Separate vmPFC Ensembles Control Cocaine Self-Administration Versus Extinction in Rats

Brandon L. Warren,^{1,2} Louisa Kane,² Marco Venniro,² Pooja Selvam,² Richard Quintana-Feliciano,^{1,2} Michael P. Mendoza,² Rajtarun Madangopal,² Lauren Komer,² Leslie R. Whitaker,² F. Javier Rubio,² Jennifer M. Bossert,² Daniele Caprioli,^{3,4} Yavin Shaham,² and Bruce T. Hope²

¹Department of Pharmacodynamics, University of Florida, Gainesville Florida 32610, ²Behavioral Neuroscience Branch, Intramural Research Program, National Institute on Drug Abuse–National Institutes of Health, Department of Health and Human Services, Baltimore, Maryland 21224, ³Department of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy, and ⁴Santa Lucia Foundation (IRCCS Fondazione Santa Lucia), Rome, Italy

Recent studies suggest that the ventral medial prefrontal cortex (vmPFC) encodes both operant drug self-administration and extinction memories. Here, we examined whether these opposing memories are encoded by distinct neuronal ensembles within the vmPFC with different outputs to the nucleus accumbens (NAc) in male and female rats. Using cocaine self-administration (3 h/d for 14 d) and extinction procedures, we demonstrated that vmPFC was similarly activated (indexed by Fos) during cocaine-seeking tests after 0 (no-extinction) or 7 extinction sessions. Selective Daun02 lesioning of the self-administration ensemble (no-extinction) decreased cocaine seeking, whereas Daun02 lesioning of the extinction ensemble increased cocaine seeking. Retrograde tracing with fluorescent cholera toxin subunit B injected into NAc combined with Fos colabeling in vmPFC indicated that vmPFC self-administration ensembles project to NAc core while extinction ensembles project to NAc shell. Functional disconnection experiments (Daun02 lesioning of vmPFC and acute dopamine D1-receptor blockade with SCH39166 in NAc core or shell) confirm that vmPFC ensembles interact with NAc core versus shell to play dissociable roles in cocaine self-administration versus extinction, respectively. Our results demonstrate that neuronal ensembles mediating cocaine self-administration and extinction come in vmPFC but have distinct outputs to the NAc core and shell that promote or inhibit cocaine seeking.

Key words: Daun02 inactivation; extinction; infralimbic cortex; operant conditioning; RNAscope; vmPFC

Significance Statement

Neuronal ensembles within the vmPFC have recently been shown to play a role in self-administration and extinction of food seeking. Here, we used the Daun02 chemogenetic inactivation procedure, which allows selective inhibition of neuronal ensembles identified by the activity marker Fos, to demonstrate that different ensembles for cocaine self-administration and extinction memories coexist in the ventral mPFC and interact with distinct subregions of the nucleus accumbens.

Introduction

Learned associations between cues, contexts, and reward play important roles in drug addiction (Wikler, 1973; Stewart et al., 1984; Siegel, 1999). In both humans and animal models, cues and contexts present during drug taking become associated with the rewarding

effects of drugs; these cues and contexts can reactivate drug-related memories during abstinence and provoke drug craving and relapse (Wikler, 1973; O'Brien et al., 1992; Bossert et al., 2013; Venniro et al., 2016). In humans and laboratory animals, the response to drug cues can be readily extinguished by repeated exposure to the drug-associated cues in the absence of the drug reward (O'Brien et al., 1992; Conklin and Tiffany, 2002; McNally, 2014). However, extinction learning does not “erase” the original drug-taking memories because learned behaviors can recover over time (spontaneous recovery; Shaham et al., 1997) or reinstate after exposure to stress, drug-associated cues and context, or re-exposure to the drug itself (Davis and Smith, 1976; de Wit and Stewart, 1981; Crombag and Shaham, 2002). Thus, the original drug taking/seeking and extinction memories are thought to be encoded by separate neural substrates in the brain (Bouton, 2002; Khoo et al., 2017).

Inactivation of whole brain regions has identified several brain regions that separately mediate drug seeking and extinction

Received April 23, 2019; revised July 2, 2019; accepted July 9, 2019.

Author contributions: B.L.W., M.V., L.R.W., J.M.B., D.C., Y.S., and B.T.H. designed research; B.L.W., L. Kane, M.V., P.S., R.Q.-F., M.P.M., R.M., L. Komer, L.R.W., F.J.R., J.M.B., and D.C. performed research; B.L.W., L. Kane, R.Q.-F., M.P.M., R.M., L. Komer, F.J.R., J.M.B., D.C., and B.T.H. analyzed data; B.L.W., L. Komer, B.T.H., L. Kane, M.V., R.Q.-F., R.M., L.R.W., F.J.R., J.M.B., D.C., and Y.S. wrote the paper.

