Neural Correlates of Appetitive Pavlovian Extinction *

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The infralimbic cortex (IL), prelimbic cortex (PL), nucleus accumbens core (NAcCore), and nucleus accumbens shell (NAcSh) have been previously implicated in operant conditioning and extinction, but less is known about their role in appetitive Pavlovian extinction. The objective of this study was to investigate c-fos immunoreactivity, an indirect marker of neuronal activity, in the IL, PL, NAcCore, medial NAcSh, and lateral NAcSh to assess their roles in early vs. late appetitive Pavlovian extinction. After appetitive Pavlovian conditioning, rats in the Paired, Inter-trial interval (ITI) Unpaired, and Home-cage (H-C) Unpaired training conditions underwent either one extinction or six extinction sessions. We observed similar c-fos immunoreactivity in the IL of Paired and ITI Unpaired conditions following one and six extinction sessions, whereas greater c-fos was found in the PL of Paired and ITI Unpaired rats following six extinction sessions. Together, these findings suggest that the IL, but not the PL, plays a role in the inhibition of conditioned responding through its continual activation in appetitive Pavlovian extinction.

Substance use disorders (SUDs) involve maladaptive behaviours which occur because of learned associations between the effects of the abused drug and discrete cues in the environment. Therefore, treatment for SUDs involves inhibiting the expression of these associations through a process known as extinction, a fundamental form of inhibitory learning. More precisely, extinction refers to a decrease in conditioned responding that is produced when the outcome an animal expects is withheld. Evidence suggests that this decrement in responding is not due to the original conditioned association being erased, but rather because animals learn a new and competing inhibitory association [1]. Accordingly, it is essential to gain a more extensive understanding of the neural mechanisms of appetitive extinction to develop better interventions for such maladaptive behaviours.

The Infralimbic Cortex to Nucleus Accumbens Shell Pathway in Appetitive Operant Extinction

In appetitive operant conditioning, the animal learns to perform an operant response (e.g., lever press) to receive a rewarding outcome (e.g., drug infusion) and then learn to inhibit responding during extinction. Previous work illustrated the important role of the infralimbic cortex (IL), but not the prelimbic cortex (PL) of the medial prefrontal cortex (mPFC), in facilitating appetitive operant extinction learning [2-4]. More specifically, the differential involvements of the mPFC subregions in extinction may be due to their specific projections to the nucleus accumbens (NAc), which is implicated in reward-motivated behaviours [5]. Findings indicate that the IL to nucleus accumbens shell (NAcSh) projection is involved in the extinction of operant conditioned responding, whereas the PL to nucleus accumbens core (NAcCore) projection is thought to be involved in promoting operant drug-seeking [6-8].

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The Infralimbic Cortex to Nucleus Accumbens Shell Pathway in Appetitive Pavlovian Extinction

Despite the evidence implicating the IL in appetitive operant extinction, less is known about its role in appetitive Pavlovian extinction. Because these are two different learning processes, the brain regions involved in operant extinction might differ from those in Pavlovian extinction [9]. In Pavlovian conditioning, the animal learns to predict the delivery of a positive unconditioned stimulus (US; e.g., sucrose) following the presentation of the conditioned stimulus (CS; e.g., auditory cue). In extinction, the CS is presented, but the US is no longer delivered, leading to a reduction in conditioned responding as measured by the number of port entries.

The IL and PL subregions of the mPFC seem to play different roles in appetitive Pavlovian extinction. Previous work has shown that activating the IL after extinction attenuates reinstatement, spontaneous recovery, and renewal [10] whereas lesioning of the IL before extinction training increases spontaneous recovery and reinstatement, suggesting that activity in the IL is sufficient to suppress appetitive Pavlovian conditioned responding after extinction [11]. Contrary to previous findings, inactivation of the IL immediately before the first extinction session seems to facilitate the acquisition of within-session extinction [9], and disrupted retrieval of the extinction memory, whereas PL inactivation had no effect [9] [12]. Together, these results suggest that the IL, but not the PL, plays an important role in the extinction of appetitive Pavlovian conditioning.

The opposite roles of the IL and PL in the extinction of Pavlovian conditioned responding may be related to their individual projections to the NAc. Ziminski and colleagues (2017) [13] found that neurons in the NAcSh are activated by the original CS-US association memory, and the number of the CS-activated neurons in the ensemble diminished after Pavlovian extinction as shown by lower c-fos expression. The diminished size of the original neuronal ensemble involved in conditioning could be explained by a new ensemble of neurons being recruited by extinction. This idea is consistent with the theory that extinction memories compete against the original CS-US association for expression. However, these limited findings underscore the necessity to further investigate the neural correlates of appetitive Pavlovian extinction learning.

The present study investigated the neural correlates of early vs. late appetitive Pavlovian extinction by comparing c-fos immunoreactivity [14] [15] in the IL, PL, NAcSh, and NAcCore. In the training phase, rats in the paired groups learned to form an association between a conditioned stimulus and sucrose solution delivery whereas the two control groups (ITI Unpaired and H-C Unpaired) received unpaired presentations of the conditioned and unconditioned stimuli. This acquisition phase was then followed by one or six extinction sessions, where the sucrose solution was no longer delivered.

