C-Fos protein expression in the brain during the extinction of a conditioned taste aversion (CTA).

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Abstract

This study investigated changes in brain activity during 3 different stages of CTA extinction (Nolan et al., 1997). 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). The neuroanatomical expression of *c-fos* protein in these rats was compared to that from subjects in several additional control groups including animals that had acquired a CTA followed by subsequent water exposures (not extinguished), rats that experienced SAC & LiCl explicitly unpaired (no CTA was formed) followed by subsequent non-reinforced SAC exposures, rats that experienced SAC & LiCl explicitly unpaired followed by subsequent water exposures, rats that were exposed only to oral, novel, SAC, and rats that were sacrificed immediately following CTA conditioning. Throughout the extinction process, elevated levels of *c-fos* protein were evident in the brainstem nuclei along the taste pathway. Increased expression within the Solitary Tract Nuclei was independent of the stage of extinction. However, the increase in expression within the Parabrachial Nucleii declined as the CTA was further extinguished. Neurons in the Basolateral Amygdala expressed less c-fos protein during the intermediate stage of extinction than during the initial or final stages. As rats achieve total reacceptance of SAC, *c-fos* expression reached its peak in the Gustatory Neocortex. These data suggest that extinction is not represented by a simple reversal of the *c-fos* activity evoked by CTA conditioning. Rather, our results identify a series of brain nuclei along the taste pathway that are sequentially activated as the CTA becomes extinguished.

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).
- 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?
- This study sought to document how functioning of brain nuclei previously associated with CTA acquisition would change during the course of extinction.
- 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).
- We measured behavioral responses during acquisition and extinction of a CTA and analyzed correlated c-Fos expression in various nuclei known to mediate this classically conditioned response.

Methods

Subjects: Adult Male Sprague-Dawley Rats

Group Designations:

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+LiCl	Water, full 60 minutes	SAC+LiCl	Water, full 60 minutes	SAC+LiCl	Water, full 60 minutes	SAC	SAC
CTA Yoked (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	SAC
Explicitly Unpaired Extinction (EU + SAC)	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	SAC	LiCl & Water, full 60 minutes	SAC	SAC
Explicitly Unpaired No Extinction (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	SAC
Conditioned Control (CTA Control)	SAC+LiCl	Water, full 60 minutes	SAC+LiCl	Water, full 60 minutes	SAC+LiCl	Water, full 60 minutes	-	SAC

Mean (+ SEM) number of rats/treatment/brain area analyzed = 5.9 + .17

Behavioral Procedures:

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

Conditioned Taste Aversion (CTA) Procedure:

- The conditioning procedure included three conditioned stimulus (CS) + unconditioned stimulus (US) trials for each rat. 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 .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 following the data provided by Nolan et.al. (1997). *Physiol & Behav*, 14, 161-170:

  CTA Extinction has been operationally defined by three distinct phases.
  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 it's yoked control; i.e., CTA + No EXT animal of similar weight was sacrificed 90 minutes 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 Nolan's 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 and sacrificed 90 minutes later.

Histology:

- Rats were sacrificed 90 minutes following their last SAC exposure.
- Rats were deeply anesthetized with Sodium Pentobarbital, i.p. (100 mg/kg, i.p.).
- Rats were intercardially perfused with heparinized saline followed by 4% paraformaldehyde.
- Brains were dissected immediately following perfusion and placed in 4% paraformaldehyde for 8-9 hours at ~4°C.
- Brains were then transferred to a 30% sucrose/ PBTh cryoprotectant until sectioned.
- Brains were sectioned at 40 µ using a freezing microtome.
- 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).
- These sections were counterstained using neutral red, mounted & coverslipped.
- Sections were viewed using the Olympus microscope, and the NIH Image software program. 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:

- Ø Nucleus of the Solitary Tract (NTS)
  Rostral (NTSr) and Intermediate (NTSi) nuclei
- Ø Parabrachial Nucleus (PBN)
  Medial and External nuclei
- Ø Amygdala
  Basolateral nucleus (BLA)
  Central nucleus (CN)
- Ø Gustatory Neocortex (GNC)

- Cells staining positive for c-Fos protein (only round, dark, uniformly stained cells) were counted per brain nucleus. The observer (CLK) was blind to the experimental condition of the rats.

