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Research Report

Spontaneous recovery of a conditioned taste aversion differentially alters extinction-induced changes in c-Fos protein expression in rat amygdala and neocortex

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ABSTRACT

Conditioned taste aversions (CTAs) may be acquired when an animal consumes a novel taste (conditioned stimulus; CS) and then experiences the symptoms of poisoning (unconditioned stimulus; US). Animals will later avoid the taste that was previously associated with malaise. Extinction of a CTA is observed following repeated, non-reinforced exposures to the CS and represents itself as a resumption of eating/drinking the once-avoided tastant. Spontaneous recovery (SR) of a CTA (a revival of the taste avoidance) occurs when the CS is offered after a latency period in which the CS was not presented. An initial study explored the experimental parameters required to produce a reliable SR following acquisition and extinction of a robust CTA in rats. A CTA was formed through 3 pairings of 0.3% oral saccharin (SAC) and 81 mg/kg i.p. lithium chloride (LiCl) followed by extinction training resulting in 90% reacceptance of SAC. After extinction training, some of the animals were also tested for SR of the CTA upon exposure to SAC following a 15-, 30-, or 60-day latency period of water drinking. We report here that latencies of 15, 30, or 60 days produced small, but reliable, SRs of the CTA—with longer latencies producing progressively more suppression of SAC consumption. A second study investigated changes in the amygdala (AMY), gustatory neocortex (GNC), and medial prefrontal cortex (mPFC) functioning during SR of a CTA. Using immunohistochemical methods, brain c-Fos protein expression was analyzed in rats that extinguished the CTA as well as those that exhibited SR of the CTA after a 30-day latency. Our previous studies indicated that the numbers of c-Fos-labeled neurons in GNC and mPFC is low following CTA acquisition and increase dramatically as rats fully extinguished the aversion. Here we report that cortical c-Fos protein expression declines significantly following SR of the CTA. Expression of c-Fos in basolateral AMY decreased significantly from EXT to SR, but control animals with an intact CTA also

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Abbreviations: AMY, amygdala; BLA, basolateral nucleus of the amygdala; CE, central nucleus of the amygdala; CS, conditioned stimulus; CTA, conditioned taste aversion; EXT, extinction; GNC, gustatory neocortex; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NST, nucleus of the solitary tract; PBN, parabrachial nucleus; PVN, periventricular nucleus of hypothalamus; PrL, prelimbic cortex; SAC, 0.3% saccharin; SR, spontaneous recovery; US, unconditioned stimulus

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decreased significantly from a short-term CTA test to a long-term CTA test. Low levels of c-Fos expression in the central nucleus of the amygdala (CE) were observed throughout EXT with little change in expression detectable following SR. These measurements reflect the dynamic nature of brain activity during acquisition and extinction of a CTA and highlight an important role for cortical neurons in the brain reorganization that occurs during SR of a CTA. The data also suggest that certain sub-nuclei of the AMY may play a relatively minor role in SR of this defensive reaction to a learned fear.

1. Introduction

1.1. *Learned fear*

Learning plays an important part in the expression of psychiatric symptoms that follow trauma. Learned fears and defensive reactions to fears, for example, characterize phobias and post-traumatic stress disorder (PTSD) (Davis and Myers, 2002; LeDoux et al., 1990; LeDoux, 2000). The symptomatology of PTSD includes unsuccessful termination of fear responses (Yehuda, 2001) and resistance to extinction (EXT) (Van der Kolk, 1994) as evidenced by spontaneous recovery (SR) of the fear between sessions of exposure therapy (Grey et al., 1979; Rachman and Lopatke, 1988). Thus, the study of EXT and SR has important implications for mental illness, therapy, and relapse (for a review, see Bouton and Swartzentruber, 1991). Although behavioral studies of EXT and SR have been ongoing since the early 20th century (Pavlov, 1927; for a review, see Rescorla and Cunningham, 1978), the literature on the neural bases of these learning phenomena are just beginning to emerge.

The basic aspects of fear acquisition are fairly well understood thanks to studies of simple forms of aversive conditioning in laboratory settings. Typically, classical conditioning has been used to produce fears or aversions by associating a neutral *conditioned stimulus* (CS) (e.g., taste, tone, light, smell) with an aversive *unconditioned stimulus* (US) (e.g., foot shock, illness, malaise) until the CS alone comes to elicit the fear or defensive reaction to fear (*conditioned response*; CR) (Pavlov, 1927). The conditioned taste aversion (CTA) has been described as 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 (US) (Garcia et al., 1955, 1961). When later given a choice between the poisoned taste and some more-familiar taste (typically water), the animal will avoid the taste that it previously associated with malaise.

The behavioral techniques that can be used to *reduce* a CR have been known for some time. One of the simplest methods is simple extinction; a form of learning that disassociates the CS and US, for example, by repeatedly presenting the CS in the absence of the US (Pavlov, 1927). EXT of a CTA usually represents itself as a resumption of eating/drinking the once-avoided tastant (Mickley et al., 2004).

1.2. *Extinction of learned fears*

Because EXT erases the behavioral signs of fear, it is tempting to assume that it erases the original learning. However, a theoretical debate has emerged regarding the extent to which EXT represents unlearning or new learning (Wagner and Rescorla, 1974; Berman and Dudai, 2001; Maren, 2005; Thomas and Ayres, 2004). If EXT is an erasure or weakening of the original associations that led to the production of the conditioned response (Bouton and Swartzentruber, 1991; Richards et al., 1984), a sufficient number of non-reinforced CS presentations will presumably reduce the net associative strength between the CS and US to zero. 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 et al., 1984).

The alternative view of EXT is to consider it as a learning process in which the original CS–US relation is never unlearned but remains intact and is supplemented by new, additional knowledge suggesting that in some contexts, or in some moments in time, the former CS–US relation does not hold (Baeyens et al., 1995; Bouton and Bolles, 1979; Rescorla, 1979; Rescorla, 2001). There is substantial evidence to support this view. For example, the spontaneous recovery (SR) of an extinguished conditioned response indicates that the CS–US association remains even after the CR disappears (Pavlov, 1927; Quirk and Gehlert, 2003). Instead of erasing the original learning, EXT may provide the CS with a second, and therefore ambiguous, meaning (Bouton and Swartzentruber, 1991; Berman and Dudai, 2001). In this case, SR may represent a deficit in the retrieval of the EXT learning (Quirk, 2002).

1.3. *Neural mechanisms of CTA acquisition and extinction*

Identifying the neural mechanisms of CTA acquisition and storage has received substantial empirical attention and theoretical speculation. Yamamoto and colleagues (Yamamoto and Fujimoto, 1991; Yamamoto et al., 1994) proposed a neural model in which the brain areas important in CTA development include the nucleus of the solitary tract (NST), parabrachial nucleus (PBN), amygdala (AMY) (especially the basolateral nucleus, i.e., the BLA), and gustatory neocortex (GNC). Lesions of the BLA impaired learning and retention of a CTA (Aggleton et al., 1981; Nachman and Ashe, 1974) and single cell recordings in the BLA responded in a facilitatory manner to the CS after CTA trials (Yasoshima et al., 1995). By way of contrast, cells of the central nucleus of the amygdala (CE) responded in an inhibitory manner to the CS after CTA trials. Finally, Yamamoto and colleagues (Yamamoto and Fujimoto, 1991; Yamamoto et al., 1994) proposed that information regarding the hedonic shift of the CS is sent from the AMY and PBN to the GNC for long-term retention. Based on their fetal brain transplant studies, Bermudez-Ratonni et al.

(Bermudez-Rattoni et al., 1986, 1987; Bermudez-Rattoni and McGaugh, 1991) argue that the GNC is most likely the area of permanent memory storage while the AMY is part of the initial storage process.

The neural mechanisms of CTA extinction have received significantly less scrutiny than have CTA acquisition and memory storage. Berman and Dudai (2001) found that EXT of CTA memory depends on protein synthesis and beta-adrenergic receptors in the insular cortex of the rat. Studies from our laboratory indicate dramatic shifts in c-Fos (the protein product of the immediate early gene c-*fos*) expression in both mPFC and GNC as CTA EXT emerges (Mickley et al., 2004, 2005). In particular, peaks in neural activity occur in both GNC and mPFC as rats achieve asymptotic levels of CTA EXT (Mickley et al., 2004, 2005). The measurement of c-Fos immunohistochemistry in these studies is especially appropriate since evidence suggests that the expression of c-Fos not only mediates sensory experience and neural activity generally but may also be instrumental in the associative aspects of a CTA specifically (Lamprecht and Dudai, 1996).

Going beyond CTA EXT, a variety of other studies have highlighted an important role for medial prefrontal cortex (mPFC) in the extinction of conditioned emotional responses (Barrett et al., 2003; Milad and Quirk, 2002; Morgan et al., 1993; Quirk et al., 2000; Santini et al., 2004). Stimulation of the infralimbic portion of mPFC reduces freezing to tones that have come to predict shock (Milad and Quirk, 2002) and inclusive lesions of mPFC impair extinction learning (Morgan et al., 1993). Quirk's laboratory (Santini et al., 2004) has described extinction-induced c-Fos expression in both prelimbic and infralimbic portions of mPFC with cell densities remarkably similar to those reported from our experiments (Mickley et al., 2004, 2005). Neurons in the mPFC are required for storage of long-term extinction memories (Barrett et al., 2003; Milad and Quirk, 2002; Santini et al., 2001, 2003). Connections between the mPFC and AMY are well established (Krettek and Price, 1977; Alheid et al., 1995; McDonald, 1991, 1998 McDonald et al., 1996; Fisk and Wyss, 2000). It should be noted, however, that some mPFC lesions have not disrupted extinction of conditioned freezing or fear-potentiated startle (Gewirtz et al., 1997).

1.4. *Spontaneous recovery of a CTA*

Although spontaneous recovery of conditioned fear represents a substantial clinical challenge, there has been relatively little exploration of either the behavioral or neurophysiological components of this phenomenon as it applies to CTA. One notable exception is a study published by Rosas and Bouton (1996) in which several different experimental CTA paradigms were explored. They reported the acquisition of a CTA following saccharin and LiCl pairings. SR of the CTA occurred following 3 EXT trials and an 18-day retention interval. Other published studies have offered only indirect (Kraemer and Spear, 1992), limited (Lubow and De La Casa, 2002), or no evidence (Bouton, 1982) for SR of a CTA. However, these studies focused on the context dependence of CTA acquisition and retention and only peripherally addressed the robustness of the SR phenomenon in CTA (for a review, see Rosas and Bouton, 1996).

In the current study (Experiment 1), we more-fully explored the experimental parameters required to produce SR of a CTA. In Experiment 2, we employed proven behavioral methods to evoke SR of a CTA and used c-Fos protein immunohistochemical techniques to label neural activity in rats that had undergone acquisition, extinction, and spontaneous recovery of this learned aversion. SR reversed EXT-induced neural activity in the mPFC and GNC. A reduction of c-Fos expression in the BLA was seen from EXT to SR, but similar changes were observed when rats with an intact CTA were tested over a comparable length of time. The level of c-Fos immunoreactivity in the CE was low and did not change significantly throughout CTA acquisition, EXT, and SR.

2. Results

2.1. *Experiment 1—Defining behavioral parameters to obtain spontaneous recovery of a CTA*

See Table 1 for a summary of group nomenclature and treatments during various stages of this study. As may be seen in Fig. 1A, on the first day of CTA conditioning, animals showed a characteristic neophobic response to SAC (mean ± SEM = 4.93 ± 0.38 ml). However, SAC drinking over the subsequent days of conditioning declined precipitously. A one-way repeated measures ANOVA (experimental day 1, 3, or 5) revealed a significant decrease in SAC consumption [$F(2,55) = 159.70$, $p < 0.001$] which was indicative of a CTA formation. Rats destined for different treatments following extinction training (CTA + EXT + SR or CTA + EXT + SAC) consumed SAC at comparable low levels during these initial conditioning days. Using this same conditioning procedure in similarly sized control animals, Mickley et al. (2004) have reported significant increases in SAC consumption across the conditioning period if the CS and US are explicitly unpaired (separated from one another by 24 h; see corroborating evidence in Experiment 2).

Fig. 1B illustrates the SR of the CTA following extinction. A one-way ANOVA comparing SAC consumption at different times (at the end of conditioning, extinction, 15-day spontaneous recovery, 30-day spontaneous recovery, and 60-day spontaneous recovery) revealed significant differences in SAC consumption [$F(4,156) = 208.69$, $p < 0.001$]. Bonferroni *post hoc* comparisons ($\alpha = 0.05$) showed that SAC consumption at extinction is significantly greater than consumption immediately after conditioning ($p < 0.001$). Spontaneous recovery tests after 15, 30, or 60 days of water-only treatment elicited a significant decrease in SAC consumption as compared with consumption levels at asymptotic extinction (15 days: $p = 0.002$; 30 days: $p < 0.001$; 60 days: $p < 0.001$).

