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Research report

Dynamic processing of taste aversion extinction in the brain

G. Andrew Mickley*, Cynthia L. Kenmuir, Colleen A. McMullen, Anna M. Yocom, Elizabeth L. Valentine, Christine M. Dengler-Crish, Bettina Weber, Justin A. Wellman, Dawn R. Remmers-Roeber

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

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Abstract

While substantial advances have been made in discovering how the brain learns and remembers, less is known about how the brain discards information, reorganizes information, or both. These topics are not only relevant to normal brain functioning but also speak to pathologies in which painful memories do not wane but are evoked time and again (e.g., post-traumatic stress disorder; PTSD). Here, we measured brain activity (as indicated by the regional expression of c-Fos protein) in rats during acquisition and throughout extinction of a conditioned taste aversion (CTA). We compared that brain activity with animals that had intact CTA memories or those that experienced an explicitly unpaired (EU) conditioned stimulus (CS; saccharin, SAC) and unconditioned stimulus (US; lithium chloride, LiCl). The data show a dynamic and nonuniform pattern of c-Fos protein expression in brain nuclei known to mediate gustation and CTAs. In particular, brainstem nuclei (e.g., nucleus of the solitary tract; NTS) and the basolateral nucleus of the amygdala (BLA) are active early as CTAs are formed and as extinction of the learned response begins. Later in the extinction process, the BLA reduces c-Fos expression relative to nonextinguished controls. Finally, as almost full reacceptance of the taste is achieved, the gustatory neocortex (GNC) expresses enhanced levels of c-Fos protein. Thus, extinction of a CTA is not represented by a simple reversal of the c-Fos activity evoked by CTA conditioning. Rather, the data demonstrate that extinction of conditioned responses is a dynamic process in which the activity levels of particular nuclei along the brain's taste pathway change depending on the extent to which the conditioned response has been extinguished.
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1. Introduction

How is the brain altered when a previously learned response is no longer reinforced (i.e., extinguished)? In the current experiments, we use the conditioned taste aversion (CTA) paradigm to reveal neurophysiological and neuroanatomical substrates of extinction and describe the dynamic nature of this form of learning as it develops. CTAs may be acquired when an animal consumes a novel taste (conditioned stimulus, CS) and then experiences the symptoms of poisoning (unconditioned stimulus, US) [14]. When later given a choice between the CS and some more-familiar gustatory stimulus (typically water), the animal will avoid the taste that it previously associated with malaise. Extinction of a CTA is observed following repeated, nonreinforced exposures to the CS [39] and represents itself as a resumption of eating/drinking the once-avoided tastant.

The neurobehavioral processes that go on during extinction have been a matter of debate. Although several different theoretical mechanisms have been proposed, two general classes of theory have emerged [43]. Extinction has sometimes been explained in terms of an ''erasure'' of the original associations that led to the production of the

* Corresponding author. Tel.: +1-440-826-2767; fax: +1-440-826-3577.
E-mail address: amickley@bw.edu (G.A. Mickley).

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conditioned response [11,32]. Hence, a sufficient number of nonreinforced CS presentations will presumably reduce the net associative strength between the CS and US to an asymptote of 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 [32]. Alternatively, extinction may be thought of as a learning process in which the original CS–US relation is probably never unlearned, but is rather supplemented by new, additional knowledge suggesting that in some contexts, or in some moments in time, the former CS–US relation does not hold [3,9,30]. If, as the preponderance of data suggest [31], extinction is indeed new learning then there must be a time course for acquisition of this memory and a corresponding temporally constrained pattern of brain activity to support it.

Much of the interest in the neural substrate of CTA has focused on the brainstem, especially the nucleus of the solitary tract (NTS) and the parabrachial nucleus (PBN) since these are the sites where taste and visceral information converge [18,19,29,40]. However, it is now recognized that forebrain structures such as the amygdala (AMY) and gustatory neocortex (GNC) contribute in significant ways to the expression and storage of the CTA memory. Information regarding a CTA is apparently transmitted from the PBN to the basolateral amygdala (BLA)—either directly or via the posterior ventromedial thalamic nucleus (VPMpc) [44]. Finally, information regarding the hedonic shift of the CS is sent from the AMY and PBN to the GNC for long-term retention [7,8,44].

Previous studies addressing the neural substrate of CTA extinction have tended to focus on one of these brain nuclei at a time and have not investigated the dynamic process of the phenomenon. Here we measured the expression of the protein product of the c-*fos* gene (as a marker of neural activity) [16,34] at three stages during the extinction process (static, dynamic and asymptotic) [26]. This method has the advantage of allowing a simultaneous behavioral and physiological assessment of the neural activity within several nuclei along the brain's taste pathway as extinction learning develops. Indicators of c-Fos expression seem especially well suited for studies of CTA encoding since the gene is apparently obligatory for the formation of this gustatory memory [22,41].

2. Materials and methods

2.1. Animals

The male Sprague–Dawley rats (451.3 ± 3.1 g) used in this study were obtained from Zivic Laboratories (Zelienople, PA). Throughout the experiment, the animals were housed individually in plastic ''shoe box'' cages (44.45 × 21.59 × 20.32 cm high). 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 was available ad libitum. All animals were placed on a 23-h water deprivation cycle for 2 days prior to the conditioning trials. To this effect, a 50 ml bottle of tap water was made available for 1 h beginning at 12:00 h. On all subsequent days, rats were allowed 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). SAC in a 0.3% concentration was chosen due to its high hedonic value in adult rats [23]. Daily fluid consumption was recorded to the nearest tenth of a gram.