This work was supported by the National Institute on Drug Abuse, Intramural Research Program, NIH, and by a Grant from the National Institute on Drug Abuse (4R00DA042102-02) to B.L.W. B.L.W. was also supported by a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation.

The authors declare no competing financial interests.

Correspondence should be addressed to Bruce T. Hope at bhope@intra.nida.nih.gov.

<https://doi.org/10.1523/JNEUROSCI.0918-19.2019>

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(Rhodes and Killcross, 2004, 2007; Peters et al., 2008a; LaLumiere et al., 2010; Ma et al., 2014). For cocaine, projections from ventral medial prefrontal cortex (vmPFC) to nucleus accumbens (NAc) shell are thought to inhibit drug seeking after extinction, while projections from the dorsal medial PFC (dmPFC) to NAc core are thought to drive drug seeking (Peters et al., 2009). However, our studies using Daun02 inactivation do not agree with this anatomical framework and demonstrate that neuronal ensembles in vmPFC and NAc shell promote rather than inhibit heroin or cocaine seeking, respectively (Bossert et al., 2011; Cruz et al., 2014). Other studies suggest that the mPFC subregions play more complex roles in drug seeking, via the efferent projections of individual neurons (Marchant et al., 2015; Moorman et al., 2015; McGlinchey et al., 2016; Gutman et al., 2017), rather than an anatomical divide between dorsal and ventral subregions.

Operant learning involves complex associations among highly specific cues and rewards that require an encoding mechanism with a comparably high degree of resolution (Warren et al., 2017). Methods targeting all neurons within a region are not capable of this high resolution. Instead, learned associations are thought to be encoded by specific patterns of neurons, called neuronal ensembles, which are selected by cues and reinforcers during learning (Hebb, 1949). The technologies to target neuronal ensembles based on their activity have just become available (Garner et al., 2012; Cruz et al., 2013, 2015). We developed the Daun02 inactivation procedure in *Fos-LacZ* transgenic rats to ablate Fos-expressing ensembles that are activated during learned behaviors. The transgene transiently coexpresses β -galactosidase (β -gal) protein with Fos in strongly activated neurons. Following intracranial injections of the inactive prodrug Daun02, the induced β -gal converts Daun02 to daunorubicin, which selectively inactivates and ablates the previously activated neuronal ensemble (Koya et al., 2009b).

Using Daun02 inactivation, we previously found that distinct neuronal ensembles within the vmPFC play a role in food self-administration and extinction of food seeking (Warren et al., 2016). Here, we first tested whether distinct vmPFC neuronal ensembles also play a role in cocaine self-administration and extinction of cocaine seeking. We then tested whether these neuronal ensembles exert their effects on cocaine seeking through different projections to distinct NAc subregions. We used retrograde tracing with cholera toxin subunit B (CTb) to label different efferent projections from the self-administration and extinction ensembles to the NAc core and shell. Finally, we performed functional disconnection experiments with Daun02 inactivation in the vmPFC and dopamine D1 receptor (Drd1) receptor blockade of NAc core and shell subregions to confirm that although self-administration and extinction ensembles converge in the vmPFC, their effects on cocaine seeking are controlled by different projections to the NAc.

Materials and Methods

Subjects

We used a total of 307 male Long-Evans rats (Charles River Laboratories) and *Fos-lacZ* transgenic rats (Koya et al., 2009a), weighing between 250 and 350 g at the start of experiments. After surgery, we housed rats individually under a reverse 12 h light/dark cycle (lights off at 8:00 A.M.). Water was freely available in the rats' home cages throughout the experiment. Food was restricted to 20 g per day of Purina rat chow (given after the daily operant sessions). All procedures followed the guidelines outlined in the *Guide for the Care and Use of Laboratory Animals* (Ed 8; <http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf>). From all experiments, we excluded 56 rats due to sickness, loss of patency, and for Experiments 2, 4,

and 5, misplaced cannulas more rostral than 3.3 mm bregma or more caudal than 2.5 mm bregma.

Surgery

We anesthetized rats with isoflurane (5% induction, 3% maintenance) and injected ketoprofen (2.5 mg/kg, s.c.; Butler Schein Animal Health) daily for 2 d following surgery to relieve pain and decrease inflammation. We allowed the rats to recover from surgery for 3–4 d.

Intravenous catheterization surgery

We implanted SILASTIC catheters into the jugular vein as described previously (Cruz et al., 2014; Rubio et al., 2015; Caprioli et al., 2017). We placed the distal end of the catheter into the jugular vein. We attached the proximal end of the catheter to a modified 22-gauge cannula. We ported the cannula through the midscapular region of the back. We flushed the catheters daily with gentamicin in sterile saline (4.25 mg/ml; APP Pharmaceuticals).

