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Spontaneous Recovery of Fear May Be Attenuated Without a Corresponding Change in *C-Fos* Expression in the Medial Prefrontal Cortex, Gustatory Neocortex, or Amygdala

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Abstract

A conditioned taste aversion (CTA) is acquired when an animal consumes a novel taste (CS) and then experiences the symptoms of poisoning (US). Following CTA training, animals will avoid the taste that was previously associated with malaise. This defensive reaction to a learned fear can be extinguished by repeated exposure to the CS alone. However, following a latency period in which the CS is not presented, the CTA will spontaneously recover (SR). Thomas *et al.* (2005) have used an explicitly unpaired (EU) extinction procedure that disassociates a light CS and footshock to thwart renewal of a conditioned emotional response. Here we applied similar procedures to the CTA paradigm and also evaluated the ability of EU extinction procedures to affect behavioral indicators of SR and *c-Fos* expression.

Fluid-deprived Sprague-Dawley rats acquired a CTA [3 pairings of 0.3% oral saccharin (SAC; the CS) and 81mg/kg i.p. lithium chloride (LiCl; the US)] followed by extinction trials consisting of multiple exposures to either, (a) CS-only, or (b) CS and US on alternate days (EU extinction). Both extinction procedures resulted in ≥90% reacceptance of SAC and were followed by a 30-day latency period of water drinking. Rats were then tested for SR with a final exposure to SAC before sacrificing. Brain *c-Fos* protein expression was evaluated via immunohistochemistry.

Rats in the CS-only group exhibited significant suppression of SAC drinking during their SR test compared to their consumption at the end of extinction. However, animals in the EU extinction group did not show such SR of the CTA and drank significantly more than the CS-only rats. The brains of EU-extinguished rats and CS-only extinguished rats did not differ in the number of *c-Fos*-labeled neurons in gustatory neocortex, medial prefrontal cortex, basolateral amygdala or the central nucleus of the amygdala. However some small, but reliable, differences were detected in Periaqueductal gray (PAG).

Thus, behavioral differences in SR between the EU and CS-only extinction animals were not represented by corresponding changes in the neural activity of several brain nuclei classically associated with extinction learning. However an analysis of PAG *c-Fos* expression may provide hints about some of the physiological changes evoked by these 2 extinction paradigms. The findings are clinically relevant as we seek the development of treatments for deficits in fear extinction (e.g. PTSD, phobias).

Introduction

- Fears may be acquired through associations of previously neutral stimuli with painful or aversive experiences.
  - Phobias
  - PTSD
- Fears may be reduced through various exposure therapies in which the object of fear (CS) is presented again, this time without the aversive stimuli (US), in an attempt to disassociate the CS – US connections (Foa, 2000).
- Fear extinction may be temporarily successful; however, spontaneous recovery (SR) and renewal of the fear (e.g. flashbacks) impede therapeutic progress (Bouton, 2002).
- Our laboratory has been studying a different model of learned fears – the conditioned taste aversion (CTA) paradigm – in which a novel taste (CS) is associated with the symptoms of poisoning (US) (Mickley et al., 2004; 2005).
- The resulting defensive aversion to, and avoidance of, the feared taste can slowly be extinguished by repeated exposure to the CS alone. However, this CS-only extinction procedure allows spontaneous recovery of the CTA (Mickley et al., 2007).
- In addition, we have used *c-Fos* immunohistochemical techniques to assess neural activity that might mediate CTA acquisition, extinction and SR (Mickley et al., 2004, 2005, 2007).
- *C-Fos* is a mediator of sensory experience and also may be instrumental in the associative aspect of a CTA (Lamprecht & Dudai, 1996).
- The current study sought to determine if, following acquisition of a CTA, employment of specifically unpaired presentations of the CS and US during extinction training (EU-EXT) might reduce or eliminate the spontaneous recovery of the CTA.
- Our previous work has focused on identifying C-Fos protein expression in brain areas important in CTA acquisition, extinction and SR (Mickley et al., 2004, 2005, 2007): medial prefrontal cortex, gustatory neocortex, and amygdala. However, the Periaqueductal gray (PAG) has also been recognized as a potentially important area for the extinction of learned fears (McNally et al., 2005) and was included in this analysis.

Methods

Subjects: Male, Sprague-Dawley rats

Conditioning

- Animals were habituated to a 23h water deprivation schedule for 2 days prior to the start of the experiment.
- **CTA animals:**
  - On days 1, 3, and 5 of CTA conditioning, animals were presented with a 0.3 % saccharin solution (SAC) for 30 min. During a 15 min latency animals were injected with lithium chloride (LiCl; 81mg/kg, i.p.) and subsequently presented with tap water for 30 min to prevent dehydration.
  - Days 2, 4, and 6 served as rest days during which the animals received two 30 min presentations of water separated by 15 min.
- **Explicitly Unpaired (control) animals:**
  - On experimental days 1, 3, and 5, rats were presented with the SAC for 30 min. Following a 15 min latency they were presented with water for 30 min.
  - On experimental days 2, 4, and 6, rats were presented with two 30 min presentations of water, separated by a 15 min latency during which LiCl (81mg/kg, i.p.) was administered.

