Material and Methods

Subjects

Male Long-Evans rats (Charles River, Raleigh, NC) or male c-fos-lacZ transgenic rats \(^1\) (total n=172) weighing 350-450 g were used. After surgery, the rats were housed individually in the animal facility under a reverse 12-h light-dark cycle (lights off at 9 a.m.). Food and water were freely available in the rats’ home cages throughout the experiment. Experimental procedures followed the guidelines of the "Principles of Laboratory Animal Care" (NIH publication no. 86-23, 1996). Sixty-two rats were excluded due to catheter problems (n=10), failure to learn to self-administer heroin (n=10), poor health (n=15), misplaced cannulae (n=10), loss of tissue (n=1), failure to meet an extinction criterion of less than 25 active lever presses per 3 h over 3 days after 26 extinction days (n=15), or lever presses during the reinstatement test that were 6.8 standard deviations above the group mean (n=1, Exp. 3, Daun02 condition). (Note: the inclusion of this outlier in the statistical analyses did not change the overall results, as indicated by significant interactions between the Daun02 Condition and the Injection Context for both active lever presses \(F_{1, \, 54}=5.6, \, p=0.021\) and \(\beta\)-gal expression \(F_{1, \, 54}=4.0, \, p=0.05\)).
Intracranial and intravenous surgery

Rats were anesthetized with sodium pentobarbital & chloral hydrate (60 & 25 mg/kg, i.p.), and permanent guide cannulae (23-gauge, Plastics One, Roanoke, VA) were implanted bilaterally 1 mm above the dorsal or ventral mPFC. The stereotaxic coordinates for dorsal mPFC were AP +3.0 mm, ML ±1.2 mm (10° angle), and DV −2.4 mm, and for ventral mPFC were AP +3.0 mm, ML ±1.5 mm (10° angle), and DV −4.3 mm; these coordinates are based on our and others’ previous work.

Following cannulae implantation, silastic catheters were inserted into the jugular vein as described previously. Catheters were attached to a modified 22-gauge cannula and mounted to the rat’s skull with dental cement. Buprenorphine (0.1 mg/kg, s.c.) was given after surgery to relieve pain, and rats were allowed to recover for 7-10 days before heroin self-administration training. During the recovery and training phases, catheters were flushed every 24-48 h with gentamicin (Butler Schein, Dublin, OH; 5 mg/ml) and sterile saline.

Intracranial injections

Muscimol (0.03 nmol/0.5 µl/side)+baclofen (0.3 nmol/0.5 µl/side) (Tocris) was dissolved in sterile saline, and Daun02 (2 µg/0.5 µl/side) was dissolved in 5% dimethyl sulfoxide (DMSO), 6% Tween 80, and 89% 0.1M PBS [for 6 rats, Daun02 was dissolved in 50% artificial cerebral spinal fluid and 50% DMSO]. The doses of muscimol+baclofen and Daun02 were based on previous studies.

Intracranial injections were administered using a syringe pump (Harvard Apparatus, Holliston, MA) and 10-µl Hamilton syringes that were attached via polyethylene-50 tubing to 30-gauge injectors (Plastics One). Muscimol+baclofen, Daun02, and their respective vehicles were injected over 1 min and the injectors were left in place for 1 min.

Fos immunohistochemistry

All experiments below were carried out at room temperature unless otherwise specified. Ninety min after exposure to Context A, the rats were deeply anaesthetized with isoflurane (approximately 80 sec) and perfused transcardially with 100 ml of 0.1M phosphate-buffered saline (PBS) followed by 400 ml of 4% paraformaldehyde in 0.1M sodium phosphate (pH 7.4). Brains were removed and post-fixed.
in 4% paraformaldehyde for 2 h before transfer to 30% sucrose in 0.1 M sodium phosphate (pH 7.4) for 48 hours at 4°C. Brains were subsequently frozen in powdered dry ice and stored at -80°C until sectioning. Coronal sections (40 µm) containing dorsal and ventral mPFC (approximately +2.5 to 3.5 mm from Bregma) were cut using a cryostat (Leica Microsystems Inc., Bannockburn, IL), collected in cryoprotectant (20% glycerol and 2% DMSO in 0.1 M sodium phosphate, pH 7.4), and stored at -80°C until further processing.

