Spontaneous Recovery (SR) of a Conditioned Taste Aversion (CTA) Reverses Extinction-Induced Changes in *c-Fos* Expression in Rat Gustatory Neocortex (GNC)

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Abstract:

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. SR of a CTA (a revival of the taste avoidance) occurs after a latency period in which the CS is not presented. This study investigated changes in GNC functioning during acquisition, extinction and SR of a CTA. Brain *c-Fos* protein expression was analyzed in fluid-deprived rats that had acquired a CTA [3 pairings of 0.3% oral saccharin (SAC) and 81mg/kg i.p. Lithium Chloride (LiCl)] followed by extinction training (i.e., subsequent non-reinforced SAC exposures) resulting in 90% reacceptance of SAC. Other animals were extinguished but were tested for SR of the CTA upon exposure to SAC following a 15-, 30-, 45- or 60-day recovery period of water drinking. Rats were sacrificed on the final day of SAC exposure and GNC *c-Fos* protein expression was evaluated. Animals allowed 30- or 60-day recovery periods exhibited a significant SR. The numbers of *c-Fos*-labeled neurons in GNC was low following CTA acquisition, increased dramatically as rats fully extinguished the aversion, and then declined significantly following SR. These data reveal behavioral parameters required to observe the SR of a CTA. Further, the *c-Fos* measurements suggest the dynamic nature of GNC activity during acquisition, extinction and SR of a CTA and further reinforce an important role for these neurons in the reorganization of learned information.

INTRODUCTION

- Conditioned Taste Aversions (CTAs) may be formed when an animal consumes a novel taste (CS) and then experiences the symptoms of poisoning (US).

- While a significant amount of work has been focused on how the brain establishes a CTA (see Yamamoto, 1993, *Neurosci. Res*., 16, 181-185, for review), relatively little has been done on how the brain adjusts during ***extinction*** of this classically conditioned response (Houpt et al., 1994, *Neurosci. Let*., 172, 1-5; Houpt et al.,1996, *Learn. & Mem*., 3, 25-30; Berman & Dudai, 2001, *Science*, 291, 2417-2419) or during ***spontaneous recovery*** of a CTA (Rosas & Bouton, 1996, *Anim. Learn. Behav*. 24, 341-348).

- Does the brain unlearn a CTA during extinction? Alternatively, does the brain retain the original information while also learning that it is no longer useful in the present context? During spontaneous recovery, does brain physiology return to the conditioned state?

- We used *c-Fos* protein immunohistochemical techniques to label neural activity. Evidence suggests that the expression of *c-Fos* (the protein product of the immediate early gene *c-fos*) not only mediates sensory experience but may also be instrumental in the associative aspects of a CTA (Lamprecht & Dudai, 1996, *Learn. & Mem*., 3, 31-41). This study sought to document if/how the number of *c-Fos*-labeled neurons would change during the course of extinction and spontaneous recovery.

- We measured behavioral responses during acquisition, extinction and spontaneous recovery of a CTA and analyzed correlated *c-Fos* expression in the Gustatory Neocortex (GNC). GNC is known to mediate CTA acquisition (Bermudez-Rattoni, 1987, *Brain Res.,* 416, 147-152) and CTA extinction (Mickley et al., 2004, *Brain Res*., 1016, 79-89).

- We also measured *c-Fos* expression in the Medial Prefrontal Cortex (mPFC) since this area of the brain has been implicated as mediating the extinction of several other learned responses (Barrett et al., 2003, *J. Neurosci*., 23, 5740-5749; Herry & Garcia, *J. Neurosci*., 2002, 22, 577-583; Milad & Quirk, *Nature*, 420, 70-74

METHOD

Subjects: Adult, male Sprague-Dawley rats.
Group Designations:
Table 1: Summary of Conditioning Procedures and Extinction/Spontaneous Recovery Timelines