Intracranial cannula implantation

We implanted permanent guide cannulas (23-gauge, Plastics One) bilaterally 1 mm above the vmPFC. The nose bar was set at -3.3 mm, and the coordinates for the vmPFC were anteroposterior: $+3.0$, mediolateral: ± 1.5 , and dorsoventral: -3.8 (10° angle). The coordinates for the NAc Core were anteroposterior: $+1.6$, mediolateral: ± 3.6 , and dorsoventral: -5.9 (20° angle). The coordinates for the NAc Shell were anteroposterior: $+1.6$, mediolateral: ± 3.5 , and dorsoventral: -7.0 (20° angle). We fixed cannulas to the rat's skull with dental cement and jeweler's screws. We used the above coordinates based on pilot and previous studies (Bossert et al., 2007, 2011, 2012; Warren et al., 2016).

Intracranial injections

We performed intracranial injections using a syringe pump (Harvard Apparatus) and 10 μ l Hamilton syringes that were attached via polyethylene-50 tubing to 30-gauge injectors (Plastics One) that extended 1 mm beyond the guide cannula. We infused 0.5 μ l over 1 min, and left the injectors in place for 1 min before removal.

Intracranial CTb injections

We contralaterally infused (0.3 μ l/side) fluorescent cholera toxin-b conjugates CTb-488 and CTb-594 (50 μ g/ μ l; Thermo Fisher, C34775, 34777) into the NAc core [anteroposterior: $+1.6$, mediolateral: ± 3.6 , and dorsoventral: -5.9 (20° angle)] or shell [anteroposterior: $+1.6$, mediolateral: ± 3.5 , and dorsoventral: -7.0 (20° angle)] using a syringe pump (World Precision Instruments) and 10 μ l Hamilton syringes. We infused over 2 min, and left the injectors in place for 5 min before removal. We chose the concentration and volume based on previous studies (Conte et al., 2009; Jin and Maren, 2015).

Drugs

We received cocaine-HCl (cocaine) from the National Institute on Drug Abuse pharmacy. We chose self-administration doses of 1.0 mg/kg/infusion and 0.5 mg/kg/infusion based on previous studies (Cruz et al., 2014). In Experiments 2, 4, and 5, on induction day, we injected Daun02 or vehicle into the vmPFC. We obtained Daun02 from Sequoia Research Products (www.seqchem.com). We dissolved Daun02 (2 μ g/0.5 μ l/side) in vehicle solution containing 5% DMSO, 6% Tween 80, and 89% 0.01 M PBS. We chose the dose of Daun02 based on previous studies (Koya et al., 2009a; Bossert et al., 2011; Fanous et al., 2012; Cruz et al., 2014). In Experiments 4 and 5, we dissolve SCH39166 (SCH; Tocris Bioscience) in sterile water (2.0 mg/ml) at a dose of 1.0 μ g/0.5 μ l/side (15 min pretreatment time) based on our previous studies (Caprioli et al., 2017; Venniro et al., 2017).

Apparatus

We trained and tested rats in Med Associates self-administration chambers; each equipped with a retractable lever located 7 cm above the grid floor and a red house light. Presses on the active retractable lever activated the injection pump, a tone, and a white discrete cue-light.

Fos immunohistochemistry

We washed coronal brain sections (40 μ m) in PBS, blocked with 3% normal goat serum (NGS) in PBS with 0.25% Triton X-100 (PBS-Tx), and incubated 24 h at 4°C with anti-Fos antibody (1:5000 dilution; Cell Signaling Technology, catalog #5348; RRID:AB_10557109) in blocking solution. We then washed sections in PBS, and incubated them with biotinylated goat anti-rabbit secondary antibody (1:600 dilution; Vector Laboratories, BA-9200; RRID:AB_2336171) in PBS-Tx and 1% NGS for 2 h. After washing in PBS, we incubated sections for 1 h in avidin-biotin-peroxidase complex (ABC Elite kit, PK-6100, Vector Laboratories) in PBS containing 0.5% Triton X-100. Finally, we washed sections in PBS and developed in 3,3'-diaminobenzidine (DAB) for ~3 min, washed with PBS, and mounted onto chromalum-gelatin-coated slides. Once dry, we dehydrated the slides through a graded series of alcohol (30, 60, 90, 100, 100% ethanol) and cleared them with Citrasolv (Fisher Scientific) before coverslipping with Permount (Sigma-Aldrich). We digitally captured bright-field images of immunoreactive (IR) cells in vmPFC using an EXi Aqua camera (QImaging) attached to a Zeiss Axioskop 2 microscope at 200 $\times$ magnification (Carl Zeiss Microscopy) and iVision software for Macintosh v4.0.15 (BioVision). Observers (B.L.W., L Kane) blind to the test conditions (inter-rater reliability: Pearson's correlate = 0.91) automatically counted labeled nuclei from two sections (bilateral) per rat (3 images per rat). We averaged the counts so that each rat was an n of 1 for each brain area.