We predicted greater c-fos immunoreactivity in the IL and the medial NAcSh since these regions have previously been implicated in the facilitation of appetitive extinction, as opposed to the PL and NAcCore. However, we predicted no effects of extinction on neural activity in the PL, lateral NAcSh and the NAcCore. We also expected greater c-fos expression in the IL and NAcSh of rats that underwent one extinction session, compared to those that underwent six extinction sessions. This prediction is supported by the Rescorla-Wagner model positing that maximal learning occurs the first time an event is surprising, in this case that is the first session where the CS is presented without the US [16]. Therefore, comparing the neuronal marker of c-fos expression following one and six extinction sessions allowed us to elucidate which brain regions are most

activated when a CS-US association is extinguished.

Method

Subjects

Thirty-eight Long-Evans male rats (220-240 grams; Charles River, Canada) were housed individually in polycarbonate cages containing a nylabone (Nylabones; Bio-Serv, Flemington, NJ), a polycarbonate tunnel (Rat Retreats, Bio-Serv), beta-chip bedding (Aspen Sani chips; Envigo, Indianapolis, IN) and shredded paper used for enrichment. The cages were maintained on a 12-hour light/ dark cycle with the lights on at 7:00 in a colony room. All rats had unlimited access to standard chow and water. All procedures performed in this experiment were approved by the Concordia University Animal Care Committee and respected the guidelines of the Canadian Council on Animal Care.

Apparatus

Behavioural testing occurred in similar conditioning chambers during each replicate of the experiment (ENV-009A, Med Associates Inc., St-Albans, VT, USA), located in a different room than the colony room. In each behavioral chamber, the floors were composed of stainless-steel rods (ENV-009 A-GF), there was a dual cup fluid port (ENV-200R3AM), a white-noise speaker (ENV-225SM), and a house light (75W, 100 mA, EV-215M) that signaled the beginning and end of each session. A sucrose solution was delivered into the fluid port via a syringe attached to a pump (PHM-100). The frequency of port entries into the fluid port was monitored by an infrared detector unit (ENV-254CB). All experiments were controlled by a computer with Med Associates interface and software (Med-PC for Windows, version IV; Med Associates Inc.).

Behavioral Procedures

Home-cage sucrose pre-exposure. 10% (w/v) sucrose and water was made available to rats in their home cage for 48 hours. Both bottles were weighed after 24 and 48 hours to measure the rats' preference of sucrose over water. The groups were counterbalanced based on body weight, sucrose preference and sucrose consumption into six experimental groups [9]: 6 Extinction (Ext) Paired (n = 7), 1 Ext Paired (n = 7), 6 Ext ITI Unpaired (n = 6), 1 Ext ITI Unpaired (n = 6), 6 Ext H-C Unpaired (n = 6), and 1 Ext H-C Unpaired (n = 6).

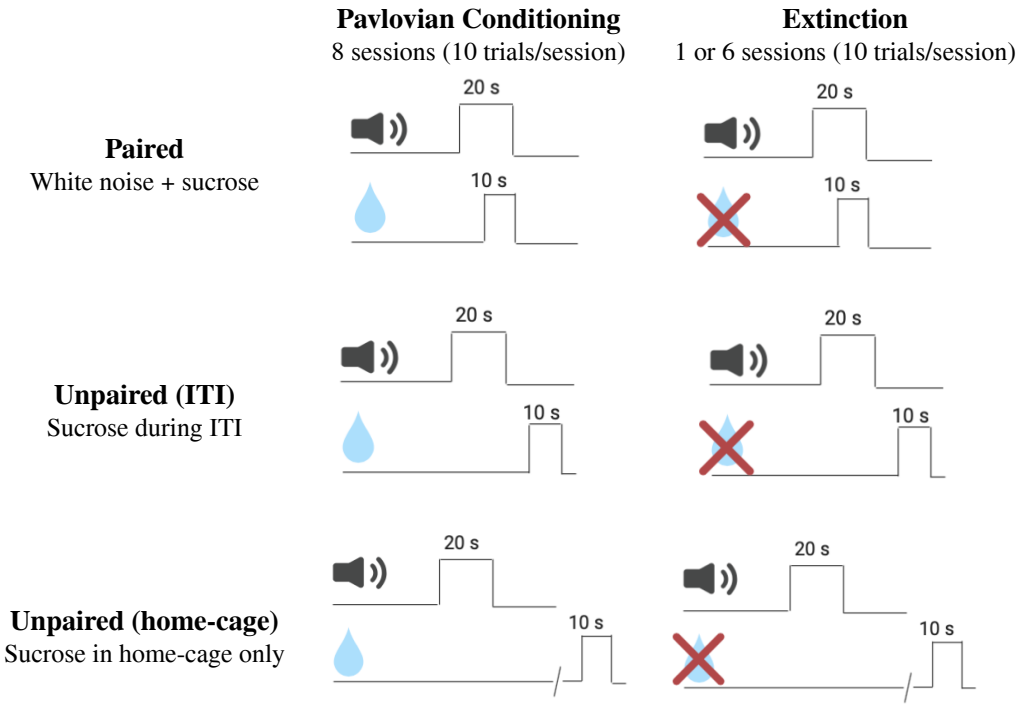
Habituation. After sucrose pre-exposure, the rats were habituated to the behavioural room and conditioning chamber for 20 minutes per day over 3 days.

