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Short Communication

A role for prefrontal cortex in the extinction of a conditioned taste aversion

G. Andrew Mickley*, Cynthia L. Kenmuir, Anna M. Yocom, Justin A. Wellman, Jaclyn M. Biada

Department of Psychology and the Neuroscience Program, Baldwin-Wallace College, 275 Eastland Road, Berea, OH 44017-2088, USA

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Abstract

This study used immunohistochemical methods to determine if the medial prefrontal cortex (mPFC) is involved in the extinction of a conditioned taste aversion (CTA). As rats reached 90% reacceptance of a tastant (saccharin: SAC) that had previously been associated with lithium chloride-induced malaise, c-*Fos* protein expression increased dramatically as compared to animals with active CTAs, animals without CTAs (i.e., explicitly unpaired CS-US exposures) or animals drinking SAC for the first time. These data indicate a role for mPFC (prelimbic and infralimbic cortex) in the formation of a CTA extinction memory.

Theme: Neural basis of behavior
Topic: Learning and memory: physiology

Keywords: Extinction; c-*fos*; Learning; Memory; Conditioned taste aversion; Immunohistochemistry; Taste; Neuroanatomy; Medial prefrontal cortex

Our laboratory has been studying the neural substrates of acquisition, extinction and spontaneous recovery of a conditioned taste aversion (CTA) [24]. Animals learn CTA by associating a novel taste (conditioned stimulus: CS) with the malaise (unconditioned stimulus: US) that follows poisoning [9]. The initially profound avoidance of the CS may be gradually reduced or eliminated upon subsequent non-reinforced samplings of the taste [24,28]. In the context of classical conditioning, this decline in the intensity of the conditioned avoidance following the withdrawal of the unconditioned stimulus represents extinction of the CTA [27].

Although once conceptualized as an elimination of the original CTA memory [32], extinction is now generally believed to be a separate learned response [27]. Studies hoping to address the neural substrates of CTA extinction learning have primarily focused on the neuronal pathways that are known to be involved in CTA acquisition [3,14,24]. However, a parallel literature has, more generally, addressed the neural basis of fear extinction. These studies have highlighted, for example, an important role for the medial prefrontal cortex (mPFC) in the extinction of conditioned emotional responses [2,25,26,31,36]. Stimulation of the infralimbic portion of mPFC reduces freezing to tones that have come to predict shock [25], and inclusive lesions of mPFC impair extinction learning [26]. However, some mPFC lesions have not disrupted the extinction of conditioned freezing or fear-potentiated startle [10].

We have reported that gustatory neocortex (GNC) is involved in the extinction of CTA—especially in the latter stages of the process [24]. While others have suggested that mPFC (but not GNC) mediates the extinction of conditioned fear [36,38]. In an attempt to determine the involvement of mPFC in extinction learning more generally, we measured the expression of the protein product of the c-*fos* gene (as a marker of neural activity) [11,33] in this brain area following the extinction of CTA. We report that c-*Fos* protein expression increases dramatically in both prelimbic and infralimbic portions of mPFC when rats have extinguished a CTA and reaccept a once-aversive taste.

The subjects were male Sprague–Dawley rats (453.48 ± 6.2 g at the beginning of the study) supplied by Zivic Laboratories (Zelienople, PA). The animals were individually housed in plastic "shoe box" cages (44.45 cm × 21.59

* Corresponding author. Fax: +1 440 826 3577.
E-mail address: amickley@bw.edu (G.A. Mickley).

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cm × 20.32 cm high). Throughout the experiment, home cage temperature was maintained at 23–26 °C under a 12:12 h light:dark cycle (lights on at 06:00 h). Rodent chow (Purina 5001) was available ad libitum. Two days prior to the conditioning trials, all animals were placed on a 23-h water deprivation cycle. To this effect, the subjects received a 50-ml bottle of tap water for 1 h beginning at 12:00 h. On all subsequent days, rats were given access to a 50-ml bottle of fluid at 12:00 h (tap water or 0.3% sodium saccharin solution [SAC] depending on the experimental condition). A 0.3% concentration of SAC was chosen due to its high hedonic value in adult rats [23]. Daily fluid consumption was recorded to the nearest tenth of a gram.

