Tittle: Chemogenetic Activation of Medial Prefrontal Cortex Projections to the Nucleus Accumbens Shell Suppresses Cocaine-Primed Reinstatement in EcoHIV Infected Mice

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Abstract

HIV is highly comorbid with cocaine use disorder (CUD). Relapse is a major challenge in the treatment of CUD, and people living with HIV (PLWH) exhibit shorter time to relapse. One driver of relapse may be re-exposure to cocaine, which can be modeled in rodents using cocaine-primed reinstatement. This process involves neuroadaptations within the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) shell, regions that mediate cocaine reward learning and relapse. HIV infection interacts with cocaine to alter corticostriatal circuits, which may further dysregulate cocaine seeking. To investigate the impact of HIV infection on cocaine reward learning and reinstatement and the role of mPFC-NAc circuits, we utilized the EcoHIV mouse model, a chimeric form of HIV-1 which can infect wild-type mice. Our findings demonstrate that EcoHIV infection enhances cocaine-primed reinstatement. We also observed increased cocaine-induced expression of the cellular activation marker cFos in the NAshell in EcoHIV-infected mice. Given the role of the mPFC-NAshell circuit in cocaine-seeking behaviors, we further demonstrated that chemogenetic activation of this circuit could reverse the behavioral deficits induced by EcoHIV. We propose that HIV infection contributes to neuroadaptations in the mPFC-NAshell circuit, and enhancing its activity may inhibit relapse-related behavior. These findings identify key neuronal circuits underlying cocaine relapse in HIV infection and suggest potential strategies for managing relapse in PLWH.

Introduction

Cocaine use disorder (CUD) and HIV infection are two global health crises that frequently co-occur (Dash et al., 2015; Lin et al., 2020). HIV infection and CUD possess independent but overlapping pathologies in the central neural system (CNS) and the immune system, which contribute to cognitive disorders and inflexible control of drug use (Hauser et al., 2007; Wayman et al., 2016). Despite the high prevalence of co-occurring CUD and HIV infection, the consequences of HIV on cocaine seeking behaviors and further, whether similar neural substrates regulate the expression of cocaine seeking in models of HIV infection, is not fully understood.

Both cocaine and HIV infection involve disruption of corticostriatal circuits that mediate motivation, cognition, and reward processing. Relapse to cocaine seeking is a primary obstacle in treating CUD and a major factor contributing to cocaine overdose (Nnadi et al., 2005). During abstinence, factors including stress, drug-associated cues, or cocaine re-exposure can lead to relapse. The medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) are essential regulators of craving and cocaine seeking (McFarland et al., 2003; Koya et al., 2009). Projections from the mPFC to NAc have distinct distributions and regulate different patterns of cocaine seeking behaviors: the prelimbic (PL) mPFC projection to NAcore is associated with reinstatement of drug seeking, while the infralimbic (IL) mPFC to NAshell projection inhibits reinstatement (McFarland et al., 2003; Peters et al., 2008).

HIV infection is associated with corticostriatal circuit dysregulation that contributes to cognitive impairment in people living with HIV (PLWH) (Plessis et al., 2015; Wayman et al., 2016; McLaurin et al., 2018, 2021). This includes synaptic damage and neuronal loss in frontal cortex areas in HIV patients with HIV-associated dementia (Heaton et al., 1999; Ru and Tang, 2017; McLaurin et al., 2021). This is also observed in preclinical models of HIV infection. For example, HIV viral protein (e.g. gp120, Tat) exposure can directly impact N-methyl-D-aspartate receptor (NMDA) glutamate receptors by potentiating the phosphorylation and synaptic trafficking of NMDARs, leading to NMDA-mediated excitatory postsynaptic stimulation and increasing the synaptic damage by overactivation (O'Donnell et al., 2006; Ru and Tang, 2017). In addition, HIV-induced pro-inflammatory cytokine (e.g. TNF-α, IL-6 and IL-1β) release from infected/reactivated microglial cells/ macrophages can cause dysregulation of glutamatergic homeostasis and promote excitatory neuronal damage (New et al., 1998; Hauser

et al., 2007; Gipson et al., 2021). HIV-induced neuropathogenesis in the corticostriatal circuit is overlapping with the reward circuits that are impacted by drug exposure. Thus, cocaine exposure in the context with HIV infection may accelerate neuropathogenesis in the reward processing regions that lead to high relapse to cocaine seeking in PLWH.

Here, we investigate cocaine reward learning and reinstatement in a conditioned place preference (CPP) paradigm within the EcoHIV mouse model. We found that EcoHIV infection did not impact cocaine CPP, but did impair extinction learning and increase cocaine-primed reinstatement, while not impacting yohimbine-induced reinstatement. We also demonstrate that EcoHIV and cocaine interacted to alter cFos induction within the NAshell. We further demonstrate that chemogenetic activation of mPFC-NAshell projections prevented cocaine-primed reinstatement in EcoHIV-infected mice. Overall, the results from current study indicate that EcoHIV infection altered cocaine-induced activity in the mPFC and NAc that was associated with extinction deficits and increased cocaine-primed reinstatement, and that the common pathways corticoaccumbens can be targeted to rescue cocaine relapse in populations with HIV infection and without.

Materials and Methods:

Subjects:

Adult male (n = 61) and female (n = 61) C57BL/6J mice (9 weeks of age upon arrival) were obtained from Jackson Laboratories. Following arrival, mice were group housed in same-sex cages for 7 days to acclimatize with *ad libitum* access to a standard chow diet and water. Mice were housed at the Drexel University College of Medicine under standard 12-hour light:12-hour dark conditions in microisolation conditions throughout the experiments. All experiments were approved by the Institutional Animal Care and Use Committee at Drexel University.

EcoHIV-NDK production and inoculation:

Plasmid DNA encoding the EcoHIV-NDK coding sequence (gift from Dr. David Volsky) was grown overnight in Stbl2 bacterial cells (ThermoFisher#10268019) and the plasmid DNA was purified using an endotoxin free plasmid purification kit (ZymoPure #D4200). Purified DNA was transfected into nearly confluent (80–90%)10 cm2 plates of low passage LentiX 293T cells (#632180, Takara Bio, San Jose, CA, USA), using a calcium phosphate transfection kit (Takara #631312). The cell culture supernatant was collected at 48 hours post-transfection using centrifugation at low speed (1500×g, 4°C), followed by passage through a cell strainer (40µm) to remove LentiX cells and cell debris. The supernatant, containing viral particles, was mixed 4:1 with a homemade lentiviral concentrator solution (4X; MD Anderson) composed of 40% (w/v) PEG-8000 and 1.2 M NaCl in PBS (pH 7.4). The supernatant–PEG mixture was incubated overnight at 4 °C on an orbital shaker (60 rpm). The mixture was centrifuged at 1500×g for 30 min at 4 °C. After centrifugation, the medium was removed, and the viral pellet was resuspended in cold, sterile PBS. The viral titer (p24 core antigen content) was determined initially using a LentiX GoStix Plus titration kit (#631280, Takara Bio, San Jose, CA, USA) and subsequently using an HIV p24 AlphaLISA detection kit (#AL291C, PerkinElmer, Waltman, MA, USA). Viral stocks were aliquoted and stored at−80 °C upon usage.