Appetitive Pavlovian conditioning. The six experimental groups of rats received eight, 57-minutes Pavlovian conditioning sessions. After a 2-minute delay, the house light turned on, signaling the start of the session. During each session, a 20 second auditory stimulus (white noise) was presented ten times and was followed by a 120-, 240- or 360-second inter-trial interval (ITI). Rats in the Paired condition received a 0.3ml sucrose reward (10% w/v) after 10 seconds of stimulus presentation, while rats in the ITI Unpaired condition received the reward half-way through the variable ITI.

Rats in the H-C Unpaired condition received sucrose delivery in their home-cage at a random time from 1 hour to up to 4 hours after the session. The formation of the CS-US association was measured by the number of fluid port entries (see Figure 1).

Figure 1

Experimental design



Note. Rats first underwent a home-cage sucrose pre-exposure phase and then they were divided into six groups: 6 Ext Paired, 1 Ext Paired, 6 Ext ITI Unpaired, 1 Ext ITI Unpaired, 6 Ext H-C Unpaired and 1 Ext H-C Unpaired. Thirty-eight rats underwent eight daily, consecutive appetitive Pavlovian conditioning sessions. Animals in the Paired condition were exposed to 10 presentations of a white-noise cue (CS), which co-terminated with the delivery of sucrose (US) into a fluid port. Animals in the ITI Unpaired condition were exposed to 10 CS presentations followed by sucrose presentation during the ITI. Animals in the H-C Unpaired condition, were exposed to 10 CS presentations followed by sucrose delivery in their home-cage at a random time after the session. After Pavlovian conditioning, they underwent either 1 extinction session or six daily, consecutive extinction sessions during which sucrose was not delivered.

Extinction. During extinction, the white-noise was presented, but the sucrose was no longer delivered. Rats were either assigned to the 6 Ext group, receiving six extinction sessions during six consecutive days, or the 1 Ext group, receiving one extinction session.

Tissue Processing & C-Fos Immunohistochemistry

The rats were anesthetized with an injection of EuthanylTM 90 minutes after the start of the final extinction session followed by transcardiac perfusions using 4% paraformaldehyde and phosphate buffer saline. The brains were stored at -80°C until they were sectioned. Coronal sections of the brains were cut into six adjacent series at 40 μm using a cryostat (Microm HM 505E). The sections were then stored in cryoprotectant at -20°C until tissue processing using c-fos immunohistochemistry. One coronal series of each brain was processed for c-fos immunoreactivity, using

a standard immunohistochemical procedure.

Microscopy and Cell Counting

The anatomical regions of interest in this experiment for statistical comparisons were the mPFC and the ventral striatum. The images of c-fos-stained sections were digitally captured at 20X magnification using a Nikon Eclipse TiE inverted C2 confocal microscope operated using NIS-Elements software. The subregions of interest in the mPFC (IL and PL) and in the ventral striatum (medial NAcSh, lateral NAcSh, and NAcCore) were then identified using the George Paxinos and Charles Watson (2007) [17] rat brain atlas. The density of c-fos-labeled nuclei was analyzed as an estimate of the number of immunoreactive cells per subregion. The normalized density was then calculated by dividing the density by the averaged density of the H-C Unpaired of the same group (6 Ext vs. 1 Ext) and multiplying this value by 100. The two experimenters were blind to the experimental conditions of each rat during quantification.

Statistical Analyses

An 8 x 2 x 3 x 2 mixed ANOVA was used to assess the acquisition of Pavlovian appetitive conditioned responding. The within-subjects factors were Session (1 to 8), and Interval (pre-CS vs. CS); and the between-subjects factors were Training (Paired, ITI Unpaired, H-C Unpaired), and Group (6 Ext, 1 Ext). A 6 x 2 x 3 mixed ANOVA across the six extinction sessions was also used to assess extinction of conditioned responding. The within-subjects factors were Session (1 to 6), and Interval (pre-CS vs. CS), and the between-subjects factor was Training (Paired, ITI Unpaired, H-C Unpaired). The dependent variable was the number of port entries for both acquisition and extinction analyses. To evaluate the final extinction session across all conditions and groups that correspond to when the brains were collected for c-fos immunoreactivity analysis, a two-way ANOVA was conducted. The dependent variable was the normalized number of port entries which represents the number of port entries made during the CS subtracted by the number of port entries made during the pre-CS, accounting for spontaneous, unconditioned port entries [10].

A two-way ANOVA was used to assess c-fos immunoreactivity for each subregion of interest separately. The between-subjects factors were Group (1 Ext, 6 Ext) and Training (Paired, ITI Unpaired, H-C Unpaired). The dependent variable was the normalized density of c-fos nuclei in each region of interest. Greenhouse-Geisser correction were reported following violations of sphericity. All data analyses were conducted using SPSS (IBM SPSS Statistics 23.0). Additionally, results were considered statistically significant at $p < .05$.

