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Environmental Enrichment during Abstinence Reduces Oxycodone Seeking and c-Fos Expression in a Subpopulation of Medial Prefrontal Cortex Neurons

Breanna Glaeser[1,2,*], **Valeria Panariello**[1,2,3,*], **Anjishnu Banerjee**[4], **Christopher M. Olsen**[1,2,5,ψ]

[1]Department of Pharmacology & Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

[2]Neuroscience Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

[3]Current Address: Department of Science and Medicine, University of Fribourg, Chemin du Musée 14 CH-1700 Fribourg, Switzerland

[4]Division of Biostatistics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

[5]Department of Neurosurgery, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

Abstract

Background: Several preclinical studies have demonstrated that environmental enrichment (EE) during abstinence reduces drug seeking for psychostimulant and opioid drugs. Drug seeking is dependent on activity within the dorsomedial prefrontal cortex, and enrichment has been able to reduce drug seeking-associated increases in c-Fos in this region. In this study, we tested the hypothesis that EE during abstinence from oxycodone self-administration would reduce drug seeking and c-Fos immunoreactivity within the prefrontal cortex in a cell-type specific manner.

Methods: Male rats self-administered oxycodone in two-hours sessions for three weeks, then underwent an initial drug seeking test under extinction conditions after one week of forced abstinence. Following this test, rats received either EE or remained individually housed in their home cage, then a second drug seeking test, with tissue collection immediately afterward.

ψCorrespondence should be addressed to: Christopher M. Olsen, PhD, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, Phone: (414) 955-7629, colsen@mcw.edu.

*denotes co-first authors

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Declarations of interest: none

Results: Compared to rats in standard housing, environmentally enriched rats had lower oxycodone seeking. In the prelimbic and infralimbic prefrontal cortices, the number of c-Fos+ cells was reduced, and this reduction was predominantly in inhibitory cells neurons, as evidenced by a reduction in the proportion of c-Fos+ cells in GAD+, but not CamKII+ cells. There was also a robust positive relationship between the number of c-Fos+ cells and persistence of oxycodone seeking in both the PrL and IL.

Conclusions: These findings further support the effectiveness of enriched environments to reduce reactivity to drug-associated stimuli and contexts and provide a potential mechanism by which this occurs.

Keywords

exercise enrichment; social enrichment; cognitive enrichment; opioid; prefrontal cortex

1. Introduction

The multifactorial etiology of drug addiction may be precipitated by the complex interaction between various genetic, environmental, and psychosocial factors (Kreek et al., 2005; Malone et al., 2022; Piazza and Le Moal, 1996). Environmental enrichment is a model achieved by housing animals in large chambers with social partners and access to novel objects. From a translational and interventional point of view, EE models positive life experiences (social, physical, and intellectual) and can decrease the impact of several disease states (reviewed in (Nithianantharajah and Hannan, 2006; van Praag et al., 2000)). Several preclinical studies have confirmed that environmental enrichment (EE) can decrease the reinforcing and conditioned rewarding effects of drug and non-drug reinforcers that ultimately influence vulnerability to addiction-like behaviors ((Venniro et al., 2018), also reviewed in (Carroll et al., 2009; Galaj et al., 2020; Grimm and Sauter, 2020; Stairs and Bardo, 2009)). More recently, EE during abstinence has been demonstrated to reduce drug seeking for psychostimulant and opioid drugs, indicating that the beneficial effects of enrichment apply to drugs of different classes and do not require concurrent exposure with the drug (reviewed in (Galaj et al., 2020; Solinas et al., 2021)). For example, EE during abstinence from cocaine self-administration blunted cocaine seeking measured during extinction testing in male rats (Chauvet et al., 2009). The same study also found that EE blunted cocaine seeking during cue- and yohimbine-associated reinstatement, but not cocaine-primed reinstatement (Chauvet et al., 2009). Similarly, EE reduced incubation of heroin craving and suppressed cued reinstatement in male and female rats that underwent either three or 15 days forced abstinence prior to extinction (Barrera et al., 2021). Despite these positive effects, there have also been reports of adverse consequences of EE in models of substance use. In male, but not female rats exposed to EE during adolescence (postnatal days 21–42 (PD 21–42)), alcohol intake was increased during intermittent access two-bottle choice testing performed on PD 46–72 (Berardo et al., 2016). This effect was also observed when EE was administered later in life, and the detrimental effects of EE were reversible (Rockman et al., 1989). Male rats that were raised in isolated conditions for 90 days after weaning showed significantly less alcohol consumption compared to those housed in EE

conditions. After this period, rats that switched housing conditions from EE to isolated housing dramatically reduced their alcohol intake (Rockman et al., 1989).

The dorsomedial prefrontal cortex (including the prelimbic cortex (PrL)) has been implicated in driving persistent drug seeking after both extinction and forced abstinence of several misused substances (Doherty et al., 2013; Peters et al., 2009; Willcocks and McNally, 2013). Moreover, EE can mitigate drug seeking-associated increases in c-Fos expression in the dorsomedial prefrontal cortex of male rats (Chauvet et al., 2011; Thiel et al., 2010). In the current study, we aimed to determine if the effects of EE on drug seeking and prefrontal c-Fos expression extended to oxycodone in male rats. Among opioids, oxycodone has the second highest number of prescriptions and the highest distribution to pharmacies in the United States (Kenan et al., 2012), but no studies to date have examined the effects of EE on oxycodone seeking. We tested the hypothesis that EE during abstinence from oxycodone self-administration would reduce drug seeking and c-Fos immunoreactivity within the prefrontal cortex of male rats.

2. Material and Methods

2.1. Animals

Male Sprague Dawley rats (n=18, 275–300 g, approximately 9 weeks of age) Envigo, Madison, WI) arrived at the Medical College of Wisconsin (MCW) and were immediately single housed and placed in a reverse light cycle housing room (lights off 0730–1930). Rats were given food and water ad libitum throughout the study, which began ~2 weeks after arrival. The only exception was during behavioral testing, which occurred during the dark (awake) lighting cycle. Testing was conducted 5 days/week. All experiments were completed in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals (8^{th} edition), and the Institutional Animal Care and Use Committee (IACUC) at MCW.

2.2. Environmental Enrichment

Control rats and EE rats prior to initiation of enrichment were single-housed in standard rat housing cages (14.5" X 10" X 7.5", Allentown Cage Products, Allentown, NJ,) with woodchip bedding covering its plastic floor and two pieces of paper towels available for nesting. All EE rats (n=9) were housed together in a 36" X 20" X 60" "Ferret Mansion" (Quality Cage Crafters, Austin, TX) with 3 levels as described (Grimm et al., 2018; Grimm et al., 2013). Plastic flooring inserts were provided at intermittent spaces of the cage, and rotation of different toys, plastic igloos, and running wheels occurred three days/week. Rats were transferred to a clean EE chamber once per week.