2.2. Experimental design, taste aversion and extinction procedures

Five treatment groups were employed in this experiment: the main experimental group and four control groups (see Table 1 for a summary of the naming conventions, timelines and procedures for each group). One hundred and ten rats

Table 1
Summary of conditioning procedures and extinction 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	Liquid consumed on the day of sacrifice
CTA extinction (CTA + EXT)	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)	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)	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)	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	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	SAC + LiCl	Water, full 60 min	–	SAC

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

were used and included in the statistical analyses of the behavioral data: CTA + EXT, *N* (static phase) = 9, *N* (dynamic phase) = 8, *N* (asymptotic phase) = 9; CTA + No EXT, *N* (static phase) = 9, *N* (dynamic phase) = 9, *N* (asymptotic phase) = 9; EU + SAC, *N* (static phase) = 7, *N* (dynamic phase) = 7, *N* (asymptotic phase) = 7; EU + No SAC, *N* (static phase) = 9, *N* (dynamic phase) = 9, *N* (asymptotic phase) = 9; CTA Control, *N* = 9. Due to some problems in the immunohistochemical procedures, not all of the brains from these animals produced useable data for the neuroanatomical analyses. The mean (± S.E.M.) number of rats/treatment/brain area analyzed for c-Fos protein expression (see below) was 5.9 ± 0.17.

The conditioned taste aversion extinction group (CTA + EXT) received three CTA conditioning trials: one every other day over the course of 6 days (experimental days 1, 3 and 5). Each trial paired the conditioned stimulus (CS) of a 30-min exposure to SAC (which was novel on the first day) with the unconditioned stimulus [US; a malaise inducing i.p. injection of 81 mg/kg lithium chloride (LiCl)]. Each day of conditioning training, rats received an additional 30 min access to tap water at 12:45 h to prevent dehydration and weight loss. On days 2, 4 and 6, rats were given access to tap water for 60 min beginning at 12:00 h.

Starting on experimental day 7, CTA + EXT rats were given access to SAC for 30 min (12:00–12:30 h), followed 15 min later by 30 min of access to tap water (12:45–13:15 h). This was repeated daily until animals reached their predetermined, randomly assigned levels of CTA extinction (static, dynamic, or asymptotic—see below) [26]. On the day that each CTA + EXT animal reached its extinction criterion, it was perfused and its brain was prepared for c-Fos protein immunohistochemistry 90 min following the last SAC exposure (i.e., at 14:00 h) (see c-Fos protein assay procedures below).

For this study, we divided the extinction process into three distinct phases based on behavioral data from Nolan et al. [26], who described the extinction process in terms of: (a) an initial static learning phase, (b) a dynamic recovery period, and (c) an asymptotic phase that was reached when rats almost completely reaccepted the once-poisoned CS. These three phases of CTA extinction were then operationalized in terms of a percentage of baseline SAC drinking. Baseline SAC drinking was determined by recording the average amount of SAC drinking (mean = 17.44 g) from an independent group of age- and weight-matched, 23-h water-deprived rats. These animals consumed 0.3% SAC over a 30-min period on their second day of SAC drinking (i.e., non-naïve consumption). The drinking criterion for each phase was then defined as follows: 10% of baseline drinking = the end of the static phase, 40% of baseline drinking = the approximate midpoint of the dynamic recovery phase, and 90% of baseline drinking = the beginning of the asymptotic phase.

The CTA, no extinction control group (CTA + No EXT) received the same 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 water for 60 min (12:00–12:30 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, a 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 for 30 min (12:00–12:30 h), and then access to SAC for 30 min (12:45–13:15 h). Rats were perfused 90 min after their last SAC exposure (14:45 h).

The CTA Control group received conditioning training as in 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 acquisition of a CTA.

Two additional control groups were included in the study. These controls did not receive CTA conditioning trials, but instead, experienced the effects of the CS and US "explicitly unpaired" from one another (i.e., separated by 24 h). This procedure for noncontingent presentation of CS and US has been shown to inhibit the production of a CTA [43] as was confirmed by our own data (see Fig. 1). The explicitly unpaired saccharin animals (EU + SAC) were given access to SAC on days 1, 3 and 5. 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, explicitly unpaired saccharin animals received SAC in a matched amount to that consumed by a CTA + EXT animal to which they were

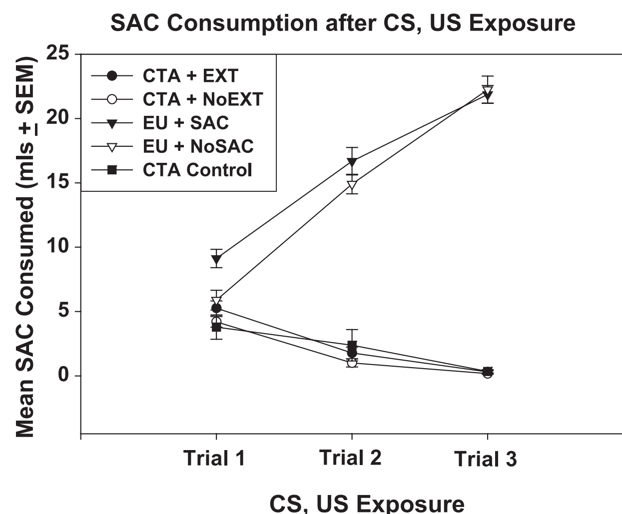

Fig. 1. Saccharin (SAC 0.3% = CS) consumption after CS, US (81 mg/kg lithium chloride, i.p.) exposure: SAC consumption in both the explicitly unpaired (EU) CS, US groups increased over the course of the three trials indicating that these rats did not acquire a CTA. Conversely, SAC consumption in all of the CTA groups (CTA + Extinction, CTA + No EXT and CTA Controls) decreased over the three trials indicating that these rats acquired a CTA. Variance indicators are the S.E.M.

yoked. These EU + SAC rats were perfused 90 min after their last SAC exposure.

Explicitly unpaired no-saccharin (EU + No SAC) animals received SAC on days 1, 3 and 5 with LiCl injections 24 h later (days 2, 4 and 6)—as in the EU + SAC group. 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. This matching of the SAC volumes consumed on the day of sacrifice reduced the chance that differences in c-Fos expression between experimental and control groups may be attributed to differences in thirst.

Although EU rats experienced a LiCl injection on the same day they drank water, they did not form an aversion to this very familiar liquid. The volume of water consumed by all EU rats (EU + SAC; EU + No SAC) remained stable before and after the EU treatments.