Following one week of acclimation, adult male and female mice were inoculated with 300 ng p24 equivalent EcoHIV-NDK or PBS (virus culture vehicle) as sham control by i.p. injection. To ensure housing consistency, all

mice were singly housed. Blood samples were collected from all mice 1, 3, and 5 weeks following EcoHIV inoculation. Five weeks after inoculation, mice were assigned for behavioral training. This dose of virus and length of infection was selected as it produces systemic infection and immune response in the CNS and periphery (Potash et al., 2005; Kelschenbach et al., 2012, 2019; Gu et al., 2018; Xie et al., 2024). Following the completion of all experiments, to confirm the terminal EcoHIV-NDK infection status, spleens were isolated and flash-frozen on dry ice prior to perfusion and stored at -80C until processing. Splenic viral DNA burden was measured using Qiagen QIAamp DNA Mini Kit (#51304, Qiagen, Germantown, MD, US). Viral DNA was analyzed by the University of Pennsylvania Center for AIDS Research (CFAR). qPCR was conducted as described (Xie et al., 2024) using primers that amply sequences with HIV-LTR provided below. The OD value was detected by a NanoDrop™ spectrophotometer (Thermo Scientific) and used to determine the input cell numbers to normalize the data.

Kumar LTR F, GCCTCAATAAAGCTTGCCTTGA

Kumar LTR R, GGGCGCCACTGCTAGAGA

Kumar LTR Probe (FAM/BHQ), 5'CCAGAGTCACACAACAGACGGGCACA 3'

Cocaine Conditioned Placed Preference (CPP) and Reinstatement test:

Six weeks after inoculation with EcoHIV, mice were trained in a three-chamber CPP paradigm. Chambers had distinct walls (black, white, or gray) and floors (grid, wire mesh, or solid). The neutral gray chamber was in the middle of black and white conditioning chambers. Photocell beam breaks were used to calculate total time spent in each chamber, latency to enter, and locomotor behavior in the boxes using Med-PC V software (Xie et al., 2019).

Mice were habituated to the CPP room for at least 1 hour before the behavioral experiment. After habituation, mice were placed individually into the neutral chamber (middle gray chamber) with both door to black and white chamber opened ("Pre-test"). The ratio of time spent in the black and white chamber during the pretest was used to determine initial side bias, and the least preferred chamber was assigned as cocaine-paired chamber.

During conditioning, mice were assigned to receive cocaine (10mg/kg, i.p.) in the cocaine-paired chamber or saline injection in the opposite chamber on alternating days. Injections were administered prior to placement in the respective chambers, and mice were confined to the chamber for 30min in each conditioning session. This pattern of conditioning was performed for 4 consecutive days, resulting in a total of 4 pairings (2 cocaine and 2 saline). After conditioning sessions, mice were tested for the preference for the cocaine-paired chamber ("Post-test"). In this session, mice were placed in the neutral chamber with both doors retracted and allowed to freely explore all chambers for 20 minutes. The time spent in each chamber was assessed. CPP scores were calculated as [Post-test time spent in cocaine-paired chamber] – [Pre-test time spent in cocaine-paired chamber]. Following the CPP post-test, mice underwent 4 days of extinction training, which were identical to the CPP test sessions. Extinction scores were calculated as [Time spent in cocaine-paired chamber in each extinction sessions] – [Post-test time spent in cocaine-paired chamber].

In the cocaine-primed reinstatement test, mice received a cocaine injection (10mg/kg, i.p.) and were tested for 20 min. The reinstatement scores were calculated as [Reinstatement time spent in cocaine-paired chamber] – [Extinction Day4 time spent in cocaine-paired chamber]. For the yohimbine-induced reinstatement test, mice underwent same CPP and extinction training but received a yohimbine injection (2mg/kg, i.p.) followed by the 20 min reinstatement test.

Immunohistochemical assessment of cFos

At least 48 hours after the cocaine reinstatement test, mice received an injection of cocaine (10mg/kg) and underwent transcardial perfusion with 4% paraformaldehyde (PFA) in 1X PBS 90 min later in order to assess cFos expression in the mPFC and NAc. Fixed brains were collected and sectioned into 40 um thick slices into triplicate wells for immunohistochemistry. Sections were blocked using 5% normal donkey serum (NDS) for 1 hour, then incubated in primary (rabbit anti-cFos, 1:10,000, Celling Signaling, 2250S) with 5% NDS overnight. After washing sections were incubated in donkey anti-rabbit secondary (1:1000, Jackson ImmunoResearch, 711-065-152) for 30 min incubation. The signal was amplified with an avidin-biotin complex (1:500, ABC kit, Vector Laboratories, PK-6200), then reacted with diaminobenzidine (Vector Laboratories, SK-4100) with nickel enhancement. Tissues were mounted on plus slides and coverslipped with DPX (Electron Microscopy, 13512).

Photomicrographs of 3 section of cFos images in the NAc (bregma: AP: +0.98mm, +1.21mm, +1.42mm) and mPFC (bregma: AP: +1.41mm, +1.69mm, +1.93mm) were obtained by light microscope (Nikon, 10X) and imported to Image J, the counts per area (total counts in the selected area, measured as counts/mm^2) were analyzed.

Chemogenetic activation of mPFC-NAshell Gq signaling:

A separate cohort of mice was used to determine the effect of chemogenetic activation of mPFC to NAshell projections on cocaine-primed reinstatement. Male and female were bilaterally injected into the mPFC (bregma: AP +1.8 mm, ML ±0.3 mm, DV –3.0 mm) with either pAAV-hSyn-hM3D(Gq)-mCherry (0.2 μL per side, 0.1μL/min, 5min diffusion, ordered from Addgene, 50474-AAV8), or control virus pAAV-hSyn-mCherry (114472-AAV8). Then a bilateral guide cannula was implanted into the NAshell (bregma: AP +1.4 mm, ML ±0.6 mm, DV –3.7mm). Mice were recovered for 7 days then underwent EcoHIV inoculation and cocaine CPP test as mentioned above. CNO infusion (500 μM, 0.2 μL/side, 0.1 μL/min) was performed over 2 min, followed by diffusion for 2 min. Mice were returned to the home cage for 10min before the cocaine-primed reinstatement test.

Immunohistochemical confirmation of AAV placement:

To verify virus expression in the mPFC and canulation placement in the NAshell, 40um brain slices from control virus- or DREADD virus-expressing mice underwent RFP (for mCherry tag) immunofluorescent staining and were examined under fluorescent scope. Slices were incubated in chicken anti-RFP primary (1:1,000, Novus Biologicals, Centennial, CO, NBP1-97371) overnight, then Alexa Fluor® 594 donkey anti-chicken secondary (1:250, Jackson ImmunoResearch, 703-585-155) for two hours. Mice with RFP signal in the mPFC region between 1.98mm and 1.34mm anterior to bregma and cannula placements between 0.98mm and 1.70mm anterior to bregma were included.