The two extended extinction groups (CTA + EXT + SAC) provided additional points of comparison with the 15- and 30-day spontaneous recovery animals. The spontaneous recovery animals having 15- or 30-day recovery intervals showed a significant lower SAC consumption as compared to the animals receiving these same periods of extended

Table 1 – Experiment 1: Group nomenclature, numbers of subjects, and summary of procedural timing

Group designation (*N*)	Stage 1 (conditioning)						Stage 2 (extinction)	Stage 3 (spontaneous recovery)	
	Treatment day 1	Treatment day 2	Treatment day 3	Treatment day 4	Treatment day 5	Treatment day 6	Liquid consumed from day 7 until end of extinction period	Liquid consumed daily during spontaneous recovery period (days)	Liquid consumed on SR test day
CTA+EXT+SR 15 (9)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	Water (15 days)	SAC
CTA+EXT+SR 30 (10)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	Water (30 days)	SAC
CTA+EXT+SR 60 (10)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	Water (60 days)	SAC
CTA+EXT+SAC 15 (5)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	SAC (15 days)	SAC
CTA+EXT+SAC 30 (12)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	SAC (30 days)	SAC

SAC = 0.3% sodium saccharin salt in deionized water.
LiCl = lithium chloride (81.0 mg/kg, i.p.).

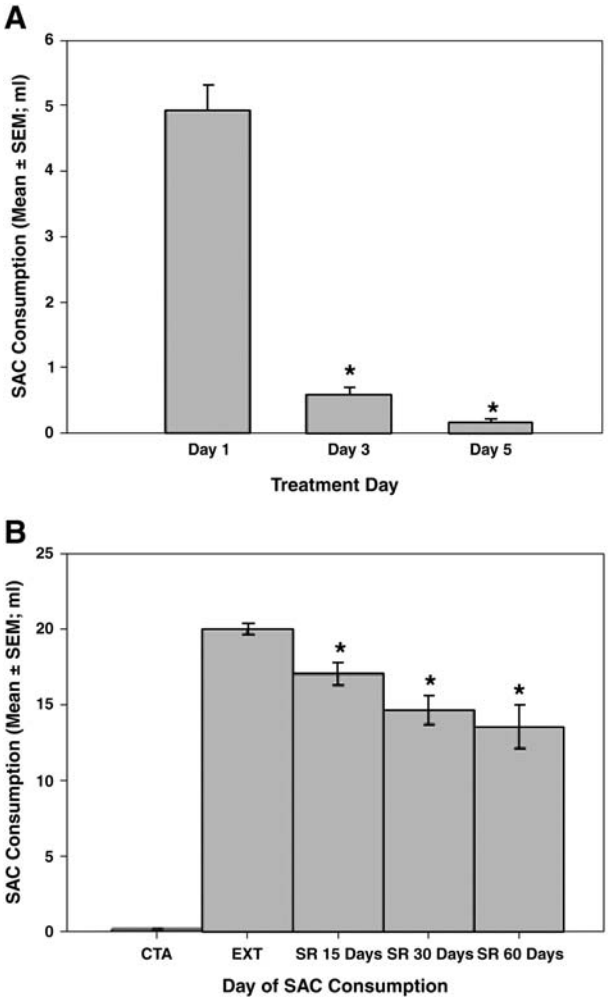


Fig. 1 – Panel A: SAC (0.3% saccharin) consumption declines over the 5-day course of CTA conditioning (Stage 1; see Table 1). By day 3 of conditioning (i.e., the first SAC drinking test after the initial SAC+LiCl pairing), animals significantly suppressed their consumption of SAC. *Significantly lower SAC consumption compared to SAC consumption on treatment day one ($p<0.001$). Variance indicators are SEM. Panel B: SAC consumption data showing changes in volume over the course of CTA acquisition, extinction, and spontaneous recovery. Consumption of SAC increased significantly after CS-only presentations (EXT; see Table 1), but this increase was partially reversed (i.e., expression of SR) after 15, 30, or 60 days of water-only treatment (no exposure to SAC). *Significantly lower than the EXT group's SAC consumption and significantly higher than CTA group's SAC consumption ($p<0.001$). Variance indicators are SEM.

extinction training [15 days: $t(22)=-6.50$, $p<0.001$; 30 days: $t(26)=-8.48$, $p<0.001$].

There was substantial variability among animals' rate of extinction with a range of 38 days between the shortest (6 days) and longest (44 days) time to reach asymptotic extinction. Variability is seen in the static phase of extinction (see Experimental procedures for definition). Once rats begin to reaccept (i.e., extinguish) SAC and enter the dynamic stage of extinction, they exhibit a similar increase

in consumption, reaching asymptotic extinction within 2 or 3 days. This natural variation in extinction length has been documented previously by Mickley et al. (2004, Fig. 2).

2.2. *Experiment 2—Behavioral data*

See Table 2 for a summary of group nomenclature and treatments during various stages of this study. Fig. 2A illustrates the long-term persistence of the CTA and the effectiveness of our explicitly unpaired CS/US CTRL manipulations. A repeated measures two-way ANOVA (conditioning day × CTA/CTRL) showed a significant change in SAC consumption over the 3 days of conditioning [$F(2, 120) = 44.59$, $p < 0.001$], a significant treatment effect between CTA and No CTA groups [$F(1, 60) = 183.44$, $p < 0.001$], and a significant interaction [$F(2, 120) = 183.12$, $p < 0.001$]. Data indicated a significant decrease in SAC consumption for rats that received CTA conditioning, while rats that received explicitly unpaired control treatment (CTRL rats) did not show a significant decrease in consumption, but a significant increase in SAC consumption signifying an abatement of neophobia and acceptance of SAC. Just as reported for Experiment 1, rats varied on their rate of CTA extinction. On average, rats extinguished in 17.38±7.66 days, with a range of 31 days (7 days minimum, 38 days maximum) observed.

Fig. 2B illustrates the reacceptance of SAC that occurs during EXT and then the decline in SAC consumption during SR of the CTA. A two-way ANOVA compared CTA animals' SAC consumption on the day of sacrifice (either at extinction or spontaneous recovery), while also factoring in their extinction history. The factors (i.e., EXT/short-term CTA X SR/long-term CTA; see Table 2) consisted of extinction treatment (Stage 2: SAC or water exposures) and spontaneous recovery treatment (Stage 3: latency period where water was consumed for 30 days or no latency period). [Note: CTRL animals that had explicitly unpaired CS/US exposures were not considered in this analysis because the focus was on the change of SAC consumption from extinction to spontaneous recovery. As can be seen in Fig. 2A, animals that experienced explicitly unpaired CS/US presentations maintained very high levels of SAC consumption throughout the study.] The two-way ANOVA revealed a significant main effect for extinction treatment [$F(1, 35) = 482.65$, $p < 0.001$] and a significant main effect for spontaneous recovery [$F(1, 35) = 13.113$, $p = 0.001$]. There was a significant interaction between extinction and spontaneous recovery treatment [$F(1, 35) = 9.875$, $p = 0.004$]. Rats that did not extinguish the CTA still exhibited very low SAC consumption even after the 30-day latency. Rats that did extinguish the CTA showed a spontaneous recovery after the 30-day latency, marked by a significant decrease in SAC consumption (see Fig. 2B).

An alternative explanation to the reduction of SAC consumption seen after the 30-day latency might be that the rats drank only the amount of fluid that is physiologically necessary, i.e., that the reduction in consumption is not a recovery of the CTA but some random fluctuation in physiological need. To investigate this possibility, the water consumed the day before the SR test was used as an indicator of physiological need, and the water consumption was com-

Table 2 – Experiment 2: Group nomenclature, numbers of subjects, and summary of procedural timing

Group designations (N)	Stage 1 (conditioning)						Stage 2 (extinction)	Stage 3 (spontaneous recovery)	
	Treatment day 1	Treatment day 2	Treatment day 3	Treatment day 4	Treatment day 5	Treatment day 6	Liquid consumed from day 7 until end of extinction period	Liquid consumed daily during latency period of 30 days	Liquid consumed on the day of sacrifice
EXT (8)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	N/A	SAC
SR (12)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	Water	SAC
Short-term CTA (9)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	Water	N/A	SAC
Long-term CTA (7)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	Water	Water	SAC
EXT-CTRL (8)	SAC	LiCl+water	SAC	LiCl+water	SAC	LiCl+water	SAC	N/A	SAC
SR-CTRL (7)	SAC	LiCl+water	SAC	LiCl+water	SAC	LiCl+water	SAC	Water	SAC
Short-term CTA-CTRL (8)	SAC	LiCl+water	SAC	LiCl+water	SAC	LiCl+water	Water	N/A	SAC
Long-term CTA-CTRL (6)	SAC	LiCl+water	SAC	LiCl+water	SAC	LiCl+water	Water	Water	SAC

SAC = 0.3% sodium saccharin salt in deionized water.
LiCl = lithium chloride (81.0 mg/kg, i.p.).
Liquid consumed during 30-day latency period not applicable (N/A) because these animals were perfused at the end of the extinction period.

pared to the amount of SAC consumed the following day during the SR test. The analysis revealed the rats drank, on average, 10.8±1.8 ml more water on the day before the SR test than SAC on the day of perfusion and that this difference is statistically significant [$t(20)=6.096$; $p<0.001$]. Specifically, rats consumed 25.4 ml of water on the day before SR test, but they only consumed 14.6 ml of SAC during the SR test. If the amount of water consumed the day before SR test is considered what is physiologically necessary to maintain homeostasis in animals of this size, then the reduction in fluid consumption observed on the SR test day may be attributed to recovery of the aversion (because the rats drank much less than what would be physiologically required).

A second possible non-associative alternative interpretation of the reduced drinking of SAC on the SR test day is that the CS might be bitter or changed in palatability during extinction or during the 30-day SR latency period. Perhaps on the SR test day, the sweetness/bitterness of SAC becomes more salient because of the long and "safe" water drinking period, resulting in the suppression of SAC consumption. However, a look at the SR-CTRL rats suggests that this may not be a strong explanation for our findings. This group controls for changes in sensation and adaptation to tastes. The SR-CTRL rats received the same number of exposures to SAC and LiCl as did the SR group during the acquisition stage of the study (Stage 1). Like the SR rats, the SR-CTRL animals were then exposed to SAC during the "extinction" phase (Stage 2). Finally, they were exposed to water for 30 days in a manner parallel to the SR rats (Stage 3). The only difference between the SR and SR-CTRL groups was the learning that occurred during the conditioning phase. At time of test, the SR rats exhibited a significant reduction in SAC drinking (as compared to their consumption at the end of EXT; see above). On average, the SR group drank 14.4±1.0 ml SAC on the day of sacrifice. The SR-CTRL rats exhibited levels of SAC drinking similar to the SR rats during EXT but did NOT reduce their drinking after the 30-day exposure to water. On average, the SR-CTRL group drank 22.6±2.4 ml SAC on the day of sacrifice. In fact, the level of SAC drinking increased somewhat in these animals. These findings are not consistent with the interpretation that the SAC somehow becomes less palatable than