Fos and NeuN immunofluorescence

We washed coronal brain sections from Experiment 1 (40 μ m) in PBS, blocked with 3% NGS in PBS with 0.25% Triton X-100 (PBS-Tx), and incubated 24 h at 4°C with anti-Fos antibody (1:5000 dilution; Cell Signaling Technology, catalog #5348; RRID:AB_10557109) and NeuN antibody (1:2000; Millipore, catalog #MAB377; RRID:AB_2298772) in blocking solution. We then washed sections in PBS, and incubated them with AlexaFluor 488-conjugated goat anti-rabbit (1:500 dilution; ThermoFisher Scientific, catalog #A-11008; RRID:AB_143165) and AlexaFluor 568-conjugated anti-mouse secondary antibodies (1:500 dilution; ThermoFisher Scientific, catalog #A-11004; RRID:AB_141371) and coverslipped with MOWIOL. We digitally captured fluorescent images of IR cells in vmPFC using a Rolera EM-C² camera (QImaging) attached to a Nikon Eclipse E800 at 200 $\times$ magnification and iVision software for Macintosh v4.0.15 (BioVision). Observers (B.L.W., L Kane) blind to the test conditions (Pearson's correlate = 0.93) automatically counted labeled nuclei from two sections (bilateral) per rat (3 images per rat). We averaged the counts so that each rat was an n of 1 for each brain area.

RNAscope in situ hybridization assay

For RNA *in situ* hybridization, we used RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics) according to the manufacturer's instructions and as described previously (Li et al., 2015; Rubio et al., 2015). Briefly, we fixed sections in 10% formalin at 4°C for 20 min, rinsed in PBS, and dehydrated in increasing concentrations of ethanol (50, 70, 100, 100%). We stored slides in 100% ethanol overnight. The next day, we dried the slides at room temperature and drew a hydrophobic barrier around the section. We then treated slides with a protease (Pretreatment 4) for 20 min and washed in distilled water. We then applied target probes for *Fos*, *Slc17a7* (*Vglut1*, a marker of pyramidal glutamatergic projection neurons), and *Slc32a1* (*Vgat*, a marker of GABAergic interneurons) designed by Advanced Cell Diagnostics (catalog #403591-C2, #317001, and #424541-C3, respectively).

We then incubated slides with a series of preamplifier and amplifier probes at 40°C (AMP1 for 30 min, AMP2 for 15 min, AMP3 for 30 min). Next, we incubated slides with fluorescently labeled probes using AMP4 Alt B to detect triplex Fos (AlexaFluor 488), *Vglut1* (Atto, 550), and *Vgat* (Atto, 647). Finally, we incubated the slides with DAPI and coverslipped them with VECTASHIELD fluorescent mounting medium (Vector Laboratories). We captured fluorescent images of labeled cells in vmPFC using a Rolera EM-C² camera (QImaging) attached to a Nikon Eclipse E800 at 200 $\times$ magnification and iVision software for Macintosh v4.0.15 (BioVision). We quantified mRNA colabeling from two hemi-sections using ImageJ (2 images per rat) in a blind manner.

Fos immunofluorescence and CTb conjugate colabeling

We washed coronal brain sections (40 μ m) in PBS, blocked with 3% NGS in PBS-Tx, and incubated 24 h at 4°C with anti-Fos antibody (1:5000 dilution; Cell Signaling Technology, catalog #5348; RRID:AB_10557109) in blocking solution. We then washed sections in PBS, and incubated them with anti-rabbit 647 (1:500; ThermoFisher Scientific, catalog #A32733; RRID:AB_2633282) for 3 h. We washed sections in PBS and mounted onto chromalum-gelatin-coated slides before coverslipping with MOWIOL. We digitally captured fluorescent images of IR cells, CTb-488 and CTb-594 (50 μ g/ μ l, ThermoFisher, C34775, C34777) in vmPFC and Nac using a Rolera EM-C² camera (QImaging) attached to a Nikon Eclipse E800 at 200 $\times$ magnification and iVision software for Macintosh v4.0.15 (BioVision). Observers (B.L.W., L Kane) blind to the test conditions (Pearson's correlate = 0.91) automatically counted labeled nuclei from two sections (bilateral) per rat (3 images per rat). We averaged the counts so that each rat was an n of 1 for each brain area. We calculated the percentage overlap between Fos-positive and CTb labeled nuclei.