Group Designation	Treatment Day 1	Treatment Day 2	Treatment Day 3	Treatment Day 4	Treatment Day 5	Treatment Day 6	Liquid Consumed from Day 7 until Sacrifice or end of Extinction period	Liquid consumed daily during Spontaneous Recovery period of 15, 30, 45 or 60 days	Liquid Consumed on the Day of Sacrifice
CTA Extinction (CTA + EXT)	SAC* + LiCl**	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, Full 60 minutes	SAC	N/A	SAC
CTA no Extinction (CTA +No EXT)	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	Water, full 60 minutes	N/A	SAC
Explicitly Unpaired Saccharin (EU + SAC)	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	SAC	N/A	SAC
Explicitly Unpaired No Saccharin (EU + No SAC)	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	Water, full 60 minutes	N/A	SAC
CTA Extinction with Spontaneous Recovery (CTA + EXT + SR)	SAC	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC	Water	SAC
Extended Extinction (CTA + EXT + SAC)	SAC	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC	SAC	SAC
CTA Control	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	SAC + LiCl	Water, full 60 minutes	N/A	N/A	SAC

*SAC = 0.3% sodium saccharin salt dissolved in water; followed by 30 minutes access to water; ** LiCl = 81mg/kg Lithium Chloride, i.p.

Table 2: Number of rats in each treatment group, stage of extinction or Spontaneous Recovery*:

Group Designation	*Static*	*Dynamic*	*Asymptotic*	*15 Day SR*	*30 Day SR*	*45 Day SR*	*60 Day SR*
CTA + EXT	9 (7)	8 (7)	9 (5)[5]				
CTA + No EXT	9 (8)	9 (6)	9 (4)				
EU + SAC	7 (5)	7 (5)	7(3)				
EU + No SAC	9 (5)	9 (5)	9 (6)				
CTA + EXT +SR				9	13(7)[7]	10	10
CTA + EXT +SAC				12 (0)	15 (3)[3]		
CTA Control	9 (7)						

*First number represents the number of rats included in the behavioral analyses. (The second number represents the number of rats in the GNC immunohistochemical analysis.) [The third number represents the number of rats in the mPFC immunohistochemical analysis.]

Procedures:

- All rats were water deprived for 23 hours per day for the duration of the experiment beginning two days prior to their conditioning trials.

Conditioned Taste Aversion (CTA) Procedure:

- The conditioning procedure included three conditioned stimulus (CS) + unconditioned stimulus (US) trials administered to each rat every other day (see Table 1). The trials were as follows:
    - For rats in the CTA groups (the CS & US were paired to create an aversion to the CS), the CTA was established by oral presentation of 0.3% Saccharin Sodium Salt (SAC) (this is the CS) followed by an 81 mg/kg Lithium Chloride (LiCl) injection, (i.p.) (US).
    - For rats in the Explicitly Unpaired groups (the CS & US were unpaired to avoid the formation of a taste aversion), SAC was presented for 30 minutes followed 24 hours later by a LiCl injection.
    - An additional control group (CTA Controls) included rats that received 3 CTA conditioning trials over 6 days, SAC on day 7, and were then sacrificed 90 minutes thereafter.

Extinction Procedure:

- Baseline drinking was computed by taking an average of familiar SAC drinking from similarly sized rats.
- Rats in the extinction groups were presented with SAC for 30 minutes daily (supplemented by water for 30 minutes) until their drinking met one of the predetermined criteria (see nomenclature proposed by Nolan et al., 1997, *Physiol. & Behav.*, 14, 161-170):
    - *Static Phase*: 10% baseline drinking
    - *Dynamic Phase*: 40% baseline drinking
    - *Asymptotic Phase*: 90% baseline drinking
- On the day that criterion was met, the rat (along with its yoked control; i.e., CTA + No EXT animal of similar weight) was sacrificed following its SAC drinking.
- Rats in the "CTA + No EXT" and "EU + No SAC" groups were presented with water for the full 60 minutes each day until the CTA + EXT rat to which they were yoked met one of the extinction criteria. On that day, these "no SAC" controls were still presented with water for their first 30 minutes. However, they were then presented with SAC for 30 minutes.
- After rats in the spontaneous recovery groups had extinguished to asymptote (90% of baseline drinking) they received water each day for 15, 30, 45 or 60 days before they had a last, single 30-minute re-exposure to SAC.