Statistics:

- Data were analyzed using a 3-way Analysis of Variance [ANOVA; Extinction Level (Static, Dynamic, Asymptotic) X Extinction Treatment (Extinction, i.e., SAC drinking, or No-Extinction, i.e., No SAC drinking) X Learning Treatment (CTA learning, Explicitly Unpaired)] followed by 1-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 expression from CTA formation to CTA extinction, we calculated additional 1-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 throughout the entire analysis.

Summary & Conclusions

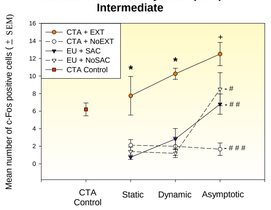
- C-Fos expression in the brains of CTA+EXT rats was frequently differentiated from that of the CTA controls suggesting the brain encodes this process in a distinctive way.
- These data suggest that extinction is not represented by a simple reversal of the c-Fos activity evoked by CTA conditioning. Rather, our results identify a series of brain nuclei along the taste pathway that are sequentially activated or inactivated as the CTA becomes extinguished.
- Levels of c-Fos protein expression are dependent on both the brain area and the level of extinction. For example, neurons in the **GNC** are most active when the once-avoided taste is fully reaccepted (Asymptotic stage of extinction). Whereas, c-Fos expression is most prominent in the **PBN** during the Dynamic stage of extinction.
- Compared to controls, rats in the CTA + EXT groups express:
  - o consistently more c-Fos-positive neurons in the **NTS** – throughout much of the extinction process.
  - o waxing and waning of c-Fos expression in the **PBN** as the extinction progresses.
  - o initially more c-Fos-positive cells in the **BLA**, followed by a waning of this response during the dynamic stage of extinction. Non-extinguished animals show an opposite response.
  - o in **CN** a differentiation between conditioned and non-conditioned animals independent of extinction treatments.
  - o 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.

Results

C-Fos immunohistochemistry:

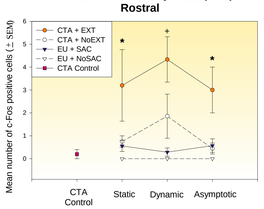
Nucleus of the Solitary Tract (NTS):

Intermediate NTS (NTSi): The cells in the "visceral" portion of the NTS increase c-Fos expression in proportion to the amount of SAC consumed. Expression of c-Fos in intermediate NTS may represent both volume of sweet taste consumed as well as CTA. Both CTA Control rats and CTA + EXT rats exhibit similar numbers of c-Fos-labeled neurons.



* = Significantly different from all other treatment groups at the same stage of extinction.
\+ = Significantly different from CTA + No EXT and EU + SAC groups.
\# = 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.

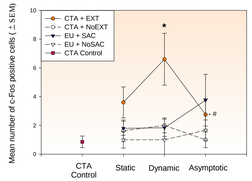
Rostral NTS (NTSr): CTA + EXT rats express more c-Fos in rostral NTS (the gustatory portion of the NTS) than do controls. However, c-Fos expression does not change as rats extinguish a CTA.



* = Significantly different from all other treatment groups at the same stage of extinction.
\+ = Significantly different from EU treatment groups.

Parabrachial Nucleus (PBN):

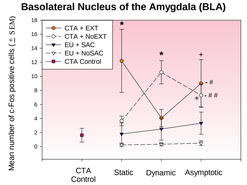
Cells of the PBN express significantly more c-Fos protein than controls only during the dynamic stage of extinction. PBN neurons of the CTA-EXT group return to pre-extinction levels of c-Fos protein expression when CTA extinction is complete.



*= 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.