X-gal histochemistry for β -gal visualization in Fos-lacZ rats

We used the X-gal assay based on previous studies (Koya et al., 2009a,b; Bossert et al., 2011; Warren et al., 2016). We collected coronal brain sections (40 μ m) of the mPFC and stored them in cryoprotectant (20% glycerol and 2% DMSO in 0.1 M PBS, pH 7.4) and stored them at -80°C until use. We washed sections in PBS and incubated them in reaction buffer (0.1 M X-gal, 100 mM sodium phosphate, 100 mM sodium chloride, 5 mM EGTA, 2 mM MgCl₂, 0.2% Triton X-100, 0.05 M K₃FeCN₆, and 0.05 M K₄FeCN₆) for 2 h at 37°C. We washed sections with PBS, and mounted onto chromalum-gelatin-coated slides. Once dry, we dehydrated the slides through a graded series of alcohol (30, 60, 90, 100, 100% ethanol) and cleared them with Citrasolv (Fisher Scientific) before coverslipping with Permount (Sigma-Aldrich). We digitally captured bright-field images of reactive cells in vmPFC using an EXi Aqua camera (QImaging) attached to a Zeiss Axioskop 2 microscope at 200 $\times$ magnification and iVision software for Macintosh v4.0.15 (BioVision). Observers (B.L.W., L Kane) blind to the test conditions (Pearson's correlate = 0.90) automatically counted labeled nuclei from two sections (bilateral) per rat (3 images per rat). We averaged the counts so that each rat was an n of 1 for each brain area.

Operant conditioning and extinction of lever pressing for cocaine

Each experiment consisted of three phases: operant cocaine self-administration (14 d), extinction training (0, 2, 7 d), and tests for drug self-administration or extinction recall (1 d; Warren et al., 2016). During the first 7 d of the self-administration phase, we trained the rats to lever press to receive infusions of cocaine (1 mg/kg/infusion) for 3 h/d with a maximum daily limit of 30 infusions. On Days 8–14, we halved the dose to 0.5 mg/kg/infusion with a daily limit of 60 infusions. If rats reached the daily limit, the lever was retracted and the house-light turned off, signaling the end of the session. Rats earned infusions on a fixed-ratio 1 and 20 s timeout reinforcement schedule. During the extinction phase, responses on the previously active lever had no programmed consequences during each 3 h/d session. During the "No Lever" period between the operant training and extinction training phases, we placed the rats in the operant conditioning chambers for 3 h/d without the retractable lever. Finally, we tested their lever pressing under extinction conditions for 30 min.

Experimental design and statistical analysis

Experiment 1: effect of recall of cocaine self-administration and extinction memory recall on Fos expression in vmPFC. The goal of Experiment 1 was to determine whether exposure to cues previously associated with cocaine self-administration and extinction would induce Fos expression in vmPFC. For this purpose, we first trained rats to self-administer cocaine for 14 d. We then exposed rats in the Test groups to a short 30 min non-reinforced test session in which the rats lever-pressed for the cue previously associated with cocaine delivery after 0, 2, and 7 extinction sessions. We reasoned that for rats in the no Prior-extinction group (0 sessions) the predominant memories recalled during the 30 min test session would be the cocaine self-administration memories, whereas for rats from the other two groups (2 or 7 extinction sessions), the predom-

Figure 1. Experiment 1: cocaine self-administration and extinction of cocaine seeking. **A**, Experimental timeline for extinction-induced Fos expression. We trained rats to self-administer cocaine for 14 d and then divided them into three groups with different number of extinction sessions: 0, 2, or 7 sessions. No Lever indicates the sessions when we confined rats to the self-administration boxes without access to the active lever. We assessed non-reinforced cocaine seeking for 30 min on the recall Test day, 24 h after the last Extinction or No-Lever session. **B**, Number of active lever presses and cocaine infusions earned during cocaine self-administration and extinction training. **C**, Number of lever presses on the active lever during the 30 min test session. Data are presented as mean $\pm$ SEM ($n = 8$ –9 per group). * $p < 0.05$ different from 0 Extinction session group. **D**, Representative images of Fos-IR nuclei in vmPFC captured at 200 $\times$ magnification. No-Test group rats were kept in home cage. Scale bar, 50 μ m. **E**, Number of Fos-IR nuclei per square millimeter in vmPFC. Data are presented as mean $\pm$ SEM ($n = 6$ –8 per group). * $p < 0.05$ different from the No-Test group for each extinction session. # $p < 0.05$ different from 0 and 7 Extinction session groups. **F**, Representative images of merged Fos + NeuN double-labeled nuclei. All neuronal nuclei were labeled red with an antibody against the general neuronal marker NeuN. Fos-expressing nuclei were labeled green. Scale bar, 100 μ m. **G**, Percentage of (Fos + NeuN double-labeled nuclei)/(NeuN-labeled nuclei) in vmPFC. Data are expressed as mean $\pm$ SEM ($n = 5$ –7 per group). * $p < 0.05$ different from the No-Test group for each Extinction session.

inant memories recalled would be the extinction memories. We left the rats in the No-Test group in their home cages on test day.