Extinction

- **CS-Only Extinction:** Animals received 30 min SAC exposure and 15 min later were presented with water for 30 min every-other day until reaching asymptotic extinction (90% baseline SAC consumption levels; Nolan et al., 1997).
- **Explicitly Unpaired Extinction (EU-EXT):** Animals received 30 min SAC exposure followed 15 min later by 30 min water presentation every-other day of the extinction phase until reaching asymptotic extinction. On alternate days these animals received two 30 min presentations of water separated by a 15min latency during which LiCl (81mg/kg, i.p.) was administered.

Spontaneous Recovery (SR)

- After reaching asymptotic extinction, animals were daily given two 30 min presentations of water for 29 days.
- On day 30 following asymptotic extinction, animals were re-exposed to SAC for 30 min.

Table 1. Group Nomenclature and Treatments

	Group Designation	Conditioning: Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Extinction[3]		30-day SR test	Number of rats per group[4]
CTA	CS-Only Extinction	SAC[1]+LiCl[2]	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC		SAC	11 / 5 7/11
CTA	Explicitly Unpaired Extinction (EU-EXT)	SAC+LiCl	Water	SAC+LiCl	Water	SAC+LiCl	Water	SAC	Water+LiCl	SAC	12 / 6 5/5
EU	EU+ CS-Only	SAC	Water+LiCl	SAC	Water+LiCl	SAC	Water+LiCl	SAC		SAC	23
EU	EU+EU	SAC	Water+LiCl	SAC	Water+LiCl	SAC	Water+LiCl	SAC	Water+LiCl	SAC	12

[1]SAC = 30min exposure to the 0.3%SAC solution
[2]LiCl = Injection of lithium chloride (81mg/kg, i.p.)
[3]Extinction = the split cells for animals designated as EU Extinction refer to the 2 different treatments received on alternate days throughout the extinction phase; the single cells refer to the single treatment that was administered daily to animals designated as CS-Only Extinction.
[4]The first number indicates the total number of rats in each group. The second number represents the number of rats that had the spontaneous recovery test. The numbers on the second line within each cell represent the number of brains analyzed for *C-Fos* protein expression following the EXT test (first number) or SR test (second number).

Histology

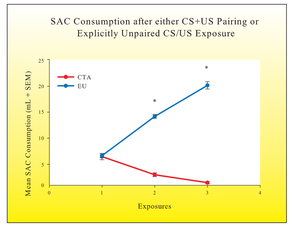
- Rats were transcardially perfused 90 min following their final SAC exposure.
- Brains were harvested and sliced at 40µm. Slices were collected and assayed for *c-fos* protein immunoreactivity, mounted and counterstained with neutral red. Cells staining positive for *C-fos* protein (only round, dark, uniformly stained cells) were counted. The observer was blind to the experimental condition of the rats (Hsu, 1981).
- Sections were viewed using light microscopy and Axiovision™ software. Nuclei were located using standard demarcations from The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1998). Brain regions were selected based on their role in fear extinction and spontaneous recovery.
  - ✓ *Brain Regions counted:* Medial Prefrontal Cortex (mPFC; prelimbic and infralimbic), Gustatory Neocortex (GNC), Amygdala (AMY; basolateral and central nuclei) and Periaqueductal gray (PAG).
- An α level of 0.05 was used to determine significance.

Results

CTA Acquisition

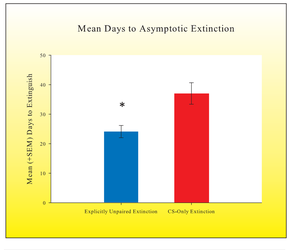
SAC Consumption After Either CS+US Pairing or Explicitly Unpaired CS/US Exposure:
The CTA group showed a significant decrease in the amount of saccharin (SAC) consumed over the three exposures. The Explicitly Unpaired (EU) group showed a significant increase in SAC consumption over the three CS/US exposures. This indicates that that CTA groups had acquired the CTA, whereas the EU (conditioning control groups) did not acquire a CTA. The SAC consumption of EU and CTA rats was the same on Exposure Day 1 but significantly different on exposure days 2 and 3. * = $p < 0.001$ (Bonferroni corrected *t*-tests)

A repeated measures ANOVA [Treatment (CTA or EU) x CS Exposure Day] revealed a significant main effect for Exposure Day [$F(1,70) = 80.982$; $p < 0.0001$] and Treatment [$F(1,70) = 413.25$; $p < 0.001$]. There was also a significant interaction [$F(1,70) = 381.743$; $p < 0.0001$].