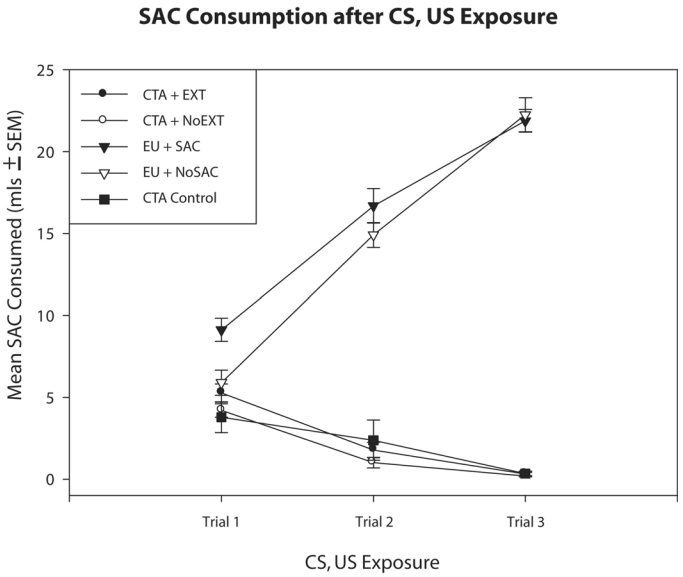
Histology:

- Rats were sacrificed 90 minutes following their last SAC exposure. Brain sections were collected and assayed for c-fos protein immunoreactivity (Hsu et.al., 1981, *Am. J. Clin. Pathol.*, 75, 734-738 & Hsu et.al., 1981, *J. Histochem. Cytochem.,* 29, 577-280).
- Brain nuclei were located using standard demarcations from The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1998, 4th ed.). Sub-nuclei were selected based on their role in gustation [see: Paxinos, (Ed.) The Rat Nervous System, 1995].
- *Brain Nuclei counted: G*ustatory Neocortex (GNC); Medial Prefrontal Cortex (mPFC)
- Cells staining positive for *c-Fos* protein (only round, dark, uniformly stained cells) were counted per brain nucleus. The observers were blind to the experimental condition of the rats.

RESULTS

CTA Acquisition

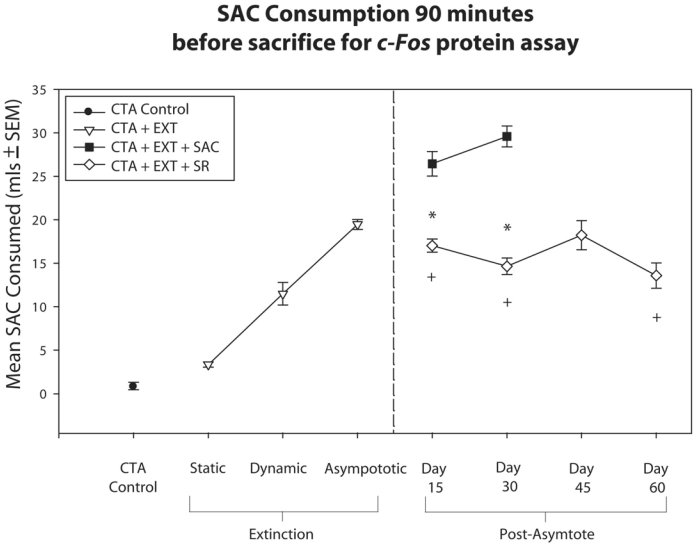
Fig. 1. Saccharin drinking during the formation of a CTA and exposure to Explicitly Unpaired CS and US: SAC consumption in both the Explicitly Unpaired (EU) CS, US groups increased over the course of the 3 trials indicating that these rats did not acquire a CTA. Conversely, SAC consumption in all of the CTA groups (CTA + Extinction, CTA + No EXT & CTA Controls) decreased over the 3 trials indicating that these rats acquired a CTA.



Saccharin (SAC, the CS) consumption after CS, US (81 mg/kg Lithium Chloride, i.p.) exposure. A repeated measures ANOVA [Treatment (CTA or EU) X Trial] revealed a significant treatment effect [$F(1,107) = 540.61$, $p < 0.001$], a significant change in SAC drinking over trials [$F(1,107) = 42.60$, $p < 0.001$], and a significant interaction [$F(1,107) = 197.18$, $p < 0.001$]. These data represent a reliable decline in SAC drinking in rats that received CS-US pairings [$F(1,35) = 24.10$; $p < 0.001$] and a reliable rise in SAC drinking in the EU animals [$F(1,50) = 234.56$; $p < 0.001$]. Variance indicators are the S.E.M.