We assessed Fos expression in the vmPFC in 44 rats using a 3 $\times$ 2 between-subjects factorial experimental design: Prior-extinction ses-

7 d of extinction training. Therefore, we predicted that post-session injections of Daun02 would inactivate ensembles encoding extinction memories and impair extinction recall in the subsequent test session 2 d

sions (0, 2, 7 sessions) $\times$ Test (No-Test, Test). The experimental timeline is shown in Figure 1A. We trained rats for 14 d to lever press for cocaine infusions as described above. We divided these rats into three groups that received 0, 2, or 7 Prior-extinction sessions with all groups previously matched based on lever pressing during the last day of self-administration training. We exposed rats in the 0 d extinction group to seven daily of No-Lever sessions. Rats in the 2 d extinction group underwent five daily No-Lever sessions, followed by two daily extinction-training sessions. Rats in the 7 d extinction group underwent seven daily 3 h extinction-training sessions. The use of variable No-Lever sessions was to ensure that each group of rats was time-matched at the time of testing and had equal handling and exposure to the chambers. On test day, 24 h after the last extinction or No-Lever session, we divided each group of rats again into two groups that we placed into the test chamber under non-reinforced conditions for 30 min (Test) or left in their home cages (No-Test). In accordance with the Fos time course, 90 min after the start of the test session, we anesthetized rats with isoflurane for 90 s and perfused them with 100 ml of PBS, followed by 400 ml of 4% paraformaldehyde in 0.1 M PBS. We postfixed the brains for an additional 90 min in paraformaldehyde and incubated them in 30% sucrose in PBS at 4°C for 2–3 d. We froze brains in powdered dry ice and kept them at -80°C until sectioning. We used these brains for Fos immunohistochemistry, as described in the Fos and NeuN Immunofluorescence section.

We repeated Experiment 1 in a separate set of rats ($n = 32$) to determine the cellular phenotype of the Fos expressing neurons using the same 3 $\times$ 2 factorial experimental design with Prior-extinction sessions (0, 2, 7) $\times$ Test (No-Test, Test). We killed these rats 30 min after the beginning of the test session, froze their brains for 20 s in -40°C isopentane and stored the brains at -80°C until use. We collected coronal brain sections (16 μ m) directly onto Super Frost Plus slides (Fisher Scientific) and stored them at -80°C until use. We used these sections for RNAscope analysis, as described above.

Experiment 2: effect of Daun02 inactivation of neurons in vmPFC activated during recall of cocaine self-administration or extinction memories. We used the Daun02 inactivation procedure (Koya et al., 2009a; Bossert et al., 2011; Cruz et al., 2014) to determine whether distinct Fos-expressing neuronal ensembles in vmPFC play a causal role in the recall of extinction memories. The experimental procedure was similar to that of Experiment 1, with the exception that added a short 30 min “induction” session, identical to the subsequent test session, to recall the putative cocaine self-administration or extinction memories. We hypothesized that extinction memories are recalled during induction day in rats that previously experienced 2 or

later, resulting in increased cocaine seeking. Conversely, to the degree that Daun02 inactivation on induction day would inactivate cocaine self-administration memories in the 0 extinction sessions group, this would lead to decreased cocaine seeking on the subsequent test for cocaine seeking 2 d later.

We examined *Fos-lacZ* rats ($n = 67$) using 3×2 between-subjects factorial experimental design: Prior-extinction sessions (0, 2, 7 d) $\times$ Injection (vehicle, Daun02). The experimental timeline is shown in Figure 3A. We anesthetized *Fos-lacZ* rats (previously bred for 45–50 generations on a Sprague-Dawley background) and implanted permanent guide cannulas bilaterally into their vmPFC as described above. After a minimum of 7 d recovery, we trained rats using the same procedure described in Experiment 1. After training the rats to lever-press for cocaine for 14 d, we divided them into three groups that received 0, 2, or seven Prior-extinction sessions with both groups previously matched based on lever pressing from the last day of self-administration training. The rats then underwent either 7 d of extinction (7 d group) 5 d of No Lever, followed by two extinction sessions (2 d group), or 7 d of No Lever (0 d group). On induction day, 24 h later, we placed the rats into the test chamber under extinction conditions for 30 min to induce β -gal protein expression. We then returned rats to their home cages, and 90 min after the start of the induction session, we injected vehicle or Daun02 into the vmPFC as described. Two days later, we tested all rats for non-reinforced lever presses during a brief 30 min recall test. Following this test, we returned all rats to their home cages. Ninety minutes after the start of the session, we deeply anesthetized the rats with isoflurane and perfused them with PBS and 4% PFA. We removed their brains and processed them for X-gal staining, as described in the 'X-gal histochemistry for -gal visualization in *Fos-lacZ* rats' section.