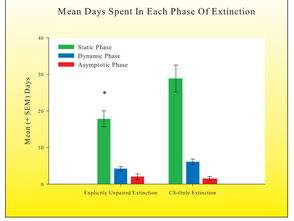


CTA Extinction

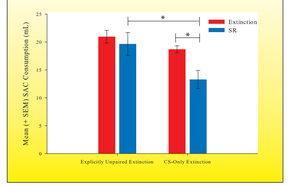
Mean Days To Extinguish a CTA:
The explicitly unpaired extinction group took significantly fewer days to extinguish the learned fear than the CS-only extinction group. * = Significantly different from the CS-Only Extinction Group: [$t(21) = 3.00$; $p = 0.007$]



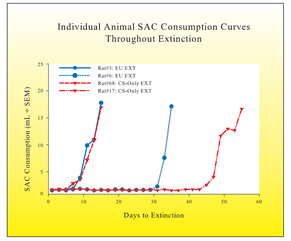
Mean Days In Each Phase Of Extinction:
Nolan et al. (1997) identified three phases of CTA extinction: static, dynamic and asymptotic. Rats experiencing the explicitly unpaired extinction procedure (EU-EXT) spent significantly fewer days in the static phase (SAC reacceptance < 10% of baseline) than the CS-only extinction group. The EU-EXT group and CS-only extinction group spent about the same number of days in the dynamic phase (SAC reacceptance ≥ 10 % but < 80% of baseline) and also the asymptotic phase (SAC reacceptance ≥ 80% of baseline). * = Significantly Different from CS-Only Extinction Group: [$t(21) = 2.52$; $p = 0.02$].



SAC Consumption on the Day of Asymptotic Extinction and Spontaneous Recovery Test:
The explicitly unpaired extinction group (EU-EXT) drank nearly the same amount of SAC on the day of extinction as they did on the 30-Day SR test day. The CS-only extinction group drank significantly more SAC on the day of extinction than on the day of the SR test [$t(5) = 2.72$; $p = 0.042$]. Likewise, SAC drinking at the SR test was significantly less in the CS-only rats than in the EU-EXT rats [$t(9) = 2.47$; $p = 0.036$]. This indicates that the CS-only extinction animals had a SR of the fear, but the EU-EXT group did not.

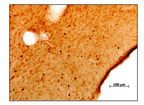


Individual Animal SAC Consumption Curves throughout Extinction:
The extinction curves of the slowest animal to extinguish and fastest animal to extinguish from each of the two main experimental groups (EU-EXT = Blue; CS-Only Extinction = Red). Rats in the EU-EXT group exhibited a smaller range of days to extinguish than did the CS-Only Extinction group.

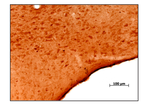


***C-Fos* Immunohistochemistry:**
No significant differences in *C-Fos* expression in mPFC (prelimbic or infralimbic), GNC, or Amygdala (basolateral or central nuclei) were observed between EU-EXT and CS-Only Extinction brains following the SR test.
The PAG from EU-EXT rats exhibited a small, but reliable, increase in *C-Fos* expression as compared to the PAG of CS-Only animals following the SR test.

Dorsolateral Periaqueductal gray (PAG) following SR test

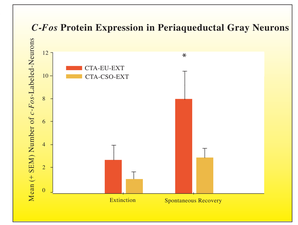


CTA-EU-EXT



CTA-CS-Only EXT

The EU-EXT procedure evokes more *C-Fos* expression in PAG than does the CS-Only Extinction procedure.



***C-Fos* expression in PAG at asymptotic extinction or Spontaneous Recovery tests.** Following full re-acceptance of the once aversive SAC (at asymptotic extinction) rats in the EU-EXT and CS-Only EXT groups did not differ in their PAG C-Fos expression. However, after the SR test, rats that underwent the EU-EXT procedure expressed more *C-Fos*-labeled neurons in the dorsolateral PAG than did CS-Only extinction control animals or CTA extinguished rats in either group. [$F(3,24) = 5.07$; $p < 0.005$]. Individual group comparisons revealed that the *C-Fos* expression in the PAG of CTA-EU-EXT rats was significantly higher than that from any other group. * $p < 0.05$

Summary & Conclusions

- Extinction learning that employed the EU-EXT procedure of disassociating the CS and US produced more rapid extinction of a CTA and also inhibited spontaneous recovery of this defensive reaction to a learned fear.
- Analyses of *C-Fos* protein expression indicate that there were no differences between the mPFC, GNC or AMY of the CS-Only brains and EU-EXT brains.
- *C-Fos* expression in PAG was low overall but the brains from the EU-EXT rats exhibited more C-Fos labeled cells than did the brains of the CS-Only rats.
- The use of EU-EXT procedures seems to produce consistent results across two pre-clinical paradigms that have employed experimental animals: CER (Thomas et al., 2005) and CTA (current study).
- Following further pre-clinical testing, health care providers treating disorders where fear is prominent may wish to consider how EU-EXT procedures may facilitate this therapy (Holmes et al., 2007; Basoglu et al., 2007).

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