Statistical Analyses:

GraphPad Prism (10) was used for statistical analysis of behavioral and molecular data. Data were analyzed using unpaired *t*-test or two-way ANOVA. Significant interactions were followed using Holm-Šídák's multiple comparisons *post hoc* analysis or Fisher's LSD multiple comparisons test where appropriate.

Results

EcoHIV did not impact cocaine preference

To investigate whether EcoHIV infection impacted the development of cocaine CPP, we assessed the time spent in the cocaine-paired chamber after 4 pairings (**Figure 1A**). We found that following conditioning, both sham and EcoHIV-infected-mice demonstrated cocaine CPP, as indicated by increase in the time spent in the cocaine-paired chamber compared to initial preference (CPP scores > 0). However, no significant difference in CPP scores between sham and EcoHIV-infected-mice were observed [$t(22)=0.5913$, $p = 0.5603$; **Figure 1B**].

EcoHIV-infected mice display impaired extinction learning

We examined the preference for the cocaine-paired chamber across 4 days of extinction training. We found that EcoHIV and sham mice showed distinct extinction patterns. Specifically, EcoHIV-infected mice were resistant to extinction during the first session when compared to sham controls [$t(22)=2.075$, $p = 0.0499$; **Figure 1C**]. To investigate the time course of this effect, change in time spent in cocaine-paired chamber was analyzed across 5-min bins on first session of extinction (**Figure 1D**). EcoHIV mice had a higher extinction score compared to sham mice [main effect of EcoHIV: $F(1, 22) = 8.211$, $p=0.009$], but there was no effect of time [$F(1.261, 27.74) = 1.208$, $p=0.2940$] or an interaction between EcoHIV and time [$F (3, 66) = 2.305$, $p=0.0848$], suggesting that differences were present across the Day1 extinction session. A post hoc Holm-Šídák's multiple comparisons test revealed that extinction score was significantly different between EcoHIV and sham mice on 10 – 15min (p's < 0.05). There was no effect of EcoHIV on the 4 days of extinction [main effect of EcoHIV: $F (1, 22) = 0.8234$, $p=0.374$; main effect of days: $F (2.807, 61.76) = 1.521$, $p = 0.22$; interaction: $F (3, 66) = 1.597$, $p=0.1985$]. These data suggested that EcoHIV-infected mice exhibited a delay of extinction learning compared to control mice but ultimately successfully extinguished the CPP.

EcoHIV infection significantly increased cocaine-primed reinstatement

Following extinction training, one cohort of mice underwent a cocaine-primed reinstatement test. EcoHIV-infected mice exhibited greater cocaine-primed reinstatement scores than sham mice [$t(22)=2.649$, $p = 0.0147$; **Figure**

1E], consistent with increased sensitivity to cocaine-primed reinstatement. To further examine this effect, we assessed the change in time spent in cocaine-paired chamber across 5-min bins which revealed that EcoHIV mice produced a higher reinstatement of cocaine seeking compared to sham mice (**Figure 1F**). A two-way ANOVA revealed a significant interaction between time and EcoHIV [$F(3, 66) = 8.231$, $p<0.0001$], as well as a main effect of EcoHIV [$F(1, 22) = 8.211$, $p=0.009$] and a trend of main effect of time [$F(3, 66) = 4.504$, $p=0.0062$]. Post hoc Holm-Šídák's multiple comparisons revealed that reinstatement significantly different between EcoHIV and sham mice on 20 min ($p=0.0016$). Locomotor activity during the reinstatement test was assessed, but no differences in locomotor activity was observed between EcoHIV and sham mice [$t(22)=0.5371$, $p= 0.5966$; **Figure 1G**], demonstrating increased reinstatement of cocaine seeking was not due to hyperlocomotor response-induced by cocaine in EcoHIV-infected mice. A separate cohort of mice underwent yohimbine-induced reinstatement. While mice established a similar CPP (**Figure 2A**), in contrast to cocaine-primed reinstatement, neither sham [$t(11)= 1.041$, $p= 0.3204$] nor EcoHIV-infected mice exhibited yohimbine-primed reinstatement [$t(11)=0.2523$, $p= 0.8055$]. Further, EcoHIV infection did not impact yohimbine-induced reinstatement [$t(22)=0.7700$, $p=0.4495$, **Figure 2B**], suggesting that potentiating effects of EcoHIV infection may be selective to drug-primed, but not stressor-primed, reinstatement.

EcoHIV infection increased cFos induction in the NAshell area

To determine if cocaine had differential impact on putative cellular activity within the mPFC and NAc of EcoHIV and sham mice, expression of the protein product of the cellular activity marker cFos was measured in mPFC and NAc subregions following cocaine or saline injection. We observed a main effect of cocaine [$F(1, 19) = 7.265$, $p=0.0143$] and a main effect of EcoHIV [$F(1, 19) = 5.094$, $p= 0.0360$] in upregulating cFos expression in the NAshell, no interaction effect was observed [$F(1, 19) = 2.973$, $p=0.1009$; **Figure 3A**]. While cocaine also increased cFos expression in the NAcore [$F(1, 19) = 4.772$, $p=0.0417$], there was no effect of EcoHIV [$F(1, 19) = 0.02334$, $p=0.8802$] or interaction between cocaine and EcoHIV in the NAcore [$F(1, 19) = 0.1771$, $p=0.6786$; **Figure 3B**]. No cFos expression differences were observed in IL [main effect of cocaine: $F(1, 19) = 0.8650$, $p=0.3640$, main effect of EcoHIV: $F(1, 19) = 0.007179$, $p=0.9334$, interaction: $F(1, 19) = 0.04101$, $P=0.8417$; **Figure 3C**] and in PrL [main effect of cocaine: $F(1, 19) = 1.035$, $p=0.3219$, main effect of EcoHIV: $F(1, 19) = 0.8100$, $p=0.3794$, interaction: $F(1, 19) = 0.1730$, $p=0.6821$; **Figure 3D**].

Chemogenetic activation of Gq signaling within mPFC to NAshell circuits suppressed cocaine-primed reinstatement in EcoHIV-infected mice

As the mPFC projections to the NAshell are known to suppress reinstatement, we assess the ability of chemogenetic activation of mPFC projections to the NAc to suppress cocaine-primed reinstatement in EcoHIV infected mice (Timeline: **Figure 4A**). A two-way ANOVA revealed a significant main effect of DREADD [$F(1, 43) = 4.300$, $p = 0.0441$] on reinstatement scores, but no main effect of EcoHIV [$F(1, 43) = 0.02411$, $P=0.8773$] or interaction between DREADD and EcoHIV [$F(1, 43) = 1.713$, $p=0.1976$, **Figure 4B**]. No difference was observed in the locomotor activity during the reinstatement across 4 groups (**Figure 4C**), indicating the DREADD-suppressed reinstatement was not associated with locomotor suppression. These findings suggested chemogenetic activation suppressed cocaine-primed reinstatement in all DREADD-expressing mice, independent of EcoHIV infection.