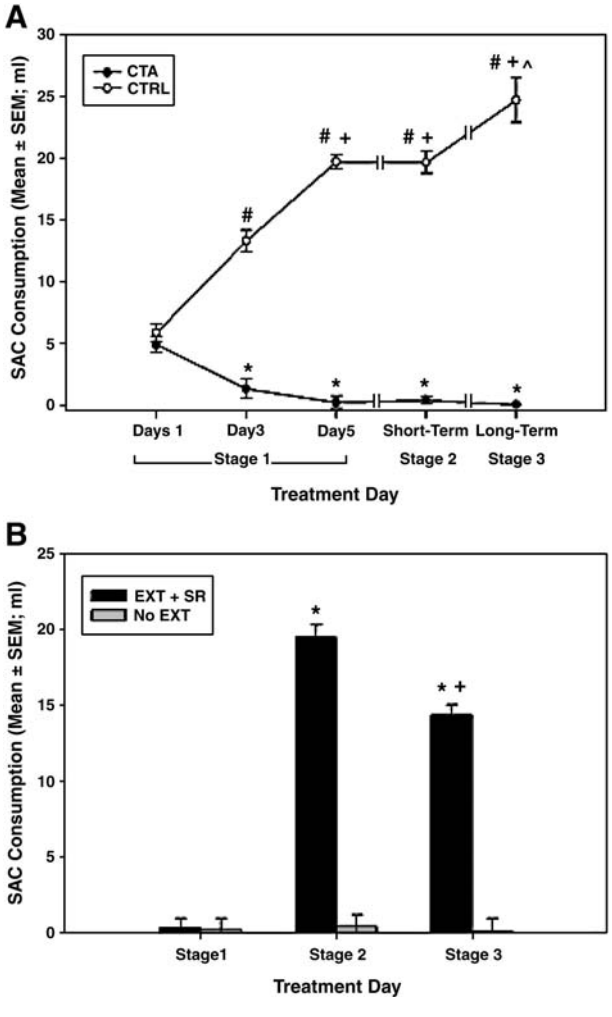


Fig. 2 – Panel A: SAC (0.3% saccharin) consumption data showing acquisition of CTA over three SAC+LiCl pairings (closed circles) or acceptance of SAC over three explicitly unpaired (EU) presentations of SAC and LiCl (open circles). The animals represented here did not receive extinction treatments and therefore were offered only water after their CTA conditioning or explicitly unpaired treatment until a final SAC consumption test. Rats that acquired a CTA continued to exhibit the aversion towards SAC throughout the study (closed circles). Likewise, CTRL rats that never learned the CTA (open circles) continued to grow in their acceptance of SAC, especially over the long term. The distance between data points for Stages 2 and 3 do not represent a standard time course since progression to these stages was determined by monitoring the animal's behavior (see line breaks). See Table 2 for group nomenclature. *Significantly lower SAC consumption than CTRL group at same time of measurement. #Significantly greater SAC consumption compared to levels consumed by both groups on treatment day 1. +Significantly greater SAC consumption compared to levels consumed by CTRL group on treatment day 3. ^Significantly greater SAC consumption compared to levels consumed by CTRL group on short-term test day. $\alpha=0.05$. Variance indicators are SEM. Panel B: SAC consumption data showing extinction and subsequent spontaneous recovery of a CTA (EXT+SR; black bars) and continued aversion to SAC (No EXT; gray bars). SAC consumption was similar at the end of conditioning (Stage 1). By the point of asymptotic extinction (Stage 2), animals that had extinction training drank more SAC than animals in Stage 1, but rats that did not undergo extinction training still exhibited a CTA. When tested for spontaneous recovery 30 days later (Stage 3), rats that once exhibited extinction (EXT+SR; black bars) showed a significant reduction in SAC consumption but drank more than rats that never received extinction training. Rats that never received extinction training (No EXT; gray bars) exhibited low, static SAC consumption throughout the experiment. *Significantly greater SAC consumption than No EXT group during the same stage of the study. +Significantly less SAC consumption than EXT+SR group at Stage 2. Variance indicators are SEM.

it was at the end of extinction training during the 30-day latency before SR test. Thus, the spontaneous recovery of the taste aversion may be best characterized as a learning phenomenon.

2.3. *Experiment 2—Immunohistochemistry data*

Rats were sacrificed at the end of either asymptotic extinction or spontaneous recovery to allow for analysis of neural activity via c-Fos immunohistochemistry. Statistical analysis of c-Fos expression was conducted as follows: Three-way ANCOVAs were used to analyze c-Fos expression density in the GNC, prelimbic cortex (PrL), infralimbic cortex (IL), BLA, and CE. The factors consisted of conditioning treatment (Stage 1: CTA or EU control), extinction treatment (Stage 2: SAC or water), and spontaneous recovery treatment (Stage 3: water or no treatment) (see Table 2 for treatments and group nomenclature). The density of c-Fos-positive cells (labeled cells/mm^2) within the brain area sampled served as the dependent variable. The covariate used was the amount of SAC consumption on the day of sacrifice. One-way ANOVAs and Bonferroni *post hoc* tests determined where the significant differences occurred between groups. For all analyses, $\alpha = 0.05$.

Fig. 3 illustrates the expression of c-Fos protein in cortical structures following CTA, EXT, SR or CTRL conditions. A three-way ANCOVA revealed a significant three way interaction [$F(1, 48) = 5.30$, $p = 0.026$] indicating that c-Fos expression in the GNC was dependent on the treatment received during all three treatment stages. Extinguished rats exhibited significantly more c-Fos expression than any other group, but after a 30-day latency resulting in spontaneous recovery, this expression decreased to a level resembling those of non-extinguished rats (see Fig. 3A). Both sets of CTRL groups showed a significant decrease in c-Fos over the 30-day latency. The covariate of SAC consumed on the day of sacrifice was not significant, indicating the amount of SAC consumed did not influence c-Fos expression in GNC.

Two different portions of mPFC were analyzed: the PrL and the IL. A three-way ANCOVA comparing PrL cell densities revealed a significant three-way interaction [$F(1, 51) = 17.92$, $p < 0.001$]. Again, c-Fos expression was significantly lower in spontaneously recovered animals than their extinguished counterparts (see Fig. 3C). The covariate of SAC consumed on the day of sacrifice was not significant, indicating the amount of SAC consumption did not influence c-Fos expression. There was a similar finding in IL; a three-way ANCOVA revealed a significant three-way interaction in IL [$F(1,51) = 45.52$, $p < 0.001$]. As with GNC and PrL, rats that spontaneously recovered the aversion expressed significantly less c-Fos than rats that only extinguished the aversion (see Fig. 3E). The amount of SAC consumed did not influence c-Fos expression in IL. In summary, our immunohistochemistry revealed that the relatively high extinction-induced c-Fos densities in the cortical regions sampled (GNC, PrL, and IL) declined during the spontaneous recovery test. In fact, at SR test, cortical cells exhibited c-Fos labeling densities similar to rats with an intact CTA. This is evident even when the amount of SAC consumed is factored out of the analyses (via the ANCOVA). See Fig. 4 for representative brain images illustrating this decrease in c-Fos expression.

Two areas within the amygdala were analyzed: BLA and CE. For c-Fos expression in BLA, a three-way ANCOVA revealed a main effect of conditioning treatment [$F(1,47) = 5.62$, $p = 0.022$], a main effect for spontaneous recovery treatment [$F(1,47) = 21.84$, $p < 0.001$], and a two-way interaction between conditioning and spontaneous recovery treatments [$F(1,47) = 5.55$, $p = 0.023$] (see Fig. 5A). The covariate of SAC consumed on the day of sacrifice was not significant, i.e., as with the cortical areas analyzed, the amount of SAC consumption did not influence c-Fos expression in BLA. Animals exhibited a relatively high level of c-Fos expression in BLA when they extinguish a CTA or retain a CTA after a comparable duration of time (i.e., the short-term CTA group). However, c-Fos expression declined during the spontaneous recovery test. A decrease in c-Fos expression was also found when non-extinguished rats were exposed to SAC after 30 days of only water exposure (i.e., the long-term CTA group). These data suggest that the difference in c-Fos expression seen between the EXT and SR groups may not be related to the spontaneous recovery observed in the behavioral data. Short-term CTA and long-term CTA rats showed a similar decline even though their SAC consumption remained low and static. For CE, a three-way ANCOVA revealed no significant differences in c-Fos expression among the eight treatment groups (see Fig. 5C).

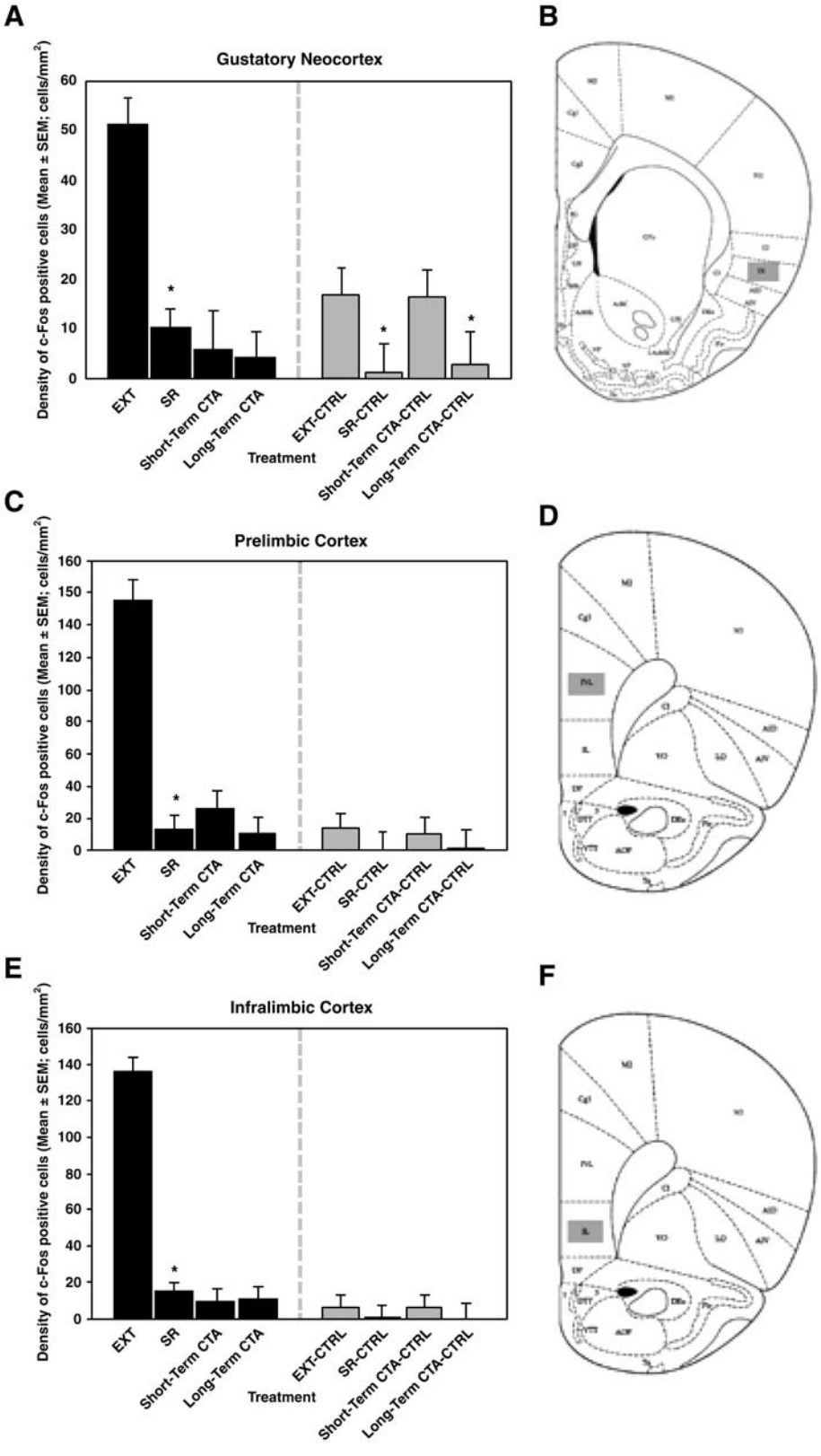
We used another series of three-way ANCOVAs to test whether the length of time for rats to extinguish the CTA influenced the c-Fos expression in GNC, PrL, IL, BLA, and CE. The ANCOVAs allowed us to determine if there was a relation between the natural variance of extinction length and c-Fos expression while factoring in the various treatments used in the experiment. The dependent variable again was the density of c-Fos-positive cells (labeled cells/mm^2) within the brain area sampled. The three factor variables were the same as in the previously mentioned three-way ANOVAs; specifically, treatments at the three experimental stages: conditioning treatment (Stage 1: CTA or EU control), extinction treatment (Stage 2: SAC or water), and spontaneous recovery treatment (Stage 3: water or no treatment). The covariate was the number of days required for a rat to extinguish (specifically, to consume an amount of SAC equal, or greater than, our defined criterion for asymptotic extinction). Results from these three-way ANCOVAs determined that the covariate (number of days to extinguish) did not have significant influence on c-Fos expression in any of the brain areas we sampled. As expected, the interaction effects and main effects of the experimental treatments (see above ANCOVAs using SAC consumption on the day of sacrifice as a covariate) remained statistically significant in the current set of ANCOVAs. In summary, this set of analyses provide evidence that variance in extinction length does not influence c-Fos expression in GNC, PrL, IL, BLA, or CE.

We also investigated correlations between the densities of c-Fos expressions found in various brain areas. A Pearson correlation (two-tailed) unveiled a significant positive relationship between c-Fos expression in the BLA and CE of EXT

rats [$r(5)=0.893$, $p=0.007$]. For SR rats, there was a significant positive correlation between c-Fos expression in GNC and PrL (Pearson, two-tailed; $r(10)=0.828$, $p=0.001$) and between c-Fos expression in PrL and IL [Pearson, two-tailed; $r(10)=0.743$, $p=0.006$].