2.3. Jugular Catheterization Surgery

Jugular catheterization was performed as described (Chiariello et al., 2023; Muelbl et al., 2022). Rats were anaesthetized with isoflurane (3%−5% induction, 1%−3% maintenance). A silicone catheter was inserted into the rat's right jugular vein towards the right atrium and exited at the interscapular region, where it was placed subcutaneously. The catheter consisted of a 22-gauge stainless steel cannula (P1 Technologies, Roanoke, VA, USA)

mounted on a base comprised of dental acrylic and polyester mesh. Rimadyl (carpofen, 5 mg/kg, Zoetis Inc) was given subcutaneously at the start of surgery and the following day. For catheter maintenance, daily flushes of 0.2 ml of heparinized saline (30 units/ml) and cefazolin (100mg/ml) were given. The recovery from the surgery consisted of at least one week before any drug self-administration.

2.4. Apparatus

All drug self-administration and seeking sessions were performed in operant conditioning chambers (31.5 × 25.4 × 26.7 cm, Med Associates, Fairfax, VT, USA) that were within sound-attenuating boxes equipped with an exhaust fan that is controlled by computer software. The chambers contained two retractable levers on the right-side wall, one on the left and one on the right side, and above each lever was a corresponding cue light. On the left wall of the chamber, a house light was present. The software MED-PC (Med Associates), was used for the programs and data collection.

2.5. Drug Self-Administration

Oxycodone self-administration was performed without prior training or food restriction in two-hour sessions. At the beginning of each session, the exhaust fan turned on and two retractable levers were presented to the rats and designated as either “active” or “inactive”, which was maintained through the entirety of the study. The side which was designated as active was counterbalanced within each group. Sessions were performed on a Fixed Ratio-1 (FR1) schedule of reinforcement, where one lever press resulted in one infusion. Active lever press also illuminated the corresponding cue light for five seconds and there was a 10-second timeout. Every press during the timeout was recorded, but there was no programmed consequence. Inactive lever presses were recorded but had no programmed consequence. Each session had a maximum number of infusions set at 96 reinforcers. Oxycodone self-administration was at a dose of 0.1 mg/kg/infusion for all 15 sessions. Three days following the final drug self-administration session, catheter patency was ascertained using Brevital (methohexital, 9 mg/kg, i.v., JHP Pharmaceuticals, Rochester, NY). Any rat that did not pass this test was eliminated from the study.

2.6. Drug Seeking, Forced Abstinence, and Enrichment

After the rats completed three weeks of drug self-administration, they went through six days of forced abstinence, within the standard isolation housing (labeled “home cage” in Fig. 1A). On the seventh day following the 15-day self-administration, rats were put back into the operant conditioning chambers to complete a seeking session. This session was done under the same conditions as the drug self-administration except that no infusions occurred. Rats were counterbalanced and divided into two groups based on the number of active lever presses during this first drug seeking session. The first group (control) was singly housed within the standard isolation housing. The second group was housed together in an Environmental Enrichment (EE) chamber. These groups were maintained in these housing conditions beginning on day eight of oxycodone abstinence until the second oxycodone seeking session, which was done 30 days later in the same manner as the first seeking session.

2.7. Immunohistochemistry

Immediately after the second drug seeking test, animals were anesthetized using Euthasol (3.85 mL/kg, i.p., Virbac, Westlake, Texas) and transcardially perfused with ice-cold 0.1 M phosphate buffered saline (PBS), pH 7.4, followed by ice-cold 4% formaldehyde in 0.1 M PBS, pH 7.4. The brains were removed and sored overnight in formaldehyde and then moved to 30% sucrose in PBS at 4°C. Coronal sections (30 μm) containing region of interest were collected at levels corresponding to approximately 2.70 and 2.20 mm from bregma (Paxinos and Watson, 1986) using a Leica CM 1860 cryostat. Sections were stored in cryoprotectant (14% 0.2M PB, 43% glycerol, 43% ethylene glycol) at 20°C until the time of immunohistochemistry.

All of the tissue sections used in this experiment were processed for c-Fos protein expression at the same time. The tissue was rinsed in 0.1M PBS and incubated in blocking solution (0.1 M PBS containing 1.0% bovine serum albumin (Millipore Sigma, St. Louis, MO) with 4% Triton X-100 (Fisher Scientific, Hampton, NH)) for 1hr. Sections were then incubated for 48 hr at 4°C in blocking solution containing rabbit anti-c-Fos antibody (1:2,000, #226003, Synaptic Systems, Goettingen, Germany) and either mouse-anti-GAD67 (1:1,000, #MAB5406, Millipore Sigma) or mouse-anti-CAMKII (1:1000, #50049, Cell Signaling Technology, Danvers, MA). This c-Fos antibody is specific and does not recognize other fos-related antigens such as delta-FosB. Thus, c-Fos immunostaining represents acute cell activity. The sections were then rinsed in 0.1M PBS and incubated in blocking solution containing secondary antibodies (1:500 donkey anti-rabbit AlexaFluor488; 1:500 donkey anti-mouse Cy3; Jackson ImmunoResearch, West Grove, PA) at room temperature for 90 mins. The sections were rinsed again using 0.1 M PBS and incubated with 0.0001 % DAPI solution in 0.1M PBS, followed by rinsing the tissue in 0.1 M PBS. Sections were then mounted onto microscope slides and coverslipped with VectaShield hard set mounting media (Vector Laboratories, Newark, CA).

Immunofluorescence images were acquired using a Leica SP8 confocal microscope and a 25×/0.95NA objective (Medical College of Wisconsin Neuroscience Research Center Microscope Core), with microscope settings kept constant between samples. Images were imported into Imaris 9.0 software (Bitplane/Oxford Instruments, Abingdon, United Kingdom), and c-Fos+ cells were identified and counted using the “spots” function, while CamKII and GAD immunoreactive regions were identified using the “surfaces” function. c-Fos+ cells were classified as being CamKII+ or GAD+ by proximity to CamKII or GAD surfaces. Classification was performed by an observer blind to group assignment.

2.8. Statistical Analysis

Data were analyzed by general linear or linear mixed effect models where appropriate. In cases where data was non-normal, a square root transformation was applied and is indicated in the text. The mixed effect model measures fixed effects while adjusting for correlations of measures within each animal, allowing us to account for measurement of two slices and hemispheres (four total measurements) for each animal. Compound symmetry structures were used for the mixed effects models. Modeling assumptions were rigorously checked, including behavior of residuals, to ensure no major departures from homoscedasticity or

normality assumptions. Multiplicity adjustments, wherever relevant, were performed using Tukey's procedure. For oxycodone self-administration, lever press data were analyzed using a three-factor mixed effects model with session (repeated measure), lever, and group as fixed effects. This analysis was done to ensure that there were no differences in self-administration history between the control and EE groups. For oxycodone seeking, active and inactive lever press data were each analyzed by a two-factor mixed effects model with session (repeated measure) and group as fixed effects. These analyses were performed to test for effects of EE on active and inactive lever pressing during oxycodone seeking. For group comparisons of cell count data (Fig.2), a one-factor mixed model with animal ID as a random effect was used to test for effects of EE while controlling for multiple measures per subject. For the persistence ratio outcome (defined as the number of active lever presses on the second oxycodone seeking session divided by the number of active lever presses on the first oxycodone seeking session), mixed effects regression failed to converge, hence an average of measurements from the same animal was used with linear regression models. Linear regressions were performed separately within each group (control or EE) and for the entire population (control+EE) to examine relationships between the numbers or proportions of c-Fos+ cells and persistence in oxycodone seeking (Fig.3 and 4).