EcoHIV infection validation

To validate the infection status of mice, we isolated and purified DNA from the spleens of all EcoHIV-infected mice and representative sham mice, and quantified HIV-1 long terminal repeat (LTR) DNA levels via qPCR. All EcoHIV-infected mice included in the dataset showed detectable levels of viral DNA. No viral DNA was detected in sham control mice. We verified that EcoHIV LTR DNA levels were in the range from 100 to 1500 viral DNA copies per 10^6 spleen cells at the end of experiments (8 weeks following inoculation) (Figure 5).

Discussion

CUD is highly comorbid with HIV infection, and infection may alter the neural substrates regulating cocaine seeking. Our findings demonstrated that EcoHIV infection impaired cocaine extinction learning and potentiated cocaine-primed reinstatement, but not stress-induced reinstatement. Given that the mPFC-NAc circuit is sensitive to both cocaine and HIV and plays a role in cocaine relapse after extinction, we investigated cellular activation in the mPFC and NAc subregions following acute cocaine exposure through cFos expression analysis. We found an interactive increase in cFos expression in the NAshell in EcoHIV-infected mice treated with cocaine, indicating greater sensitivity to cocaine treatment following infection. Moreover, chemogenetic activation of the mPFC-NAshell circuit rescued cocaine-primed reinstatement in EcoHIV-infected mice, suggesting that – as is observed in the absence of infection - activation of this pathway can suppress reinstatement.

Cocaine re-exposure is a common factor for relapse in people living with CUD (Baum et al., 2009; Mimiaga et al., 2013). A priming dose of cocaine is often sufficient to trigger craving and relapse by reactivating extinguished memories formed through maladaptive plasticity during cocaine exposure (Farrell et al., 2018). The NAshell plays an important role in inhibiting cocaine-primed and cue-induced reinstatement, and its activity is required for extinction-induced suppression of cocaine seeking. Evidence suggests that inactivation of the NAshell does not block cocaine reinstatement in the absence of extinction training, highlighting the circuit's specific role in extinction-driven inhibition of drug-seeking behavior (Peters et al., 2008; Lalumiere et al., 2012). Cocaine exposure and withdrawal has profound molecular and synaptic altercations in NAc. Our results show that EcoHIV-infected mice were resistant to extinction on Day 1, spending more time in the cocaine-paired chamber compared to the post-test (**Figure 1D**). Additionally, EcoHIV-infected mice exhibited a higher rate of cocaine-primed reinstatement than sham controls. We observed increased cFos expression in the NAshell in response to cocaine in EcoHIV-infected mice, suggesting that EcoHIV infection may induce neuroplastic changes in these regions associated with behavioral alterations.

Glutamate system alterations have been linked to extinction learning, with studies showing that extinction training induces an increase in AMPA glutamate receptor expression in the NAc, facilitating the retrieval of drug-associated memories and enhancing inhibitory control during relapse (Sutton et al., 2003; Self and Choi, 2004;

Panopoulou and Schlüter, 2022). This upregulation of AMPA receptors may serve as a compensatory mechanism to counteract the decreased basal glutamate levels during cocaine withdrawal. Repeated cocaine exposure with extinction training enhances AMPA and NMDA receptor trafficking to synaptic membranes and elevates glutamate transmission in the NAc, priming long-term potentiation (LTP) and promoting reinstatement of cocaine-seeking when re-exposed to the drug (Yao et al., 2004; Panopoulou and Schlüter, 2022). HIV infection may exacerbate glutamatergic adaptation in the NAc. Studies show that HIV viral proteins promote NMDA receptor phosphorylation and potentiate glutamate excitotoxicity, leading to neuronal apoptosis (Haughey et al., 2001; Eugenin et al., 2007; Gorska and Eugenin, 2020). These findings suggest that EcoHIV may disrupt glutamatergic neuroadaptations in the NAc, contributing to deficits in extinction learning and memory consolidation, ultimately enhancing reinstatement of cocaine-seeking behavior.

mPFC-NAc circuitry is particularly vulnerable to both cocaine and HIV infection, though the specific mechanisms by which circuit dysfunction modulates cocaine-seeking behavior in HIV-infected individuals remain unclear. Circuit manipulation techniques, such as chemogenetics, can help reveal projection-specific mechanisms underlying cocaine-induced dysfunction in the context of HIV infection. For example, chemogenetic activation of the ventromedial PFC-to-NAshell pathway has been shown to reduce cue-induced reinstatement of cocaine-seeking, suggesting that activating these circuits could be a potential strategy to mitigate relapse triggered by cocaine-associated cues (Augur et al., 2016b). We found chemogenetic activation of the mPFC-NAshell pathway rescued cocaine-seeking reinstatement in EcoHIV-infected mice. These findings may suggest that EcoHIV infection induced neuroadaptations in mPFC-NAshell circuitry, leading to extinction learning deficits and heightened cocaine-primed reinstatement. This chemogenetic dissection of mPFC-NAshell contributions to HIV-induced dysregulation of cocaine seeking behaviors reveal crucial insight into the development of targeted medications to treat CUD in the context of comorbid HIV. Though a limitation of this study is unable to precisely control microinjection and DREADD expression across mPFC subregions (e.g., IL or PrL) of mouse. Future study is necessary to dissect the precise circuit-level contributions to observed suppressing EcoHIV-induced reinstatement. This could be addressed using strategies such as retrograde targeting of the NAshell combined with optogenetic stimulation of the IL subregion. Overall, findings from current study highlight the importance of incorporating circuit-level mechanisms into preclinical SUD research in PLWH which could accelerate the identification of effective targets for SUD prevention and treatment.

One consideration is that the enhanced cFos induction in the NAshell in response to acute cocaine in EcoHIV-infected mice may contrast with the chemogenetic data, which suggests that activation of the mPFC-NAshell glutamatergic circuit reduces reinstatement to cocaine seeking. One possibility is that selective activation of mPFC inputs to the NAshell is required for the modulation of reinstatement, and increased mPFC glutamatergic input to the Nashell selectively can suppress reinstatement. It is also possible that cocaine-driven increases in NAshell activity could reflect alterations in dopaminergic activity that could compete with mPFC inputs (Aragona et al., 2008). Thus, the observed cFos expression may not fully represent the functional outcomes of neuronal activity in relation to cocaine-seeking behaviors. Further studies are needed to clarify EcoHIV-associated cell-type and circuit specific alterations in response to cocaine that underlying enhanced reinstatement to cocaine seeking.