Free-floating sections were washed (3 times for 10 min each) in PBS, incubated for 1 h in 3% normal goat serum (NGS) in PBS with 0.20% Triton X-100 (PBS-Tx), and incubated overnight at 4°C with rabbit anti-c-Fos primary antibody (c-Fos sc-52, Lot F2209, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:4000 in 1% NGS in PBS-Tx. The sc-52 antibody was raised against amino acids 3-16 of human c-Fos: SGFNADYEASSSRC. Sections were then washed in PBS and incubated for 2 h with biotinylated anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories, Burlingame, CA) diluted 1:600 in 1% NGS in PBS-Tx. Sections were again washed in PBS and incubated in avidin-biotin-peroxidase complex (ABC Elite kit, PK-6100, Vector Laboratories) in PBS containing 0.5% Triton X-100 for 1 h and washed in PBS. Sections were developed in 3,3'-diaminobenzidine (DAB) for approximately 4 min, washed in PBS, mounted onto chrom-alum/gelatin-coated slides, and air-dried. Slides were dehydrated through a graded series of alcohol concentrations (30, 60, 90, 95, 100, 100% ethanol), cleared with Citrasolv (Fisher Scientific, Hampton, NH), and coverslipped with Permount (Fisher Scientific).

Brightfield images of dorsal and ventral mPFC were digitally captured using a CCD Camera (CoolSnap Photometrics, Roper Scientific Inc., Trenton, NJ, USA) attached to a Zeiss Axioskop 2 microscope with a 5x objective. Labeled Fos-immunoreactive (IR) nuclei from both left and right hemispheres of 1-2 sections per rat in the Control (A-A-A) and Renewal (A-B-A) groups (n=9-11) were automatically counted using IPLab software (version 3.9.4 r5; Scanalytics Inc., Fairfax, VA) for Macintosh. Each hemisphere’s approximate size was 0.98 and 0.25 mm² for dorsal and ventral
mPFC, respectively. Image capture and quantification of Fos-positive nuclei were conducted in a blind manner by JMB.

Double labeling of Fos and NeuN immunohistochemistry (Exp. 1)

A double-labeling experiment for Fos and NeuN, a marker for neuronal-specific nuclear protein\textsuperscript{10}, was used to determine the proportion of neurons expressing Fos. Brain sections from 8 rats from Exp. 1 (Control and Renewal; n=4 per group) were thawed and washed (three times for 10 min each) in Tris-buffered saline (TBS; 0.025 M Tris-HCl, 0.5 M NaCl, pH 7.5) and incubated for 20 min in TBS with 0.2\% Triton X-100 (TBS-Tx). Sections were washed in TBS and incubated for 48 h with the rabbit anti-Fos primary antibody (1:500 dilution, sc-52) and mouse anti-NeuN primary antibody (1:2,000 dilution of MAB377, Millipore, Temecula, CA) in TBS-Tx. Sections were again washed in TBS and incubated for 1 h with secondary antibodies Alexa 488-labeled donkey anti-rabbit and Alexa 568-labeled goat anti-mouse antibody (1:200 dilution in TBS-Tx for both antibodies, Invitrogen, Carlsbad, CA). Finally, sections were washed in TBS, mounted on chrom-alum/gelatin coated slides, air-dried, and coverslipped with Vectashield fluorescent mounting medium (H-1400, Vector Laboratories).

Fluorescent images of dorsal and ventral mPFC were captured with Qimaging Exi Aqua camera (Exton, PA) attached to a Zeiss Axioskop 2 microscope using a 20x objective. Sampled areas for dorsal and ventral mPFC were 0.27 mm\textsuperscript{2}. The number of Fos-labeled, NeuN-labeled, and double-labeled immunoreactive nuclei in these images were manually counted using iVision MacOS 10.62 (version 4.0.15) in a blind manner by JMB.

Double labeling of Fos and CamKII or Fos and GAD67 immunohistochemistry (Exp. 1)

We double-labeled Fos with GAD67 (glutamic acid decarboxylase 67, a marker of GABAergic neurons)\textsuperscript{11,12} or CamKII (calcium/calmodulin-dependent protein kinase II, a marker of cortical glutamatergic pyramidal projection neurons)\textsuperscript{13} to characterize the phenotype of the Fos-activated neurons. We used two sections per rat from 3 rats from the ABA renewal group, one section for Fos+CamKII and one section for Fos+GAD67. The sections were thawed and washed (three times
for 10 min each) in PBS and incubated for 30 min in 1% H$_2$O$_2$ in PBS. Sections were washed in PBS, incubated for 1 h in a blocking solution of 5% NGS and 2.5% bovine serum albumin in PBS-Tx, and then incubated for 48 h with the rabbit anti-Fos primary antibody (1:1800 dilution, sc-52) and mouse anti-CamKIIα primary antibody (1:100 dilution, catalog # MA1-048, Pierce Biotechnology Rockford, IL) or mouse anti-GAD67 primary antibody (1:1000 dilution, catalog # MAB377, Millipore, Temecula, CA) in a blocking solution. Sections were again washed in PBS and incubated for 2 h in the blocking solution with secondary antibodies Alexa 488-labeled donkey anti-rabbit (1:200) and Alexa 568-labeled goat anti-mouse antibody (1:400). Sections were again washed in PBS, mounted on chrom-alum/gelatin coated slides, air-dried, and coverslipped with Vectashield fluorescent mounting medium.