3. Results

Rats began the experiment by acquiring self-administration over 15 sessions, then having a drug seeking session under extinction conditions on abstinence day 6 (Fig. 1). Group assignment to undergo further abstinence in either the home cage or EE was based on the number of active lever presses on drug seeking session 1. This strategy minimized differences between groups when comparing active and inactive lever pressing during the self-administration phase (main effect of EE: $F(91,32)=0.11$, $p=0.74$; all interactions with EE $p>0.27$, square root transformation applied). Rats that underwent EE had a strong suppression of active lever pressing during drug seeking test 2 (main effect of session: $F(1,16)=31.8$, $p<0.0001$; main effect of EE: $F(1,16)=1.2$, $p=0.29$; EE x session interaction: $F(1,16)=8.7$, $p=0.0096$). EE rats had fewer active lever presses than control rats on drug seeking test 2 ($t(32)=2.4$, $p=0.022$) and reduced active lever presses between drug seeking tests 1 and 2 ($t(16)=6.1$, $p<0.0001$), while control rats did not show a reduction between drug session tests 1 and 2 ($t(16)=1.9$, $p=0.074$). Inactive lever pressing was not different between the groups (main effect of group: $F(1,16)=1.34$, $p=0.26$, interaction: $F(1,16)=1.73$, $p=0.21$), indicating that EE selectively reduced the response associated with prior oxycodone self-administration.

To examine the impact of EE on activation of excitatory and inhibitory neurons in the prefrontal cortex, we performed immunohistochemistry for c-Fos, calcium/calmodulin-dependent protein kinase II (CamKII), and glutamic acid decarboxylase-67 (GAD) (Fig. 2). CamKII is selective for glutamatergic neurons in the cortex, while GAD is selective for GABAergic neurons in the cortex. In the prelimbic cortex, EE sharply reduced the number of c-Fos+ cells ($p<0.001$, Fig. 2C) and the number of c-Fos+CamKII+ cells ($p<0.001$, Fig. 2D), although EE did not change the proportion of c-Fos+ cells that were CamKII+ ($p=0.79$, Fig. 2E, full statistical details in Table 1). EE also reduced the number of c-Fos+ cells in tissue used for GAD analysis ($p<0.001$, Fig. 2F) and the number of c-Fos+GAD+ cells

(p<0.001, Fig. 2G), as well as the proportion of c-Fos+ cells that were GAD+ (p<0.001, Fig. 2H, full statistical details in Table 1). We found very similar patterns in the IL, where EE rats had fewer c-Fos+ cells (p<0.001, Fig. 2I) and c-Fos+CamKII+ cells (p<0.05, Fig. 2J), while there was no difference in the proportion of c-Fos+ cells that were CamKII+ (p=0.85, Fig. 2K, full statistical details in Table 1). As observed in the PrL, EE reduced the number of c-Fos+ cells in tissue used for GAD analysis (p<0.001, Fig. 2L) and the number of c-Fos+GAD+ cells (p<0.001, Fig. 2M), as well as the proportion of c-Fos+ cells that were GAD+ (p<0.001, Fig. 2N, full statistical details in Table 1). These data indicate that drug seeking after EE was associated with far fewer c-Fos+ cells in the PrL and IL, but there was a selective reduction in the population of GAD+ cells.

Next, we explored the relationships between cell activation and oxycodone seeking in the PrL and IL. In the PrL, we found a significant positive association between the number of c-Fos+ cells and persistence of oxycodone seeking in both the tissue stained for CamKII (r^2=0.73, p<0.0001, Fig. 3A) and GAD (r^2=0.72, p<0.0001, Fig 3B). To determine if this relationship was also present specifically within excitatory or inhibitory neurons, we measured the percentage of the c-Fos+ cells that were also positive for CamKII or GAD, respectively. In the PrL, there was no significant relationship between the proportion of c-Fos+ cells that were CamKII+ (Fig. 3C), but there was in GAD+ cells (r^2=0.30, p=0.019, Fig 3D). The association between the proportion of these inhibitory c-Fos+ cells was not observed within either the control or EE group, suggesting that the dual reduction of oxycodone seeking and c-Fos+GAD+ cells by EE drives this relationship. Data from the IL largely paralleled those from the PrL. There was a significant positive association between the number of c-Fos+ cells and persistence of oxycodone seeking in both the tissue stained for CamKII (r^2=0.54, p=0.0005, Fig. 4A) and GAD (r^2=0.67, p<0.0001, Fig 4B), although the effects were not as large as in the PrL. Similarly, there was no significant relationship between the proportion of c-Fos+ cells that were CamKII+ (Fig. 4C), but there was in GAD+ cells (r^2=0.41, p=0.0042, Fig 4D). As in the PrL, the dual reduction of oxycodone seeking and c-Fos+GAD+ cells by EE appears to be a major contributor to this relationship.

4. Discussion

In the present study, we found that compared to rats singly housed in standard laboratory caging during abstinence, environmentally enriched rats had lower oxycodone seeking. EE began one week after drug cessation, indicating that the beneficial effects of enrichment can occur even when administered after abstinence has begun. We also found that EE reduced the reactivity of the PrL and IL as measured by the number of c-Fos+ cells in each region. The reduction in c-Fos+ cells was predominantly in inhibitory cells of the PrL and IL, as evidenced by a reduction in the proportion of c-Fos+ cells in GAD+, but not CamKII+ cells. Examination of relationships between c-Fos+ cells and oxycodone seeking identified a robust positive relationship between the number of c-Fos+ cells and persistence of oxycodone seeking in both the PrL and IL. This was confirmed in two distinct sets of tissue (tissue co-stained for CamKII or GAD). There were also positive relationships between the % of c-Fos+ cells that were GAD+, but not CaMKII+ in the PrL and IL, although these relationships were less robust and largely driven by EE-associated reductions in both factors examined.

The experimental evidence for the preventive effects of EE on misused substances derives from many well-established models used for studying addiction-like behaviors that expose the animals to EE before any contact with the drug. EE administered before drug self-administration reduced amphetamine intake in both male and female rats (Bardo et al., 2001; Green et al., 2002). EE also reduced acquisition of heroin self-administration, but steady state intake during the fifth week was similar to isolated rats (Bozarth et al., 1989). Similarly, EE reduced escalation of cocaine intake, but only for a lower unit dose of cocaine (0.1 mg/kg/infusion, but not 0.5 mg/kg/infusion). EE also did not impact acquisition or steady state intake during cocaine self-administration of 1 mg/kg/infusion (Bozarth et al., 1989).

EE has had more consistent effects on drug seeking when enrichment is administered during abstinence. EE reduced the incubation of both cocaine and heroin craving following protracted abstinence (Barrera et al., 2021; Chauvet et al., 2012). Cued reinstatement of methamphetamine seeking was also depressed in rats that received EE compared to those in standard isolation housing (Hofford et al., 2014). The beneficial effects of EE during abstinence are also observed for cued, contextual, and stress-induced reinstatement of cocaine seeking, although drug primed reinstatement is not affected (Chauvet et al., 2009; Ranaldi et al., 2011; Thiel et al., 2009). In an electric barrier conflict model, EE reduced the time to voluntary abstinence from drug seeking in rats with a history or either heroin or cocaine self-administration (Ewing and Ranaldi, 2018; Peck et al., 2015). These prior reports indicate that EE is effective at suppressing drug seeking behavior in a variety of conditions for both psychostimulants and opioids. Our current data using a forced abstinence model following oxycodone seeking are consistent with this literature.