This experiment employed six treatment groups: the main experimental group and five control groups (see Table 1 for a summary of the naming conventions, timelines, procedures and number of subjects in each group). The conditioned taste aversion extinction group (CTA + EXT) received three CTA conditioning trials: one every other day (on experimental days 1, 3 and 5) for a total of six days. Each trial began at 12:00 noon and consisted of a 30-min exposure to SAC (novel on the first day) as the conditioned stimulus (CS) paired with a malaise inducing i.p. injection of 81 mg/kg lithium chloride (LiCl) as the unconditioned stimulus (US). To prevent dehydration and weight loss, the animals received an additional 30 min of access to tap water at 12:45 h on each day of conditioning training. For the same reason, rats were given 60 min of access to tap water beginning at 12:00 h on days 2, 4, and 6.

Starting on experimental day 7, CTA + EXT rats were given 30 min of access to SAC at 12:00–12:30 h followed 15 min later by 30 min of access to tap water at 12:45–13:15 h. This was repeated daily until animals reached 90% of baseline SAC drinking (asymptotic extinction) [28] as determined by calculating the average amount of SAC drinking (mean = 17.44 g) from an independent group of age- and weight-matched, 23-h water-deprived rats who consumed SAC for 30 min on their second day of SAC drinking (i.e., non-naive consumption). On the day that each CTA + EXT animal reached its extinction criterion, it was perfused at 14:00 h (90 min following the last SAC exposure) and its brain was prepared for c-*Fos* protein immunohistochemistry (see c-*Fos* protein assay procedures below).

The CTA, no extinction control group (CTA + No EXT) received identical conditioning training as the CTA extinction animals (SAC + LiCl pairings on experimental days 1, 3 and 5). However, on the 7th day and each day thereafter, they were given access to only water for 60 min (12:00–12:30 h and 12:45–13:15 h). Each CTA + No EXT rat was randomly paired (i.e., yoked) to a rat in the CTA + EXT group so that on the day that a CTA + EXT rat reached its extinction criterion, the yoked CTA + No EXT rat was also given access to SAC and perfused. Therefore, on the day that a CTA + No EXT rat's paired CTA + EXT rat reached its criterion, the CTA + No EXT rat received access to water

Table 1
Summary of conditioning procedures and extinction timelines

Group designation	Number of rats	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	Liquid consumed on the day of sacrifice
CTA Extinction (CTA + EXT)	5	SAC[a] + LiCl[b]	Water, full 60 min	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	SAC	SAC
CTA no extinction (CTA + No EXT)	9	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	Water, full 60 min	SAC
Explicitly unpaired saccharin (EU + SAC)	8	SAC	LiCl and water, full 60 min	SAC	LiCl and Water, full 60 min	SAC	LiCl and Water, full 60 min	SAC	SAC
Explicitly unpaired no saccharin (EU + No SAC)	7	SAC	LiCl and water, full 60 min	SAC	LiCl and Water, full 60 min	SAC	LiCl and Water, full 60 min	Water, full 60 min	SAC
CTA control (CC)	6	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	–	SAC
SAC only (SAC)	7	SAC	–	–	–	–	–	–	SAC

[a] SAC = 0.3% sodium saccharin salt dissolved in water; followed by 30-min access to water.
[b] LiCl = 81 mg/kg lithium chloride, i.p.

for 30 min (12:00–12:30 h), access to SAC for 30 min (12:45–13:15 h), and then was perfused 90 min after his last SAC exposure (14:45 h).

The CTA Control group received the same conditioning training as the CTA + EXT and CTA + No EXT groups but were perfused on day 7 (90 min after their last SAC exposure). This was done to document the brain c-*Fos* levels in animals that were sacrificed immediately after CTA acquisition.