Unexpectedly, EcoHIV infection did not affect yohimbine-induced reinstatement of cocaine-seeking. Yohimbine, an α2A-adrenergic receptor antagonist, functions by blocking the inhibitory effect of the α2A receptor on norepinephrine release, thereby increasing the stress- and anxiety-like responses (Charney et al., 1983). Yohimbine has been reported to induce cocaine craving and relapse (Mantsch et al., 2010; Feltenstein et al., 2011), and this phenomenon has been blocked by the α2A receptor agonist (Lee et al., 2004), suggesting the role of α2A receptor in cocaine reinstatement. The studies examining HIV effects on α2A receptor are very limited. However, noradrenergic signaling in response to stress stimuli is impaired by HIV-associated immune defect (Søndergaard et al., 2000), In addition, as HIV infection may increase dopamine level in the striatum, and dopamine (DA) is the precursor of norepinephrine and epinephrine (Gaskill and Khoshbouei, 2022), it is possible HIV-associated neuroimmune responses have indirect impact on noradrenergic system that contributes to stress activation. As no effect was observed on yohimbine-induced reinstatement of cocaine seeking, it is possible that EcoHIV did not affect α2A receptor at this experimental timepoint.

Conclusion

We demonstrated that EcoHIV infection induced extinction learning deficits and increased cocaine-primed reinstatement, which was associated differential activation of the NAshell. Chemogenetic activation of mPFC-

NAshell projection Gq signaling was able to suppress cocaine-primed reinstatement of cocaine seeking in EcoHIV-infected mice, which suggests that similar circuits may regulate drug seeking in EcoHIV infection. These findings provide valuable insights into potential strategies for managing cocaine relapse among people living with HIV.

Figure 1. EcoHIV induced cocaine-primed reinstatement. (**A**) Timeline of experiment. Mice underwent EcoHIV or sham inoculation 5 weeks prior to undergoing a cocaine CPP test. Mice were tested for their CPP chamber bias during a 20-min pre-test session. Cocaine (10 mg/kg, i.p.) and saline pairings (2 each) occurred on alternating days followed by CPP the CPP post-test. Mice then underwent 4 days of extinction training. In the cocaine-primed reinstatement test, mice received a cocaine injection (10mg/kg, i.p.) and were tested for 20 min. (**B**) CPP was acquired in both sham (n=12) and EcoHIV-infected mice (n=12), no effect of EcoHIV was detected. (**C**) EcoHIV and sham mice showed distinct extinction patterns. EcoHIV-infected mice were showing a delay of extinction compared to sham controls. (**D**) To determine the time course of this effect, change in time spent in cocaine-paired chamber was analyzed across 5-min bins on first session of extinction. EcoHIV mice had a higher extinction score compared to sham mice. (**E, F**) EcoHIV-infected mice showed higher cocaine-primed reinstatement, as measured by increase reinstatement score across the session (**E**) and in total (**F**) compared to sham mice. (**G**) No locomotor activity difference was observed between sham and EcoHIV-infected mice after cocaine injection during reinstatement. Timeline created with Biorender.com. Closed and open symbols represent male and female mice, respectively. Bars represent means ± SEM. *$p < 0.05$, **$p<0.01$.

Figure 2. EcoHIV did not impact yohimbine-induced reinstatement. (**A**) CPP was acquired in both sham (n=12) and EcoHIV-infected mice (n=12). (**B**) No difference was observed between sham and EcoHIV-infected mice in yohimbine-induced reinstatement test. (**C**) No locomotor activity difference was observed between sham and EcoHIV-infected mice after yohimbine injection during reinstatement. Closed and open symbols represent male and female mice, respectively. Bars represent means ± SEM.

Figure 3. Expression of cFos in subregions of the mPFC and NAc. Cocaine and EcoHIV increased the cFos expression (counts/mm^2) in the NAshell (**A**) cocaine increased cFos expression in the NAcore (**B**). While no main effect of EcoHIV or cocaine was observed in IL (**C**) and PrL (**D**). (**E**) Representative immunohistochemistry images of cFos in the NAc. n=6/group Bars represent mean +/- SEM. *$p<0.05$

Figure 4. Chemogenetic activation of mPFC-NAshell projections. (A) Timeline of DREADD study. Anterograde neuronal Gq-DREADD (pAAV-hSyn-hM3D(Gq)-mCherry) or control (pAAV-hSyn-mCherry) virus was injected to the mPFC of mice prior to EcoHIV inoculation. Five weeks after inoculation, mice underwent the

cocaine CPP and cocaine-primed reinstatement test. On the day of reinstatement, mice received an injection of CNO to activate the mPFC-NAshell circuit prior to cocaine injection. (**B**) Gq DREADD activation by CNO administration significantly attenuated cocaine-primed reinstatement in both sham and EcoHIV-infected mice. (**C**) No difference was observed in the locomotor activity during the reinstatement across four groups. (**D**) Representative immunofluorescence images of hM3Dq-mCherry or mCherry in the mPFC sections. Data represent mean +/- SEM, Closed and open symbols represent CNO and saline treatment, respectively. n= 7-9 /group. *$p < 0.05$.

Figure 5. EcoHIV splenic DNA viral load. Copies of EcoHIV-NDK spleen viral DNA at the end of experiment (week 8 of infection). The number of copies were in the range from 100 to 1500 viral DNA copies per 106 spleen cells. Closed and open symbols represent male and female mice, respectively. Data represent means ± SEM.