Extinction and Spontaneous Recovery of a CTA

Fig. 2: Rats avoided SAC when it was previously associated with LiCl. But this aversion extinguished after subsequent, non-reinforced SAC exposures. Spontaneous Recovery of a CTA (i.e., a significant decrease in SAC drinking) follows a latency of 15, 30 or 60 days of daily water consumption.

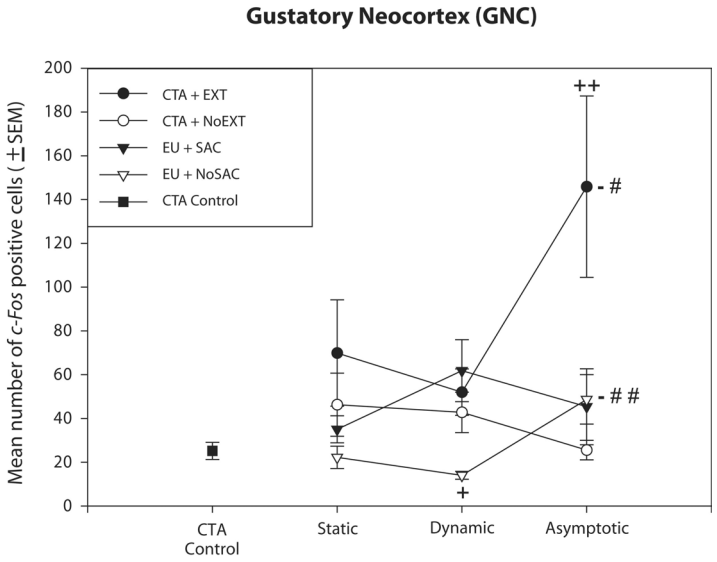


+ = Significant decrease in amount of SAC consumed as compared to the asymptotic level of extinction. [Day 15: $t(16) = 2.584$, $p = 0.01$; Day 30: $t(20) = 3.89$, $p < 0.001$; Day 45: NS; Day 60: $t(17) = 3.610$, $p = 0.001$]

* = Significant decrease in amount of SAC consumed as compared to the CTA + EXT + SAC (extended extinction control) group. [Two-way ANOVA (SR or Extended EXT X 15 or 30 recovery): $F(1,45) = 43.25$, $p < 0.001$]. Variance indicators are the S.E.M.

c-Fos Expression in GNC During Extinction of a CTA

Fig. 3: As compared to controls, extinction of a CTA causes an increase in the number of *c-Fos*-labeled neurons in GNC - but only during the **Asymptotic** stage of extinction.



The statistical analysis revealed significant main effects for Extinction treatment [F (1,55) 9.98, p = 0.003] and Learning treatment [F (1,55) = 5.42, p = 0.024] as well as a significant 3-way interaction (Extinction level X Extinction treatment X Learning treatment) [F (2,55) = 4.26, p = 0.018]. Post-hoc tests ($p < 0.05$):

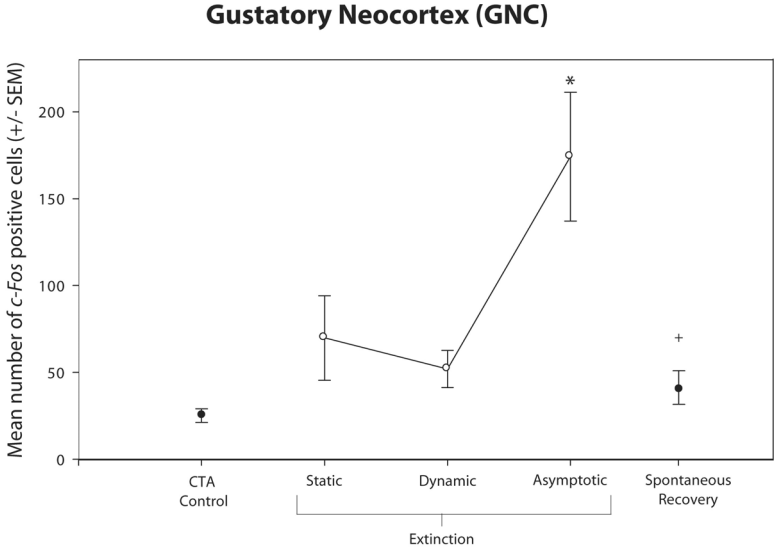
+ = Significantly different from EU + SAC group.
++ = Significantly different from CTA + No EXT group.
# = Treatment group shows a significant increase in *c-Fos* expression (CTA Control to Asymptotic and Dynamic to Asymptotic) depending on the stage of extinction.