Results

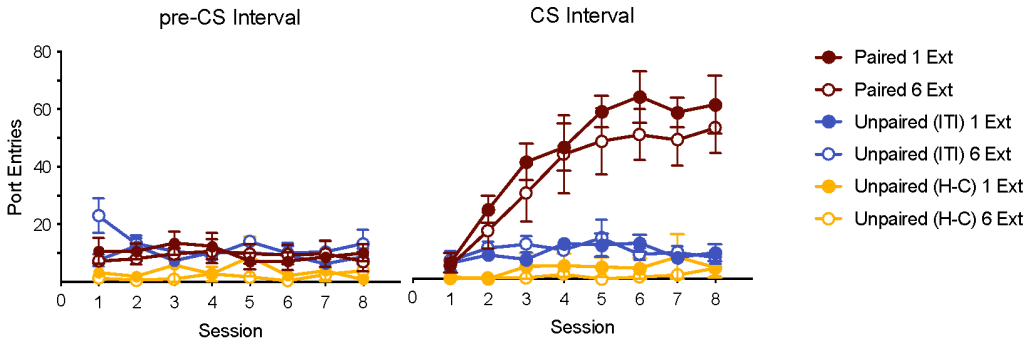
Summary Statistics

Acquisition. The mixed ANOVA revealed a main effect of Session [$F(4.42, 141.49) = 7.45$, $p < .005$, $\eta^2 = .03$; see Figure 2]. There was also a significant main effect of Training on the number of port entries [$F(2,32) = 49.92$, $p < .005$, $\eta^2 = .26$]. Bonferroni-corrected post-hoc comparisons revealed that the Paired group made significantly more port entries than both Unpaired groups ($p < .005$). This difference is because only the Paired training condition was exposed to a white-noise CS that co-terminated with sucrose. The ITI Unpaired training condition also displayed a significantly higher numbers of port entries than the H-C Unpaired training condition ($p = .009$). This result can be explained by the fact that the ITI Unpaired training condition received sucrose in the

conditioning chamber during the intertrial interval, whereas the H-C Unpaired condition received sucrose in the home-cage only. A main effect of Interval was also uncovered [$F(1, 32) = 48.04$, $p < .005$, $\eta^2 = .07$]. Bonferroni-corrected post-hoc comparisons showed that the number of port entries during the pre-CS was significantly different than during the CS ($p < .005$), with more port entries made during the CS interval. The analysis also detected an Interval x Session x Training interaction [$F(7.01, 112.16) = 9.38$, $p < .005$, $\eta^2 = .05$], indicating that the Interval x Session interaction described previously differed between the three training conditions. Bonferroni-corrected post-hoc comparisons discerned that port entries significantly increased during the CS for the Paired condition across conditioning sessions 1 to 8 ($p < .005$). However, no significant differences were found in the number of port entries of ITI Unpaired and H-C Unpaired during the CS across sessions 1 to 8 ($p = 1.000$).

Figure 2

Acquisition of appetitive Pavlovian conditioning.

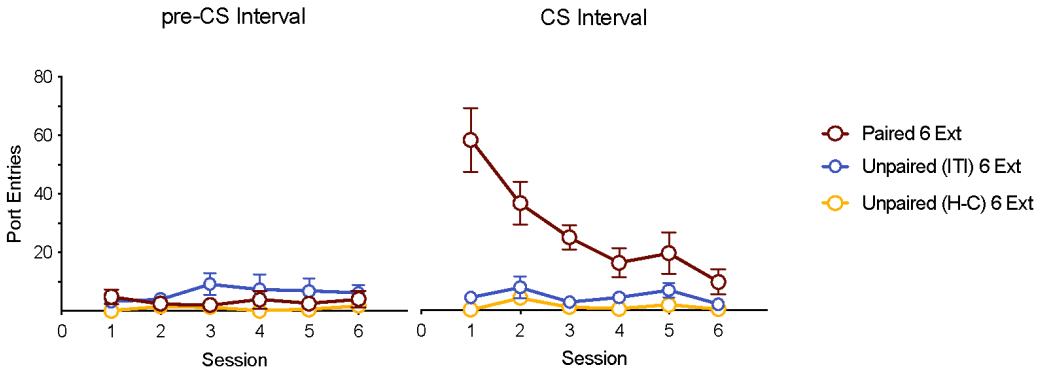


Note. This figure depicts the pre-CS and CS port entries made across conditioning sessions. An Interval x Session x Training interaction was detected, whereby responding to the discrete sucrose cue increased during the CS interval across conditioning sessions for the Paired conditions ($p < .005$), but not for either of the Unpaired conditions ($p = 1.000$). Port entries also did not differ during the pre-CS interval across sessions for the training conditions ($p = 1.000$). Error bars represent the SEM.