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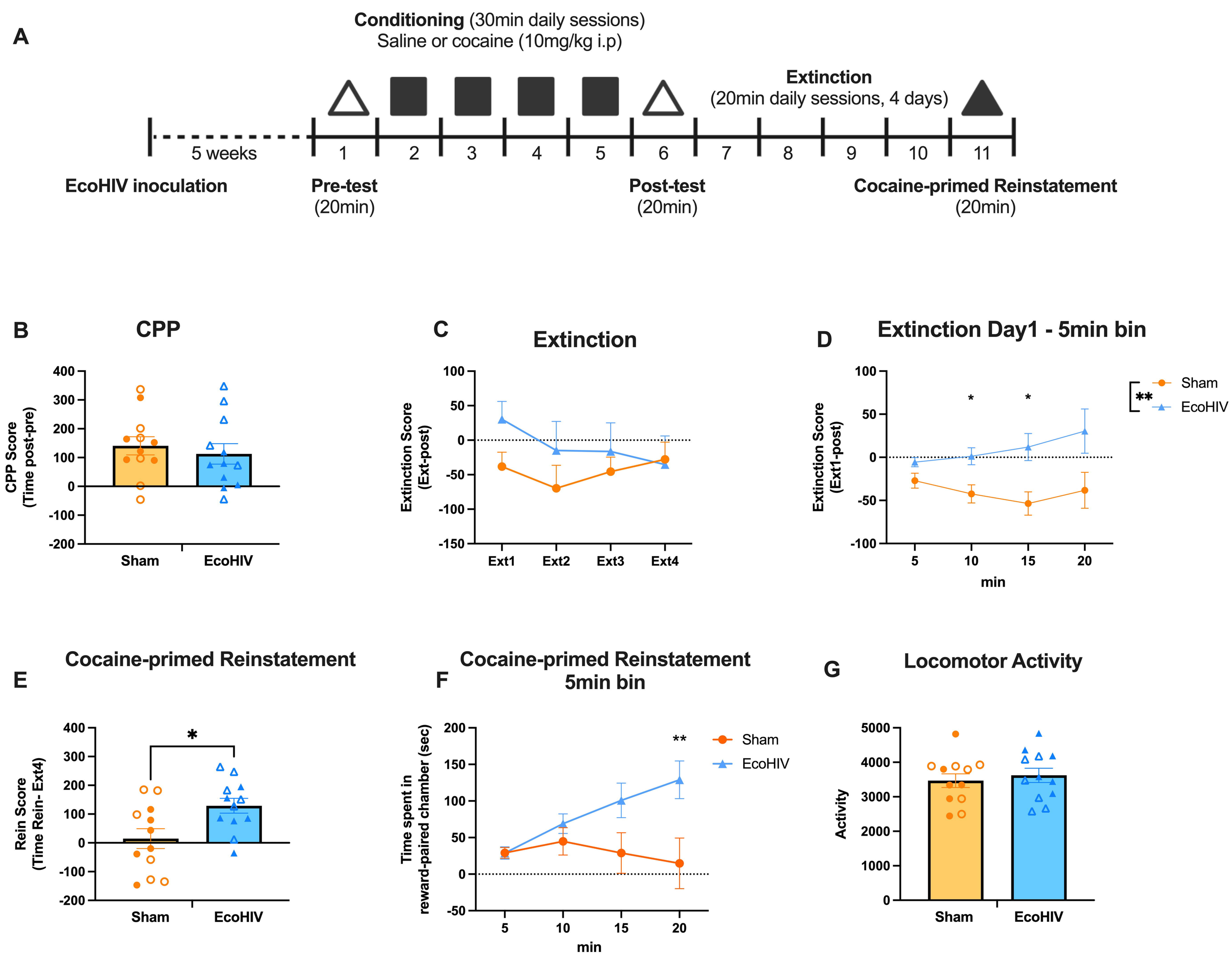
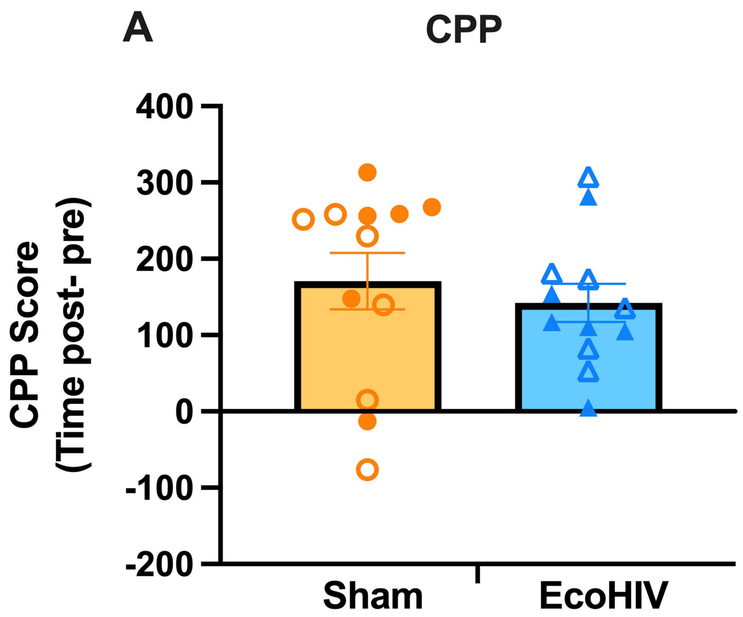

