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Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin

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Abstract

In a rat model of context-induced relapse to heroin, we identified sparsely distributed ventral medial prefrontal cortex (mPFC) neurons that were activated by the heroin-associated context. Selective pharmacogenetic inactivation of these neurons inhibited context-induced drug relapse. A small subset of ventral mPFC neurons forms neuronal ensembles that encode the learned associations between heroin reward and heroin-associated contexts; re-activation of these neuronal ensembles by drug-associated contexts during abstinence provokes drug relapse.

Keywords

Conditioned cues; Daun02 inactivation method; drug environment; extinction; heroin self-administration; medial prefrontal cortex; neuronal ensembles; opiates; reinstatement

Exposure to environmental contexts previously associated with heroin reward often provokes drug relapse in humans 1. We previously adapted an ABA renewal procedure 2 to study context-induced relapse to drug seeking in a rat model 3. Here, we examined the role of dorsal and ventral mPFC in context-induced reinstatement of heroin seeking, because results from recent studies with cocaine-experienced rats led to the hypothesis that dorsal and ventral mPFC sub-regions play opposite roles—facilitation and inhibition, respectively—in drug relapse 4.

We trained rats to self-administer intravenous heroin for 3 h/day for 12 d in one context (A) (Fig. S1a). During training, “active” lever-presses led to heroin infusions that were paired with a discrete tone-light cue, while “inactive” lever-presses had no programmed consequences. We then extinguished active lever-presses in the presence of the discrete tone-light cue in a non-drug context (B) over 12–26 days (Fig. S1b). The contexts differed in their tactile (narrow/wide floor grids), visual (houselight ON/OFF), auditory (chamber fan

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Author contributions: JMB designed the experiments, ran the experiments, and wrote the paper; ALS ran the experiments, managed the data, and made the figures; FRMT and CC helped perform the behavioral experiments and the molecular assays; EK and BTH provided input on experimental design and the write-up of the paper, and helped perform Exp. 3 (Daun02 method) and the molecular assays; YS supervised the project, designed the experiments, and wrote the paper with JMB.

ON/OFF), and circadian (session onset at 8–9 AM/2–3 PM) features. After extinction, we assessed context-induced reinstatement of heroin seeking by re-exposing rats to the drug-associated (A) context. During reinstatement tests, presses on the previously active lever led to contingent presentations of the discrete tone-light cue but not heroin. We inferred that context-induced reinstatement of heroin seeking had occurred if the number of active lever-presses during testing in the previously heroin (A) context was higher than in the extinction (B) context. Experimental procedures were approved by the NIDA-IRP Animal Care and Use Committee.

In Exp. 1, we measured the neuronal activation marker Fos (the protein product of the immediate early gene *c-fos*) to determine whether context-induced reinstatement is associated with activation of dorsal or ventral mPFC. Exposure to context A after extinction in context B reinstated heroin seeking (Fig. 1a); this reinstatement was associated with Fos induction in both dorsal and ventral mPFC (Fig. 1c and Fig. S2a). We also assessed the percentage of Fos-activated neurons by double-labeling Fos with the neuronal marker NeuN. The percentage of double-labeled Fos+NeuN neurons in the *Control* group (AAA; training, extinction, and reinstatement in context A) was 2.0±0.4% and 1.9±0.5% in dorsal and ventral mPFC, respectively; the percentage in the *Renewal* group (ABA; training in A, extinction in B, testing in A) was 6.9±0.5% and 6.0±0.8% in dorsal and ventral mPFC, respectively (Fig. 1c). We also examined the phenotype of the Fos-positive nuclei in dorsal and ventral mPFC by assessing the percentage of Fos-positive nuclei that are co-localized with CamKII or GAD67, markers for glutamatergic pyramidal projection neurons and GABAergic interneurons, respectively. We found that the percentage of Fos+CamKII and Fos+GAD67 double-labeling in dorsal mPFC was 76.5±4.9 and 8.6±0.6, respectively, and in ventral mPFC the percentage was 71.2±2.5 and 12.8±1.6, respectively. These data suggest that re-exposure to the heroin-associated context primarily activates mPFC glutamatergic projection.