Extinction. The three-way mixed ANOVA revealed a main effect of Session [$F(2.91, 46.51) = 4.69$, $p = .007$, $\eta^2 = .03$; see Figure 3]. However, Bonferroni-corrected post-hoc comparisons showed that the number of port entries made in the 1st session was not significantly different compared to the 6th session ($p = .118$). A main effect of Training was also detected [$F(2,16) = 13.97$, $p < .005$, $\eta^2 = .20$]. Bonferroni-corrected post-hoc comparisons showed that the number of port entries was significantly different for the Paired compared to the ITI Unpaired ($p = .008$) and H-C Unpaired ($p < .005$) training conditions, with more port entries exhibited by the Paired condition. Additionally, the analysis revealed a main effect of Interval [$F(1,16) = 25.44$, $p < .005$, $\eta^2 = .07$]. Bonferroni-corrected post-hoc comparisons showed that the number of port entries made during the CS was significantly higher than those made during the pre-CS ($p < .005$).

Figure 3

Extinction of appetitive Pavlovian conditioned responding



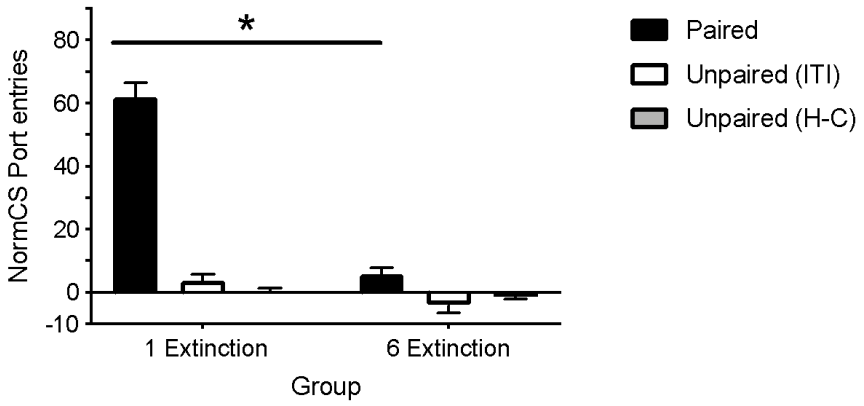
Note. This figure depicts the pre-CS and CS port entries made across extinction sessions. An Interval x Session x Training interaction was detected, whereby responding to the discrete sucrose cue decreased during the CS interval across extinction sessions for the Paired condition ($p < .005$), but not for either of the Unpaired conditions ($p = 1.000$). The number of port entries did not differ during the pre-CS interval across extinction sessions for the three training conditions ($p > .05$). Error bars represent the SEM.

The three-way mixed ANOVA also revealed an Interval x Session x Training interaction [$F(5.50, 43.99) = 5.73$, $p < .005$, $\eta^2 = .06$], indicating that the Interval x Session interaction described previously differed between the three training conditions. Bonferroni-corrected post-hoc comparisons showed that port entries significantly decreased during the CS for the Paired condition across extinction session 1 to 6 ($p < .05$). However, there was no significant difference across extinction sessions during the CS for both Unpaired groups ($p > .05$). Additionally, the number of port entries did not differ during the pre-CS across extinction sessions for the three training conditions ($p > .05$).

Final extinction. The two-way ANOVA revealed a main effect of Group [$F(1,32) = 102.91$, $p < .005$, $\eta^2 = .16$; see Figure 4]. Bonferroni-corrected post-hoc comparisons showed that the number of normalized port entries during the final extinction session was significantly higher for the subjects that had 1 extinction session compared to those that had 6 extinction sessions ($p < .005$). Additionally, the analysis detected a main effect of Training [$F(2,32) = 113.86$, $p < .005$, $\eta^2 = .36$], indicating that the training condition had a significant influence on the normalized number of port entries made during the final extinction session. Bonferroni-corrected post-hoc comparisons revealed that the Paired training condition made significantly more normalized port entries than the ITI Unpaired, and H-C Unpaired ($p < .005$) training conditions.

Figure 4

Number of normalized port entries made during the final extinction session following appetitive Pavlovian conditioning.



Note. This figure depicts the number of normalized port entries made during the final extinction session for each training condition. A Group x Training interaction was detected, whereby the number of normalized port entries was significantly higher for subjects in the Paired 1 Ext group compared to the Paired 6 Ext group ($p < .005$). No differences were found between the ITI Unpaired 1 Ext compared to ITI Unpaired 6 Ext group ($p = .051$) or for subjects in the H-C Unpaired 1 Ext compared to H-C Unpaired 6 Ext group ($p = .700$). Error bars represent the SEM. (*) Significant difference between Paired 1 Ext group and Paired 6 Ext group (Two-way ANOVA, $p < .05$).

Finally, the analysis discerned a Group x Training interaction [$F(2,32) = 68.02$, $p < .005$, $\eta^2 = .21$]. This result indicates that the number of normalized port entries made during the final extinction session across the different levels of group differed between the three training conditions. Bonferroni-corrected post-hoc comparisons showed that normalized port entries were significantly higher for the Paired training condition in the 1 Ext group compared to the 6 Ext group ($p < .005$). However, there were no significant differences for the ITI Unpaired 1 Ext group vs. 6 Ext group ($p = .051$), nor the H-C Unpaired 1 Ext vs. 6 Ext group ($p = .700$).