Experiment 3: activation of vmPFC to NAC projections during cocaine self-administration and extinction recall. The goal of Experiment 3 was to determine whether neuronal ensembles associated with cocaine self-administration or extinction preferentially project to different NAC subregions. We hypothesized that neuronal ensembles associated with self-administration of cocaine project to the core, whereas those associated with extinction project to the shell (Peters et al., 2008a, 2009). We used an experimental procedure similar to Experiment 1, except that 24 h after the last cocaine self-administration session, we contralaterally infused CTb conjugates (CTb488 and CTb594) into the core and shell ($n = 21$). We then subjected the rats to either 0 or 7 d of extinction sessions as described. Based on the results of Experiment 2, we did not include a 2 d of extinction group. Twenty-four hours after the last extinction or No-Lever session, we placed rats into the test chambers for a 30 min test of recall under extinction conditions to induce Fos. Ninety minutes after the start of the session, we deeply anesthetized the rats with isoflurane and perfused them with PBS and 4% PFA. We removed their brains and processed them for immunofluorescence staining, as described in the Fos immunofluorescence and CTb conjugate colabeling section.

Experiment 4: disconnection of vmPFC neuronal ensembles to shell and core during recall of extinction memories. To test whether an interaction of activated vmPFC ensembles to shell are necessary for extinction of co-

Figure 2. Cell-type characterization of Fos-expressing cells in vmPFC on recall Test day following 0, 2, and 7 of Prior-extinction sessions. **A**, Representative images of RNAscope *in situ* hybridization for Fos and DAPI, Fos and *Slc17a7* (*Vglut1*), and Fos and *Slc32a1* (*Vgat*). Fos, *Vglut1*, and *Vgat* labeling are indicated by white, red, and green dots, respectively; DAPI is blue. Arrows indicate cells double-labeled with Fos (white dots) and either DAPI (blue) or *Vglut1* (red dots) or *Vgat* (green dots). Scale bar, 50 μ m. **B**, Number of Fos-labeled nuclei per square millimeter in vmPFC. Data are expressed as mean $\pm$ SEM ($n = 3–8$ per group). * $p < 0.05$ different from the No-Test group for each Extinction session. **C**, Percentage of (Fos + *Vglut1* double-labeled cells)/(Fos single-labeled cells) in vmPFC. **D**, Percentage of (Fos + *Vgat* double-labeled cells)/(Fos single-labeled cells) in vmPFC.

caine seeking, we performed a disconnection experiment using *Fos-LacZ* transgenic rats ($n = 26$). This experiment is a variation of an anatomical asymmetric disconnection procedure. In classical asymmetric disconnection procedure, unilateral manipulations are performed either in the same hemisphere (unilateral) or in opposite hemispheres (contralateral). The role of the pathway is inferred when behavior is disrupted by the contralateral, but not ipsilateral manipulation. Here, we injected Daun02 unilaterally into the vmPFC on induction day to inactivate neuronal ensembles associated with extinction of cocaine seeking, and then 2 d later on test day, we injected the Drd1 antagonist SCH at a dose of 1.0 μ g/0.5 μ l/side into the contralateral or ipsilateral shell of the same rats to inhibit neurons that would normally be activated during reactivation of the extinction memory. We used SCH, because blockade of Drd1 decreases NAc Fos expression induced by exposure to drug-associated cues and contexts (Ciccocioppo et al., 2001; Hamlin et al., 2007). The experiment was a single-factor between-subjects design with three groups: No-Daun02, ipsilateral, or contralateral. The No-Daun02 control received a vehicle injection into vmPFC on induction day and a unilateral SCH injection on test day. The experimental timeline is shown in Figure

5A. We trained rats to self-administer cocaine as described in Experiments 1–2 and extinguished the lever pressing for 7 d. On induction day, 24 h after the last extinction session, we exposed the rats to a brief (30 min) session of extinction recall. We waited 90 min for Fos and β -gal expression, and then infused either vehicle or Daun02 unilaterally into the vmPFC to inactivate neuronal ensembles associated with extinction recall. Two days later on test day, we infused SCH either ipsilaterally or contralaterally into the NAc shell to inhibit local neural activity. We then waited 10 min after SCH infusions before initiating a 30 min test of extinction recall.

We also performed an identical experiment targeting extinction-associated ensembles projecting to the NAc core to test whether activated projections from vmPFC to core are necessary for extinction of cocaine seeking using *Fos-LacZ* transgenic rats ($n = 24$). The goal of this anatomical control experiment was to rule out that the effect of SCH infusions into the shell was due to diffusion to the nearby core.

Experiment 5: disconnection of vmPFC neuronal ensembles to core during recall of cocaine self-administration memories. To test whether an interaction of activated vmPFC ensembles to core are necessary for recall of the memories of cocaine self-administration, we performed a disconnection experiment using *Fos-LacZ* transgenic rats ($n = 37$). This experiment is identical to Experiment 4 except that we performed the disconnection between vmPFC neuronal ensembles and NAc core in rats that did not undergo extinction training. The experiment was a single factor between-subjects design with three groups: No-Daun02, ipsilateral, or contralateral. The No-Daun02 control received a vehicle injection into vmPFC on induction day and a unilateral SCH injection into the core on test day. The experimental timeline is shown in Figure 6A. We trained rats to self-administer cocaine as described in Experiments 1–4. On induction day, 1 week after the last training session, we exposed the rats to a brief (30 min) session of cocaine seeking recall. We waited 90 min for Fos and β -gal expression, and then infused either vehicle or Daun02 unilaterally into the vmPFC (counterbalanced across left and right hemispheres) to inactivate neuronal ensembles associated with cocaine self-administration. Two days later on test day, we infused SCH either ipsilaterally or contralaterally into the NAc core to inhibit neurons that would normally be activated during reactivation of the cocaine self-administration memory. We then waited 10 min after SCH infusions before initiating a 30 min test of cocaine seeking.