Fos induction data are correlational and therefore do not indicate whether increased neuronal activity is a cause or a consequence of context-induced reinstatement. Fos induction during reinstatement tests may also be due to the context switch or motor components of lever-pressing rather than increased motivation to seek heroin. Therefore, in Exp. 2 we determined a causal role of context-induced mPFC activation in context-induced reinstatement by using a mixture of muscimol+baclofen (GABAa+GABAb agonists) to non-selectively inactivate the *majority* of dorsal or ventral mPFC neurons 5–10 min prior to context-induced reinstatement testing. Muscimol+baclofen injections into ventral, but not dorsal, mPFC decreased context-induced reinstatement of heroin seeking (Fig. 2). This finding extends previous results on ventral mPFC's role in reinstatement of heroin seeking induced by discrete cues or heroin priming 5, 6.

Early theories 7 and subsequent electrophysiology and cellular imaging studies led to the hypothesis that learned associations between environmental cues and unconditioned rewards are encoded by specific patterns of sparsely distributed neurons 8 called *neuronal ensembles* 9. Based on this hypothesis, we speculated that context-induced reinstatement of heroin seeking is mediated by activation of neuronal ensembles encoding the learned associations between heroin rewarding effects and the drug self-administration context. We hypothesized

that these neuronal ensembles are comprised of some of the 6% of sparsely distributed ventral mPFC Fos-positive neurons activated by heroin-context exposure (Fig. 1) during the reinstatement tests.

Although theoretically appealing, demonstrating the putative role of ventral mPFC neuronal ensembles in context-induced reinstatement or other learned behaviors has until recently presented an intractable challenge. Neither traditional methods (e.g., excitotoxic lesions or intracranial injections of selective receptor or molecular inhibitors) nor newer approaches (e.g., optogenetic neuronal inhibition/activation of neurons based on a neurochemical phenotype or time-locked genetic control over signaling molecule expression) are suitable for studying causal roles of neuronal ensembles in learned behaviors. This is because these methods invariably inhibit or activate both activated and non-activated neurons within a particular brain area.

Therefore, in Exp. 3 we used the novel Daun02 inactivation method 10 to selectively inactivate a minority of ventral mPFC neurons that presumably form neuronal ensembles that mediate context-induced reinstatement (see description of the experimental method in Fig. 3a). This approach allowed us to examine the causal role of these putative neuronal ensembles in context-induced reinstatement.

The Daun02 inactivation method employs *c-fos-lacZ* transgenic rats where beta-galactosidase (β-gal) and Fos are co-expressed within behaviorally activated neurons (we found that 84±1.8 % of β-gal are co-localized with Fos in ventral mPFC, Fig. S3). Ninety minutes after rats perform a behavioral task, these activated neurons are inactivated by injecting the prodrug Daun02 into a given brain area 11. β-gal within the behaviorally activated neurons converts Daun02 into daunorubicin, which disrupts normal function of these neurons for at least 3 days 10 (Fig. S3). Ventral mPFC Daun02 injections 90 min after exposure to the heroin-associated (A) context decreased subsequent (3 d later) context-induced reinstatement and β-gal expression (a Fos induction marker) (Fig. 3). In contrast, ventral mPFC Daun02 injections 90 min after exposure to the extinction-associated (B) context had no effect on subsequent context-induced β-gal expression and, surprisingly, somewhat *increased* (p=0.15) lever-presses during the reinstatement test (Fig. 3). These data negate the possibility that Daun02 injections non-specifically decrease ventral mPFC activity.

Our data suggest that a small subset of ventral mPFC neurons form neuronal ensembles that encode the learned associations between heroin reward and the context in which the drug is self-administered. After prolonged abstinence and extinction of the heroin-reinforced responding in a different context, reactivation of these neuronal ensembles by re-exposure to the heroin-associated context causes relapse to heroin seeking. Based on results from studies using cocaine-experienced rats, a dichotomy in mPFC function was proposed: dorsal (prelimbic, cingulate sub-regions) mPFC promotes drug seeking, while ventral (infralimbic region) mPFC inhibits drug seeking 4. Our data suggest that this dichotomy does not generalize to context-induced reinstatement of heroin seeking, which is promoted by a minority of selectively activated ventral mPFC neurons. Based on our previous data on the role of accumbens shell, but not core, in context-induced reinstatement 3, 13, we speculate