C-fos immunoreactivity. The c-fos immunoreactivity was analyzed in the mPFC (IL, PL) and the ventral striatum (NAcCore, medial NAcSh, lateral NAcSh).

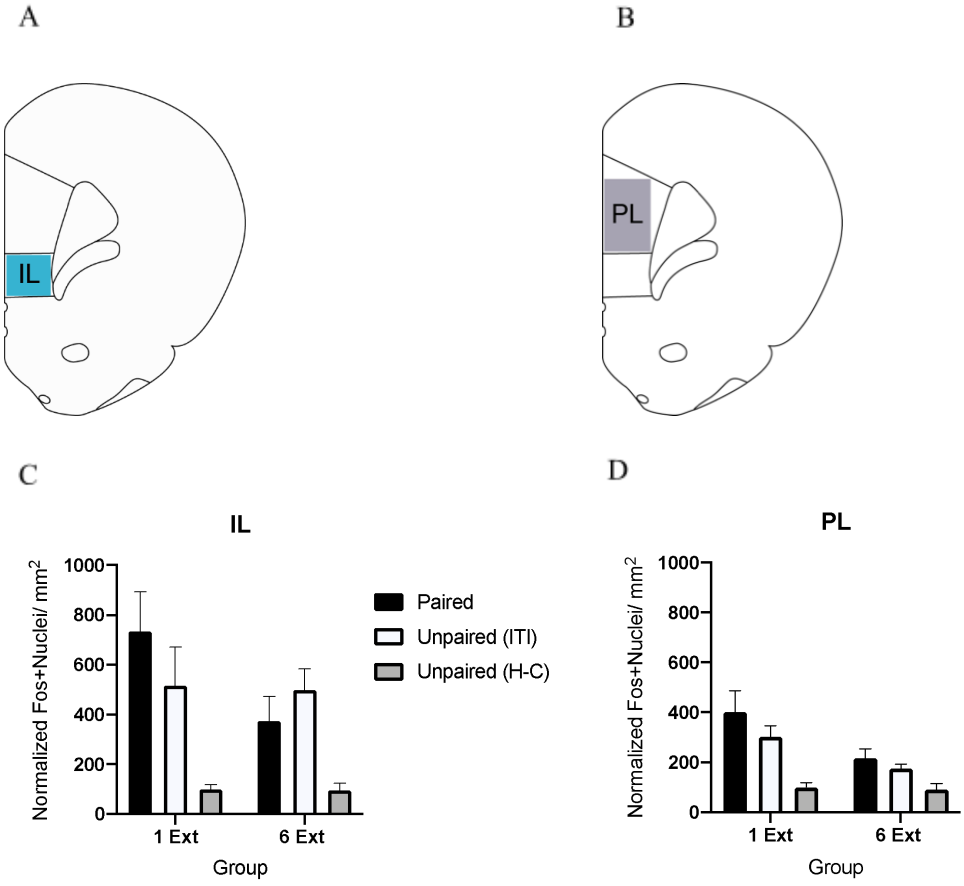
Medial prefrontal cortex.

The two-way ANOVAs revealed a main effect of Training in the IL [$F(2, 32)= 10.43$, $p < .005$, $\eta^2 = .36$; see Figure 5C]. Bonferroni-corrected post-hoc comparisons showed that the normalized density of c-fos nuclei in the IL was significantly higher for the Paired ($p = .001$) and ITI Unpaired ($p = .003$) conditions compared to the H-C Unpaired condition, while there was no significant difference in the normalized density of c-fos nuclei between the Paired and ITI Unpaired conditions ($p = 1.000$). There was no significant main effect of Group [$F(1, 32) = 2.03$, $p = .164$, $\eta^2 = .04$], nor a significant interaction between Group and Training [$F(2, 32) = 1.80$, $p = .182$, $\eta^2 = .06$] on the normalized density of c-fos nuclei in the IL. Further, a main effect of Training was also detected in the PL [$F(2, 32) = 10.11$, $p < .005$, $\eta^2 = .32$; see Figure 5D]. Bonferroni-corrected post-hoc comparisons showed that the normalized density of c-fos nuclei was significantly higher for the Paired ($p < .005$) and ITI Unpaired ($p = .022$) conditions compared to the H-C Unpaired condition. Ad-

ditionally, the analysis detected a Group main effect in the PL [$F(1, 32) = 7.23$, $p = .011$, $\eta^2 = .12$]. Bonferroni-corrected post-hoc comparisons showed that the normalized density of c-fos nuclei was significantly higher for the 1 Ext group compared to the 6 Ext group ($p = .011$). There was no significant Group x Training interaction [$F(2, 32) = 1.74$, $p = .192$, $\eta^2 = .06$] in the PL, indicating that the normalized density of c-fos nuclei across the different levels of group did not differ between the three training conditions.

Figure 5

c-fos immunoreactivity in the medial prefrontal cortex



Note. **A.** Normalized number of c-fos-immunoreactive nuclei per square millimeter in the infralimbic cortex (IL). **B.** Normalized number of c-fos-immunoreactive nuclei per square millimeter in the prelimbic cortex (PL). **C.** Mean ± SEM normalized number of c-fos immunoreactive nuclei per square millimeter in the IL. **D.** Mean ± SEM normalized number of c-fos immunoreactive nuclei per square millimeter in the PL. A Group main effect in the prelimbic cortex was detected, whereby the normalized density of c-fos nuclei was significantly higher for the 1 Ext group compared to the 6 Ext group ($p = .011$).

