Extinction Phase - Group Designation	Group N	Extinction Training		30-day Latency before SR test	SR Group N	SR Test Solution
		Days 1, 3, 5	Days 2, 4, 6			Odd Days	Even Days			
CTA	23	SAC <sup>3</sup> + LiCl <sup>4</sup>	H <sub>2</sub> O	CTA+EU-EXT <sup>1</sup>	11	SAC	H <sub>2</sub> O+LiCl	H <sub>2</sub> O	5	SAC
				CTA+CSO-EXT <sup>2</sup>	12	SAC	H <sub>2</sub> O+ SAL <sup>5</sup>	H <sub>2</sub> O	6	SAC
NO CTA	24	SAC	H <sub>2</sub> O + LiCl	NO CTA+ "EU-EXT"	12	SAC	H <sub>2</sub> O+LiCl	H <sub>2</sub> O	6	SAC
				NO CTA+ "CSO-EXT"	12	SAC	H <sub>2</sub> O+ SAL	H <sub>2</sub> O	6	SAC

<sup>1</sup> CTA-EU-EXT = "Explicitly Unpaired" extinction procedure in which both the CS and US are presented on alternate days

<sup>2</sup> CTA-CSO-EXT = "Conditioned Stimulus Only" extinction procedure in which only the CS is presented every other day

<sup>3</sup> SAC = saccharin salt dissolved in DI H<sub>2</sub>O (0.3% w/v; p.o.)

<sup>4</sup> LiCl = lithium chloride injection dissolved in sterile, physiological saline (81 mg/ml; 81 mg/kg; i.p.)

<sup>5</sup> SAL = sterile, physiological saline (0.9% w/v; NaCl; 1ml/kg; i.p.)