To control for the unconditioned exposure to the CS and US, two additional groups were included in the study. Instead of receiving the CTA conditioning trials, these control groups experienced the effects of the CS and US "explicitly unpaired" from one another (i.e., separated by 24 h). This procedure for non-contingent presentation of CS and US has been shown to inhibit the production of CTA [40] as was confirmed by our own data (see Fig. 1). On days 1, 3, and 5, the explicitly unpaired saccharin animals (EU + SAC) were given access to SAC for 30 min. In order to reduce the likelihood of a CS–US association, LiCl injections were administered 24 h later (on experimental days 2, 4 and 6). On the 7th day and each day thereafter, EU + SAC animals received SAC in a matched amount to that consumed by a CTA + EXT animal to which they were yoked. Ninety minutes after their last SAC exposure, these EU + SAC rats were perfused.

Explicitly unpaired no-saccharin (EU + No SAC) animals received conditioning training in the same manner as in the EU + SAC group (SAC on days 1, 3 and 5 with LiCl injections 24 h later on days 2, 4 and 6). On the 7th day and each day thereafter, EU + No SAC animals received water (as in the CTA + No EXT group). On the day that a particular CTA + EXT animal met its criterion, the yoked EU + No SAC rat was given SAC (in a matched amount to what the CTA + EXT rat consumed) and was perfused 90 min after the last SAC exposure. Matching the SAC volumes consumed by the CTA + EXT animals to the EU + No SAC rats on the day of sacrifice (by limiting the time these animals had access to the drinking bottles) reduced the chance that differences in c-*Fos* expression between the experimental and control groups may be attributed to differences in thirst.

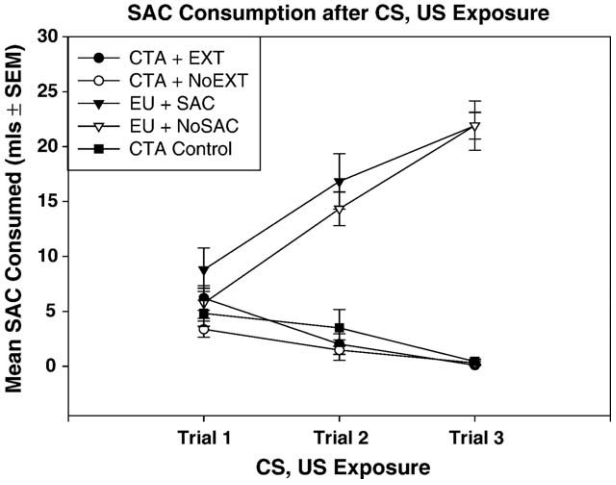

Fig. 1. Saccharin (SAC 0.3% = CS) consumption on the 3 conditioning days. See Table 1 for the group treatments and nomenclature. Day 1 data represent initial (neophobic) SAC drinking before the administration of the US (81 mg/kg Lithium Chloride, i.p.) to the conditioned animals. SAC consumption in both the explicitly unpaired (EU) CS and US groups increased over the course of the 3 trials, indicating that these rats did not acquire CTA. Conversely, SAC consumption in all of the CTA groups (CTA + Extinction, CTA + No EXT, and CTA Controls) decreased over the 3 trials, indicating that these rats acquired CTA. Variance indicators are the SEM.

Although EU rats drank water and then experienced a LiCl injection on days 2, 4, and 6, they did not form an aversion to this very familiar liquid. The volume of water consumed by the EU + SAC and EU + No SAC rats remained stable before and after the EU treatments.

A final control group (SAC only) allowed us to determine the neural representation associated with the sensation of drinking novel SAC. Before the study started, these animals were placed on 23-h water deprivation for 2 days as described above. On day 1 of the study they were allowed access to SAC (single bottle test) for 1/2 h and were then perfused 90 min later.