Immediate early genes such as *c-fos* (and its product c-Fos) have been used as activity-dependent markers for decades (Cruz et al., 2013; Kawashima et al., 2014; Sheng and Greenberg, 1990)). The PrL is particularly sensitive to increases in c-Fos following drug seeking. The number of c-Fos+ cells in the PrL is elevated following drug seeking in rats that have self-administered either a psychostimulant or opioid (Doherty et al., 2013; McGlinchey et al., 2016), even within the same animal that has self-administered both drugs (Rubio et al., 2019). Furthermore, the number of c-Fos+ cells in the PrL, but not other brain regions examined, was correlated with intensity of cocaine seeking (Zhou et al., 2014). In rats that experienced EE during forced abstinence from cocaine self-administration, EE suppressed cocaine seeking and the associated increase in c-Fos+ cells in both the prelimbic and infralimbic prefrontal cortices (Thiel et al., 2010).

Studies have also found preferential expression of c-Fos in specific populations of prefrontal neurons during drug seeking. An increase in c-Fos+ PrL neurons that project to the nucleus accumbens was observed with cued reinstatement of cocaine seeking, and the number of these cells was positively associated with seeking (McGlinchey et al., 2016). As our identification of excitatory projection neurons relied on CamKII, we were not able to focus on a specific projection pathway, which may explain why we did not observe a correlation between the percentage of c-Fos+ cells that were CamKII+ and oxycodone seeking. Alternatively, PrL and IL GABAergic neurons may represent corticofugal neurons which modulate reactivity to drug-associated stimuli by regulation of downstream subcortical

projections (Caputi et al., 2013; Melzer and Monyer, 2020). In this case, alterations in activity of GAD+ neurons would not be expected to influence local CamKII+ neurons.

Miller and Marshall found that expression of cocaine conditioned place preference was associated with an increase in c-Fos+ cells of the PrL, and this was selectively observed in GAD+, but not CamKII+ cells (Miller and Marshall, 2004). These data suggest that without prior extinction, drug seeking is largely controlled by GAD+ cells in the PrL. This notion is supported by the finding that after forced abstinence, re-exposure to heroin self-administration-associated cues resulted in an increase in GABAergic inhibition of pyramidal cells as reflected by an increase in the frequency of inhibitory postsynaptic potentials (Van den Oever et al., 2010). This unique role of GABAergic signaling in seeking without prior extinction is further illustrated by the ability of PrL GABA-A receptor agonist infusion to enhance heroin seeking (Schmidt et al., 2005). Our use of an experimental design that did not provide full extinction prior to oxycodone seeking may explain why we observed positive associations between the proportion of c-Fos+ cells that were GABAergic and oxycodone seeking, which was also the population of cells that was affected by EE.

One limitation of this study is that CamKII and GAD immunostaining were performed in separate tissues. We tested several antibodies for each of these antigens and determined that the best results were obtained using mouse monoclonal antibodies for each. This precluded the effective use of co-labeling c-Fos, CamKII and GAD in the same tissues. As a result, these measurements came from different tissues. However, our use of different tissues allowed us to have replicate studies for our c-Fos measurements, and these studies produced highly consistent results (e.g., Figs. 2C and 2F, Figs. 3A and 3B). Another limitation is the use of only male rats. Prior studies found that EE can reduce opioid seeking in both male and female rats (Barrera et al., 2021; Barrera et al., 2023), suggesting that we would expect to see similar results in females. Future studies are needed to determine if EE also reduces oxycodone seeking in females, and if this is also accompanied by selective reduction of c-Fos in GAD+ cells. Another limitation is that we did not test the effects of EE on acquisition or maintenance of oxycodone self-administration. EE has been shown to reduce progressive ratio breakpoints in heroin self-administration (Imperio et al., 2018) and reduce essential value and reinforcer intensity of remifentanil in a behavioral economics-based self-administration study (Hofford et al., 2017), so it is likely that EE would also oxycodone self-administration. It is notable that we did not observe incubation of craving between our two sessions (Grimm et al., 2001; Pickens et al., 2011). There are a few potential reasons for this. First, the initial oxycodone seeking session was conducted on day 7 of abstinence, whereas incubation of craving is typically measured relative to seeking on day 1 of abstinence. Incubation of heroin seeking peaks at approximately 7 days of abstinence (Pickens et al., 2011), so our initial measurement on abstinence day 7 may have prevented observance of an increase relative to this baseline. Additionally, our seeking tests were done using a within-subjects design with 2-hour seeking sessions, which could promote extinction of drug seeking between sessions.

In conclusion, we find that environmental enrichment reduces oxycodone seeking, similar to reports for heroin and psychostimulants. Activity of inhibitory, but not excitatory neurons of the PrL and IL during drug seeking was specifically reduced by EE. These findings further

support the effectiveness of enriched environments to reduce reactivity to drug-associated stimuli and contexts and provide a potential mechanism by which this occurs.

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7. References

Bardo MT, Klebaur JE, Valone JM, Deaton C, 2001. Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. Psychopharmacology (Berl) 155(3), 278–284. [PubMed: 11432690]

Barrera ED, Loughlin L, Greenberger S, Ewing S, Hachimine P, Ranaldi R, 2021. Environmental enrichment reduces heroin seeking following incubation of craving in both male and female rats. Drug Alcohol Depend 226, 108852. [PubMed: 34225225]

Barrera ED, Timken PD, Lee E, Persaud KRS, Goldstein H, Parasram DN, Vashisht A, Ranaldi R, 2023. Environmental enrichment facilitates electric barrier induced heroin abstinence after incubation of craving in male and female rats. Drug Alcohol Depend 244, 109799. [PubMed: 36774806]

Berardo LR, Fabio MC, Pautassi RM, 2016. Post-weaning Environmental Enrichment, But Not Chronic Maternal Isolation, Enhanced Ethanol Intake during Periadolescence and Early Adulthood. Frontiers in behavioral neuroscience 10, 195. [PubMed: 27790100]

Bozarth MA, Murray A, Wise RA, 1989. Influence of housing conditions on the acquisition of intravenous heroin and cocaine self-administration in rats. Pharmacol Biochem Behav 33(4), 903–907. [PubMed: 2616610]

Caputi A, Melzer S, Michael M, Monyer H, 2013. The long and short of GABAergic neurons. Curr Opin Neurobiol 23(2), 179–186. [PubMed: 23394773]

Carroll ME, Anker JJ, Perry JL, 2009. Modeling risk factors for nicotine and other drug abuse in the preclinical laboratory. Drug Alcohol Depend 104 Suppl 1, S70–78. [PubMed: 19136222]

Chauvet C, Goldberg SR, Jaber M, Solinas M, 2012. Effects of environmental enrichment on the incubation of cocaine craving. Neuropharmacology 63(4), 635–641. [PubMed: 22634364]

Chauvet C, Lardeux V, Goldberg SR, Jaber M, Solinas M, 2009. Environmental enrichment reduces cocaine seeking and reinstatement induced by cues and stress but not by cocaine. Neuropsychopharmacology 34(13), 2767–2778. [PubMed: 19741591]