3. Discussion

Following acquisition of a potent CTA and protracted extinction, rats will spontaneously recover the CTA if the



latency period is of appropriate length. Here, we report that, in a progressive fashion, 15-, 30-, and 60-day latencies are sufficient to cause small but reliable SRs of a CTA (when compared to SAC consumption on the last day of EXT). Although SR did not intensify significantly as post-extinction latencies extended from 30 to 60 days, the data were generally consistent with several other studies demonstrating that spontaneous recovery increases as the length of time between extinction and the SR test becomes greater (for a review, see Rescorla, 2004). Based on these data, and practical considerations aimed at keeping the latency period relatively short, we selected a 30-day latency to be used in the immunohistochemical study (Experiment 2).

Animals in the CTA+EXT+SAC groups (Experiment 1; see Table 1) displayed an increased level of SAC reacceptance beyond what was expected. In fact, these animals drank more SAC than did the non-conditioned weight-matched rats that were used to determine baseline SAC drinking. The CTA+EXT+SAC animals represent an extended extinction procedure not documented in the CTA literature before now. These data may suggest that the CTA memory can be completely extinguished (see Thomas and Ayres, 2004) in which case further research should be done to determine whether or not such dramatic extinction is capable of SR.

We wanted to ensure that rats in all groups had similar taste experiences before they were perfused for c-Fos analysis. Although we had shown previously that the amount of SAC consumed seemed to have little affect on c-Fos expression in our brain areas of interest (Mickley et al., 2004, 2005), we worried that rats that acquired a CTA, but did not undergo extinction training, might entirely avoid consuming SAC. If the rats did not (at least) taste the SAC, it would be impossible to attribute the brain changes we observed to that CS. In an attempt to produce similar sensory experiences in rats that retained a CTA, we instituted a procedure whereby we gave 1 ml SAC, via oral lavage, if a rat drank no SAC at SR test. This procedure raises some issues in light of the interesting work published by Bernstein and colleagues (Schafe et al., 1998; Wilkins and Bernstein, 2006) indicating that different conditioning methods (i.e., voluntary vs. intra-oral infusion of a tastant) can influence the neural processing of the CTA. Specifically, voluntary drinking engages much of the putative CTA circuit (including BLA, CE, and GNC), whereas oral infusion of the CS leads only to activation of the CE (Wilkins and Bernstein, 2006). Fortunately, on the final day of the study, only 2 of 16 rats (13%) failed to sample the SAC and were therefore exposed to the taste via oral lavage. Second, we confirmed our previous finding that the *amount* of SAC consumed is not a good predictor of c-Fos expression. In the current analysis, we employed an ANCOVA with the amount of SAC drunk on the last day of the study as a covariate. This covariate was not a significant predictor of the c-Fos expression in any of the brain areas sampled.

The immunohistochemical data indicate that the extent of c-Fos protein expression during SR of a CTA depends on the brain area sampled. The number of c-Fos labeled neurons in GNC and mPFC parallels behavioral changes observed as rats acquire, extinguish, and spontaneously recover a CTA. Corroborating our previous findings (Mickley et al., 2005), rats that are exhibiting a CTA have low c-Fos densities in GNC and mPFC. However, c-Fos expression increases dramatically as extinction of the CTA approaches asymptotic levels and the CS becomes reaccepted. Here we report a significant reduction of cortical c-Fos expression that accompanies SR of a CTA. For SR rats, there were also significant positive correlations between c-Fos expression in GNC and PrL and between c-Fos expression in PrL and IL suggesting that these brain areas are functioning consistently to produce recovery of a taste aversion following a post-extinction latency of 30 days.

The interpretation and generalizability of our data may be somewhat limited by the fact that we sampled within a constrained anterior-posterior (AP) and medial–lateral (ML) extent of the brain areas of interest. We chose the specific portions of the mPFC, GNC, and AMY because of their role in gustation, CTA acquisition, or extinction (Alheid et al., 1995; Norgren, 1995; Saper, 1995; Santini et al., 2004;

Fig. 3 – Panel A: c-Fos expression within the sample region of gustatory neocortex (GNC) for all treatment groups. Expression of c-Fos in GNC is relatively high when animals extinguish the CTA, but expression returns to levels resembling those of a continued CTA when animals undergo spontaneous recovery. *Significantly less c-Fos expression than the EXT group ($p<0.001$), comparable to the short- and long-term CTA groups. #Significantly less c-Fos expression than the EXT-CTRL and the short-term CTA CTRL groups ($p<0.001$). See Table 2 for group nomenclature and treatment descriptions. Variance indicators are SEM. Panel B: Coronal brain diagram containing GNC adapted from Paxinos and Watson (1998); 1.70 mm anterior to bregma. The gray box within the diagram is the region of GNC that was sampled for c-Fos counting. Panel C: c-Fos expression within the sample region of prelimbic cortex (PrL) of mPFC for all treatment groups. Expression of c-Fos in PrL is relatively high when animals extinguish CTA, but expression returns to levels resembling those of a continued CTA when animals undergo spontaneous recovery. *Significantly less than the EXT group ($p<0.001$), comparable to the short- and long-term CTA groups. See Table 2 for group nomenclature and treatment descriptions. Variance indicators are SEM. Panel D: Brain diagram containing PrL adapted from Paxinos and Watson (1998); 3.20 mm anterior to bregma. The gray box within the diagram is the region of PrL that was sampled for c-Fos counting. Panel E: c-Fos expression within the sample region of Infralimbic cortex (IL) of mPFC for all treatment groups. Expression of c-Fos in IL is relatively high when animals extinguish CTA, but expression returns to levels resembling those of a continued CTA when the animal undergoes spontaneous recovery. *Significantly less than the EXT group ($p<0.001$), comparable to the short- and long-term CTA groups. See Table 2 for group nomenclature and treatment descriptions. Variance indicators are SEM. Panel F: Brain diagram containing IL adapted from Paxinos and Watson (1998); 3.20 mm anterior to bregma. The gray box within the diagram is the region of IL that was sampled for c-Fos counting.

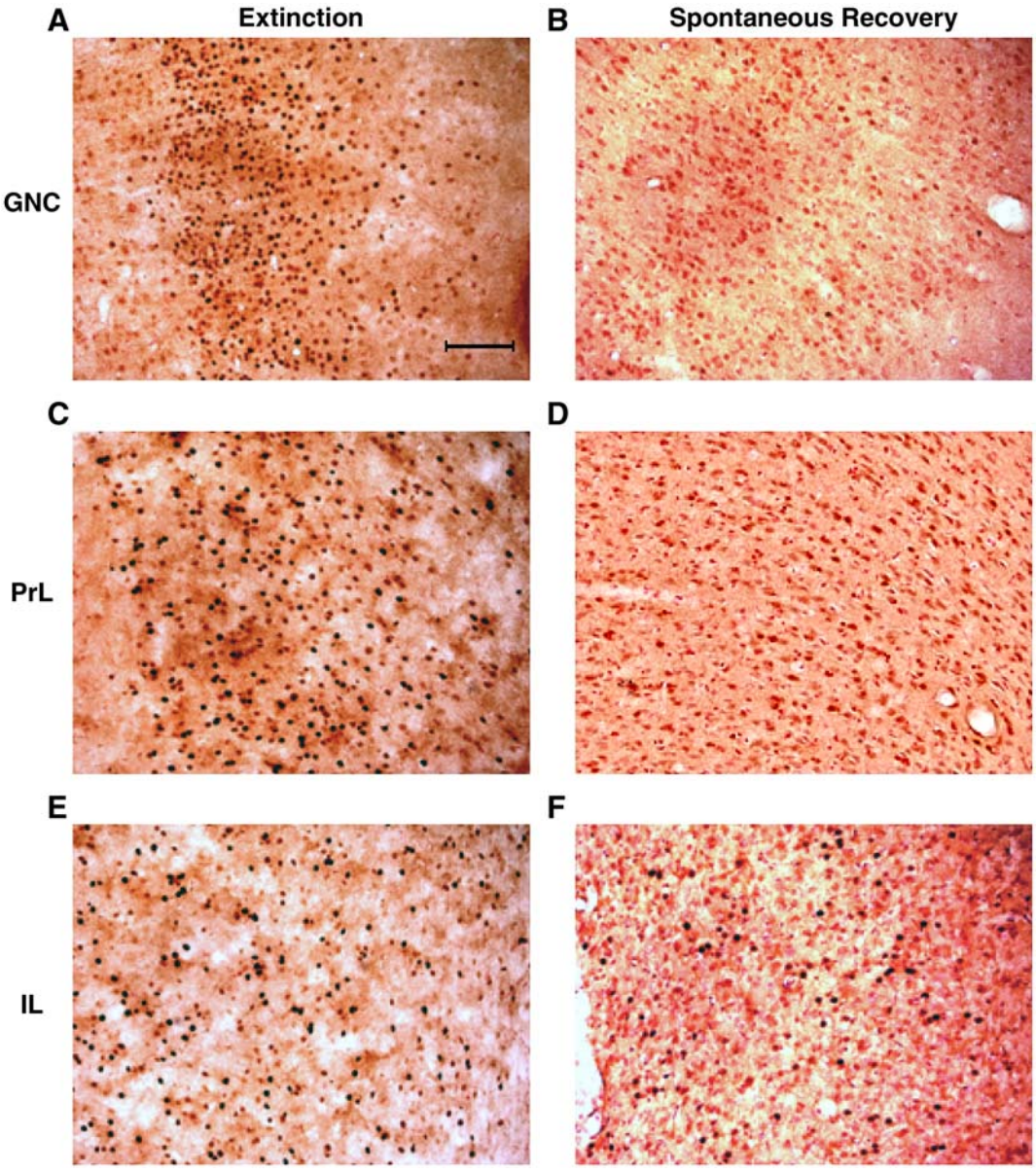


Fig. 4 – Photomicrographs (20× magnification for all images; scale bar = 100 μm.) of representative coronal brain sections from rats that exhibited either asymptotic extinction of a CTA (panels A, C, and E) or spontaneous recovery of the aversion (panels B, D, and F). In the GNC (panel A), PrL (panel C), and IL (panel E), c-Fos expression was high in rats that have acquired a CTA and then extinguished the aversion. A significant reduction of cortical cells expressing c-Fos in GNC (panel B), PrL (panel D), and IL (panel F) accompanied spontaneous recovery of the CTA.

Bermudez-Rattoni and McGaugh, 1991). For example, we counted cells in the dysgranular zone of the GNC since prominent gustatory neurons are found there (Norgren, 1995). Further, we used this selection method since it is similar to other published studies from our laboratory focusing on extinction of CTAs and may allow comparisons between the current, and previous, data (Mickley et al., 2004, 2005). However, AP and ML differences in anatomical connections and functionality are well established for the brain areas examined here (see, for example, Adamec and Shallow, 2000; Accolla et al., 2007). Therefore, caution should be exercised in generalizing these results to all portions of the GNG, mPFC, or AMY.

Unlike the GNC and mPFC areas sampled, a change in expression of c-Fos labeled BLA cells does NOT accompany the transition from CTA acquisition to extinction (Mickley et al., 2004). Compared to EXT, spontaneous recovery of the CTA significantly reduces the expression of c-Fos protein in this portion of the amygdala, but a reduction of c-Fos expression in BLA is also seen when non-extinguished rats are given an SAC test after a 30-day latency as well. This finding suggests that c-Fos reduction in BLA is weakly related spontaneous recovery of a CTA. By way of contrast, c-Fos-labeled cells in the CE are generally low, independent of the CTA acquisition (Mickley et al., 2006), extinction, or spontaneous recovery history of the animal.