A
Conditioning (30min daily sessions)
Saline or cocaine (10mg/kg i.p)
Extinction
(20min daily sessions, 4 days)
5 weeks
1
2
3
4
5
6
7
8
9
10
11
EcoHIV inoculation
Pre-test
(20min)
Post-test
(20min)
Cocaine-primed Reinstatement
(20min)
B
CPP
CPP Score
(Time post-pre)
400
300
200
100
0
-100
-200
Sham
EcoHIV
C
Extinction
Extinction Score
(Ext-post)
100
50
0
-50
-100
-150
Ext1
Ext2
Ext3
Ext4
D
Extinction Day1 - 5min bin
Extinction Score
(Ext1-post)
100
50
0
-50
-100
5
10
15
20
min
*
*
**
Sham
EcoHIV
E
Cocaine-primed Reinstatement
Rein Score
(Time Rein- Ext4)
400
300
200
100
0
-100
-200
*
Sham
EcoHIV
F
Cocaine-primed Reinstatement
5min bin
Time spent in
reward-paired chamber (sec)
200
150
100
50
0
-50
5
10
15
20
min
**
Sham
EcoHIV
G
Locomotor Activity
Activity
5000
4000
3000
2000
1000
0
Sham
EcoHIV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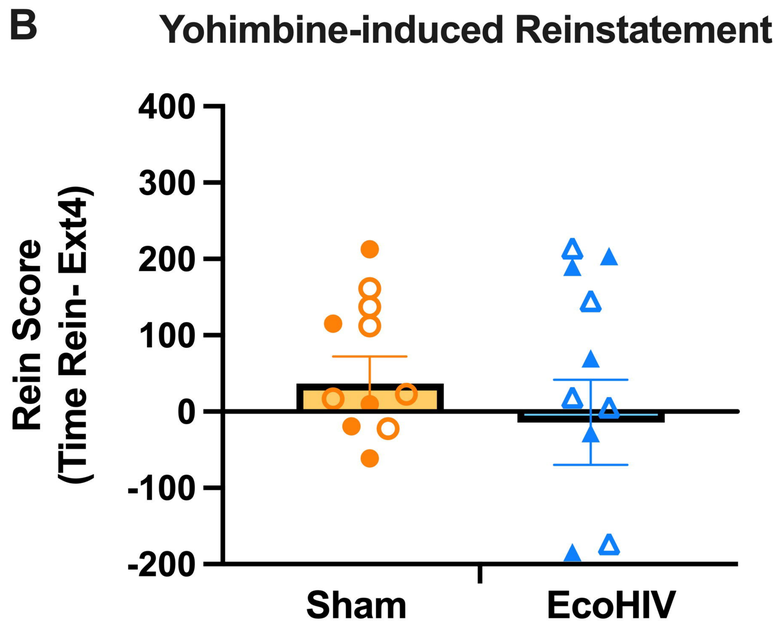
CPP
CPP Score
(Time post- pre)
400
300
200
100
0
-100
-200
Sham
EcoHIV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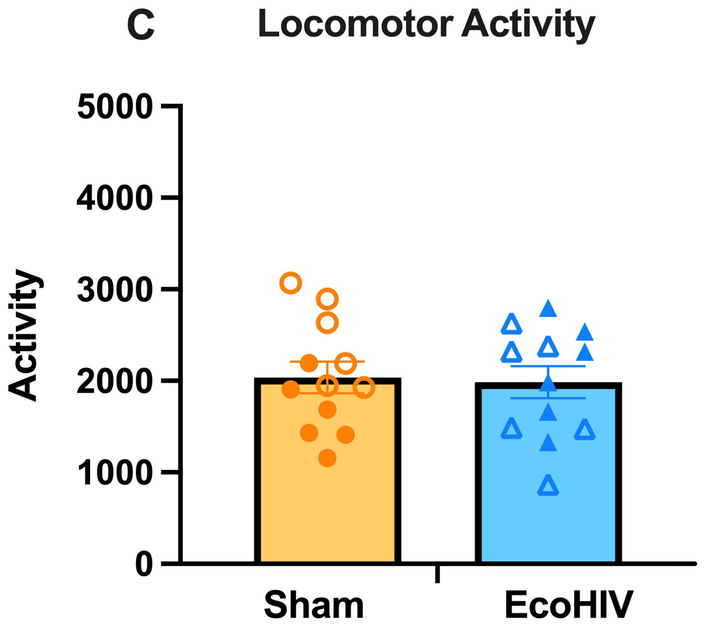
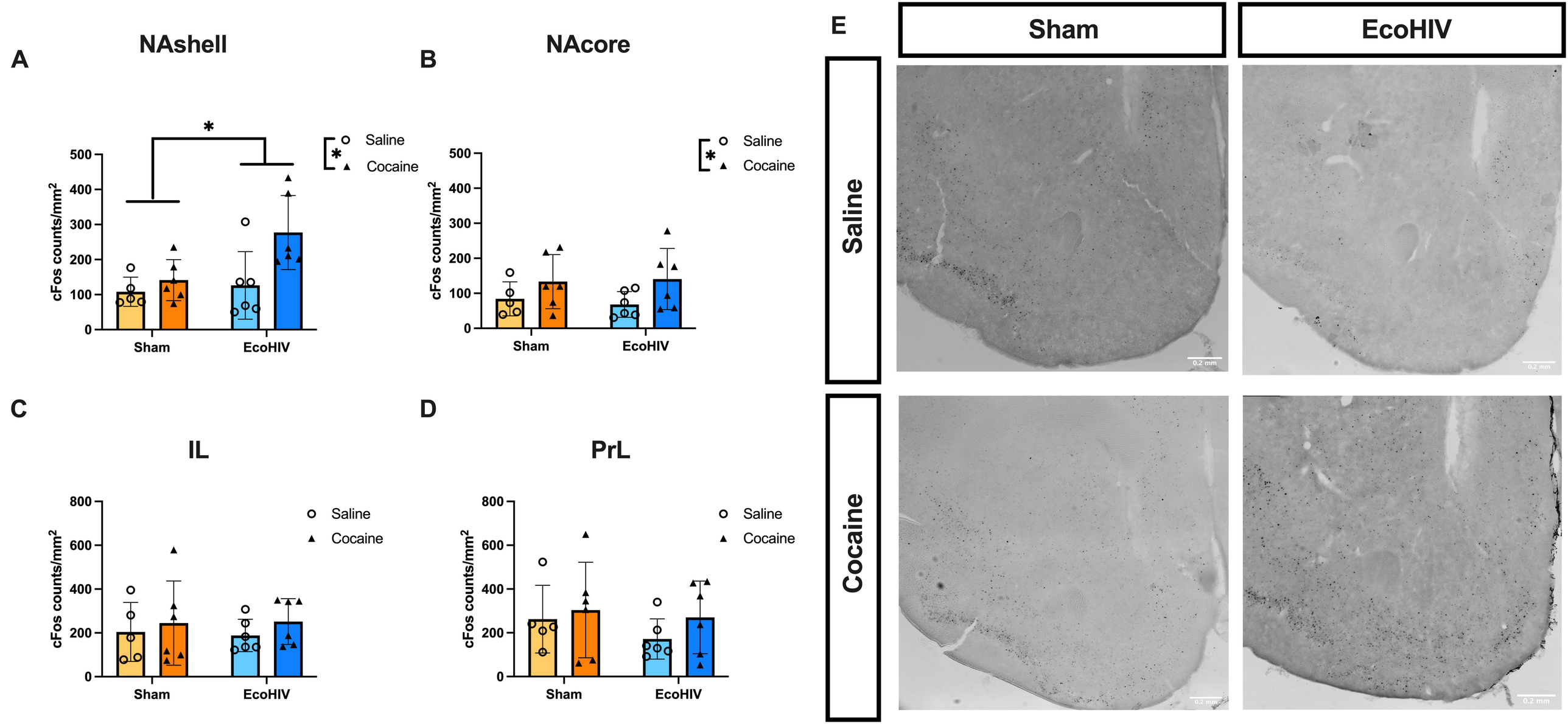
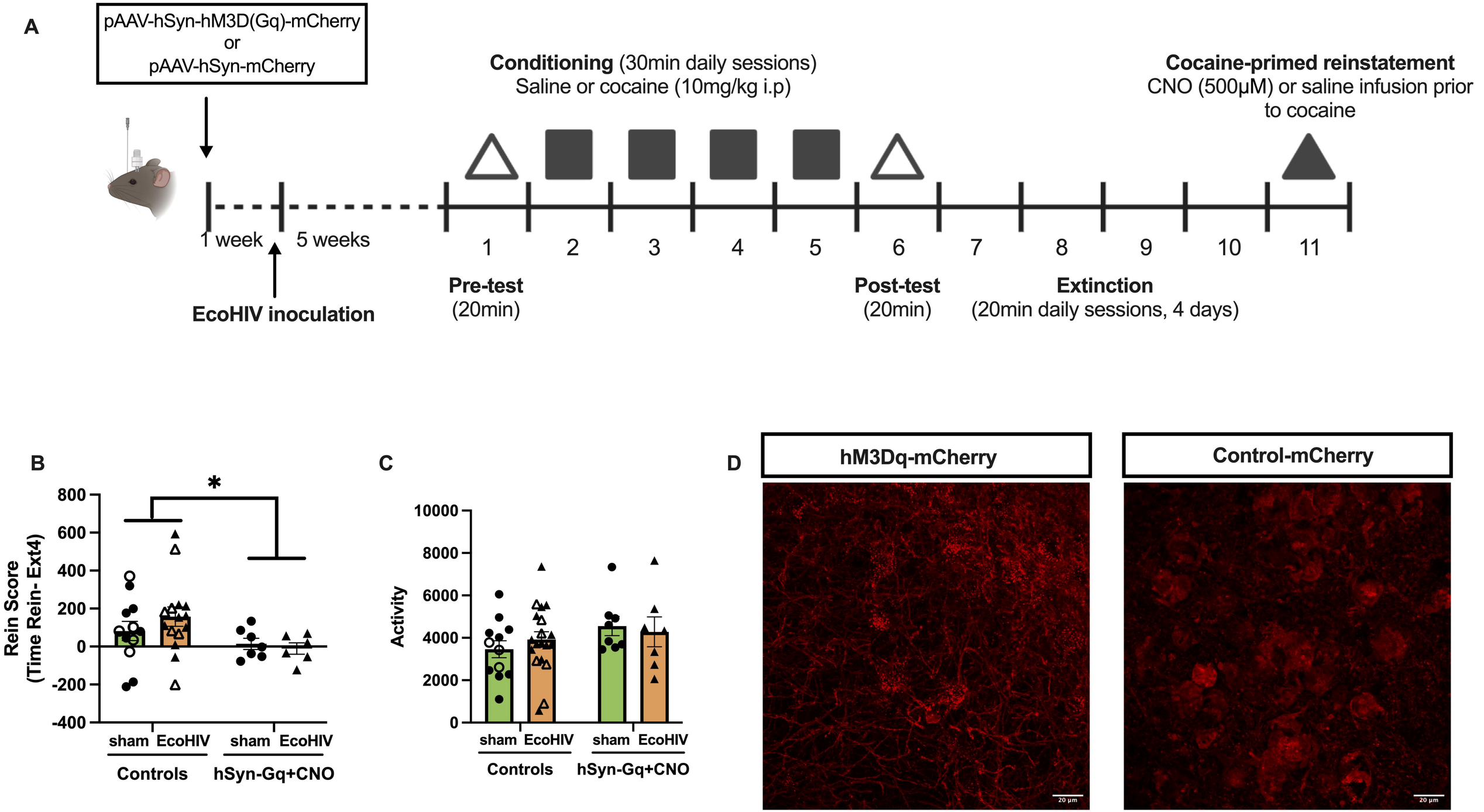
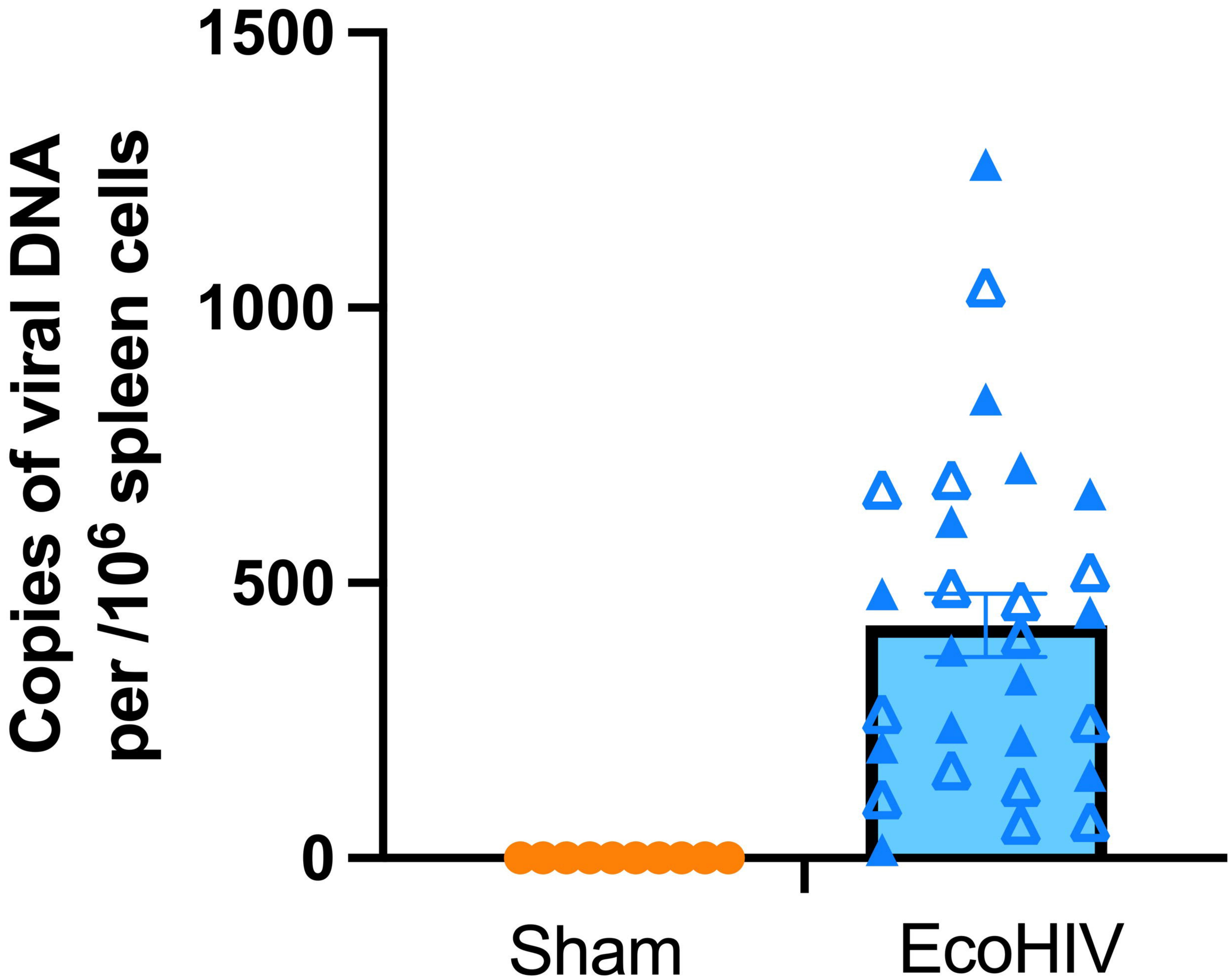
Yohimbine-induced Reinstatement
Rein Score
(Time Rein- Ext4)
400
300
200
100
0
-100
-200
Sham
EcoHIV