Chauvet C, Lardeux V, Jaber M, Solinas M, 2011. Brain regions associated with the reversal of cocaine conditioned place preference by environmental enrichment. Neuroscience 184, 88–96. [PubMed: 21477638]

Chiariello R, McCarthy C, Glaeser BL, Shah AS, Budde MD, Stemper BD, Olsen CM, 2023. Chronicity of repeated blast traumatic brain injury associated increase in oxycodone seeking in rats. Behav Brain Res 438, 114181. [PubMed: 36330906]

Cruz FC, Koya E, Guez-Barber DH, Bossert JM, Lupica CR, Shaham Y, Hope BT, 2013. New technologies for examining the role of neuronal ensembles in drug addiction and fear. Nat Rev Neurosci 14(11), 743–754. [PubMed: 24088811]

Doherty JM, Cooke BM, Frantz KJ, 2013. A role for the prefrontal cortex in heroin-seeking after forced abstinence by adult male rats but not adolescents. Neuropsychopharmacology 38(3), 446–454. [PubMed: 23072838]

Ewing S, Ranaldi R, 2018. Environmental enrichment facilitates cocaine abstinence in an animal conflict model. Pharmacol Biochem Behav 166, 35–41. [PubMed: 29407873]

Galaj E, Barrera ED, Ranaldi R, 2020. Therapeutic efficacy of environmental enrichment for substance use disorders. Pharmacol Biochem Behav 188, 172829. [PubMed: 31778722]

Green TA, Gehrke BJ, Bardo MT, 2002. Environmental enrichment decreases intravenous amphetamine self-administration in rats: dose-response functions for fixed- and progressive-ratio schedules. Psychopharmacology (Berl) 162(4), 373–378. [PubMed: 12172690]

Grimm JW, Glueck E, Ginder D, Hyde J, North K, Jiganti K, 2018. Sucrose Abstinence and Environmental Enrichment Effects on Mesocorticolimbic DARPP32 in Rats. Sci Rep 8(1), 13174. [PubMed: 30181585]

Grimm JW, Hope BT, Wise RA, Shaham Y, 2001. Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature 412(6843), 141–142. [PubMed: 11449260]

Grimm JW, Sauter F, 2020. Environmental enrichment reduces food seeking and taking in rats: A review. Pharmacol Biochem Behav 190, 172874. [PubMed: 32084492]

Grimm JW, Weber R, Barnes J, Koerber J, Dorsey K, Glueck E, 2013. Brief exposure to novel or enriched environments reduces sucrose cue-reactivity and consumption in rats after 1 or 30 days of forced abstinence from self-administration. PLoS One 8(1), e54164. [PubMed: 23342096]

Hofford RS, Chow JJ, Beckmann JS, Bardo MT, 2017. Effects of environmental enrichment on self-administration of the short-acting opioid remifentanil in male rats. Psychopharmacology 234(23–24), 3499–3506. [PubMed: 28916995]

Hofford RS, Darna M, Wilmouth CE, Dwoskin LP, Bardo MT, 2014. Environmental enrichment reduces methamphetamine cue-induced reinstatement but does not alter methamphetamine reward or VMAT2 function. Behav Brain Res 270, 151–158. [PubMed: 24821405]

Imperio CG, McFalls AJ, Hadad N, Blanco-Berdugo L, Masser DR, Colechio EM, Coffey AA, Bixler GV, Stanford DR, Vrana KE, Grigson PS, Freeman WM, 2018. Exposure to environmental enrichment attenuates addiction-like behavior and alters molecular effects of heroin self-administration in rats. Neuropharmacology 139, 26–40. [PubMed: 29964093]

Kawashima T, Okuno H, Bito H, 2014. A new era for functional labeling of neurons: activity-dependent promoters have come of age. Front Neural Circuits 8, 37. [PubMed: 24795570]

Kenan K, Mack K, Paulozzi L, 2012. Trends in prescriptions for oxycodone and other commonly used opioids in the United States, 2000–2010. Open Med 6(2), e41–47. [PubMed: 23696768]

Kreek MJ, Nielsen DA, Butelman ER, LaForge KS, 2005. Genetic influences on impulsivity, risk taking, stress responsivity and vulnerability to drug abuse and addiction. Nat Neurosci 8(11), 1450–1457. [PubMed: 16251987]

Malone SG, Shaykin JD, Stairs DJ, Bardo MT, 2022. Neurobehavioral effects of environmental enrichment and drug abuse vulnerability: An updated review. Pharmacol Biochem Behav 221, 173471. [PubMed: 36228739]

McGlinchey EM, James MH, Mahler SV, Pantazis C, Aston-Jones G, 2016. Prelimbic to Accumbens Core Pathway Is Recruited in a Dopamine-Dependent Manner to Drive Cued Reinstatement of Cocaine Seeking. J Neurosci 36(33), 8700–8711. [PubMed: 27535915]

Melzer S, Monyer H, 2020. Diversity and function of corticopetal and corticofugal GABAergic projection neurons. Nat Rev Neurosci 21(9), 499–515. [PubMed: 32747763]

Miller CA, Marshall JF, 2004. Altered prelimbic cortex output during cue-elicited drug seeking. J Neurosci 24(31), 6889–6897. [PubMed: 15295023]

Muelbl MJ, Glaeser BL, Shah AS, Chiariello RA, Nawarawong NN, Stemper BD, Budde MD, Olsen CM, 2022. Repeated blast mild traumatic brain injury and oxycodone self-administration produce interactive effects on neuroimaging outcomes. Addict Biol 27(2), e13134. [PubMed: 35229952]

Nithianantharajah J, Hannan AJ, 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7(9), 697–709. [PubMed: 16924259]

Peck JA, Galaj E, Eshak S, Newman KL, Ranaldi R, 2015. Environmental enrichment induces early heroin abstinence in an animal conflict model. Pharmacol Biochem Behav 138, 20–25. [PubMed: 26368843]

Peters J, Kalivas PW, Quirk GJ, 2009. Extinction circuits for fear and addiction overlap in prefrontal cortex. Learn Mem 16(5), 279–288. [PubMed: 19380710]

Piazza PV, Le Moal ML, 1996. Pathophysiological basis of vulnerability to drug abuse: role of an interaction between stress, glucocorticoids, and dopaminergic neurons. Annu Rev Pharmacol Toxicol 36, 359–378. [PubMed: 8725394]

Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y, 2011. Neurobiology of the incubation of drug craving. Trends Neurosci 34(8), 411–420. [PubMed: 21764143]

Ranaldi R, Kest K, Zellner M, Hachimine-Semprebom P, 2011. Environmental enrichment, administered after establishment of cocaine self-administration, reduces lever pressing in

extinction and during a cocaine context renewal test. Behav Pharmacol 22(4), 347–353. [PubMed: 21673566]

Rockman GE, Gibson JE, Benarroch A, 1989. Effects of environmental enrichment on voluntary ethanol intake in rats. Pharmacol Biochem Behav 34(3), 487–490. [PubMed: 2623006]