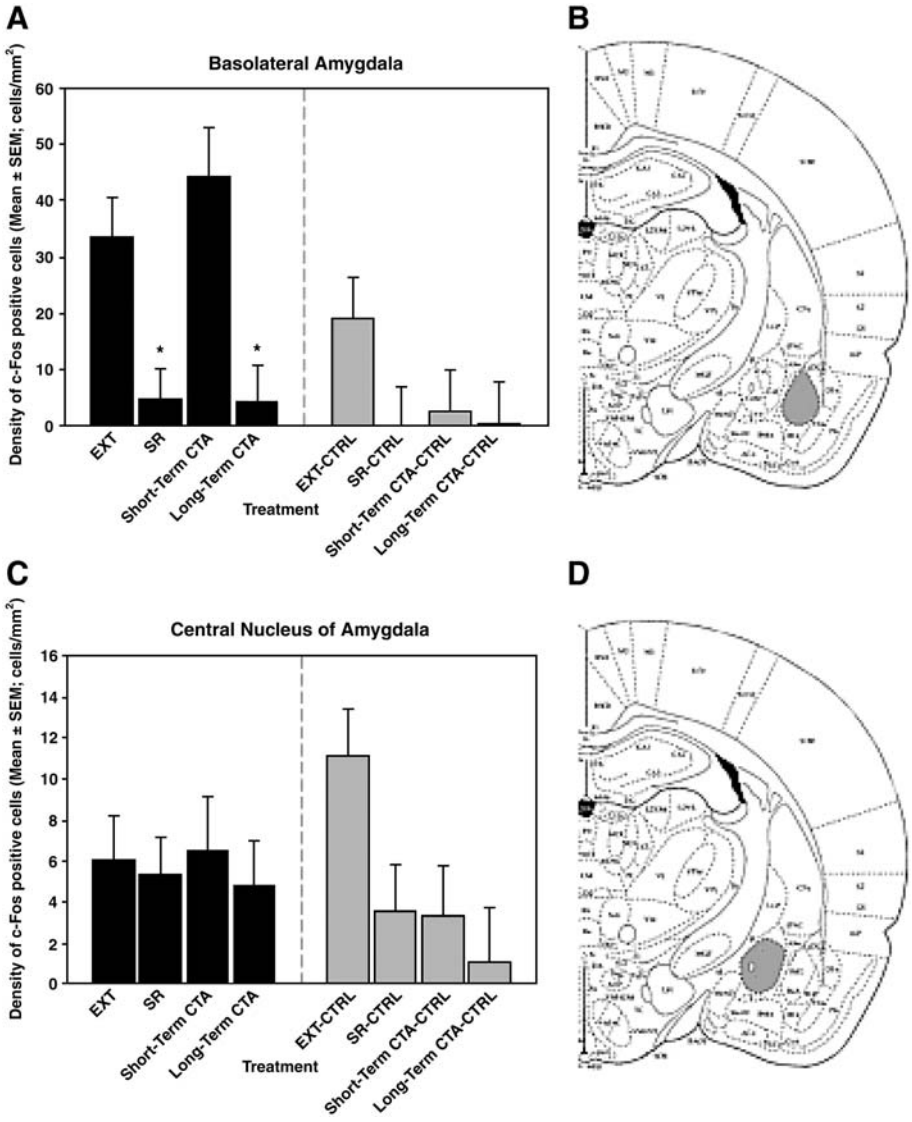


Fig. 5 – Panel A: c-Fos expression within the basolateral nucleus (BLA) of the amygdala for all treatment groups. Expression of c-Fos in BLA was relatively high when animals extinguish CTA (EXT), but expression returned to levels resembling those of a continued long-term CTA when animals exhibited spontaneous recovery (SR). C-Fos expression was also relatively high in the short-term CTA group, but declined when tested 30 days later. *Significantly less than the EXT and short-term CTA groups (SR: $p=0.025$; long-term CTA: $p=0.043$). All c-Fos-labeled neurons within the BLA of a coronal section of the amygdala were counted. See Table 2 for group nomenclature and treatment descriptions. Variance indicators are SEM. Panel B: Brain diagram containing BLA (shaded gray) adapted from Paxinos and Watson (1998); 2.30 mm posterior from bregma. Panel C: c-Fos expression within the central nucleus (CE) of the amygdala for all treatment groups. Statistical analysis revealed no significant differences in c-Fos expression among any of the treatment groups, indicating little change in this measure of neural activity during CTA acquisition, EXT, or SR. All c-Fos-labeled neurons within the CE of a coronal section of the amygdala were counted (see Paxinos and Watson (1998). See Table 2 for group nomenclature and treatment descriptions. Variance indicators are SEM. Panel D: Brain diagram containing CE (shaded gray) adapted from Paxinos and Watson (1998); 2.30 mm posterior from bregma.

3.1. *Are extinction and spontaneous recovery new learning?*

A theoretical perspective outlined in the introduction to this paper suggests that EXT depends on new learning that competes with the original fear memory (for a review, see Maren, 2005). Related issues raised by the current data are whether SR of a CTA is, (a) a deficit in retrieval of the EXT memory, (b) a simple retrieval of the original memory, or (c) a reconsolidation of the original memory (perhaps identifying still-another temporal context in which the original conditioning applies). Using a conditioned fear paradigm, Quirk (2002) has demonstrated that, given a sufficiently long latency period, extinguished freezing to a tone CS sponta-

neously recovers to full strength (something we did not observe in the current CTA study). The extinction memory persists however (as revealed by an enhanced rate of re-extinction), suggesting that conditioned fear and extinction exist as separate memories. Although Stollhoff et al. (2005) suggest that reconsolidation of an acquisition memory underlies SR, it is not yet clear if the mechanisms of reconsolidation are the same (or even similar) to the mechanisms of the original consolidation (Bahar et al., 2004; Taubenfeld et al., 2001; Dudai, 2006). Further, "simple" retrieval of a CTA may not actually be a simple process with perhaps several memory traces competing for control of consummatory behaviors. Bahar et al. (2003) have shown that CTA consolidation depends on protein synthesis in the CE of the amygdala while EXT of CTA depends on protein synthesis in the BLA. Further, inhibition of protein synthesis in either CE or BLA does not interfere with the reconsolidation of a CTA memory (Bahar et al., 2004). These findings were dependent on the training history of the animals but suggest that reconsolidation does not accurately recapitulate consolidation and that there may be significant differences between the neural mechanisms of reconsolidation and extinction.

If SR is a recapitulation of the original conditioned response then we would expect that neural activity associated with both the CTA and the SR to be similar, if not identical. The c-Fos expression in GNC and mPFC provide some tepid support for this hypothesis. There was a clear tendency for c-Fos protein expression to decelerate from the high levels observed during EXT and move towards those low levels seen in rats having a robust CTA. Likewise, the SAC drinking of the SR rats decreased significantly (as compared to the consumption of rats at asymptotic EXT). Thus, this one indicator of cortical activity seems to indicate similarity between the brain mechanisms of acquisition and SR of a CTA. However, the orders of magnitude of these 2 behavioral and immunohistochemical responses were quite different. The behavioral SR reflected a modest but reliable decline in SAC drinking, whereas the levels of c-Fos expression decreased dramatically from EXT to SR. This non-congruency between behavioral and measures of cortical activity may suggest that additional brain areas are involved in the SR of a CTA.

3.2. *Neural components of extinction*

There is a clear role for both cortical and subcortical brain structures in extinction learning and the expression (or inhibition of expression) of learned behaviors. Our previous data (Mickley et al., 2004, 2005, replicated here) indicate a significant increase in GNC and mPFC (both IL and PrL) activity as CTA EXT becomes well established. Beyond CTA, evidence suggests that the long-term storage of extinction of a conditioned fear occurs in the mPFC, and the IL in particular, since lesions of the IL impair the recall of extinction (Morgan et al., 1993; Quirk et al., 2000). Perhaps even more convincing, an extinguished CS evokes firing of IL neurons and electrical stimulation of IL induces an extinction-like behavioral response (Milad and Quirk, 2002). Since there are robust projections from the mPFC (both IL and PrL) and insular (GNC) cortex to AMY (McDonald et al., 1996; for a review, also see Sotres-Bayon et al., 2004) and a feedback loop from AMY to mPFC (Garcia et al., 1999), it has been proposed that these circuits exhibit inhibitory control to limit behavioral responding during extinction learning.

This proposal has received substantial support. Stimulation of mPFC neurons decreases the output of CE cells (Quirk et al., 2003). Moreover, the excitatory connections from cortex to various amygdalar nuclei may ultimately result in inhibition of behavioral responding via activation of GABAergic intercalated neurons of the AMY and dampening the output of the CE (Royer and Paré, 2002; Sotres-Bayon et al., 2004; Quirk et al., 2003). The central nucleus of the AMY has divergent projections to multiple fear expression centers (Royer and Paré, 2002) as well as brainstem centers (e.g., PBN) that are part of the taste neural pathway (Huang et al., 2003) and therefore have the potential to play an important role in the modulation of behavioral responding associated with CTA EXT and possibly SR (although the data presented in the current paper suggest otherwise).

3.3. *Role for inhibition in the neural control of extinction and spontaneous recovery*

Rescorla (2004, 2005) has offered some thoughtful observations regarding SR and acknowledges the important theoretical role that "inhibition" has played in accounting for it. If during EXT new learning is superimposed on the original learning, that new learning may involve acquisition of an inhibitory tendency. SR then is accounted for by the dissipation of that inhibition with the passage of time. Although one might not necessarily expect a perfect correspondence between inhibition at the behavioral level and inhibition at the cellular or molecular level, Harris and Westbrook (1998) have shown that inhibitory synapses are involved in EXT. Descriptions of the neural mechanisms for SR are in their infancy but, as described above, GABAergic inhibition mediates mPFC-to-AMY projections (Royer and Paré, 2002). Paré et al. (2004) have suggested that subsets of the amygdala's intercalated neurons may exhibit increased or decreased responsiveness after conditioning, EXT, or SR. What remains unclear is exactly what is being inhibited or stimulated. The saliency of the CS? The CR? The CS–US association itself?

The current studies inform several of the theoretical ideas regarding the neural substrate of EXT and SR. The decreases in c-Fos expression in mPFC, GNC, and BLA that we observed during SR of a CTA corroborate several findings from other laboratories. For example, SR of a CTA may be disrupted by manipulation of β-adrenergic or cAMP systems within insular cortex of the rat (Berman et al., 2003). Going beyond the CTA paradigm, SR of a conditioned fear has been associated with sustained prefrontal long-term depression (LTD) (Herry and Garcia, 2002, 2003). However, when EXT of conditioned fear was accompanied by stimulation of mPFC neuronal activity, it was not followed by an SR of extinguished responses (Herry and Garcia, 2002; Milad and Quirk, 2002). More recently, Garcia's laboratory has also reported that SR of a conditioned fear is accompanied by a failure to observe induction of c-Fos and *zif288* early gene expression in both the mPFC and BLA

(Herry and Mons, 2004). Remarkably, SR of an extinguished fear may be induced by lesioning portions of the ventromedial PFC (Quirk et al., 2000). Therefore, the current data provide support for the hypothesis that GNC, mPFC, and (perhaps to a lesser extent) BLA are engaged in the SR of a CTA.

Perhaps the most surprising finding in this study was the lack of change in CE activity throughout EXT and SR. There are also cortical efferents that bypass the amygdala and project directly from the mPFC to the hypothalamus and other portions of the brain that may be involved in drinking (the behavioral outcome measured here) (Hurley et al., 1991; Takagishi and Chiba, 1991; Sesack et al., 1989). Perhaps these other circuits were engaged in order to produce the behavioral inhibition observed in our animals. The relatively low levels of c-Fos expression in the CE of the amygdala, independent of CTA treatment or spontaneous recovery, suggest a less important role for this nucleus in the processing of CTA learning and memory.

Consistent with this hypothesis was the finding of a significant positive relationship between c-Fos expression in the BLA and CE of EXT rats. When BLA neurons are active, CE neurons are also active. Although there are well-known connections between BLA and CE through the inhibitory intercalated neurons of the AMY (Royer and Paré, 2002; Sotres-Bayon et al., 2004; Quirk et al., 2003), our data do not seem to indicate that activation of BLA induces inhibition of CE. As suggested above, these findings may be indicative of alternative circuits being used to induce the behavioral changes associated with EXT and SR. However, it should be noted that c-Fos expression in CE was remarkably low under all treatment conditions employed here. Perhaps, "basement" effects have obscured our ability to discern functional relationships between BLA and CE.

3.4. *Neural c-Fos expression as a potential indicator of related learning phenomena*

It is worth noting that, non-conditioned control rats also exhibited relatively low levels of c-Fos expression in the cortical areas sampled. Therefore, exposures to SAC that predict either malaise (short- or long-term CTA groups) or have no negative association (sort- or long-term CTA CTRL), yield similar levels of c-Fos expression. The origins of this result remain uncertain. However, animals in all of these groups are exhibiting very stable behaviors—either well-established avoidance or well-established acceptance of SAC. Perhaps it is possible that low levels of c-Fos expression in GNC and mPFC reflect some level of certainty associated with overtraining. If so, these data are consistent with others suggesting that portions of mPFC play a role in shifting from voluntary, goal-directed actions to reflexive, habit-based responding (Coutureau and Killcross, 2003).