that a neuronal projection from ventral mPFC to accumbens shell, recently implicated in inhibition of cocaine seeking 4, 12 promotes this reinstatement. This difference between brain mechanisms of heroin and cocaine relapse in a rat model extends previous reports that cocaine- and heroin-taking behaviors are mediated by different neural systems 6, 14, 15 and has implications for future research on neurobiological mechanisms of human drug relapse and craving across different classes of abused drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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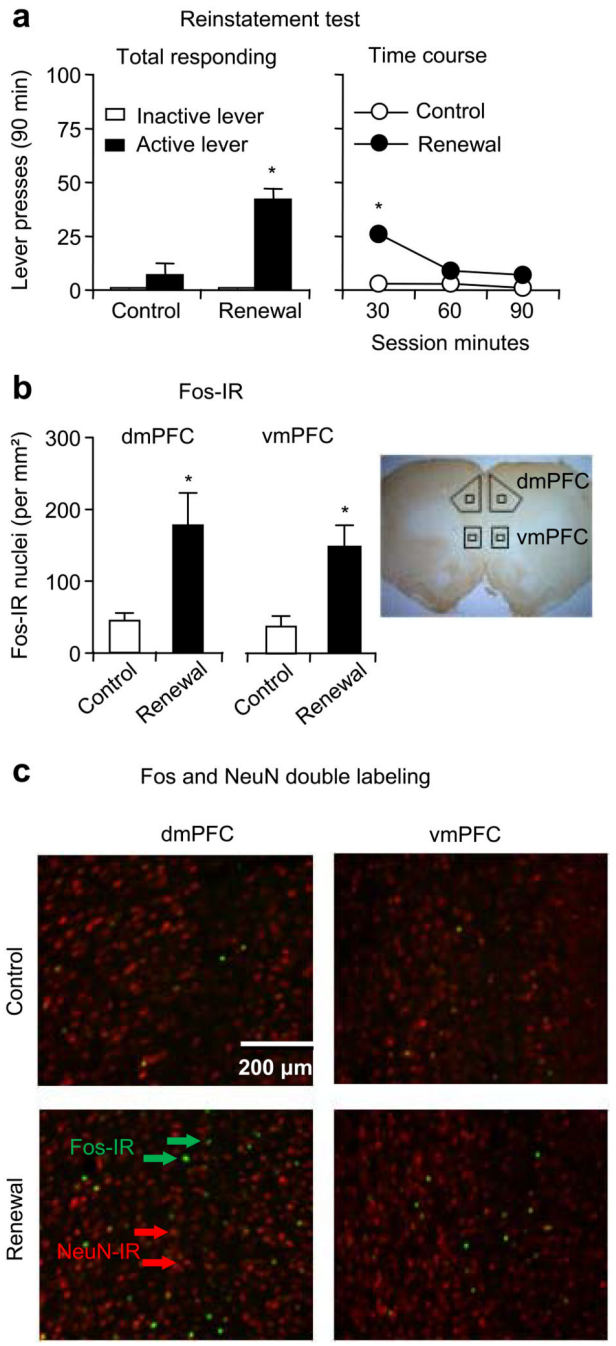

Figure 1. Context-induced reinstatement of heroin seeking is associated with Fos induction in dorsal and ventral mPFC

(**a**) Reinstatement test: *Left column:* Total number of active lever and inactive lever presses in rats in which heroin seeking was extinguished in the same (*Control* A-A-A) or different (*Renewal* A-B-A) context as their heroin self-administration context. ANOVA showed a significant Experimental Group (*Control [AAA], Renewal [ABA]*) × Lever (Active, Inactive) interaction effect ($F_{1,18}$=39.9, p=0.0001). *Right column:* Time course of active lever-presses. (**b**) Fos: Number of Fos-immunoreactive nuclei per mm^2 in dorsal and ventral mPFC (right

panel: area of quantification). ANOVA shows a significant effect of Experimental Group ($F_{1,18}$=9.4, p=0.007), but not mPFC region (dorsal, ventral) or Experimental Group × mPFC region. (**c**) Fos+NeuN double labeling: Representative photomicrographs of Fos+NeuN for dorsal and ventral mPFC (see small squares in the picture in 1b for approximate areas). Data are depicted as mean±SEM. * Different from Control, p<0.05, n=9–11 per group in 1a and 1b, and n=4 per group in 1c.