C
Locomotor Activity
Activity
5000
4000
3000
2000
1000
0
Sham
EcoHIV


A
NAshell
B
NAcore
C
IL
D
PrL
E
Sham
EcoHIV
Saline
Cocaine
cFos counts/mm²
Saline
Cocaine
*
0.2 mm


A
pAAV-hSyn-hM3D(Gq)-mCherry
or
pAAV-hSyn-mCherry
1 week
5 weeks
EcoHIV inoculation
Conditioning (30min daily sessions)
Saline or cocaine (10mg/kg i.p)
1
2
3
4
5
6
7
8
9
10
11
Pre-test
(20min)
Post-test
(20min)
Extinction
(20min daily sessions, 4 days)
Cocaine-primed reinstatement
CNO (500μM) or saline infusion prior to cocaine
B
Rein Score
(Time Rein- Ext4)
800
600
400
200
0
-200
-400
*
sham
EcoHIV
sham
EcoHIV
Controls
hSyn-Gq+CNO
C
Activity
10000
8000
6000
4000
2000
0
sham
EcoHIV
sham
EcoHIV
Controls
hSyn-Gq+CNO
D
hM3Dq-mCherry
Control-mCherry
20 μm
20 μm


Copies of viral DNA
per /10^6 spleen cells
1500
1000
500
0
Sham
EcoHIV