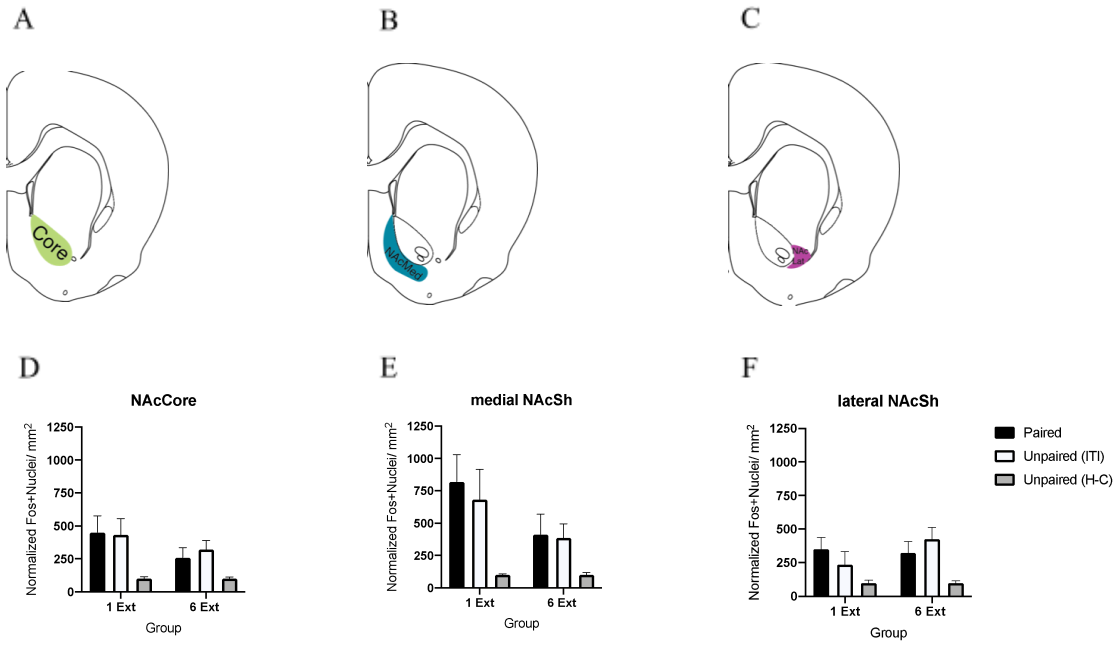
Ventral striatum.

The two-way ANOVAs revealed a main effect of Training in the NAcCore [$F(2,32) = 6.08$, $p = .006$, $\eta^2 = .26$; see Figure 6D], the medial NAcSh [$F(2,32) = 6.18$, $p = .005$, $\eta^2 = .25$; see Figure 6E], and in the lateral NAcSh [$F(2,32) = 6.51$, $p = .004$, $\eta^2 = .27$; see Figure 6F]. Bonferroni-corrected

post-hoc comparisons showed that the normalized density of c-fos nuclei in the NAcCore was significantly higher for the Paired (p = .017) and ITI Unpaired (p = .012) conditions compared to the H-C Unpaired training condition. Additionally, Bonferroni-corrected post-hoc comparisons showed that the normalized density of c-fos nuclei in the medial NAcSh was significantly higher for the Paired (p = .007) and ITI Unpaired (p = .033) compared to the H-C Unpaired training condition. The Paired and ITI Unpaired conditions did not significantly differ in their normalized density of c-fos nuclei (p = 1.000). Moreover, Bonferroni-corrected post-hoc comparisons showed that the normalized density of c-fos nuclei in the lateral NAcSh was significantly higher for the Paired (p = .008) and ITI Unpaired (p = .014) conditions compared to the H-C Unpaired training condition. There was also no significant difference in the normalized density of c-fos nuclei between the Paired and ITI Unpaired conditions (p = 1.000). Overall, these results indicate that the training condition had a similar effect on the normalized density of c-fos nuclei in each of these regions of interest. Furthermore, a trend towards a Group main effect in the medial NAcSh was also detected [$F(1, 32)$= 3.33, p = .077, η^2 = .07]. The analyses did not, however, detect a main effect of Group for the NAcCore [$F(1, 32)$= 2.02, p = .165, η^2 = .04], or the lateral NAcSh [$F(1, 32)$= .85, p = .364, η^2 = .02].