Fluorescent images of ventral mPFC were captured with a Qimaging Exi Aqua camera (Exton, PA) attached to a Zeiss Axioskop 2 using a 40x objective. Fos-labeled and double-labeled (Fos+CamKII or Fos+GAD67) immunoreactive nuclei were manually counted by CC using iVision MacOS 10.62 (version 4.0.15). Sampled areas from ventral mPFC were 0.07 mm$^2$.

X-gal histochemistry for β-galactosidase (β-gal) visualization with c-fos-lacZ rats (Exp. 3)

We used transgenic c-fos-lacZ rats that had been bred for 35-40 generations from a Sprague-Dawley background $^1$. 90 min after exposure to Context A (see Exp. 3 below) rats were deeply anaesthetized with isoflurane (approximately 80 sec) and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed, and sectioned the same as described above.

Free-floating sections were washed three times for 10 min each in PBS and incubated in reaction buffer (0.1 M X-gal, 100 mM sodium phosphate, 100 mM sodium chloride, 5 mM EGTA, 2 mM MgCl$_2$, 0.2% Triton X-100, 0.05 M K$_3$FeCN$_6$, 0.05MK$_4$FeCN$_6$) for 5 h at 37 °C with gentle shaking. Sections were washed three times for 10 min each in PBS and mounted onto chrom-alum/gelatin-coated slides and air-dried. The slides were dehydrated through a graded series of alcohol (30, 60, 90, 95, 100, 100% ethanol), cleared with Citrasolv, and coverslipped with Permount.
Brightfield images of ventral mPFC were captured with a Qimaging Exi Aqua camera (Exton, PA) attached to a Zeiss Axioskop 2-light microscope using a 5x objective. β-gal-expressing nuclei, characterized by blue nuclear staining, were counted using iVision MacOS 10.62 (version 4.0.15) (Fig. 3d); quantification and determination of threshold level was similar to that described previously 3. We counted nuclei in sampling areas (approximately 1.08 mm²) around ventral mPFC injection site (left and right hemispheres) in 2 coronal sections per rat. Image capture and quantification were conducted in a blind manner by JMB.

**Double labeling of β-gal and Fos immunohistochemistry with c-fos-lacZ rats (Exp. 3)**

We double-labeled β-gal with Fos to determine the proportion of β-gal positive neurons that express Fos in c-fos-lacZ rats 3. We used brain sections from 4 rats that were exposed to the heroin self-administration context on induction day and were given vehicle injections 90 min after the onset of the session. Brain sections were thawed and washed (three times for 10 min each) in TBS and incubated for 20 min in TBS-Tx. Sections were washed in TBS and incubated for 48 h with the rabbit anti-Fos primary antibody (1:500 dilution, sc-52) and goat anti-β-gal primary antibody (1:1,000 dilution, 4600-1409, Biogenesis, Poole, England) in TBS-Tx. Sections were again washed in TBS and incubated for 1 h with secondary antibodies Alexa 488-labeled donkey anti-rabbit and Alexa 568-labeled donkey anti-goat (1:200 dilution in TBS-Tx for both antibodies, Invitrogen). Finally, sections were washed in TBS, mounted on chrom-alum/gelatin coated slides, air-dried, and coverslipped with Vectashield fluorescent mounting medium.

Fluorescent images of ventral mPFC were captured with Qimaging Exi Aqua camera (Exton, PA) attached to a Zeiss Axioskop 2 using a 40x objective. The number of Fos-labeled, β-gal labeled, and double-labeled immunoreactive nuclei were manually counted using iVision MacOS 10.62 (version 4.0.15). Sampled areas from ventral mPFC were 0.07 mm² and were near the injection site.