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 CTA [15,16,21,39]. All rats in the current study were sacrificed 90 min following the end of their last SAC exposure, since c-*Fos* protein expression by the c-*fos* gene is highest between 90 and 120 min after post-synaptic neuronal activity [11]. In order to control for c-*Fos* expression that may be directly due to the sensation of a sweet taste, all rats were given SAC before perfusion. The amounts of SAC consumed by rats in the EU groups were artificially matched to that of the CTA + EXT group by limiting the time these animals had access to the 50 ml bottles. Before perfusion, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p. injection). Each rat was intercardially perfused with heparinized saline followed by 4% paraformaldehyde. Their brains were immediately dissected and placed in a 118.5 ml polystyrene jar filled with 4% paraformaldehyde and kept at a temperature of ~4° C. Approximately 8 h later, the brains were transferred to a polystyrene jar containing a cryoprotectant solution (30% sucrose mixed in phosphate buffer with 0.01% thimerosal) and kept at ~4 °C until they were sliced. Using a freezing microtome, brains were coronally sectioned at 40 μm. All slices were stored in vials containing phosphate buffered saline with 0.2% sodium azide until they were assayed. The brain sections were assayed for c-*Fos* protein immunoreactivity as previously described [17,24] and then mounted onto slides. [Note: We failed to see c-*Fos* protein expression in the positive control sections run in the assay along with brain sections from four rats in the CTA + EXT group.

Therefore, these animals were excluded from the analysis. The number of subjects represented in Table 1 represents the animals used in the statistical computations.]

Using a 546-nm filter, slides were viewed via an Olympus™ microscope connected to a computer running NIH Image software. Selecting a coronal section 3.2 mm anterior to the bregma, we counted immunoreactive neurons in the prelimbic and infralimbic portions of the mPFC (each area = 0.81 ± 0.02 mm^2). The brain structures were identified consistent with the anatomical demarcations specified by Paxinos and Watson [30]. We selected this particular anterior–posterior plane since sections from the anterior portion of mPFC contain both infralimbic and prelimbic cortices. Cell densities (see Fig. 2) were calculated by dividing the cell counts by the area analyzed. To ensure that the cell counts from this section analyzed were similar to those in other sections from the same brain, we counted c-*Fos*-labeled cells in an additional brain section from each of the rats in the main experimental group (CTA + EXT) (i.e., the group showing the most variability) and then additional sections chosen at random from each of the control groups (total $N = 13$). The cell counts from the 2 brain sections of the 2 portions of mPFC analyzed were highly correlated (prelimbic cortex: $r(11) = 0.999$, $P < 0.001$; infralimbic cortex: $r(11) = 0.970$, $P < 0.001$).

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 255-step gray scale (0 = clear; 255 = opaque), we counted cells that had a mean density of 230.55 (±7.67, standard deviation; SD) against a background density of 91.60 (± 38.10 SD). The average c-*Fos*-labeled cell was 3.3 SD units darker than the background. The observer (G.A.M.) was blind to the experimental condition of the rats. See Fig. 3.

SAC consumption was recorded on a daily basis during the conditioning days. The data show that all rats in the three CTA groups acquired a strong CTA by the end of the third conditioning trial. Additionally, the two explicitly unpaired (EU) groups (where the CS and US exposures were non-contingent; see description below) had not acquired a CTA by the end of this same "conditioning" period (Fig. 1). A repeated measures ANOVA [Conditioning treatment (CTA or EU) × Extinction treatment (Extinction/SAC or No Extinction/water) × Conditioning Trial (Trial 1, 2 or 3)] revealed a significant conditioning treatment effect