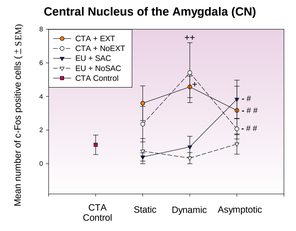
Amygdala: The Basolateral Nucleus (BLA) and Central Nucleus (CN) of the amygdala exhibited very different patterns of c-Fos expression throughout the course of the study.

Basolateral Nucleus (BLA): At the early (Static) and late (Asymptotic) stages of extinction, rats expressed more c-Fos than did controls. However, c-Fos expression is reduced in the CTA + EXT (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 (yoked) 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.



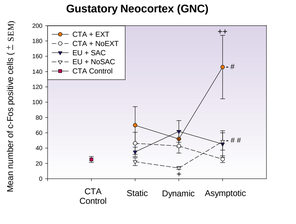
* = Significantly different from all other treatment groups at the same stage of extinction.
\+ = Significantly different from EU + No SAC group.
\# = Treatment group shows a significant increase in c-Fos expression (CTA Control to Static) depending on the stage of extinction.
= Treatment group shows a significant increase (CTA Control to Dynamic and Asymptotic) or decrease (Static to Dynamic) in c-Fos expression depending on the stage of extinction.

Central Nucleus (CN): CN c-Fos expression does not change as CTA extinction progresses. Conditioned rats express more c-Fos than the non-conditioned (EU) rats – but only during the dynamic phase of extinction.



\+ = Significantly different from EU + No SAC group.
++ = Significantly different from both EU + SAC and EU + No SAC groups.
\# = 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.

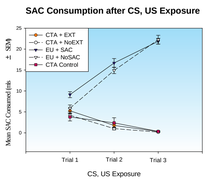
Gustatory Neocortex (GNC): *Neurons in the GNC express more c-Fos than control animals - but only during the Asymptotic stage of extinction.*

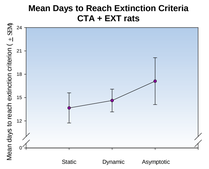


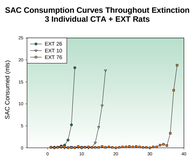
\+ = Significantly different from EU + SAC group. ++ = Significantly different from CTA + No EXT group.
\# = Treatment group shows a significant increase in c-Fos expression (CTA Control to Asymptotic and Dynamic to Asymptotic) depending on the stage of extinction.
= EU + No SAC group shows a significant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

Drinking Behavior:

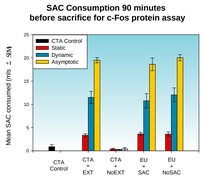
SAC consumption after CS, US exposure: Saccharin consumption in both the Explicitly Unpaired CS,US groups increased over the course of the 3 trials indicating that these rats did not acquire a CTA. Conversely, SAC consumption in all of the CTA groups (CTA/Extinction, CTA/Yoked & Conditioned Controls) decreased over the 3 trials indicating that these rats acquired a CTA.







SAC Consumption before c-Fos protein measurements: CTA + EXT rats indeed extinguished the CTA, while those receiving water (CTA + No-EXT) retained the CTA. The rats that received explicitly unpaired 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.



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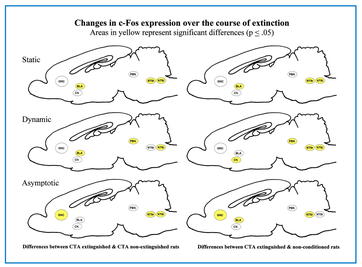

(Left column) Highlighted are brain areas where significant changes in c-Fos protein expression were observed between animals that were conditioned and extinguished (CTA + EXT) as compared to animals that were conditioned but never extinguished (CTA + No EXT). Therefore, these differences should represent changes associated specifically with the extinction process. Over the course of extinction, 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.

(Right column) Highlighted are areas where significant changes in c-Fos protein expression were observed between animals that were conditioned and extinguished (CTA + EXT) as compared to animals that were never conditioned (EU). A lack of difference between these groups in a particular brain area may illustrate an attenuation of the CTA engram (i.e., a reversal of learning). Differences between these groups may represent the extinction process as a new learning.