= EU + No SAC group shows a significant increase in *c-Fos* expression from "Dynamic" to "Asymptotic" stages of the study.

Variance indicators are the S.E.M.

c-Fos Expression in GNC during Extinction and Spontaneous Recovery of a CTA

Fig. 4. Spontaneous Recovery of a CTA (following a 30-day latency) caused a significant decrease** in the expression of *c-Fos* in the GNC.



** In line with the criteria set up by (Rosas & Bouton, 1996, *Anim. Learn. Behav*. 24, 341-348), this analysis included only animals that decreased their SAC drinking ≥ 20%.

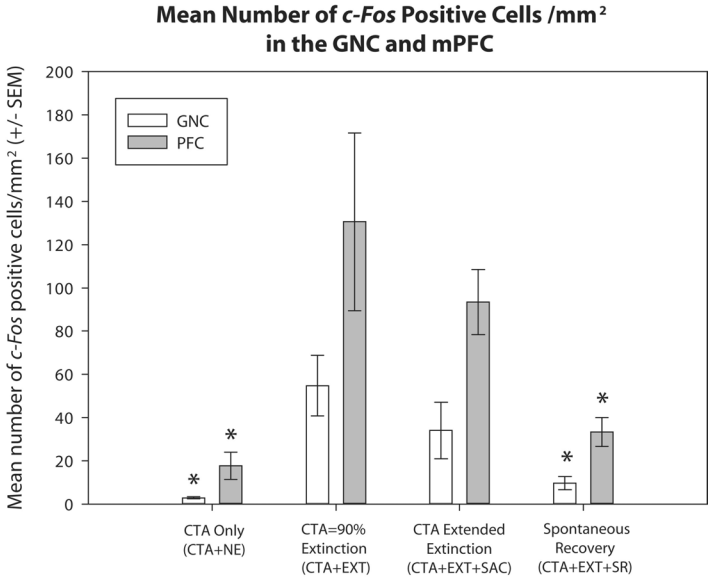
One-Way ANOVA (CTA controls, Static, Dynamic, Asymptotic, SR): $F(4,26) = 8.045$, $p < 0.001$. Tukey post hocs, $p < 0.05$.

* = Significantly different from CTA controls, Static and Dynamic stages of EXT.
+ = Significantly different from Asymptotic stage of EXT.
Variance indicators are the S.E.M.

Cell densities in GNC and mPFC following Extinction and Spontaneous Recovery of a CTA

Fig. 5: As compared to rats that had an extinguished CTA, spontaneous recovery of a CTA (following a 30-day latency) was accompanied by a significant drop in the density of *c-Fos*-labeled neurons in the both the GNC and mPFC.

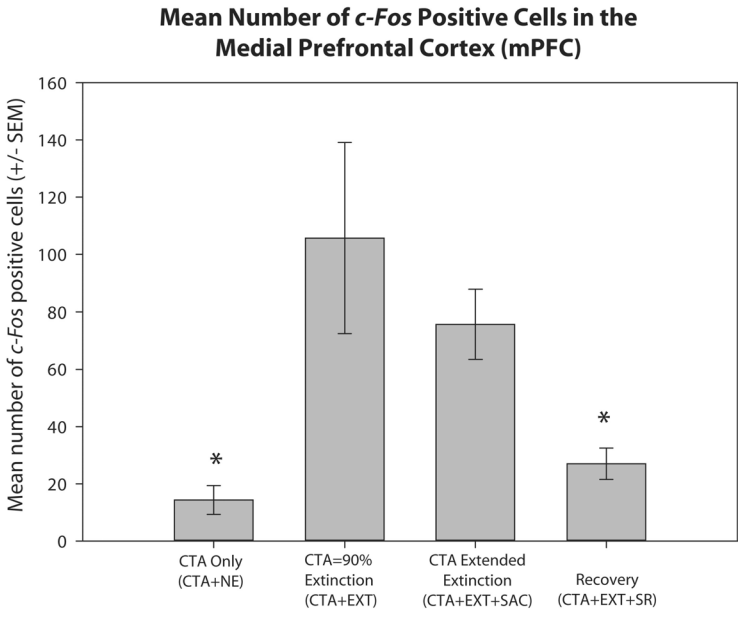
Densities of *c-Fos* positive cells were similar for rats that had retained a strong CTA and those that spontaneously recovered following extinction of a CTA.