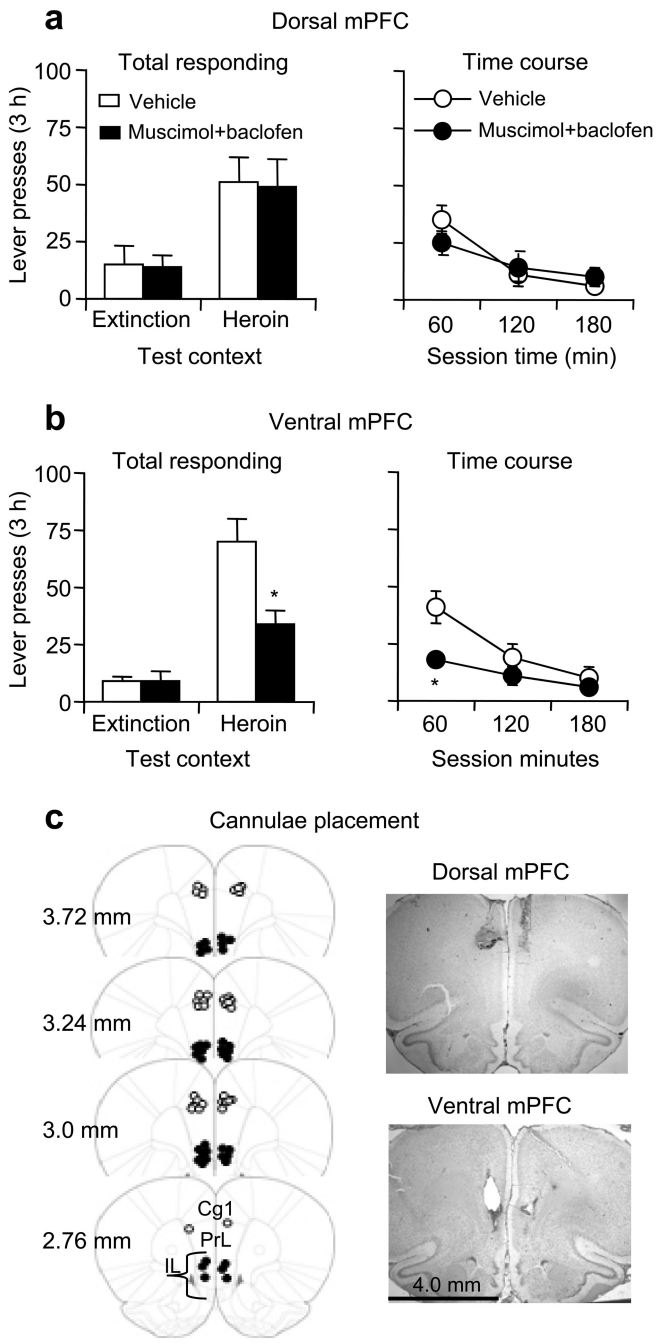

Figure 2. Non-selective global inhibition of the majority of ventral but not dorsal mPFC neurons by muscimol+baclofen decreased context-induced reinstatement of heroin seeking

Total lever-presses (left panel): Number of active lever-presses after bilateral injections of vehicle or muscimol+baclofen (0.03 nmol+0.3 nmol/side) into dorsal (**a**) or ventral (**b**) mPFC 5–10 min before exposure to the heroin or extinction context. ANOVA showed a significant Drug Dose (vehicle or muscimol+baclofen) × mPFC Area (dorsal or ventral) × Test Context (Heroin [A] or Extinction [B]) interaction effect ($F_{1,29}$=4.0, p=0.05). Time course (right panel): Number of active lever-presses (**c**) Cannulae placement: Approximate

placement and representative pictures of injector tips. Data are depicted as mean±SEM. * Different from Vehicle, $p<0.05$, n=7–10 per group. Cg1, cingulate area 1; PrL, prelimbic cortex; IL, infralimbic cortex.