2.3. Perfusion, histology and immunohistochemistry

Since c-Fos protein expression by the c-*fos* gene is highest between 90 and 120 min after post-synaptic neuronal activity [16], all rats in the current study were sacrificed 90 min following the end of their last SAC exposure. All rats were given SAC before perfusion in order to control for c-Fos expression that may be directly due to the sensation of a sweet taste. The amounts consumed by rats in the EU groups were artificially matched to that of the CTA + EXT group by limiting the time these animals had with the drinking tubes. 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. Brains were dissected immediately and placed in 4% paraformaldehyde for 8–9 h at ~ 4 °C. Brains were then transferred to a cryoprotectant solution (30% sucrose mixed in phosphate buffer with 0.01% thimersol) until they were sliced. Brains were sectioned coronally at 40 μm using a freezing microtome. All sections were stored in phosphate buffered saline with 0.2% sodium azide until they were assayed.

The brain sections were assayed for c-Fos protein immunoreactivity as follows [20]. The tissue was first rinsed in phosphate buffered saline (PBS) to remove any residual fixative, cryoprotectant, or azide. This was followed by a 30-min wash in 0.3% hydrogen peroxide. The sections were rinsed with PBS and incubated for 1 h in a blocking solution (1.5% normal goat serum/0.2% Triton/PBS). The tissue was then rinsed in PBS and incubated for 1 h in 1.0 μg/ml c-Fos primary antibody (rabbit polyclonal, Ab-5, Oncogene, San Diego, CA). Sections were then chilled to 4 °C for 18 h. After rinsing with PBS to remove any residual antibody, the sections were incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (Oncogene). Tissue was again rinsed to remove any residual antibody, and then incubated for 1 h in avidin–biotin peroxidase complex (Elite ABC kit, Vector Laboratories, Burlington, CA). Finally, the tissue was washed in 0.5% Triton and then antibody binding was revealed with a final 2–3 min incubation in a 0.1% diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO)/0.02% hydrogen peroxide solution. Staining was quenched in deionized water and then sections were placed into PBS at 4 °C. These sections were mounted on gelatin and chrom-alum coated slides, dehydrated, counterstained with neutral red and cover slipped with Permount™.

Slides were viewed via an Olympus™ microscope using a 546 nm filter connected to a computer running NIH Image software. Brain structures were identified consistent with the anatomical demarcations specified by Paxinos and Watson [28]. The neuroanatomical abbreviations: GNC = gustatory neocortex; BLA = basolateral nucleus of the amygdala; CN = central nucleus of amygdala; PBN = parabrachial nucleus; NTSr = nucleus of the solitary tract (rostral); NTSi = nucleus of the solitary tract (intermediate). According to our measurements, the mean (± S.E.M.) areas of these nuclei in coronal section were: GNC = 3.328 ± 0.053 mm^2; BLA = 0.768 ± 0.019 mm^2; CN = 0.297 ± 0.003 mm^2; PBN = 0.121 ± 0.005 mm^2; NTSr = 0.070 ± 0.003 mm^2; NTSi = 0.073 ± 0.002 mm^2. These areas (when combined with the data from Figs. 4 and 5) may be used to calculate the c-Fos-labeled cell densities of each brain area for each experimental condition during each phase of the study.

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; S.D.) against a background density of 91.60 (± 38.10 S.D.). The average c-Fos-labeled cell was 3.3 S.D. units darker than background. The observer (C.L.K.) was blind to the experimental condition of the rats. Previous assessments of inter-rater reliability in our laboratory have always revealed correlation statistics $r > 0.90$. In this particular study, the intra-rater reliability was $r = 0.98$.

2.4. Statistical analyses

Unless otherwise stated, immunohistochemical data collected from each brain area were analyzed using a three-way between subjects analysis of variance [ANOVA; extinction level (static, dynamic, asymptotic) × extinction treatment (extinction, i.e., SAC drinking, or No-Extinction, i.e., No SAC drinking) × learning treatment (CTA learning, explicitly unpaired)] [21] with the number of c-Fos-positive cells serving as the dependent variable. Brain areas analyzed

were those known to be involved in gustation and CTA [27,32,44].

Follow-up analyses were conducted using one-way ANOVAs and Tukey HSD post hoc tests to determine where significances fell between treatment groups. In order to further explore changes in c-Fos protein expression from CTA formation to CTA extinction, we calculated additional one-way ANOVAs (including CTA controls, static, dynamic and asymptotic levels of extinction) per treatment, per brain area. An α level of 0.05 was used to determine significance for all analyses.

3. Results

3.1. Taste aversion and extinction data

The design of the conditioning days allowed for the daily recording of SAC consumption data. Therefore, we are able to report that all rats in the three CTA groups had acquired a strong CTA by the end of the third conditioning period, and that the two explicitly unpaired (EU) groups (where the CS and US exposures were noncontingent; see description below) had not acquired a CTA by the end of this same "conditioning" period (Fig. 1). A repeated measures ANOVA [treatment (CTA or EU) × 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 [$F(1,50)=234.56$; $p<0.001$].

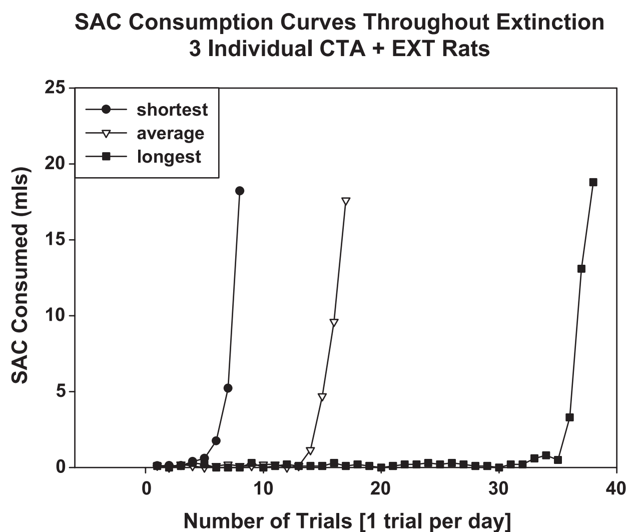

Fig. 2. CTA extinction curves for three individual CTA + EXT rats illustrating the shortest (●), longest (■) and average (▽) number of days to reach asymptote (i.e., 90% of baseline SAC drinking) following three CS + US pairings and then daily nonreinforced presentations of the CS. The data illustrate the behavioral variability that was controlled for by our yoking of the CTA + No EXT rats to our CTA + EXT animals.