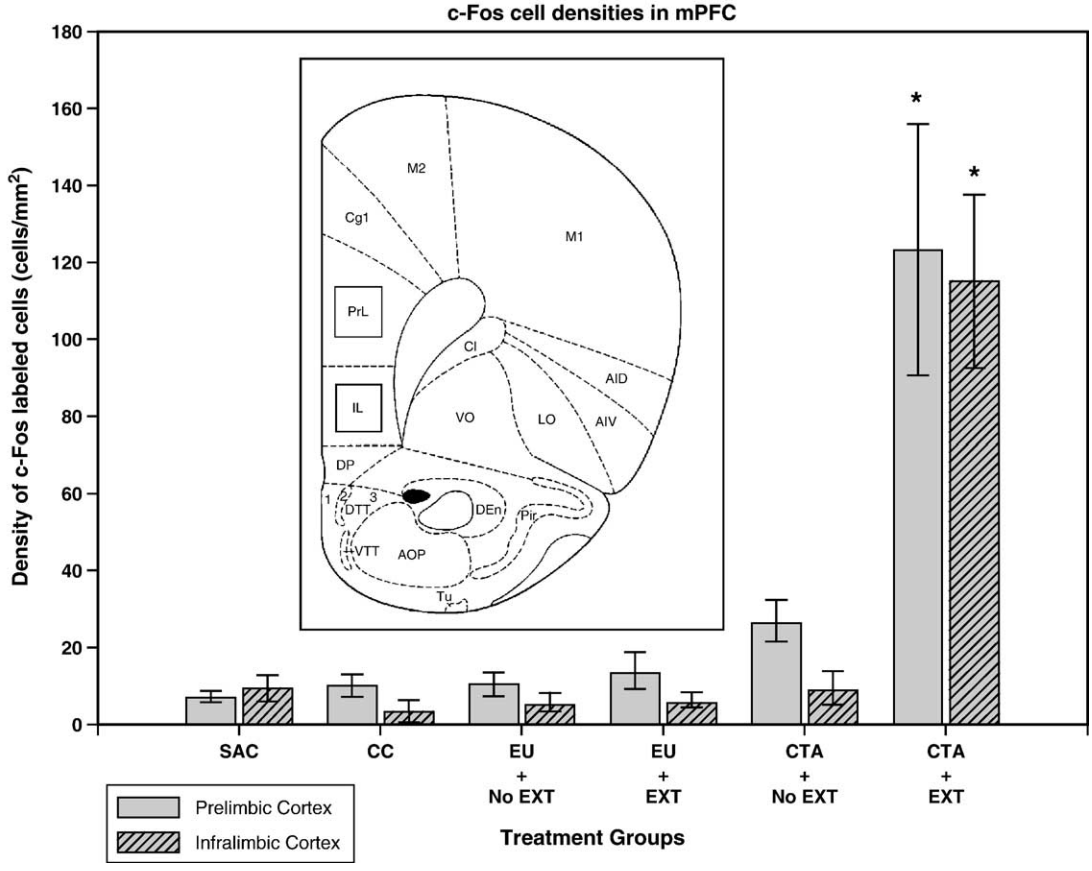

Fig. 2. Mean ± SEM densities of cells expressing c-*Fos* protein immunoreactivity in either prelimbic (PrL) or infralimbic (IL) portions of mPFC following CTA conditioning and extinction (CTA + EXT) or various control treatments. See Table 1 for the group treatments and nomenclature. The densities of c-*Fos*-labeled neurons in both portions of mPFC were significantly higher in CTA + EXT animals than in any of the control groups. [*Significantly different ($P < 0.05$) from all other groups within the same brain area analysis.] PrL and IL cells were counted in a coronal brain slice 3.2 mm anterior to bregma (illustration is adapted from Paxinos and Watson [30]). The area counted/brain area was 0.81 mm^2 (see boxes).