Rubio FJ, Quintana-Feliciano R, Warren BL, Li X, Witonsky KFR, Valle FSD, Selvam PV, Caprioli D, Venniro M, Bossert JM, Shaham Y, Hope BT, 2019. Prelimbic cortex is a common brain area activated during cue-induced reinstatement of cocaine and heroin seeking in a polydrug self-administration rat model. Eur J Neurosci 49(2), 165–178. [PubMed: 30307667]

Schmidt ED, Voorn P, Binnekade R, Schoffelmeer AN, De Vries TJ, 2005. Differential involvement of the prelimbic cortex and striatum in conditioned heroin and sucrose seeking following long-term extinction. Eur J Neurosci 22(9), 2347–2356. [PubMed: 16262673]

Sheng M, Greenberg ME, 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4(4), 477–485. [PubMed: 1969743]

Solinas M, Chauvet C, Lafay-Chebassier C, Jaafari N, Thiriet N, 2021. Environmental enrichment-inspired pharmacological tools for the treatment of addiction. Curr Opin Pharmacol 56, 22–28. [PubMed: 32966941]

Stairs DJ, Bardo MT, 2009. Neurobehavioral effects of environmental enrichment and drug abuse vulnerability. Pharmacol Biochem Behav 92(3), 377–382. [PubMed: 19463254]

Thiel KJ, Pentkowski NS, Peartree NA, Painter MR, Neisewander JL, 2010. Environmental living conditions introduced during forced abstinence alter cocaine-seeking behavior and Fos protein expression. Neuroscience 171(4), 1187–1196. [PubMed: 20933585]

Thiel KJ, Sanabria F, Pentkowski NS, Neisewander JL, 2009. Anti-craving effects of environmental enrichment. Int J Neuropsychopharmacol 12(9), 1151–1156. [PubMed: 19691875]

Van den Oever MC, Lubbers BR, Goriounova NA, Li KW, Van der Schors RC, Loos M, Riga D, Wiskerke J, Binnekade R, Stegeman M, Schoffelmeer AN, Mansvelder HD, Smit AB, De Vries TJ, Spijker S, 2010. Extracellular matrix plasticity and GABAergic inhibition of prefrontal cortex pyramidal cells facilitates relapse to heroin seeking. Neuropsychopharmacology 35(10), 2120–2133. [PubMed: 20592718]

van Praag H, Kempermann G, Gage FH, 2000. Neural consequences of environmental enrichment. Nat Rev Neurosci 1(3), 191–198. [PubMed: 11257907]

Venniro M, Zhang M, Caprioli D, Hoots JK, Golden SA, Heins C, Morales M, Epstein DH, Shaham Y, 2018. Volitional social interaction prevents drug addiction in rat models. Nat Neurosci 21(11), 1520–1529. [PubMed: 30323276]

Willcocks AL, McNally GP, 2013. The role of medial prefrontal cortex in extinction and reinstatement of alcohol-seeking in rats. Eur J Neurosci 37(2), 259–268. [PubMed: 23106416]

Zhou L, Pruitt C, Shin CB, Garcia AD, Zavala AR, See RE, 2014. Fos expression induced by cocaine-conditioned cues in male and female rats. Brain Struct Funct 219(5), 1831–1840. [PubMed: 23832598]

Highlights:

- Environmental enrichment during abstinence sharply reduced oxycodone seeking
- Environmental enrichment reduced c-Fos in prelimbic and infralimbic cortices
- Environmental enrichment selectively reduced c-Fos in GAD67+ neurons

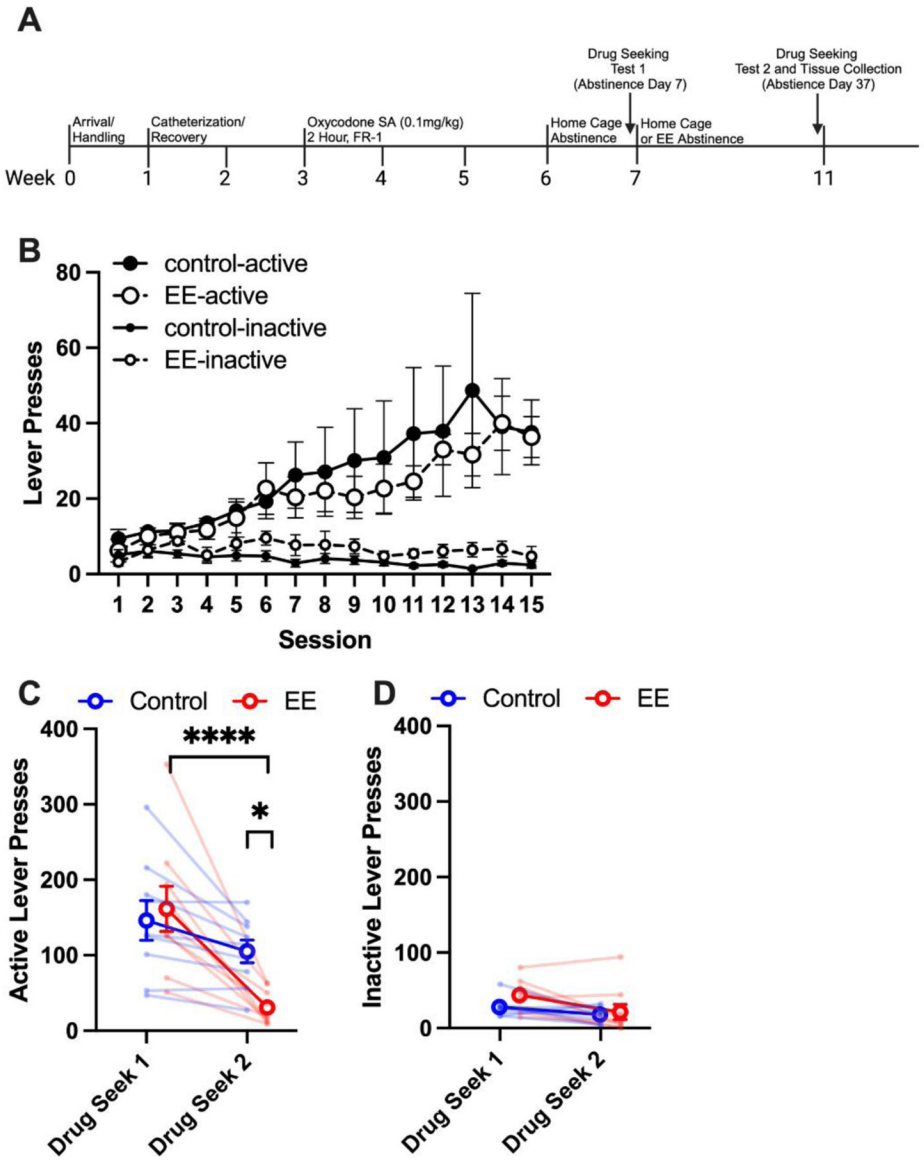

Figure 1: Oxycodone self-administration and seeking in rats exposed to control or enriched environment during abstinence.