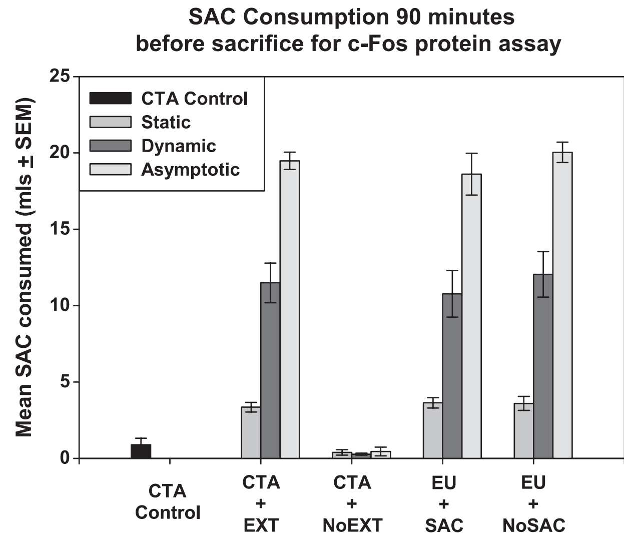

Fig. 3. Levels of saccharin consumption 90 min before sacrifice for c-Fos protein measurements: CTA + EXT rats indeed extinguished the CTA, while those receiving water (CTA + NoEXT) retained the CTA. The rats that received explicitly unpaired (EU) exposures to SAC, LiCl early in the study, later received the same volume of SAC or H_2O as their matched experimental rats in the CTA + EXT group. Variance indicators are the S.E.M.

There was substantial variability in the number of trials/days that it took animals randomly assigned to a particular extinction phase to reach the same drinking criterion. However, as expected, on average it did take rats slightly longer to reach the asymptotic stage, than it did to reach the dynamic stage, than it did to reach the static stage of extinction (mean ± S.E.M.: static = 13.67 ± 1.94 days; dynamic = 14.63 ± 1.46 days; asymptotic = 17.11 ± 3.01 days).

No matter how many days it took for an individual rat to reach its particular extinction criterion, the shapes of their extinction curves looked nearly identical. Typically, the variability in the amount of time needed to reach a criterion was determined in the static phase of extinction when rats avoided the CS assiduously. Rats took approximately the same amount of time to dynamically recover and reach asymptote (see Fig. 2).

All rats were given SAC before perfusion in order to control for c-Fos expression that may be directly due to the sensation of a sweet taste. The amounts consumed by rats in the EU groups were artificially matched to that of the CTA + EXT group. Therefore, as expected, the average consumption of these EU animals on the day of sacrifice closely matched that of the CTA + EXT rats (mean ml consumed ± S.E.M.; static = 3.35 ± 0.31, dynamic = 11.49 ± 1.30, asymptotic = 19.48 ± 0.57). The CTA + No EXT rats

learned a CTA (Fig. 3) and were never extinguished (Mean ml consumed ± S.E.M.; static = 0.39 ± 0.18, dynamic = 0.28 ± 0.06, asymptotic = 0.45 ± 0.29). The amount of SAC consumed by the CTA + No EXT animals on the day of sacrifice was not significantly different from the CTA Controls (Mean ml consumed ± S.E.M. = 0.89 ± 0.43). As expected, the amount of voluntary SAC consumption of these animals on the day of sacrifice confirms that they did retain a CTA throughout the experiment. It should be noted that our procedure of giving CTA + No EXT rats access to water before they tasted SAC on the last day of the experiment may have artificially reduced SAC consumption in these partially saited rats. In order to further confirm that CTA + No EXT animals retained the CTA until time of sacrifice, we ran nine additional CTA + No EXT animals but, in this case, did not give them a preliminary drink of water before their final taste of SAC. This experiment verified that the CTA was indeed intact (mean ± S.E.M. SAC consumed = 0.2 ± 0.14 ml).

3.2. Immunohistochemical data

Expression of c-Fos protein was dependent on the brain area analyzed, the conditioning history of the animal, and the level of extinction (see Figs. 4–6).