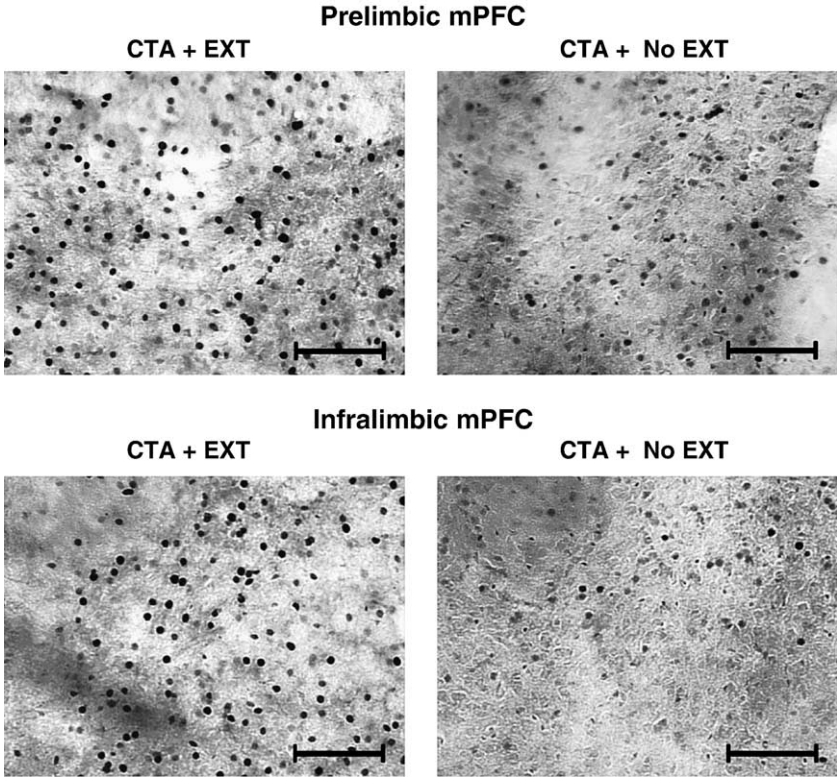

Fig. 3. Microphotographs of representative coronal rat brain sections assayed to illustrate the expression of the c-*fos* protein. Extinction of CTA (CTA + EXT) causes extensive c-*Fos*-labeling in both prelimbic and infralimbic portions of mPFC. For comparison, we also show brains from animals that had acquired CTA but did not extinguish the avoidance (CTA + No EXT). c-*Fos* protein is evidenced by the appearance of a dark spot in the nucleus of the cell (see text for details regarding cell counting). Scale bars represent 0.01 mm.

[$F(1,25) = 87.04$, $P < 0.001$], a significant change in SAC drinking over trials [$F(1,25) = 47.92$, $P < 0.001$], and a significant Trial × Conditioning treatment interaction [$F(1,25) = 186.23$, $P < 0.001$] [19].

On the first day of conditioning, the SAC was novel, and rats exhibited a typical neophobic response (see Fig. 1). Because animals drank SAC before receiving the first presentation of the US, consumption was similar for all treatment groups on this initial day. However, one-way ANOVAs comparing both CTA groups with the EU groups revealed significant differences on conditioning days 2 [$F(3,28) = 22.53$; $P < 0.001$] and 3 [$F(3,28) = 79.61$; $P < 0.001$]. Post hoc tests (Tukey HSD; $\alpha = 0.05$) indicated that pairing of the SAC + LiCl caused animals to drink significantly less SAC than did the rats exposed to the explicitly unpaired procedure.

On average, it took 16.00 ± 1.37 (mean ± SEM) days for our CTA + EXT animals to achieve 90% reacceptance of SAC. In order to control for c-*Fos* expression that may be directly due to the sensation of a sweet taste, all rats were given SAC 90 min before the perfusion on their day of sacrifice. The amounts of SAC consumed by rats in the EU groups (Mean ml consumed ± SEM = 18.99 ± 0.84 ml) were artificially matched to animals of the CTA + EXT group (19.46 ± 0.95 ml). The CTA + No EXT rats learned a CTA (Fig. 1) that was never extinguished (drinking 0.45 ± 0.29 ml of SAC on the last day of the study). In fact, the amount of SAC consumed by the CTA + No EXT animals on the day of sacrifice was not significantly different from the CTA Controls (0.77 ± 0.69 ml) and confirmed that they retained a potent CTA throughout the experiment. The amount of SAC consumed by the SAC-only rats 90 min before sacrifice was 14.84 ± 1.45 ml.

A two-way ANOVA [mPFC Area (prelimbic, infralimbic) × Treatment group (CTA + EXT, CTA + NO EXT, CTA Control, EU + SAC, EU + No SAC, SAC only)] comparing the number of neurons expressing c-*Fos* protein revealed a significant treatment effect [$F(5,72) = 39.38$, $P < 0.001$] but no difference between sub-portions of mPFC. The number of immunoreactive cells was significantly higher in both the prelimbic and infralimbic portions of mPFC in rats with an extinguished CTA—as compared to all other control groups [Tukey HSD post hoc test, $P < 0.05$] [19] (see Fig. 2). The levels of c-*Fos* expression in all of the control groups were statistically indistinguishable ($P > 0.05$).