**Apparatus and procedures**

Rats were trained and tested in standard Med Associates (St. Albans, VT) self-administration chambers. Each chamber was equipped with two levers located 9 cm above the grid floor. Presses
on the active retractable lever activated the infusion pump while presses on the inactive non-retractable lever had no programmed consequences. The two contexts differed from each other in terms of their auditory, visual, tactile, and circadian (i.e., morning [onset at 8-9 am] vs. afternoon [onset at 2-3 pm] sessions) cues, using procedures identical to those described in our previous studies. The contexts are referred to as A and B, whereby A is the heroin self-administration (training) and reinstatement (testing) context, and B is the extinction context (but see Control group in Procedures and Exp. 1 below). The physical environments that constituted Contexts A and B were counterbalanced.

Experiments 1 and 2 consisted of 3 phases: self-administration training (12 d), extinction training (14-22 d), and tests for context-induced reinstatement of heroin-seeking (1 or 2 d). The experimental sequence for Exp. 1 was Context A (training)—Context B (extinction)—Context A (test) for the Renewal group, and Context A (training)—Context A (extinction)—Context A (test) for the Control group. The experimental sequence for Exp. 2 was Context A (training)—Context B (extinction)—Contexts A and B (test). Experiment 3 consisted of five phases: self-administration training (12 d), extinction training (12-26 d), induction day (30 min exposure to either Context A or B followed 60 min later by Daun02 or vehicle injections), extinction training (2 d), and test day (90 min exposure to Context A followed by immediate perfusion). To simplify, the experimental sequence for Exp. 3 was Context A (training)—Context B (extinction)—Contexts A or B (induction)—Context B (extinction)—Context A (test) (Fig. 3a).

Heroin self-administration training and extinction

Rats were trained to self-administer heroin for 3-h/d for 12 days. Heroin (diacetylmorphine HCl; NIDA) was dissolved in sterile saline and infused at a volume of 65 µl over 2.3 sec at a dose of 0.1 (first 6 sessions) or 0.05 (last 6 sessions) mg/kg/infusion. During training, heroin infusions were earned on a fixed-ratio-1 (FR1), 2.3 sec timeout reinforcement schedule and were paired with a compound tone-light cue lasting 2.3 sec. During the extinction phase, responses on the previously active lever led to presentation of the tone-light cue but not heroin. Tests for context-induced
reinstatement were conducted under extinction conditions and started after a minimum of 12 daily extinction sessions when the rats met the extinction criterion of a mean of less than 25 presses on the previously active lever over the last 3 extinction sessions.

Exp. 1: Effect of context-induced reinstatement on Fos induction in dorsal and ventral mPFC

In Exp. 1, we measured Fos-IR in rats exposed to the heroin context after extinction of lever responding in the same or a different context to assess whether context-induced reinstatement is associated with neuronal activation in mPFC. For this purpose, we used two groups of rats (n=9-11 per group). The Control group (A-A-A) underwent heroin self-administration training (3 h/day), extinction training (3 h/day), and reinstatement testing (90 min), all in context A. The Renewal (A-B-A) group underwent heroin self-administration training in context A, extinction training in context B, and reinstatement testing in context A. The reinstatement test was 90 min because of the known time course of Fos induction after exposure to drug or non-drug stimuli. At the end of the test session, the rats were anaesthetized, perfused with PBS and 4% paraformaldehyde, and their brains were removed for subsequent Fos-IR immunohistochemical determination. Rats in the Control and Renewal groups were matched for their heroin intake and number of active lever presses during training and extinction.

Exp. 2: Effect of dorsal or ventral mPFC injections of muscimol+baclofen on context-induced reinstatement

In Exp. 1, we found that context-induced reinstatement is associated with Fos-IR induction in both dorsal and ventral mPFC. In Exp. 2, we assessed the causal role of these mPFC subregions in context-induced reinstatement by reversibly inactivating them with muscimol+baclofen (0.03 nmol+0.3 nmol/side) 5-10 min prior to the reinstatement tests. For this purpose, we used four groups of rats (n=7-10 per group). Rats were injected with vehicle or muscimol+baclofen into dorsal (2 groups) or ventral (2 groups) mPFC. Each rat was tested twice with their assigned dose (vehicle or muscimol+baclofen): once in Context A (heroin context) and once in Context B (extinction context). The order of testing in Context A and B was counterbalanced, and tests were separated by 48 h (rats
remained in animal housing room between tests). After testing, rats were deeply anesthetized, decapitated, and their brains were removed. Coronal sections (50 µm) were sliced on a cryostat and stained with Cresyl Violet. Brains were then verified for cannulae placement under a light microscope.