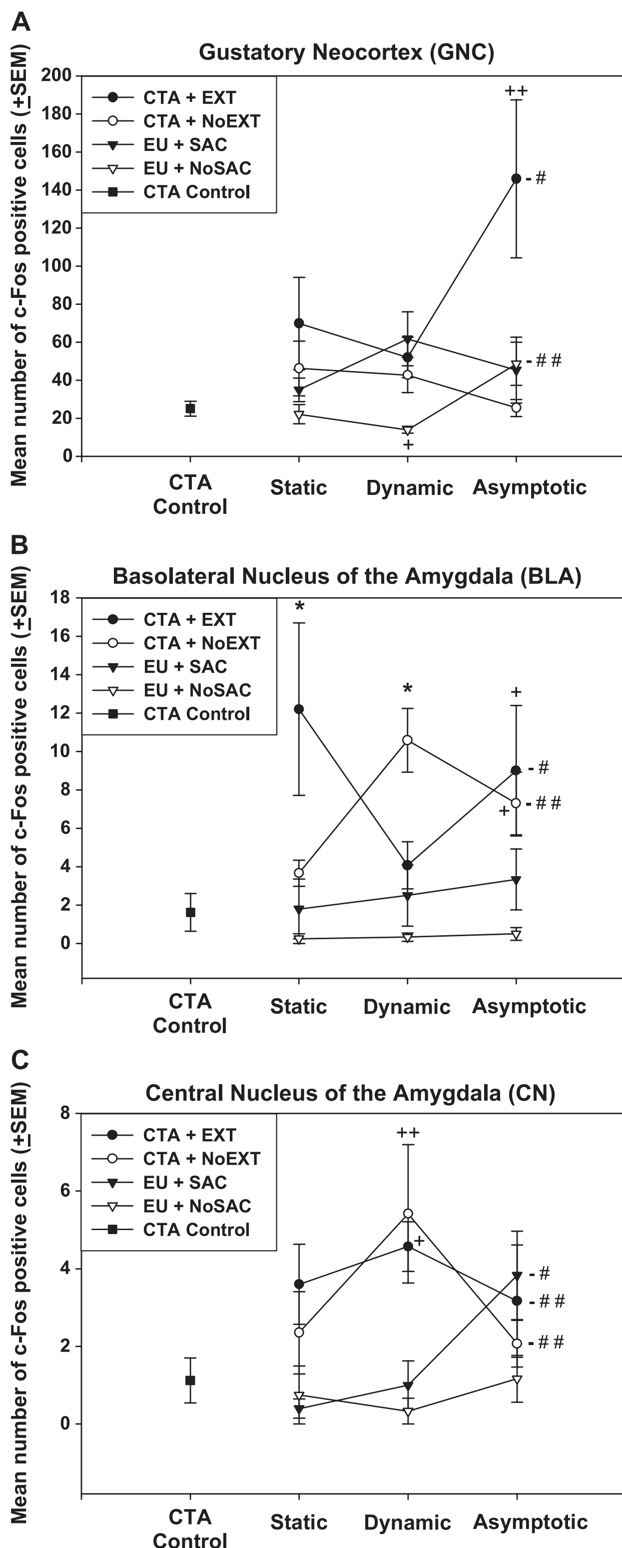

Fig. 4. c-Fos protein expression in the forebrain. Animals either acquired a CTA followed by extinction of the aversion (CTA + EXT); learned a CTA and then drank water over a series of days such that the CTA was not extinguished (CTA + No EXT); never learned a CTA but received explicitly unpaired (EU) exposure to the taste of SAC and an injection of the LiCl US followed by either daily access to SAC (EU + SAC) or water (EU + No SAC); or, learned a CTA and then were sacrificed for the c-Fos assay (CTA Control). (A) Gustatory neocortex (GNC): neurons in the GNC express more c-Fos than control animals—but only during the asymptotic stage of extinction. $^{+}$Significantly different from EU + SAC group at same stage of extinction. $^{++}$Significantly different from CTA + No EXT group at same stage of extinction. $^{\#}$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. (B) Basolateral nucleus (BLA) of the amygdala: at the early (static) stage of extinction, conditioned rats expressed more c-Fos than did nonextinguished controls. However, c-Fos expression is reduced in the CTA + EXT rats (and increased in the CTA + No EXT) during the dynamic stage of extinction. When rats have fully extinguished, their c-Fos expression in the BLA is similar to that of the CTA + No EXT animals. Rats exposed to the explicitly unpaired CS and US do not change their c-Fos expression in the BLA upon subsequent re-exposure to SAC or to water. *Significantly different from all other treatment groups at the same stage of extinction. $^{+}$Significantly different from EU + No SAC group at same stage of extinction. $^{\#}$Treatment group shows a significant increase in c-Fos expression (CTA Control to static) depending on the stage of extinction. $^{\#\#}$Treatment group shows a significant increase (CTA Control to dynamic and asymptotic) or decrease (static to dynamic) in c-Fos expression depending on the stage of extinction. (C) Central nucleus of the amygdala (CN): CN c-Fos expression does not change as CTA extinction progresses. Conditioned rats express more c-Fos than the nonconditioned (EU) rats—but only during the dynamic phase of extinction. $^{+}$Significantly different from EU + No SAC group at same stage of extinction. $^{++}$Significantly different from both EU + SAC and EU + No SAC groups at same stage of extinction. $^{\#}$Treatment group shows a significant increase in c-Fos expression (static to asymptotic) depending on the stage of extinction. $^{\#\#}$Treatment group shows a significant increase in c-Fos expression (CTA Control to dynamic) depending on the stage of extinction. $\alpha = 0.05$ throughout. Variance indicators represent the S.E.M.

3.2.1. GNC

A comparison of animals that maintained a CTA versus those that experienced extinction revealed that the GNC becomes activated only when rats have fully reaccepted the CS. 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 three-way interaction (extinction level × extinction treatment × learning treatment) [$F(2,55)=4.26$, $p=0.018$]. See Fig. 4 for the results of the individual group comparisons.