These data also point out the fact that expression of c-Fos is not merely an indicator of volume of SAC consumed. Rats in the short- or long-term CTA groups drank very little SAC before sacrifice. Whereas, rats in the short- or long-term CTA CTRL groups drank 20–30 ml SAC (See Fig. 2A). Despite this wide variation in the volume of SAC consumed, the number of c-Fos-labeled neurons was very low in all of these animals. Moreover, the ANCOVAs employed to assess the significance of the changes in c-Fos expression used the amount of SAC consumed before sacrifice as the covariate. Uniformly, this covariate was not a statistically significant factor. Therefore, c-Fos expression in the brain areas sampled is not merely an indicator of the neural processing associated with the sensory experience or the quantity of sweet liquid consumed. A more likely explanation for the changes in c-Fos expression we observed as rats extinguished and spontaneously recovered the CTA is that the changes reflect the brain's reorganization of learned information.

A puzzling aspect of our data is the finding that, depending on the brain area under investigation, there were sometimes distinct differences between the densities of c-Fos expressed following our short-term and long-term treatments. Perhaps the most prominent example of this may be found in the BLA c-Fos data (short-term CTA and long-term CTA groups). Rats that had acquired a potent CTA and then retained the CTA for an average of almost 19 days (short-term) expressed significantly more c-Fos labeling of BLA neurons than did rats that retained that CTA for 30 days longer (long-term). Likewise, in the GNC, non-conditioned control rats (EXT-CTRL and short-term CTA CTRL) that were involved in the study for 30 days less than SR-CTRL or long-term CTA CTRL animals exhibited significantly more c-Fos expression in GNC than did their long-term counterparts. Since these time-dependent changes seem to occur in both conditioned and non-conditioned animals, the data suggest a transition in GNC and BLA physiology that occurs from 20 to 50 days, independent of CTA training. Of course, beyond the conditioning that the experimenters are imposing, the animals are likely learning all sorts of things associated with laboratory routine that may sensitize or habituate a variety of brain systems over the short/long term. The fact that these effects were most reliably seen in GNC and BLA may provide additional clues as to the nature of these functional adjustments in neurophysiology.

Our use of the CTA paradigm allows us to broaden the testing of EXT and SR theories within a methodology that goes beyond the ubiquitous barpress suppression test. Like barpress suppression, CTA represents a defensive reaction to a learned fear. However, the natural preparedness for CR acquisition and the time scales for extinction and SR are very different in these two paradigms (Bahar et al., 2004). Thus, the further investigation of CTA may be useful as we determine the extent to which our knowledge about the neural mechanisms of extinction and SR is general in nature.

3.5. *Summary and conclusions*

In summary, the data presented here further implicate the mPFC and GNC in the spontaneous recovery of a CTA. Neural activity in cerebral cortex, previously heightened as CTA extinction reached asymptote, is then significantly attenuated as rats once again reduce their SAC drinking during SR. The expression of c-Fos in the CE of the amygdala is not altered during SR from extinction and there is a poor correlation between SAC consumption and c-Fos expression in the BLA. These data suggest a relatively minor role for these amygdala structures in the suppression of SAC drinking that accompanies SR of a CTA.

Behavioral and pharmacological therapies for PTSD, panic disorders and phobias may be challenged by "flashbacks", relapses, and other intrusions of the fearful emotions that were present during the acquisition of the fear (Watts, 1974; Koren et al., 2001). Given its clinical relevance, it is therefore surprising that so little attention has been given the study of the brain mechanisms that underlie the spontaneous recovery of learned fears. This said, our work with pre-clinical models seems to corroborate findings from two studies that have tested clinical populations. Functional imaging studies have shown low activity in ventromedial prefrontal cortex during exposure to stimuli (CSs) that induced spontaneous recovery of fear responses in individuals with PTSD (Bremner et al., 1999; Shin et al., 1997). It is perfectly conceivable that different learned fears, and circumstances surrounding the acquisition and extinction of those fears, will affect SR in clinical populations. However, the current data point to a prominent role for cortical structures in mediating SR in the context of CTA.

4. Experimental procedure

4.1. *Experiment 1—Subjects*

Forty-six adult male Sprague–Dawley rats (483.26±7.12 g; see Table 1 for a description of nomenclature, experimental treatments, and number of subjects in each group) were obtained from Zivic Laboratories (Zelienople, PA). Animals were individually housed in plastic "shoebox" cages (44.45 cm×21.59 cm×20.32 cm) at 23–26 °C. The animals were kept on a 12/12-h light/dark cycle (lights on at 06:00). Rodent chow was available *ad libitum* for the entire study. All phases of the experiment were conducted in each animal's home cage.

4.2. *Experiment 1—Experimental design and procedures*

Two days prior to conditioning, all animals were placed on a 23-h water deprivation cycle and maintained on this schedule throughout the study. On these days, a 50-ml bottle of tap water was available for 1 h starting at 12:00 h. Throughout the study, animals were given access to a 50-ml bottle of either tap water or 0.3% sodium saccharin salt dissolved in water (SAC), depending on the experimental condition. Daily fluid consumption data were recorded to the nearest tenth of a gram by an observer blind to the treatment group of the animals.

The study itself was divided into 3 stages: Stage 1, conditioning; Stage 2, extinction; and Stage 3, spontaneous recovery. See Table 1 for a summary of the different treatments that each of 5 groups of rats experienced in the 3 experimental stages. In brief, each group experienced three CTA conditioning trials during Stage 1 followed by extinction to asymptote (CS alone; Stage 2). After this near-complete extinction, rats in 3 different groups (designated as CTA+EXT+SR 15, CTA+EXT+SR 30, or CTA+EXT+SR 60) received water for 15, 30, or 60 days (Stage 3). On the last day of their previously assigned recovery period, animals in each group received SAC (SR test).

The Stage 1 conditioning phase took place over 6 days. On days 1, 3, and 5 all animals received the CS (SAC) for 30 min (starting at 12:00) followed by the US (81 mg/kg LiCl, i.p.) followed by 30-min access to water. On days 2, 4, and 6 (rehydration days) all animals received water for one full hour beginning at 12:00. All fluids were made available in a 50-ml drinking bottle placed onto the animal's home cage.

On experimental day 7 all animals entered the extinction phase of the study (Stage 2). For this phase, animals received 30-min access to SAC beginning at 12:00 followed by 30-min access to water beginning at 12:45. An animal remained within the extinction phase until the day it reached an "asymptotic" level of extinction (mean±SEM=18.76±1.23 days). Asymptote was operationally defined as reaching 90% reacceptance of SAC as compared to baseline SAC consumption [i.e., an average of the volume of SAC consumed by rats similar (in weight and SAC experience) to the rats at the beginning of the current study; mean±SEM=17.6±1.4 ml SAC]. This procedure was developed based on the results of previous behavioral experiments from our laboratory (Mickley et al., 2004). Across extinction trials, rats begin to reaccept SAC over a highly variable number of days (ranging from 5 to 35). Despite this variability, the EXT process was consistently characterized as a stepwise function showing an initial *static* period of no or little reacceptance followed by a *dynamic recovery* period in which SAC was quickly reaccepted (over 2–3 days) followed finally by an *asymptotic* period where the animal reached a plateau of almost fully reaccepting the SAC CS (Nolan et al., 1997; Mickley et al., 2004).

Following asymptotic extinction, the spontaneous recovery phase began (Stage 3). CTA+EXT+SR 15, CTA+EXT+SR 30, or CTA+EXT+SR 60 animals received water for 15, 30, or 60 days, respectively. The length of the SR period was randomly assigned to individual rats at the beginning of the experiment. Throughout this phase, each animal was presented with water for 30 min beginning at 12:00 and again beginning at 12:45. On the last day of the recovery period, each animal received SAC for 30 min beginning at 12:00.

Two additional control groups were included to assess how much SAC would be consumed by rats that had first acquired a CTA, experienced 90% reacceptance ("asymptotic extinction" of the CTA) and then continued to receive daily SAC for either 15 or 30 additional days: CTA+EXT+SAC 15, CTA+EXT+SAC 30 (see Table 1). The treatment of these animals was as described for the SR rats except these animals received SAC during the SR latency period instead of water. Like all rats in the study, on the last day of the experiment each animal received a final exposure to SAC for 30 min beginning at 12:00.

Throughout each phase of the study all contextual cues remained constant except for the handling associated with the injection during the CTA conditioning phase, which was not reintroduced during the extinction or the spontaneous recovery phases. All animals were handled briefly during weighing, which took place prior to the experiment each day regardless of conditioning phase.

4.3. *Experiment 2—Subjects*

Sixty-five adult male Sprague–Dawley rats (437.2±5.3 g; see Table 2 for a description of nomenclature, experimental treatments, and number of subjects in each group) were

obtained from Zivic Laboratories (Zelienople, PA) and maintained as described in Experiment 1.

4.4. *Experiment 2—Experimental design and procedures*

Two days prior to conditioning, all animals were placed on a 23-h water deprivation cycle and maintained on this schedule throughout the study. As described above, the study itself was divided into 3 stages: Stage 1, conditioning; Stage 2, extinction; and Stage 3, spontaneous recovery. See Table 2 for a summary the different treatments that each of 8 groups of rats experienced in each of the 3 experimental stages. Also see timing and measurement details under Experiment 1 (above).

Eight groups were employed in Experiment 2. A CTA extinction group (EXT) included animals that (during Stage 1) acquired the CTA and then extinguished the aversion (during Stage 2). EXT animals received three CTA conditioning trials administered every-other day over the course of 6 days (experimental days 1, 3, and 5). Each trial began at 12:00 h and paired a 30-min exposure of 0.3% SAC (CS) with a malaise-inducing i.p. injection of 81 mg/kg LiCl, (US). At 12:45 h, rats were given 30-min access to tap water to prevent dehydration and weight loss. For the same reasons, on days 2, 4, and 6, rats were given 1 h access to tap water beginning at 12:00 h. Beginning on experimental day 7, EXT animals were given 30-min access to SAC (12:00–12:30 h) followed 15 min later by 30-min access to tap water (12:45–13:15 h). This was repeated daily until rats consumed 90% of their baseline SAC consumption (calculated as described for Experiment 1, above). After each EXT animal met its extinction criterion, it was anesthetized, perfused, and the brain was harvested for c-Fos immunohistochemical analysis as described below.

A spontaneous recovery group (SR) included animals that acquired the CTA in Stage 1, extinguished it (in Stage 2), and then spontaneously recovered the aversion (in Stage 3). SR animals received the same conditioning and extinction trials as the EXT group. However, following asymptotic extinction, SR animals entered Stage 3. Here, SR animals received water for 60 min (12:00–12:30 and 12:45–13:15 h) for 30 consecutive days. The 30-day latency period was chosen based on the data from Experiment 1 indicating that a reliable spontaneous recovery could be achieved using this time period. On the last day of the spontaneous recovery period, each SR animal received SAC for 30 min beginning at 12:00 and was then anesthetized and perfused as described below.

A short-term CTA group (short-term CTA) included animals that acquired the CTA (in Stage 1) but received water during the extinction period (Stage 2) and thus did not extinguish the aversion. Short-term CTA animals received the same conditioning trials as the EXT group. However, beginning on the 7th experimental day, short-term CTA animals were given access to water for 60 min (12:00–12:30 and 12:45–13:15 h). Each short-term CTA animal was yoked to an animal of similar weight in the EXT group so that on the day an EXT animal reached its extinction criterion and was perfused, a short-term CTA rat was also given access to SAC and perfused. On the day the EXT rat reached its criterion, the yoked short-term CTA rat received access to SAC for 30 min (12:00–12:30 h) and was anesthetized and perfused as described below. Rats that did not voluntarily drink any SAC on this last day of the study received oral lavage of 1 ml SAC to induce a sensory experience similar to the EXT and SR rats.

A long-term CTA group (long-term CTA) included animals that acquired the CTA (in Stage 1) but received water during Stages 2 and 3. Thus, long-term CTA animals neither extinguished nor spontaneously recovered the aversion. long-term CTA animals received the same conditioning trials as the EXT group. However, beginning on the 7th experimental day, long-term CTA animals were given access to water for 60 min (12:00–12:30 and 12:45–13:15 h). Each long-term CTA animal was yoked to an animal of similar weight in the SR group so that on the day that an SR animal reached its extinction criteria and entered Stage 3, the long-term CTA animal also entered the Stage 3 and for an additional 30 days received access to water for 60 min (12:00–12:30 and 12:45–13:15 h). On the last day of Stage 3, each long-term CTA animal received access to SAC for 30 min beginning at 12:00 and was then anesthetized and perfused as described below. As described above, rats that did not voluntarily drink any SAC on this last day of the study received oral lavage of 1 ml SAC to induce a sensory experience similar to the EXT and SR rats.