Figure 6

c-fos immunoreactivity in the ventral striatum



Note. **A.** Normalized number of c-fos-immunoreactive nuclei per square millimeter in the nucleus accumbens core (NAcCore). **B.** Normalized number of c-fos-immunoreactive nuclei per square millimeter in the medial nucleus accumbens shell (medial NAcSh). **C.** Normalized number of c-fos-immunoreactive nuclei per square millimeter in the lateral nucleus accumbens shell (lateral NAcSh). **D.** Mean ± SEM normalized number of c-fos-immunoreactive nuclei per square millimeter in the NAcCore. **E.** Mean ± SEM normalized number of c-fos-immunoreactive nuclei per square millimeter in the medial NAcSh. The two-way ANOVA revealed a trend main effect of Group on the dependent variable in the medial NAcSh (p = .077). **F.** Mean ± SEM normalized number of c-fos-immunoreactive nuclei per square millimeter in the lateral NAcSh.

Discussion

In the present study, we examined neural activity in regions of the mPFC and ventral striatum following early and late extinction of appetitive Pavlovian conditioned responding using c-fos immunohistochemistry. More specifically, we sought to identify differences in neural activity in the IL, PL, and subregions of the NAc, following one or six extinction sessions.

The normalized density of c-fos nuclei was significantly higher for the 1 Ext group compared to the 6 Ext group, suggesting that the PL was more active in early extinction than late extinction. Thus, our results support the hypothesis of the opposing roles of the IL and PL subregions of the mPFC as demonstrated in both aversive extinction [18] and appetitive extinction paradigms [12]. This is consistent with our results, suggesting that as inhibitory learning occurs in extinction training, the PL is no longer recruited to facilitate the expression of conditioned responding.

Additionally, the normalized density of c-fos nuclei was similar between the 1 Ext and 6 Ext groups for the IL, suggesting that that the number of extinction sessions did not influence c-fos immunoreactivity. We predicted greater c-fos expression in the IL and NAcSh of rats who underwent early extinction compared to those who underwent late extinction. Our data do not suggest a differential involvement of the IL during early compared to late extinction training as was found in an appetitive operant extinction [4]. In our experiment, the IL subregion of the medial PFC was continually active to inhibit conditioned responding during appetitive Pavlovian extinction. One possibility for this difference is that the IL mediates extinction differently in appetitive operant compared to appetitive Pavlovian extinction. Thus, these results provide novel information on the role of the IL in appetitive Pavlovian extinction and highlights potential differences in the neural correlates of the two types of extinction learning.

Previous research has also shown that the subregions of the NAc play differential roles in extinction learning explained by the projections they received from different regions of the medial PFC. Particularly, the NAcSh mediates appetitive extinction via its input from the IL, whereas the NAcCore promotes appetitive conditioned responding through its input from the PL [8, 6, 2]. Therefore, we expected higher c-fos expression in the NAcSh compared to the NAcCore. Contrary to our expectation, no differences of c-fos expression were found following early vs. late extinction for any regions of the NAc for the Paired training condition. Although, not statistically significant, upon visual inspection of the data, we did observe greater c-fos expression in the IL and medial NAcSh, compared to the other regions. Thus, this result seems to align with previous research supporting the role of the IL to NacSh projection in extinction, but further research should verify this result. Nevertheless, it is important to keep in mind that c-fos immunoreactivity alone cannot determine neural pathways, but region activation studies could. Similar to the c-fos immunoreactivity results observed for the IL, we did not find any c-fos expression differences following early vs. late extinction training for the medial NAcSh, suggesting that the continual activation of this subregion of the NAc is necessary for the inhibition of conditioned responding.

Interestingly, greater c-fos expression was found in the regions of the mPFC, and ventral striatum of Paired and ITI Unpaired conditions, compared to the H-C Unpaired condition. We had hypothesized that both Unpaired control conditions would show low levels of c-fos immunoreactivity compared to the Paired conditions following extinction training. This was because the ITI Unpaired and H-C Unpaired conditions did not acquire the CS-US association during conditioning, and therefore did not extinguish this association when sucrose was omitted. However, the

rats in the ITI Unpaired condition showed similar patterns of neural activation to the Paired condition. This suggests that rats in the ITI Unpaired condition may have acquired and extinguished a learned association leading to c-fos expression. Rather than learning a CS-US association, the ITI Unpaired rats may have learned a context-US association, an association between the context of the conditioning chamber and the sucrose delivery during the intertrial interval. For this reason, it was crucial to include the H-C Unpaired control group, which never learned a context-US association, to compare baseline c-fos immunoreactivity in these regions to conditions that underwent extinction of either a CS-US association, or a context-US association.

Limitations

One limitation of the present study is the use of c-fos immunoreactivity as an indirect marker of neuronal activity and the assumption that little or no c-fos protein is expressed under baseline conditions, which allows visualization of active neurons. Further, c-fos expression alone does not provide information about brain connectivity. Therefore, the brain pathways of learning and memory can be investigated only by combining c-fos immunoreactivity with other methods such as retrograde tracers [19]. Additionally, stimulus activation does not always lead to the activation of immediate early genes that lead to the transcription of the c-fos protein in neurons. Therefore, a lack of c-fos expression does not necessarily translate into lack of neuronal activity.

Future Directions

Future research should further investigate appetitive Pavlovian extinction using optogenetics which allows for direct manipulation of the neural activity of regions of interest with high temporal resolution. Importantly, this method would enable us to investigate not only regions, but pathways in the brain, such as the IL-NAcSh pathway in appetitive Pavlovian extinction.

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