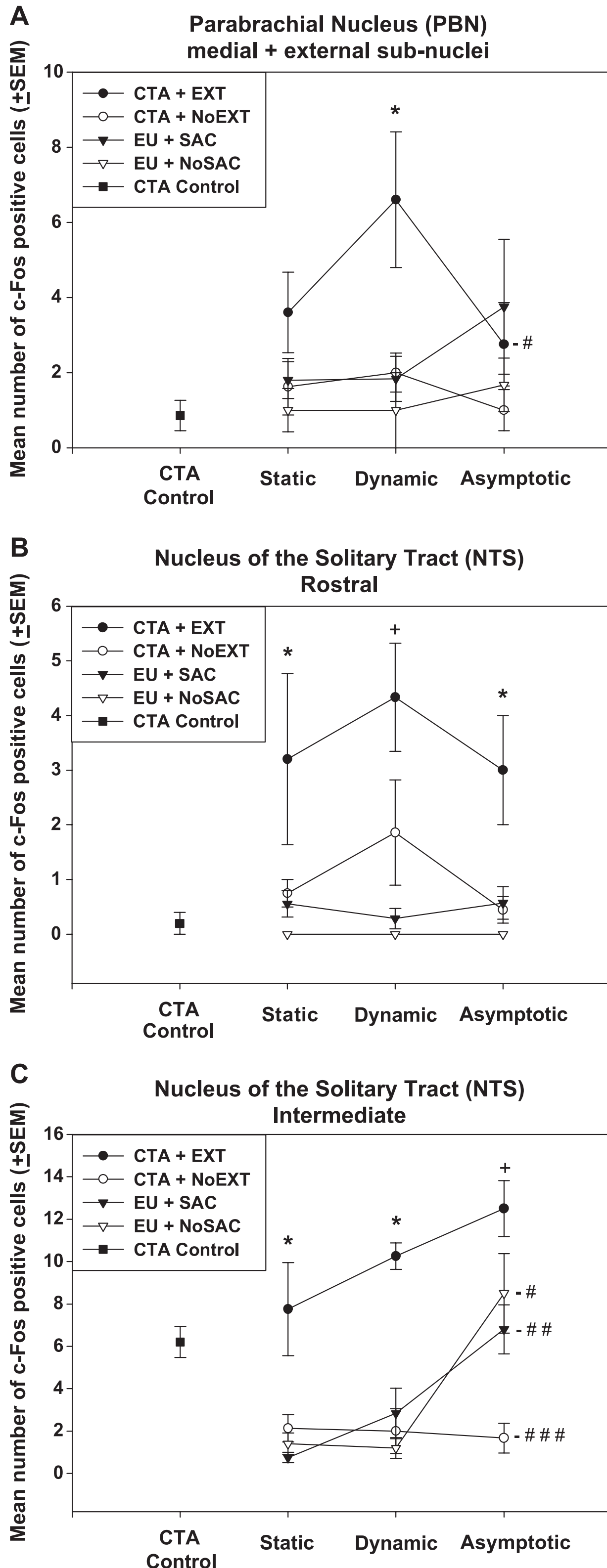

Fig. 5. c-Fos protein expression in brainstem nuclei: nuclei of the solitary tract (NTS) and parabrachial nucleus (PBN). Depending on the level of CTA extinction, rats exhibit different patterns of c-Fos protein expression than do control animals that still have a CTA or have never learned a taste aversion. See text and Fig. 4 for group nomenclature. (A) Cells of the PBN express significantly more c-Fos protein than controls only during the dynamic stage of extinction. PBN neurons of the CTA + EXT group return to pre-extinction levels of c-Fos protein expression when CTA extinction is complete. *Significantly different from all other treatment groups at the same stage of extinction. [#]Treatment group shows a significant increase in c-Fos expression (CTA Control to dynamic) depending on the stage of extinction. (B) Rostral NTS (NTSr): CTA + EXT rats express more c-Fos-labeled neurons in rostral NTS (the gustatory portion of the NTS) [15,24,37,42] than do controls. However, c-Fos expression does not change as rats extinguish a CTA. *Significantly different from all other treatment groups at the same stage of extinction. [+]Significantly different from EU treatment groups at same stage of extinction. (C) Intermediate NTS (NTSi): expression of c-Fos in the NTSi is similar for CTA Control and CTA + EXT rats. In this portion of the NTS known to encode visceral inputs [12,15,24,37,42], the number of immunoreactive cells increases as volume of fluid consumed rises to peak levels during the asymptotic stage of the study. *Significantly different from all other treatment groups at the same stage of extinction. [+]Significantly different from CTA + No EXT and EU + SAC groups at same stage of extinction. [#]Treatment group shows a significant decrease (CTA control to static) or increase (static and dynamic to asymptotic) in c-Fos expression depending on the stage of extinction. [##]Treatment group shows a significant increase in c-Fos expression (static to asymptotic) depending on the stage of extinction. [###]Static, dynamic and asymptotic levels of "extinction" significantly lower than the CTA controls. $\alpha=0.05$ throughout. Variance indicators represent the S.E.M.

3.2.2. BLA and CN

The BLA and CN of the amygdala exhibited very different patterns of c-Fos expression over the course of extinction (see Fig. 4). Compared to nonextinguished rats and CTA controls, animals that are undergoing extinction exhibit an initial burst of BLA neural activity during the static stage of extinction. This is followed by a dramatic waning of this response. On the other hand, the neural activity of the CN is not significantly altered by extinction but, instead, exhibits a pattern of responding that consistently differentiates conditioned and nonconditioned animals—especially during the beginning and middles stages of the CS re-exposure.

The statistical analyses of c-Fos expression in the BLA revealed a significant main effect for learning treatment [$F(1,57)=38.23$, $p<0.001$], as well as a significant two-way interaction (extinction level × extinction treatment) [$F(2,57)=4.34$, $p=0.018$], and a significant three-way interaction (extinction level × extinction treatment × learning treatment) [$F(2,57)=4.94$, $p=0.01$]. On the other hand, the analysis of c-Fos expression in the CN showed a significant