Four additional control (CTRL) groups were included in this study: EXT-CTRL, SR-CTRL, short-term CTA CTRL, and long-term CTA CTRL (see Table 2 for a description of nomenclature, experimental treatments, and number of subjects in each group). The purpose of these groups was to detect the changes in SAC consumption and c-Fos expression caused by the mere exposure to the CS and US—but not in a manner that creates an association between the two. Animals in each of these groups *did not* receive CTA conditioning trials (in Stage 1) but instead received the CS and US "explicitly unpaired" from one another (i.e., separated by 24 h). This procedure foils the acquisition of a CTA (Wagner and Rescorla, 1974; Mickley et al., 2004). Thus, on experimental days 1, 3, and 5, animals in each of the control groups were given access to SAC for 30 min. Following a 15-min latency, the rats were allowed access to water for an additional 30 min. On experimental days 2, 4, and 6, animals were administered LiCl injections (81.0 mg/kg, i.p.). Beginning on the 7th experimental day, each control group received the same treatments as its corresponding experimental group during Stages 2 and 3 of the study (see Table 2.). Data from these groups that have had a non-associative exposure to SAC and LiCl provide a comparison point with the experimental animals that have learned that SAC predicts malaise. Thus, they allow us to differentiate the non-associative changes in SAC consumption and c-Fos expression, following Stage 1 of the study, from those changes in behavior and physiology that are caused by CTA acquisition.

Throughout each phase of the study all contextual cues remained constant except for the handling associated with the injection during the CTA conditioning phase, which was not reintroduced during the extinction or the spontaneous recovery phases. All animals were handled briefly during weighing, which took place prior to the experiment each day regardless of conditioning phase.

4.5. *Experiment 2—Perfusion, histology, and immunohistochemistry*

Rats were sacrificed 90 min following the end of the last SAC exposure. This timing was employed because c-Fos expression is highest between 90 and 120 min after post-synaptic neuronal activity (Herrera and Robertson, 1996). All rats were given access to SAC before perfusion to control for c-Fos expression that may be directly caused by the sensation of a sweet taste. Note that almost all rats in this study drank at least some SAC on the last day of the experiment. A small number (13%) of the long- and short-term CTA rats failed to drink SAC and instead received oral lavage 90 min before perfusion. Previous experiments from our laboratory indicate that c-Fos expression in the brain areas studied here is not altered by the volume of SAC consumed (Mickley et al., 2004). Similar findings are reported in the current paper.

Before perfusion, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p. injection). Each rat was then transcardially perfused with 300–500 ml of heparinized saline followed by 300–500 ml of 4% paraformaldehyde. The brains were immediately removed, placed in 4% paraformaldehyde, and stored at ~4 °C. Eight hours later, the brains were transferred to a cryoprotectant solution (30% sucrose mixed in phosphate buffered saline with 0.01% thimerosal) and kept at ~4 °C until they were sliced. Brains were sliced in the coronal plane at 40 μm using a freezing microtome and stored in phosphate buffered saline containing 0.2% sodium azide until they were assayed.

Coronal sections (see Table 3 for group Ns) were assayed for c-Fos protein immunoreactivity as previously described (Hsu et al., 1981; Mickley et al., 2004). C-Fos protein immunohistochemistry was used to identify neural activity. Evidence suggests that expression of c-Fos not only mediates sensory experience but may also be instrumental in the associate aspects of a CTA (Houpt et al., 1996a,b; Lamprecht and Dudai, 1996; Swank et al., 1996). Afterward immunohistochemistry, brain sections were mounted on gelatin and chrom-alum-coated slides, dehydrated, counterstained with neutral red, and cover slipped with Permount™.

To ensure that negative c-Fos expression is not a result of faulty staining procedures, we employed a positive control procedure as described by Rinaman et al. (1997). Adult male rats received an injection of 4% saline solution (2.0 ml/100 g, i.p.) to produce an osmotic thirst, which leads to certain c-Fos expression in the periventricular nucleus (PVN) of the hypothalamus. Ninety minutes later, the rats were given a supra-lethal dose of sodium pentobarbital (100 mg/kg, i.p.), perfused, and prepared for c-Fos immunohistochemical procedures. Brain slices including the PVN from these animals were included in all the immunohistochemical assays of the experimental and control groups to ensure that successful staining had occurred. If positive staining was not observed in the positive control sections, the entire assay was discarded. Likewise, in order to ensure that artifacts associated with counterstaining were not counted, negative control brain sections were included along with each assay and treated as described above but were not incubated in the primary antibody.

The analysis of c-Fos immunoreactivity focused on GNC which is known to mediate CTA acquisition (Bermudez-Rattoni et al., 1987) and CTA extinction (Mickley et al., 2004). Further, SR of a CTA may be blocked by microinfusion of a β-adrenergic antagonist into the insular cortex (Berman et al., 2003). Analysis of medial prefrontal cortex (PrL and IL) was also included since this brain area has been implicated as mediating the extinction of several learned responses (Barrett et al., 2003; Herry and Garcia, 2002; Milad and Quirk, 2002) including CTA (Mickley et al., 2005). Further, SR of conditioned fear has been associated with sustained long-term depression (LTD) in prefrontal cortex (Herry and Garcia, 2002, 2003). Both the BLA and CE were included as they have known functional interconnections with cortical structures (see Likhtik et al., 2005, and review, above) and play a significant role in extinction (Maren and Quirk, 2004; Bahar et al., 2003).

Slides were viewed with 20× magnification using an Olympus™ BX-60 microscope equipped with an AxioCam MRc5 camera connected to a computer running Carl Zeiss AxioVision™ v. 4.5 software. Brain structures were identified consistent with the anatomical demarcations specified by Paxinos and Watson (1998). All coronal sections analyzed were at the following A/P coordinates: GNC: 1.70 mm anterior to bregma (see Fig. 3B); PrL and IL: 3.20 mm anterior to bregma (see Figs. 3D and F, respectively); BLA and CE: 2.3 mm posterior from bregma (see Fig. 5B and D, respectively). Although a single coronal section was analyzed per brain area for each animal, we have determined that this method produces cell counts not significantly different from those in close-by sections. Parametric studies indicate a high correlation [$r(15)=0.98$; $p<0.01$] between the cell counts in one of our brain sections and counts from the same brain area found in sections 80–160 μm anterior or posterior. Moreover, the cell counts in these close-by sections are not significantly different from one another.

A measurement frame of 342,100 μm^2 (676 μm × 506 μm) was used for cell counting mPFC and GNC. Entire AMY nuclei (BLA and CE) were counted in each coronal section selected. Cells were counted as expressing positive c-Fos protein immunoreactivity based on the visualization of a black, punctate, round, and uniformly stained neuronal nucleus. On a 4095-step gray scale (4095=clear; 0=opaque) we counted cells that had a mean density of 470.55 (±100.61, standard error of the mean; SEM) against a background density of 2370.10 (±213.28 SEM).

Table 3 – Number of brains included in the immunohistochemical analyses of each treatment group

	GNC	PrL	IL	BLA	CE
EXT	7	5	5	7	7
SR	12	12	12	11	11
Short-term CTA	4	9	9	6	7
Long-term CTA	7	7	7	7	7
EXT-CTRL	8	8	8	6	6
SR-CTRL	7	7	7	7	7
Short-term CTA CTRL	7	7	7	6	6
Long-term CTA CTRL	5	5	5	6	6

GNC=gustatory neocortex; PrL=prelimbic area of medial prefrontal cortex; IL=infralimbic area of medial prefrontal cortex; BLA=basolateral nucleus of the amygdala; CE=central nucleus of the amygdala.

The average c-Fos-labeled cell was 2.8 SD units darker than the background. The average diameter of cells included in this analysis was 7.25 (±0.19 SEM) μm. The observer (G.A.M.) was blind to the experimental condition of the rats.

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REFERENCES

Accolla, R., Bathellier, B., Peterson, C.C.H., Carleton, A., 2007. Differential spatial representation of taste modalities in the rat gustastory cortex. J. Neurosci. 27, 1396–1404.

Adamec, R., Shallow, T., 2000. Rodent Anxiety and kindling of the central amygdala and nucleus basalis. Physiol. Behav. 70, 177–187.

Aggleton, J.P., Petrides, M., Iversen, S.D., 1981. Differential effects of amygdaloid lesions on conditioned taste aversion learning in rats. Physiol. Behav. 27, 397–400.

Alheid, G.F., de Olmos, J.S., Beltramino, C.A., 1995. Amygdala and extended amygdala, In: Paxinos, G. (Ed.), The Rat Nervous System, 2nd edition. Academic Press, San Diego, pp. 495–578.

Baeyens, F., Eelen, P., Crombez, G., 1995. Pavlovian associations are forever: on classical conditioning and extinction. J. Psychophysiol. 9, 127–141.

Bahar, A., Samuel, A., Hazvi, S., Dudai, Y., 2003. The amygdalar circuit that acquires taste aversion memory differs from the circuit that extinguishes it. Eur. J. Neurosci. 17, 1527–1530.

Bahar, A., Dorfman, N., Dudai, Y., 2004. Amygdalar circuits required for either consolidation or extinction of taste aversion memory are not required for reconsolidation. Eur. J. Neurosci. 19, 1115–1118.

Barrett, D., Shumake, J., Jones, D., Gonzalez-Lima, F., 2003. Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. J. Neurosci. 23, 5740–5749.

Berman, D.E., Dudai, Y., 2001. Memory extinction, learning anew, and learning the new: dissociations in the molecular machinery of learning in cortex. Science 291, 2417–2419.

Berman, D.E., Hazvi, S., Stehberg, J., Bahar, A., Dudai, Y., 2003. Conflicting processes in the extinction of conditioned taste aversion: behavioral and molecular aspects of latency, apparent stagnation and spontaneous recovery. Learn. Mem. 10, 16–25.

Bermudez-Rattoni, F., McGaugh, J.L., 1991. Insular cortex and amygdala lesions differentially affect acquisition on inhibitory avoidance and conditioned taste aversion. Brain Res. 549, 165–170.

Bermudez-Rattoni, F., Grijalva, C.V., Kiefer, S.W., Garcia, J., 1986. Flavor-illness aversions: the role of the amygdala in the acquisition of taste-potentiated odor aversions. J. Physiol. Behav. 38, 503–508.

Bermudez-Rattoni, F., Fernandez, J., Sanchez, M.A., Aguilar Roblero, R., Drucker Colin, R., 1987. Fetal brain transplants induce recuperation of taste aversion learning. Brain Res. 416, 147–152.

Bouton, M.E., 1982. Lack of reinstatement of an extinguished taste aversion. Anim. Learn. Behav. 10, 233–241.

Bouton, M.E., Bolles, R.C., 1979. Contextual control of the extinction of conditioned fear. Learn. Motiv. 10, 445–466.

Bouton, M.E., Swartzentruber, D., 1991. Sources of relapse after extinction in Pavlovian and instrumental learning. Clin. Psychol. Rev. 11, 123–140.

Bremner, J.D., Staib, L.H., Kaloupek, D., Southwick, S.M., Stofer, R., Charney, D.S., 1999. Neural correlates of exposure to traumatic pictures and sounds in Vietnam combat veterans with and without post-traumatic stress disorder: a positron emission tomography study. Biol. Psychiatry 45, 806–816.

Coutureau, E., Killcross, S., 2003. Inactivation of the infralimbic prefrontal cortex reinstates goal-directed responding in overtrained rats. Behav. Brain Res. 146, 167–174.

Davis, M., Myers, K.M., 2002. The role of glutamate and gamma-aminobutyric acid in fear extinction: clinical implications for exposure therapy. Biol. Psychiatry 52, 998–1007.

Dudai, Y., 2006. Reconsolidation: the advantage of being refocused. Curr. Opin. Neurobiol. 16, 174–178.

Fisk, G.D., Wyss, J.M., 2000. Descending projections of infralimbic cortex that mediate stimulation-evoked changes in arterial pressure. Brain Res. 859, 83–95.

Garcia, J., Kimeldorf, D.J., Knelling, R.A., 1955. Conditioned aversion to saccharin resulting from exposure to gamma radiation. Science 122, 157–158.

Garcia, J., Kimeldorf, D.J., Hunt, E.L., 1961. The use of ionizing radiation as a motivating stimulus. Radiat. Res. 12, 719–727.

Garcia, R., Vouimba, R.M., Baudry, M., Thompson, R.F., 1999. The amygdala modulates prefrontal cortex activity relative to conditioned fear. Nature 402, 294–296.

Gewirtz, J., Falls, W., Davis, M., 1997. Normal conditioned inhibition and extinction of freezing and fear-potentiated startle following electrolytic lesions of medial prefrontal cortex. Behav. Neurosci. 111, 712–726.

Grey, S., Sartory, G., Rachman, S., 1979. Synchronous and dysynchronous changes during fear reduction. Behav. Res. Ther. 17, 137–147.