* = significantly different from respective extinguished controls (CTA+EXT and CTA+EXT+SAC): Two-way ANOVA [Brain area (GNC, mPFC) X Treatment (CTA+NE, CTA+EXT, CTA+EXT+SAC, CTA+EXT+SR)]; $F_{Treatment}$ (3,26) = 11.08; $p < 0.001$; Post-hoc t-tests ($p < 0.05$). Variance indicators are the S.E.M.

c-Fos Expression in mPFC during Extinction and Spontaneous Recovery of a CTA

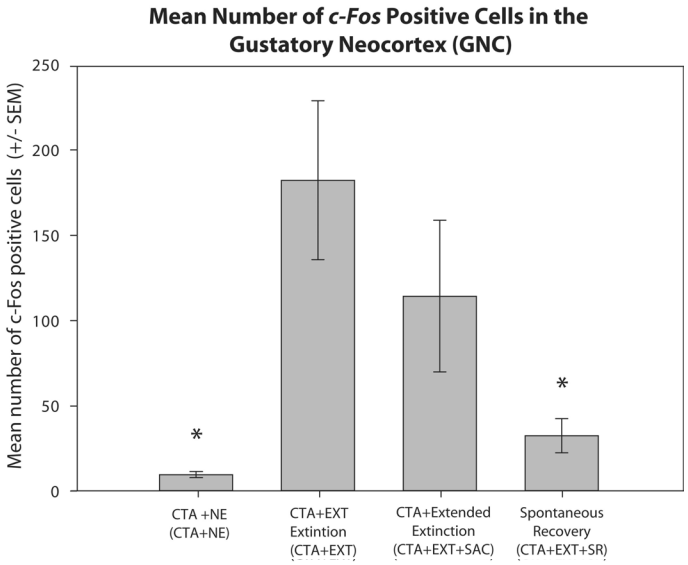
Fig. 6: Extinction of a CTA caused an increase in the number of *c-Fos* positive cells in the mPFC. Spontaneous recovery of a CTA (following a 30-day latency) produced a significant drop in the number of *c-Fos*-labeled cells in mPFC – numbers that paralleled those observed in animals with full-blown CTAs.



* = significantly different from extinguished controls (CTA+EXT and CTA+EXT+SAC) One-way ANOVA; $F_{Treatment}$ (3,13) = 6.081; p = 0.008; Post-hoc t-tests ($p < 0.05$). Variance indicators are the S.E.M.

c-Fos Expression in GNC during Extinction andSpontaneous Recovery of a CTA

Fig. 7: Extinction of a CTA caused an increase in the number of *c-Fos* positive cells in the GNC. Spontaneous recovery of a CTA (following a 30-day latency) produced a significant drop in the number of *c-Fos*-labeled cells in GNC – numbers that paralleled those observed in animals with full-blown CTAs.



* = significantly different from extinguished controls (CTA+EXT and CTA+EXT+SAC) One-way ANOVA; $F_{Treatment}$ (3,13) = 7.816; p = 0.003; Post-hoc t-tests ($p < 0.05$). Variance indicators are the S.E.M.

SUMMARY & CONCLUSIONS

- GNC c-*Fos* expression in the brains of CTA-EXT rats was differentiated from that of the CTA controls suggesting the brain encodes extinction in a distinctive way.
- Compared to controls, rats in the CTA + EXT group expressed relatively few *c-Fos*-positive cells in the GNC during the initial stages of extinction but significant increases as the animals move towards near-complete reacceptance of the SAC (Asymptotic stage of extinction).
- These changes in GNC activity are apparently not a reflection of volume of SAC consumed since EU control rats, drinking similar volumes, did not exhibit a rise in *c-Fos*-labeled cells.
- During Spontaneous Recovery, *c-Fos* expression in both GNC and mPFC neurons was significantly lower than on the last extinction day.
- These data reflect a dynamic role for the GNC in acquisition, extinction, and Spontaneous Recovery of a CTA.
- By indicating that mPFC neuronal activity is altered by extinction of a CTA, our observations extend an already established role for mPFC in extinction. Further, a role for mPFC in Spontaneous Recovery of a CTA is also suggested by our data.

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