**Exp. 3: Effect of ventral mPFC injections of Daun02 on context-induced reinstatement**

Four groups of rats (n=10-18 per group) were used. The experimental sequence for two groups was ABAA (training in A, extinction in B, induction in A, test in A); the experimental sequence for the other two groups was ABBA (Fig. 3a). On induction day, rats were exposed to Context A (heroin context; 2 groups) or Context B (extinction context; 2 groups) for 30 min. 60 min after the end of the session, rats were injected with vehicle or Daun02 (2 µg/side) into ventral mPFC and returned to their home cage. For the next two days, all rats were given two 3-h extinction sessions in Context B, and one day later they were tested for context-induced reinstatement in context A (90 min session). At the end of the test session, rats were anaesthetized, perfused with PBS and 4% paraformaldehyde, and their brains were removed for subsequent β-gal and Fos-IR immunohistochemical determination.

Rats in the vehicle and Daun02 groups were matched for their heroin intake and number of active lever presses during training, extinction, and induction day (induction day: ABAA vehicle: 29.3±4.6 active lever presses/30 min; ABAA daun02: 29.1±4.7; ABBA vehicle: 5.7.X±1.3; ABBA daun02: 6.0±1.7).

**Statistical analyses**

Data were analyzed using mixed ANOVAs with the statistical program SPSS (GLM procedure), and significant main effects and interaction effects (p< 0.05) were followed by Fisher PLSD post-hoc tests. The between- and within-subjects factors used in the analyses are described in the text. Because the intracranial injections of the vehicles, muscimol+baclofen, and Daun02 had no effect on very low inactive lever presses (means of less than 4 presses during the test sessions), these data are not presented.
Figure legends

**Figure S1.** *Heroin self-administration training and extinction of the drug-reinforced responding.* (a) **Training:** Number of heroin infusions, and active and inactive lever presses during the 12 days of heroin self-administration training. (b) **Extinction:** Number of presses on the previously active lever and on the inactive lever during the first 12 extinction sessions conducted in the absence of heroin in either the heroin self-administration context (Exp. 1 control group, n=9) or in a different (extinction) context (Exp. 1 renewal group and Exp. 2-3, n=101). Data are depicted as mean±SEM.

**Figure S2. (a)** *Fos induction in Exp. 1.* Representative photomicrographs (20X) of Fos-immunoreactivity for the Control and Renewal groups in ventral and dorsal mPFC. See Figure 1 for additional experimental details and quantification of Fos-immunoreactivity. (b) Fos+CamKII and Fos+GAD67 double labeling in Exp. 1. Representative photomicrographs (40X) of Fos+CamKII and Fos+GAD67 in ventral mPFC.

**Figure S3.** *Schematic mechanism for Daun02 inactivation in c-fos–lacZ rats (adapted from Figure 2 in Koya et al. 3).* (a) The *c-fos–lacZ* transgene contains a *c-fos* promoter that drives expression of *c-fos-lacZ*, which encodes the bacterial protein β-galactosidase (β-gal). β-gal can catalyze the conversion of the prodrug Daun02 into daunorubicin, which decreases subsequent cellular excitability. (b) Exposure to the heroin-associated context induces β-gal expression (red-labeled nuclei) and Fos expression (green-labeled nuclei) in ventral mPFC of *c-fos–lacZ* rats. Nuclei double-labeled for both β-gal and Fos appear yellow to orange in the merged image panel and indicate co-localization of β-gal and Fos proteins.
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Supplemental Figure 1. Training and Extinction

**a**
Training

- Active lever
- Infusions
- Inactive lever

Infusions or lever presses (3 h)

Training session

0 20 40 60 80

1 2 3 4 5 6 7 8 9 10 11 12

0.1 mg/kg/infusion

0.05 mg/kg/infusion

**b**
Extinction

- Active lever
- Inactive lever

Lever presses (3 h)

Extinction session

0 20 40 60

1 2 3 4 5 6 7 8 9 10 11 12
Supplemental Figure 2. Fos expression in Experiment 1

a  
Fos  

dmPFC  
vmPFC  

Control  

Renewal  

Fos-CamKII and Fos-GAD67 (ventral mPFC)

b  
Fos-CamKII and Fos-GAD67 (ventral mPFC)  

Fos-CamKII Merged  

Fos-GAD67 Merged
Supplemental Figure 3. The Daun02 inactivation method

Context-induced neuronal activation

a

\[ \text{c-fos promoter} \rightarrow \text{c-fos} \rightarrow \text{lacZ} \rightarrow \text{Daunorubicin (reduces excitability)} \]

b

\[ \text{β-gal} \]

\[ \text{Merged} \]

50 µm