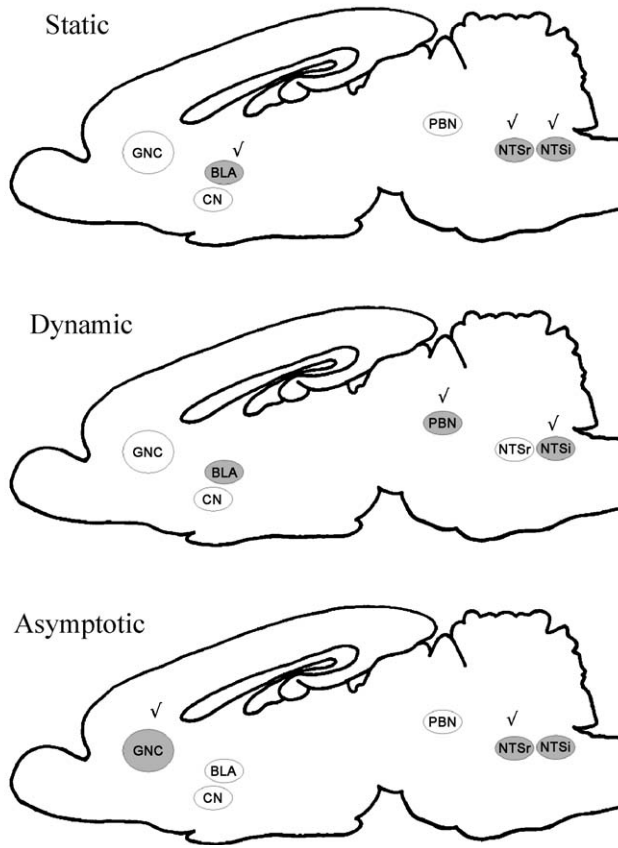

Fig. 6. Highlighted are areas of the rat brain where significant ($\alpha = 0.05$) changes in c-Fos protein expression were observed between animals that were conditioned and extinguished (CTA + EXT) as compared to animals that were conditioned but never extinguished (CTA + No EXT). Therefore, these differences represent changes associated specifically with the extinction process. Over the course of extinction (static, dynamic and asymptotic stages), each brain area was found to have a different pattern of c-Fos expression, perhaps indicating the temporal role each area has in extinction learning. For example, BLA expressed changes in c-Fos levels only during the earliest stages of extinction, whereas PBN expressed changes only during the middle stage of extinction. Moreover, GNC expressed changes only during the final, asymptotic stage of extinction. A comparison between groups that were conditioned and extinguished (CTA + EXT) and animals that were never conditioned (EU + SAC) provides an answer to the question ''Does brain activity revert back to a preconditioning configuration following extinction?''. A lack of difference between these groups in a particular brain area may suggest that extinction produces an attenuation of the CTA engram (i.e., an erasure of a memory)—since the brain looks like one that has never acquired a CTA. Conversely, differences between these groups may indicate that the CTA + EXT brain does not return to its naïve configuration but rather, continues to represent a change that may reflect its conditioning history. Thus, differences between the c-Fos expression seen in a particular brain area of CTA + EXT versus EU + SAC animals are most revealing when that same brain area also exhibits different levels of activity when extinction occurs (i.e., when CTA + EXT and CTA + No EXT animals show different levels of c-Fos expression in this same nuclei where differences between CTA + EXT and EU + SAC are also detected). The existence of this dual difference is represented by a ''√'' beside particular brain areas illustrated in the figure. GNC = gustatory neocortex; BLA = basolateral nucleus of the amygdala; CN = central nucleus of amygdala; PBN = parabrachial nucleus; NTSr = nucleus of the solitary tract (rostral); NTSi = nucleus of the solitary tract (intermediate).

main effect for learning treatment [$F(1,57) = 16.08$, $p < 0.001$] and a significant two-way interaction (extinction level × learning treatment) [$F(2,57) = 4.72$, $p = 0.013$]. See Fig. 4 for the results of the individual group comparisons.

3.2.3. PBN

The brainstem nuclei of the PBN are selectively active only during the dynamic (middle) stage of extinction (Fig. 5). A significant main effect for extinction treatment [$F(1,48) = 11.58$, $p = 0.001$] was revealed by the statistical analysis—as was a significant two-way interaction (extinction level × learning treatment) [$F(2,48) = 3.15$, $p = 0.052$]. See Fig. 5 for the results of the individual group comparisons.

3.2.4. NTsr and NTSi

At the early and final stages of extinction, the rostral portion of the NTS in CTA + EXT rats expresses more c-Fos protein than does the NTSr of CTA + NoEXT controls. Whereas, the neurons that make up the intermediate NTS express more c-Fos protein in proportion to the levels of fluid consumed by rats in the different treatment groups (Fig. 5). However, this effect was only observed at the asymptotic stage of the study (when volumes of fluid consumption were highest) and was independent of conditioning history. NTSi neurons are active following CTA acquisition and remain active throughout extinction learning. The statistical analysis of NTSr c-Fos expression revealed significant main effects for extinction treatment [$F(1,66) = 17.07$, $p < 0.001$] and learning treatment [$F(1,66) = 31.97$, $p < 0.001$] and a significant two-way interaction (extinction treatment × learning treatment) [$F(1,66) = 7.95$, $p = 0.006$] However, in the NTSi, there were significant main effects for extinction level [$F(2,61) = 13.17$, $p < 0.001$], extinction treatment [$F(1,61) = 29.33$, $p < 0.001$] and learning treatment [$F(1,61) = 11.18$, $p = 0.001$] as well as two separate significant two-way interactions (extinction level × learning treatment) [$F(2,61) = 4.17$, $p = 0.020$]; (extinction treatment × learning treatment) [$F(1,61) = 32.94$, $p < 0.001$]. See Fig. 5 for the results of the individual group comparisons.

See Fig. 6 for a summary of the main differences between the spatiotemporal patterns of brain c-Fos expression in CTA + EXT versus CTA + No EXT animals (also outlined above and in Figs. 4 and 5). These comparisons reveal how extinction learning alters brain activity.

Further, to help answer the question ''Does regional brain activity return to pre-learning levels as extinction becomes complete?'', we also provide a graphical representation of the statistical comparisons that were made between the c-Fos expression in the brains of animals that acquired and then experienced an extinction of a CTA (CTA + EXT), versus those rats that never acquired a CTA (EU + SAC) (see Fig. 6). These comparisons reveal that c-Fos protein expression in PBN does indeed return to levels comparable to rats that never acquired a CTA. However, as extinction reaches asymptote, c-Fos expression in PBN neurons is also

similar to that of conditioned controls—again suggesting that the PBN may play a role in extinction that is limited to the middle stage of this process (see Fig. 5). More to the point, when animals reaccepted the previously avoided taste during the asymptotic stage of extinction the neurons of the GNC, BLA and NTSr express significantly more c-Fos than do the same brain areas of nonconditioned rats.

Our analysis focused on the number of c-Fos labeled cells per brain area of interest. However, the density of cells (cell/ mm^2) expressing c-Fos protein may be an indirect clue to the biological and behavioral impact of this gene product. We calculated the average area (mm^2) of each brain nucleus we analyzed (see above). Therefore, an estimate of the density of c-Fos labeled neurons of animals in each experimental/ control condition, at each stage of extinction, is readily accessible by dividing the cell counts expressed in Figs. 4 and 5, by these areas. While cell densities in some areas (under some conditions) were low, the responses to experimental manipulation were dynamic. The following densities of c-Fos labeled cells represent the lowest and highest means (across experimental conditions, across stages of extinction) we observed in our studies: GNC: 1.335–43.816 cell/mm^2; BLA: 0.325–15.879 cell/mm^2; CN: 1.123–18.257 cell/ mm^2; PBN: 7.043–54.231 cell/mm^2; NTSr: 0–61.904 cell/mm^2; NTSi: 10.232–170.532 cell/mm^2.