A) Timeline of experiment. After arrival and handling, rats underwent jugular catheterization and recovery, then oxycodone self-administration, and an initial drug seeking session. Rats were counterbalanced to group assignment based on active lever presses during drug seeking test 1, then underwent forced abstinence in the home cage (control) or environmental enrichment chambers. Drug seeking test two was conducted 4 weeks after initiation of home cage or enrichment housing. B) Active and inactive lever responses during the oxycodone self-administration sessions. C) Active lever presses during oxycodone seeking test 1 and 2. D) Inactive lever presses during oxycodone seeking test 1 and 2. N=9 rats/group. Symbols represent mean±SEM. *p<0.05 between groups comparison of presses during drug seeking test 2. ****p<0.0001 within-subjects comparison from seeking test 1 to 2. Panel A was created with Biorender.com.

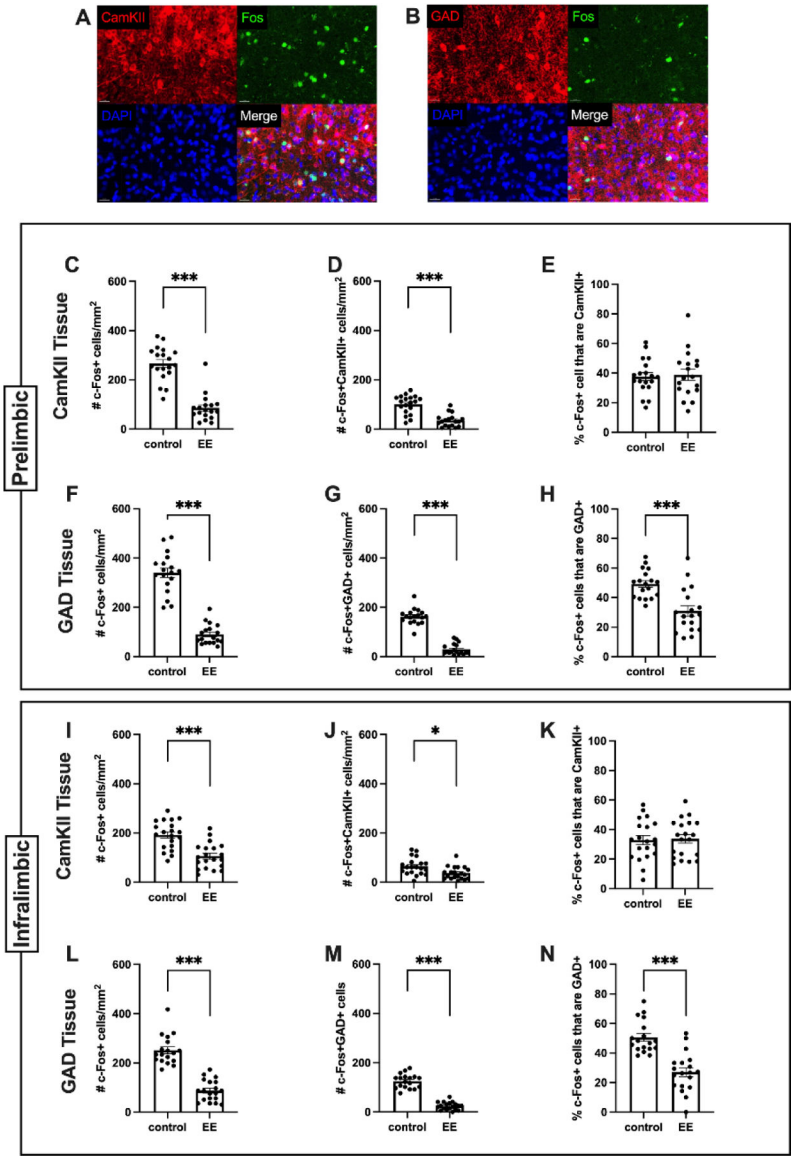

Figure 2: c-Fos+ cell counts and colocalization with CamKII and GAD in prelimbic and infralimbic prefrontal cortices.

A) Maximum projection from prelimbic cortex of a control rat. B) Maximum projection from prelimbic cortex of an environmental enrichment rat. Panels C-E and I-K are from tissue stained with CamKII, panels F-H and L-N are from tissue stained with GAD. C) In the PrL, EE reduced the number of c-Fos+ cells and D) the number of c-Fos+ cells that were CamKII+. E) EE had no effect on the % of c-Fos+ cells that were CamKII+. F) EE reduced the number of c-Fos+ cells and G) the number of c-Fos+ cells that were GAD+. H) EE reduced the percentage of c-Fos+ cells that GAD+. I) In the IL, EE reduced the number of c-Fos+ cells and J) the number of c-Fos+ cells that were CamKII+. K) EE had no effect on the % of c-Fos+ cells that were CamKII+ in the IL. L) EE reduced the number of c-Fos+ cells and M) the number of c-Fos+ cells that were GAD+. N) EE reduced the percentage of c-Fos+ cells that GAD+. n=18 images from 9 rats per group. Full statistical details in Table 1. ***p<0.001.