Different laboratories, using diverse behavioral paradigms, are reporting surprisingly similar results regarding common aspects of the neural substrate of extinction. For example, Quirk and colleagues, using a conditioned fear paradigm, have demonstrated that basal amygdala lesions impair short-term (i.e., within session) extinction [1], whereas neurons in the medial prefrontal cortex are

required for the storage of long-term extinction memories [25,34,35]. This laboratory also described [36] extinction-induced c-*Fos* expression in both prelimbic and infralimbic portions of mPFC with cell densities remarkably similar to those reported here. These previous findings support our data that c-*Fos* protein expression in mPFC increases dramatically when rats have reaccepted the once-avoided taste. The current results add to evidence suggesting that insular cortex is critical to the storage of long-term taste memory [3–5,24] and that, more generally, frontal cortical areas may be involved in the permanent storage of extinction learning [2].

We counted cells expressing c-*Fos* in the prelimbic and infralimbic portions of mPFC [30] and found extinction-induced increases in both brain areas. Previous neurophysiological studies have shown that neurons in the prelimbic cortex do not significantly change their rate of firing during the presentation of an extinguished CS (tone that once predicted shock) whereas neurons in the infralimbic cortex increase their firing as conditioned fear extinguished [25]. This disparity in findings between conditioned fear extinction and CTA extinction may simply reflect the different hedonic value or temporal aspects of the dependent variables employed and the fact that prelimbic cortex is more involved in goal-directed responding (e.g., consummatory behaviors), more generally, than is infralimbic cortex [6]. Alternatively, the prelimbic cortex may be more closely tied to the extinction of taste aversion learning since this cortical area receives inputs from amygdalar structures [20] known to be involved in CTA formation [29] and extinction [1]. Further, the prelimbic cortex sends efferents to brain stem nuclei (parabrachial nucleus and nucleus of the solitary tract) also involved in gustation and CTA formation [18,29,37,41,42]. The exact manner in which infralimbic and prelimbic mPFC regulate learning and memory is still being debated [22,38].

The use of lesion techniques to study the role of mPFC in CTA extinction is almost absent in the literature. Understandably, most of the focus on the neural mechanisms of CTA has been placed on the brain structures in the taste pathway [41] and the ultimate destination of these neurons in the gustatory neocortex. The extinction of CTA is impaired by small lesions of this portion of insular cortex [8]. Using experimental methods distinctly different from those described here (i.e., a single conditioning day with brief, 1.25-min exposure to the CS), Fresquet, Yamamoto and Sandner [7] reported that transection of the frontal portions of the brain fails to disrupt the extinction of CTA. However, the extinction curves produced by this group's procedure are very different from those in our animals (for details see, Ref. [24]). Moreover, the frontal transactions disconnected much more than just the prelimbic infralimbic cortex from the rest of the brain. Additional lesion experiments, more precisely targeting prelimbic and infralimbic cortices, would be necessary to further explore the role of these portions of mPFC on CTA extinction.

CTA extinction induces a dramatic increase in c-*Fos*-labeled neurons of the mPFC and (from our previous work) GNC [24]. However, it would be inappropriate to assume that increased neural activity occurs throughout the brain as CTA extinction develops. Other midbrain and brainstem structures known to be involved in gustation and acquisition of CTAs exhibit nuclei-specific waxing and waning of neural activity throughout the course of extinction [24].

CTA extinction may impose, on the initial learning, depressive processes that interfere with the aversion [27]. Therefore, the enhancement of certain circuitry and/or the inhibition of others may be involved in the neural substrate of extinction [12,13]. It should be emphasized that c-*Fos* immunoreactivity, while a very useful indicator of neural activity, has limitations (e.g., inhibitory responses may not be detected by this method) [42]. The study of additional neural processing markers can provide further insight on the development of extinction learning in the brain.

The expression of c-*Fos* in the mPFC of the CTA + EXT animals and the EU + SAC rats was quite dissimilar despite the fact that levels of SAC drinking were closely matched in these 2 groups of animals. When rats consume SAC at the end of CTA extinction, the level of brain activity evoked by this behavior does not retreat to a pre-CTA configuration representing the CS as a neutral, or benignly familiar, stimulus. Our data are consistent with others suggesting that extinction is not the erasure of a learned response, but may instead reflect new learning [3,32]. Further, the data presented here indicate that both the prelimbic and infralimbic portions of mPFC are involved in this CTA extinction learning.

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