4. Discussion

Our data represent a look at the dynamic nature of extinction learning over time and are consistent with the conclusion that the neural substrates of short-term and long-term extinction phenomena are not uniform. Certainly, many nuclei in the taste pathway become involved during the extinction of a CTA. However, the spatial-temporal pattern of cortical, subcortical and brainstem activation shifts as extinction learning develops (see summary in Fig. 6).

Different laboratories, using different behavioral paradigms, are reporting surprisingly similar results regarding the neural substrate of extinction. Using a conditioned fear paradigm, Anglada-Figueroa et al. [1] have demonstrated that basal amygdala lesions impair short-term (i.e., within session) extinction. Whereas, neurons in the medial prefrontal cortex are required for storage of long-term extinction memories [24,35,36]. These previous findings are consistent with those reported here—showing that c-Fos protein expression in BLA is most prominent in the early stages of CTA extinction but cortical (GNC) neurons become activated only when rats have fully reaccepted the once-avoided taste. Our data add to evidence suggesting that insular cortex is critical to long-term taste memory storage [7,8] and that, more generally, frontal cortical areas are involved in permanent storage of extinction learning [5].

The BLA and CN of the amygdala exhibited very different patterns of c-Fos expression over the course of extinction. Compared to nonextinguished rats and CTA controls, animals that are undergoing extinction exhibit an initial burst of BLA neural activity during the static stage of extinction. This is followed by a dramatic waning of this response—perhaps suggesting that activity in this structure provides a temporary store of extinction memory. On the other hand, the neural activity of the CN is not significantly altered by extinction but, instead, exhibits a pattern of responding that consistently differentiates conditioned and nonconditioned animals—especially during the beginning and middles stages of the CS re-exposure. These data are consistent with others indicating that the CN plays a much larger role in CTA acquisition rather than CTA extinction, whereas the BLA is critical for extinction learning [4]. Moreover, they point to the interdependent role these two subnuclei may play in the extinction of conditioned aversive reactions [33].

The expression of c-Fos protein in the rostral portion of the NTS (NTSr; primarily receiving gustatory afferents) [15,27,37,42] in CTA + EXT animals is significantly different from CTA + NoEXT controls #-# but only during the early and final stages of extinction learning. On the other hand, the neurons that make up the intermediate NTS (NTSi; receiving primarily visceral afferents) [12,15,27,37,42] express more c-Fos protein in proportion to the levels of fluid consumed by rats in the different treatment groups (Fig. 5). However, this effect was only observed at the asymptotic stage of the study when volumes of fluid consumption were highest and was independent of conditioning history. NTSi neurons are active following CTA acquisition (confirming the work of Houpt et al. [17]) and remain active throughout extinction learning. Previous experiments have reported a decline in the number of NTSi neurons expressing c-Fos as a CTA is extinguished; but this previous work administered the CS via intraoral infusion [17] which, when compared to voluntary drinking, is known to limit the number of c-Fos immunoreactive neurons [45]. It should also be noted that c-Fos expression in NTSi seems inconsistent between CTA controls and CTA + No EXT animals (Fig. 5) both of which show strong behavioral indicators of SAC avoidance. CTA retention time and the opportunity for forgetting differentiate these two groups. The NTSi may play a special role in forgetting and can influence the potency of CTAs [19].

The measurement of c-Fos protein expression is indicative of neuronal activity [16,34] but the minimum number of active cells required for functional change is unknown. Might the density of cells expressing c-Fos protein offer a clue about the extent to which a particular brain area is involved in behavioral change? The mean c-Fos cell densities calculated in these studies ranged from 0 to 170 cells/ mm^2. Perhaps equally important are the experimentally induced dynamic responses of particular brain areas. The ratios of the maximum c-Fos cell densities/minimum c-Fos cell densities observed per brain area ranged from 7.7 (in PBN) to 61.9 (in NTSr). Thus, as a minimum, our exper-

imental manipulations produced over a seven-fold change in c-Fos expression. Additional studies would be required to determine the functional impact of these dynamic responses.

Our data are consonant with several lines of behavioral evidence suggesting that the original associations are not erased following extinction even though conditioned responding disappears. For example, when a CS that is completely extinguished in a context different from the acquisition context and is next re-introduced in the acquisition context, a strong recovery of conditioned responding towards the CS can be observed [2,9,10]. Secondly, when due to the application of an extinction procedure, conditioned responding has been completely abolished, the mere passage of time may cause conditioned responses to spontaneously recover [11,13]. Thirdly, rapid reacquisition of a previously extinguished conditioned response may suggest that the original CS–US association is not unlearned [38]. The current data provide a rich neuroanatomical substrate by which these behavioral phenomena may be better understood.

Similarly, recent neurophysiological data seem to support the conclusion that extinction is a learning process. For example, CTA extinction shares molecular mechanisms typically associated with learning (e.g., protein synthesis and involvement of β-adrenergic receptors) [6]. Glucose metabolism increases in the prefrontal cortex of animals following the extinction of a conditioned emotional response [5]. Further, the neural circuit in the amygdala that is involved in CTA acquisition is different from that involved in CTA extinction [4].

CTA extinction imposes on the initial learning depressive processes that interfere with performance [25]; but the neural substrate of extinction may involve the enhancement of certain circuitry and/or the inhibition of others. It should be stressed 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) [45]. The study of additional markers of time-dependent neural processing will be able to shed further light on the development of extinction learning in the brain.

When rats drink SAC at the end of CTA extinction, the patterns of brain activity evoked by this behavior do not uniformly retreat to a pre-CTA configuration representing the CS was a neutral stimulus. Instead, our data represent extinction as a time dependent process wherein different brain nuclei (e.g., GNC) become active, or inactive, as extinction becomes well established.

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