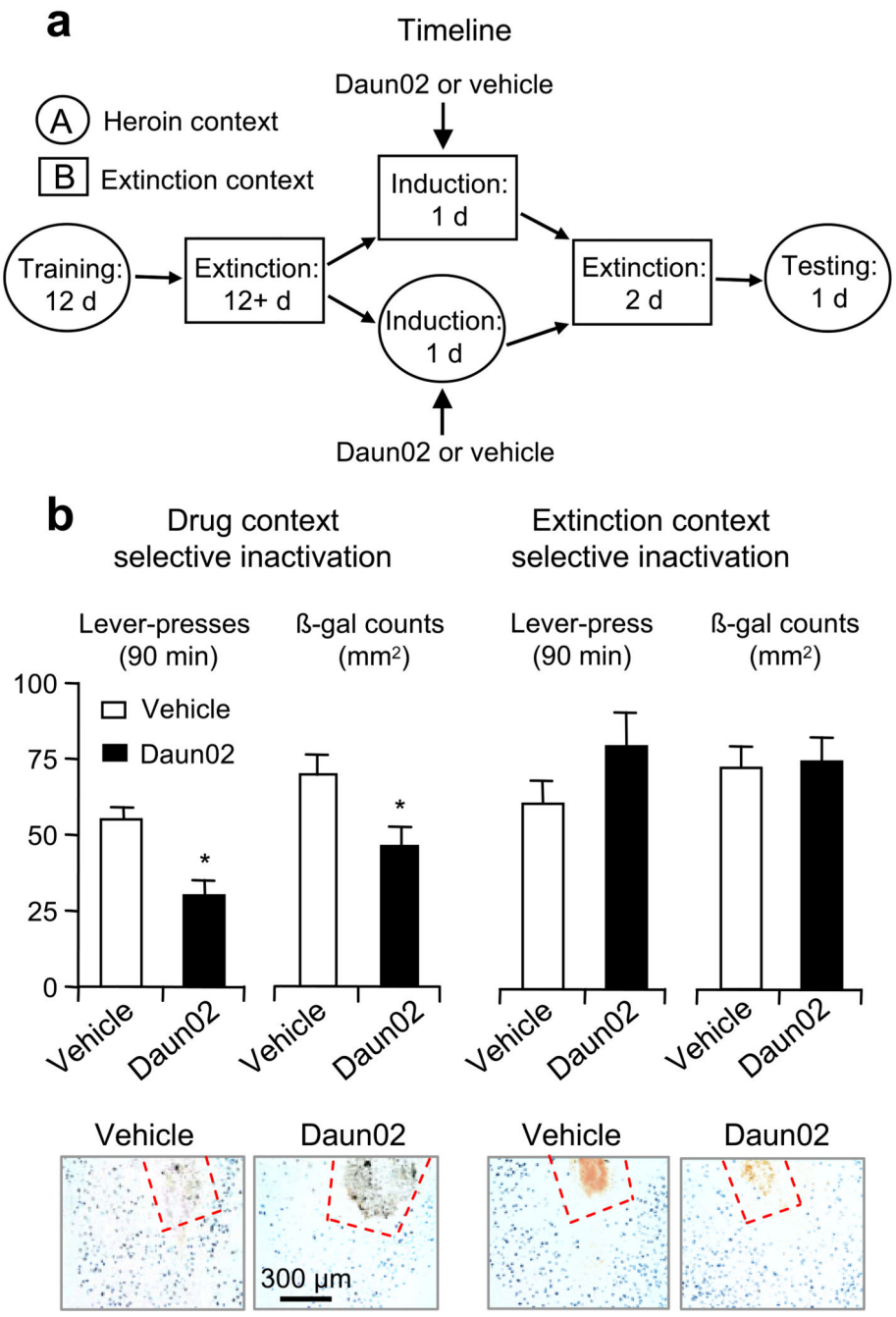

Figure 3. Ventral mPFC Daun02 injections after exposure to the heroin-associated context during induction day decreased subsequent context-induced reinstatement of heroin seeking

(**a**) Timeline of the experimental procedure: Heroin self-administration training (A context), extinction of heroin seeking (B context), induction (A or B context), and context-induced reinstatement test (A context). During the "induction" day, Daun02 (2 µg/side) or vehicle was injected into the ventral mPFC 90 min after 30 min exposure to the Heroin (A) context or the Extinction (B) context. (**b–c**) Daun02 injections in the heroin-associated context or the extinction-associated context: Total active lever-presses and ventral mPFC β-gal

expression. ANOVAs of active lever-presses and β-gal counts revealed significant Daun02 Condition (vehicle, Daun02) × Induction Day Context (Heroin [A] context, Extinction [B] context) interaction effects ($F_{1,53}$=12.6, p=0.001 and $F_{1,53}$=4.3, p=0.042, respectively). (**d**) Representative images of X-gal staining. Visualization of β-gal -labeled nuclei in ventral mPFC. Dotted red lines indicate approximate area of injector tip. Data are depicted as mean ±SEM. * Different from Vehicle, p<0.05, n=10-18 per group.