Harris, J.A., Westbrook, R.F., 1998. Evidence that GABA transmission mediates context-specific extinction of learned fear. Psychopharmacology 140, 105–115.

Herry, C., Garcia, R., 2002. Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice. J. Neurosci. 22, 577–583.

Herry, C., Garcia, R., 2003. Behavioral and paired-pulse facilitation analyses of long lasting depression at excitatory synapses in the medial prefrontal cortex. Behav. Brain Res. 146, 89–96.

Herry, C., Mons, N., 2004. Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. Eur. J. Neurosci. 20, 781–790.

Herrera, D.G., Robertson, H.A., 1996. Activation of c-*fos* in the brain. Prog. Neurobiol. 50, 83–107.

Houpt, T.A., Philopena, J.M., Joh, T.H., Smith, G.P., 1996a. C-fos induction in the rat nucleus of the solitary tract by intraoral

quinine infusion depends on prior contingent pairing of quinine and lithium chloride. Physiol. Behav. 60, 1535–1541.
Houpt, T.A., Philopena, J.M., Joh, T.H., Smith, G.P., 1996b. C-fos induction in the rat nucleus of the solitary tract correlates with the retention and forgetting of a conditioned taste aversion. Learn. Mem. 3, 25–30.
Hsu, S.M., Raine, L., Fangler, H., 1981. Use of avidin–biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.
Huang, T., Yan, J., Kang, Y., 2003. Role of the central amygdaloid nucleus in shaping the discharge of gustatory neurons in the rat parabrachial nucleus. Brain Res. Bull. 61, 443–452.
Hurley, K.M., Herbert, H., Moga, M.M., Saper, C.B., 1991. Efferent projections of the infralimbic cortex of the rat. J. Comp. Neurol. 308, 249–276.
Koren, D., Arnon, I., Klein, E., 2001. Long term course of chronic posttraumatic stress disorder in traffic accident victims: a three-year prospective follow-up study. Behav. Res. Ther. 39, 1449–1458.
Kraemer, P.J., Spear, N.E., 1992. The effect of nonreinforced stimulus exposure on the strength of a conditioned taste aversion as a function of retention interval: do latent inhibition and extinction involve a shared process? Anim. Learn. Behav. 20, 1–7.
Krettek, J., Price, J., 1977. Projections from the amygdaloid complex to the cerebral cortex and thalamus in the rat and cat. J. Comp. Neurol. 172, 687–722.
Lamprecht, R., Dudai, Y., 1996. Transient expression of c-*fos* in rat amygdala during training is required for encoding conditioned taste aversion memory. Learn. Mem. 3, 31–41.
LeDoux, J.E., 2000. Emotion circuits in the brain. Annu. Rev. Neurosci. 23, 155–184.
LeDoux, J.E., Cicchetti, P., Xagoraris, A., Romaniski, M., 1990. The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J. Neurosci. 10, 1062–1069.
Likhtik, E., Pelletier, J.G., Paz, R., Pare, D., 2005. Prefrontal control of the amygdala. J. Neurosci. 25, 7429–7437.
Lubow, R.E., De La Casa, L.G., 2002. Superlatent inhibition and spontaneous recovery: differential effects of pre- and postconditioning CS-alone presentations after long delays in different contexts. Anim. Learn. Behav. 30, 376–386.
Maren, S., 2005. Building and burying fear memories in the brain. Neuroscientist 11, 89–99.
Maren, S., Quirk, G.J., 2004. Neuronal signaling of fear memory. Nat. Rev., Neurosci. 5, 844–852.
McDonald, A.J., 1991. Organization of amygdaloid projections to the prefrontal cortex and associated striatum in the rat. Neuroscience 44, 1–14.
McDonald, A.J., 1998. Cortical pathways to the mammalian amygdala. Prog. Neurobiol. 55, 257–332.
McDonald, A.J., Mascagni, F., Guo, L., 1996. Projections of the medial and lateral prefrontal cortices to the amygdala: a phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience 71, 55–75.
Mickley, G.A., Kenmuir, C.L., McMullen, C.A., Yocom, A.M., Valentine, E.L., Dengler-Crish, C.M., Weber, B., Wellman, J.A., Remmers-Roeber, D.R., 2004. Dynamic processing of taste aversion extinction in the brain. Brain Res. 1061, 79–89.
Mickley, G.A., Kenmuir, C.L., Yocom, A.M., Wellman, J.A., Biada, J.M., 2005. A role for prefrontal cortex in extinction of a conditioned taste aversion. Brain Res. 105, 176–182.
Mickley, G.A., Hoxha, Z., Bacik, S., Kenmuir, C.L., Wellman, J.A., Biada, J.M., DiSorbo, A., 2006. Spontaneous Recovery (SR) of a Conditioned Taste Aversion (CTA) Differentially Alters Extinction-Induced Changes in c-Fos Protein Expression in rat Amygdala (AMY) and Neocortex. Program No. 752.7. 2006 Neuroscience Meeting Planner. Society for Neuroscience, Atlanta, GA. Online. http://www.abstractsonline.com/viewer/%3Fmkey%3D%257BD1974E76%252D28AF%252D4C1C%25D8AE8%252D4F73B56247A7%257D.
Milad, M.R., Quirk, G.J., 2002. Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420, 70–74.
Morgan, M., Romanski, L., LeDoux, J., 1993. Extinction of emotional learning: contribution of medial prefrontal cortex. Neurosci. Lett. 163, 109–113.
Nachman, M., Ashe, J., 1974. Effects of basolateral amygdala lesions on neophobia, learned taste aversions, and sodium appetite in rats. J. Comp. Physiol. Psychol. 87, 622–643.
Nolan, J., McCaughey, S.A., Giza, K.K., Rhinehart-Doty, J.A., Smith, J.C., Scott, T.R., 1997. Extinction of a conditioned taste aversion in rats: I. Behavioral effects. Physiol. Behav. 61, 319–323.
Norgren, R., 1995. Gustatory system. In: Paxinos, G. (Ed.), The Rat Nervous System. Academic Press, New York, pp. 751–771.
Paré, D., Quirk, G.J., LeDoux, J.E., 2004. New vistas on amygdala networks in conditioned fear. Neurophysiology 92, 1–9.
Parker, L.A., 2003. Taste avoidance and taste aversion: evidence for two different processes. Learn. Behav. 31, 165–172.
Pavlov, I.P., 1927. Conditioned Reflexes. Oxford Univ. Press, London. Trans. by G.V. Anrep.
Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, 4th ed. Academic Press, San Diego.
Quirk, G.J., 2002. Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery. Learn. Mem. 9, 402–407.
Quirk, G.J., Gehlert, D.R., 2003. Inhibition of the amygdala: key to pathological states? Ann. N. Y. Acad. Sci. 985, 263–272.
Quirk, G.J., Russo, G., Barron, J., Lebron, K., 2000. The role of ventromedial prefrontal cortex in the recovery of extinguished fear. J. Neurosci. 20, 6225–6231.
Quirk, G.J., Likhtik, E., Guillaume, J., Pare, D., 2003. Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. J. Neurosci. 23, 8800–8807.
Rachman, S., Lopatke, C., 1988. Return of fear: underlearning and overlearning. Behav. Res. Ther. 26, 99–104.
Rescorla, R.A. (Ed.), 1979. Conditioned Inhibition and Excitation. Erlbaum, Hillside, NJ.
Rescorla, R.A., 2001. Experimental extinction. In: Mowrer, R.R., Klein, S.B. (Eds.), Handbook of Contemporary Learning Theories. Lawrence Erlbaum, Mahwah, pp. 119–154.
Rescorla, R.A., 2004. Spontaneous recovery. Learn. Mem. 11, 501–509.
Rescorla, R.A., 2005. Spontaneous recovery of excitation but not inhibition. J. Exp. Psychol. 31, 277–288.
Rescorla, R.A., Cunningham, C.L., 1978. Recovery of the US representation over time during extinction. Learn. Motiv. 9, 373–391.
Richards, W.G., Farley, J., Alkon, D.L., 1984. Extinction of associative learning in Hermissenda: behavior and neural correlates. Behav. Brain Res. 14, 161–170.
Rinaman, L., Stricker, E.M., Hoffman, G.E., Verbalis, J.G., 1997. Central c-Fos expression in neonatal and adult rats after subcutaneous injection of hypertonic saline. Neuroscience 79, 1165–1175.
Rosas, J.M., Bouton, M.E., 1996. Spontaneous recovery after extinction of a conditioned taste aversion. Anim. Learn. Behav. 24, 341–348.
Royer, S., Paré, D., 2002. Bidirectional synaptic plasticity in intercalated amygdala neurons and the extinction of conditioned fear responses. Neuroscience 115, 455–462.
Santini, E., Muller, R.U., Quirk, G.J., 2001. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. J. Neurosci. 21, 9009–9017.
Santini, E., Burgos-Robles, A., Quirk, G.J., 2003. Protein Synthesis in the Medial Prefrontal Cortex is Required for Consolidation of Extinction Memory. 2003 Abstract Viewer/Itinerary Planner.

Society for Neuroscience. Online: http://web.sfn.org/ACSplash.cfm.
Santini, E., Ge, H., Ren, K., Pena, S., Quirk, G., 2004. Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. J. Neurosci. 24, 5704–5710.
Saper, C.B., 1995. Central autonomic system. In: Paxinos, G. (Ed.), The Rat Nervous System. Academic Press, New York, pp. 107–135.
Schafe, G.E., Thiele, T.E., Bernstein, I.L., 1998. Conditioning method dramatically alters the role of amygdala in taste aversion learning. Learn. Mem. 5, 481–492.
Sesack, S.R., Deutch, A.Y., Roth, R.H., Bunney, B.S., 1989. Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: and anterograde tract-tracing study with phaseolus vulgaris leucoagglutinin. J. Comp. Neurol. 290, 213–242.
Shin, L.M., Kosslyn, S.M., McNally, R.J., Alpert, N.M., Thompson, W.L., Rauch, S.L., Macklin, M.L., Pitman, P.K., 1997. Visual imagery and perception in post-traumatic stress disorder. A positron emission tomography investigation. Arch. Gen. Psychiatry 54, 233–241.
Sotres-Bayon, F., Bush, D.E.A., LeDoux, J.E., 2004. Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction. Learn. Mem. 11, 525–535.
Stollhoff, N., Menzel, R., Eisenhardt, D., 2005. Spontaneous recovery from extinction depends on the reconsolidation of the acquisition memory in an appetitive learning paradigm in the honey bee (*Apis mellifera*). J. Neurosci. 25, 4485–4492.
Swank, M.W., Ellis, A.E., Cochran, B.N., 1996. C-Fos antisense blocks acquisition and extinction of conditioned taste aversion in mice. NeuroReport 7, 1866–1870.
Takagishi, M., Chiba, T., 1991. Efferent projections of the infralimbic cortex (area 25) region of the medial prefrontal cortex in the rat: an anterograde tracer PHA-L study. Brain Res. 566, 26–39.
Taubenfeld, S.M., Milekic, M.H., Monti, B., Alberini, C.M., 2001. The consolidation of new but not reactivated memory requires hippocampal C/EBP beta. Nat. Neurosci. 4, 813–818.
Thomas, B.L., Ayres, J.J.B., 2004. Use of the ABA fear renewal paradigm to assess the effects of extinction with co-present fear inhibitors or excitors: Implications for theories of extinction and for treating human fears and phobias. Learn. Motiv. 35, 22–52.
Van der Kolk, B.A., 1994. The body keeps the score: memory and the evolving psychobiology of posttraumatic stress. Harv. Rev. Psychiatry 5, 253–265.
Wagner, A.R., Rescorla, R.A. (Eds.), 1974. Inhibition in Pavlovian Conditioning. Academic Press, London.
Watts, F.N., 1974. The control of spontaneous recovery of anxiety in imaginal desensitization. Behav. Res. Ther. 12, 57–59.
Wilkins, E.E., Bernstein, I.L., 2006. Conditioning method determines patterns of c-*fos* expression following novel taste-illness pairing. Behav. Brain Res. 169, 93–97.
Yamamoto, T., Fujimoto, Y., 1991. Brain mechanisms of taste aversion learning in the rat. Brain Res. Bull. 27, 403–406.
Yamamoto, T., Shimura, T., Sako, N., Yasoshima, Y., Sakai, N., 1994. Neural substrates for conditioned taste aversion in the rat. Behav. Brain Res. 65, 123–137.
Yasoshima, Y., Shimura, T., Yamamoto, T., 1995. Single unit responses of the amygdala after conditioned taste aversion in conscious rats. NeuroReport 6, 2424–2448.
Yehuda, R., 2001. Biology of posttraumatic stress disorder. J. Clin. Psychiatry 61 (Suppl. 17), 41–46.