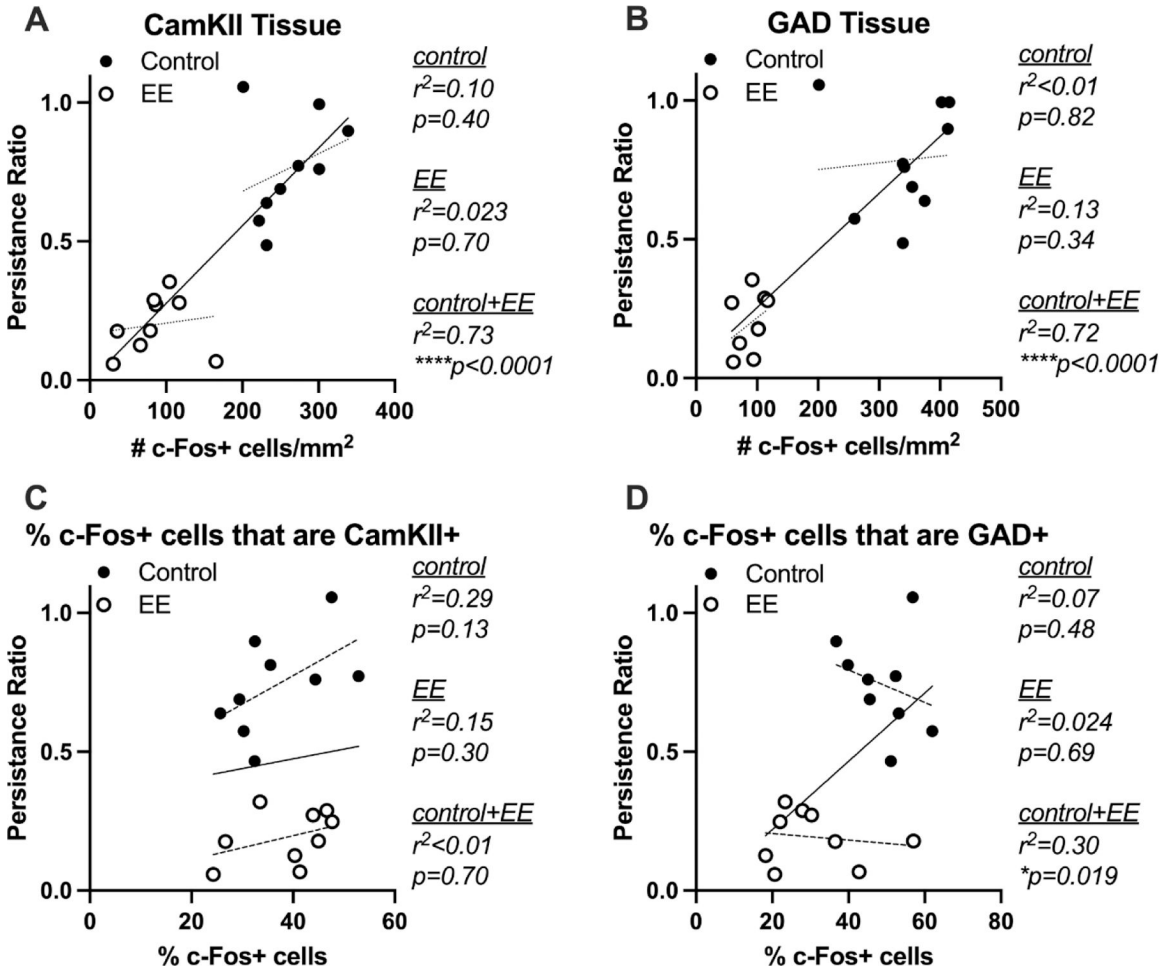

Figure 3: Correlations between the c-Fos immunoreactivity and persistence of oxycodone seeking in the prelimbic cortex.

A) Correlation between the number of c-Fos+ cells and persistence ratio in CamKII stained tissue. B) Correlation between the number of c-Fos+ cells and persistence ratio in GAD stained tissue. C) Correlation between the percentage of c-Fos+ cells that were CamKII+ and persistence ratio. D) Correlation between the percentage of c-Fos+ cells that were GAD+ and persistence ratio. N=9 rats/group. *p<0.05, ****p<0.0001.

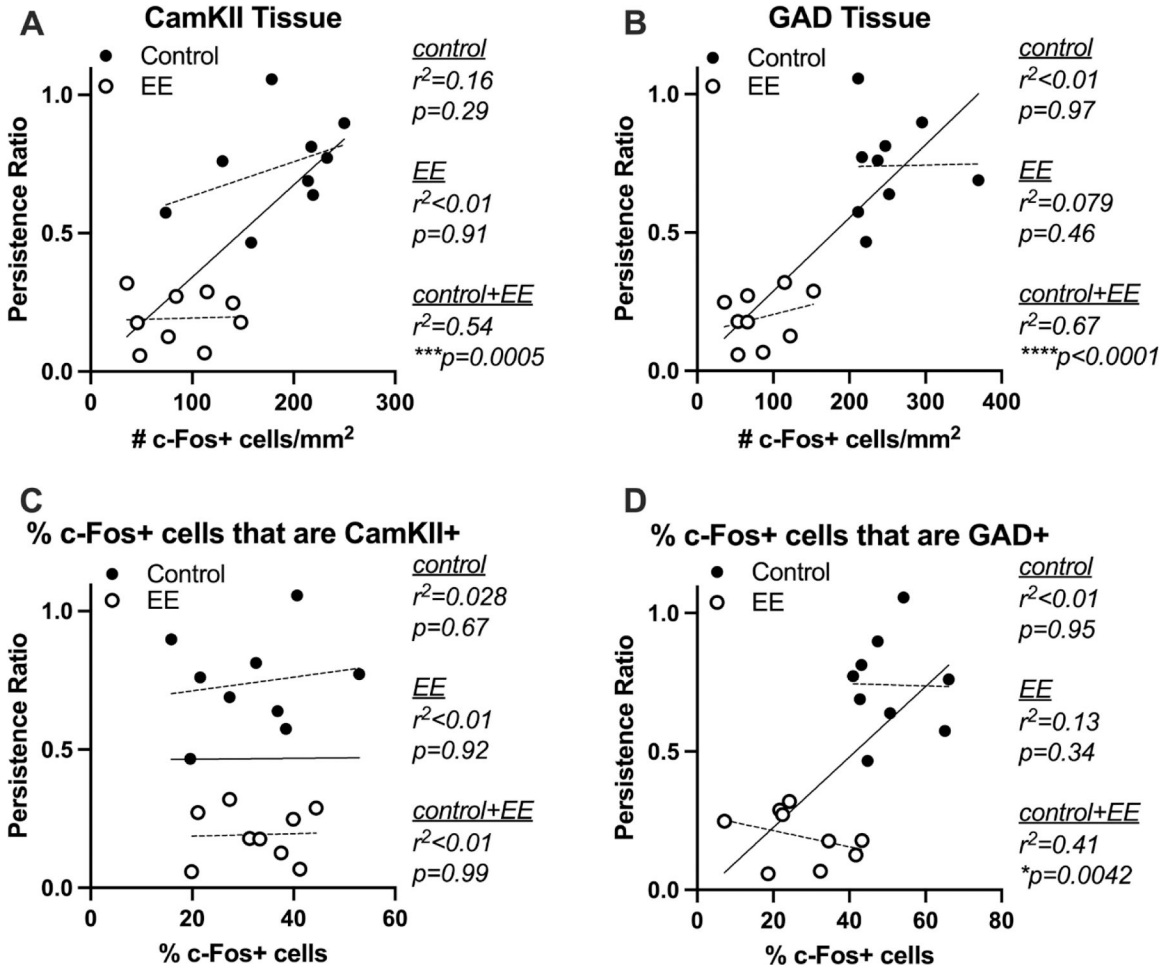

Figure 4: Correlations between the c-Fos immunoreactivity and persistence of oxycodone seeking in the infralimbic cortex.

A) Correlation between the number of c-Fos+ cells and persistence ratio in CamKII stained tissue. B) Correlation between the number of c-Fos+ cells and persistence ratio in GAD stained tissue. C) Correlation between the percentage of c-Fos+ cells that were CamKII+ and persistence ratio. D) Correlation between the percentage of c-Fos+ cells that were GAD+ and persistence ratio. N=9 rats/group. *p<0.05, ****p<0.0001.

Table. 1

Region	Tissue	Outcome Measure	B	SE	DF	P value
Prelimbic	CAMKII	# c-Fos+ cells	−180.3	21.4	33	**<0.001**
Prelimbic	CAMKII	# c-Fos+CamKII+ cells	−64.5	11.9	33	**<0.001**
Prelimbic	CAMKII	% c-Fos+ cells that are CamKII+	1.24	4.6	33	0.79
Prelimbic	GAD	# c-Fos+ cells	−249.5	24.2	32	**<0.001**
Prelimbic	GAD	# c-Fos+GAD+ cells	−132.5	9.1	32	**<0.001**
Prelimbic	GAD	% c-Fos+ cells that are GAD+	−18.2	5.0	32	**<0.001**
Infralimbic	CAMKII	# c-Fos+ cells	−87.1	23.1	36	**<0.001**
Infralimbic	CAMKII	# c-Fos+CamKII+ cells	−26.8	12.4	36	**<0.001**
Infralimbic	CAMKII	% c-Fos+ cells that are CamKII+	0.98	5.0	36	0.85
Infralimbic	GAD	# c-Fos+ cells	−167.1	21.7	33	**<0.001**
Infralimbic	GAD	# c-Fos+GAD+ cells	−101.1	9.4	33	**<0.001**
Infralimbic	GAD	% c-Fos+ cells that are GAD+	−23.3	5.0	33	**<0.001**