Statistical analysis

We analyzed the behavioral and immunohistochemical data in Experiments 1 and 2 by one-way and two-way ANOVAs using Prism (GraphPad Software). For test-day behavior following Daun02 in Experiment 2, we used a two-way ANCOVA with active lever pressing on induction day as the covariate. For these experiments, we used Holm–Sidak for *post hoc* analyses when the prior AN(C)OVAs indicated significant main or interaction effects ($p < 0.05$). For the CTb tracing experiment in Experiment 3, we analyzed test-day lever presses with an unpaired *t* test. For the disconnection experiments in Experiments 4 and 5, we analyzed test-day lever presses by one-way ANOVAs. We used Dunnett's test for *post hoc* analysis when the ANOVA indicated significant main or interaction effects ($p < 0.05$).

Results

For all experiments in our study, we hypothesized that the cocaine “self-administration” memory was recalled on Test day following 0 Prior-extinction sessions, whereas the “extinction” memory was recalled on Test day following 2 and 7 Prior-extinction sessions.

Experiment 1: effect of recall of cocaine self-administration memories and extinction memories on Fos expression in vmPFC

The design of Experiment 1 is shown in Figure 1A. Figure 1B shows the mean $\pm$ SEM number of infusions earned and active lever presses during the self-administration and extinction phases. We performed two separate one-way ANOVAs with repeated measures to assess lever pressing across self-administration sessions and across

extinction sessions. The rats learned to lever press for cocaine during the self-administration phase ($F_{(13,520)} = 56.6, p < 0.0001$) and decreased lever pressing when infusions were withheld during the extinction phase ($F_{(6,78)} = 28.5, p < 0.0001$). These were the “Prior-extinction sessions” before test day.

On recall test day, non-reinforced lever pressing was assessed for 30 min (Fig. 1C). One-way ANOVA indicated a significant effect of Prior-extinction sessions ($F_{(2,23)} = 21.2, p < 0.0001$). *Post hoc* analysis indicated that either two or seven Prior-extinction sessions reduced active lever pressing on test day compared with rats that received zero extinction sessions ($p < 0.0001$).

We analyzed Fos protein expression in the vmPFC following the test recall sessions; sample images from vmPFC are shown in Figure 1D. Two-way ANOVA indicated a significant main effect of Test day exposure (No-Test, Test; $F_{(1,34)} = 34.42, p < 0.0001$), but not Prior-extinction sessions ($F_{(2,34)} = 2.9, p = 0.067$), and a significant interaction between the two factors ($F_{(2,34)} = 3.7, p = 0.034$). *Post hoc* analysis indicated that Fos expression was increased in Test rats compared with No-Test controls after 0 ($p = 0.047$), 2 ($p < 0.0001$), and 7 ($p = 0.047$) days of extinction (Fig. 1E). Subsequent double labeling for Fos and the general neuronal marker NeuN (Fig. 1F) indicated that Fos was expressed at a basal range of 0.7–0.8% and an induced range of 2–2.5% of neurons in vmPFC (Fig. 1G). Two-way ANOVA indicated a significant main effect of Test-day exposure (No-Test, Test; $F_{(1,31)} = 13.02, p = 0.0011$), but not Prior-extinction sessions ($F_{(2,31)} = 0.32, p = 0.73$), and no significant interaction between the two factors ($F_{(2,31)} = 0.24, p = 0.79$).

To determine the phenotype of Fos-expressing neurons in the vmPFC, we used *in situ* hybridization to label *Fos*, *Slc17a7* (*Vglut1*), and *Slc32a1* (*Vgat*) mRNA in a separate set of rats trained identically to the first experiment. *Vglut1* is a marker of glutamatergic pyramidal neurons in PFC (Bellocchio et al., 2000; Geisler et al., 2007), whereas *Vgat* is a marker of GABAergic interneurons (Meister, 2007). Sample images from vmPFC are shown in Figure 2A. Figure 2B shows *Fos* mRNA expression patterns 30 min after the start of the test. Two-way ANOVA indicated a significant main effect of Test-day exposure ($F_{(1,26)} = 51.4, p < 0.0001$), but not Prior-extinction sessions ($F_{(2,26)} = 0.2, p > 0.05$) and no significant interaction between the two factors ($F_{(2,26)} = 0.1, p > 0.05$). Approximately 90% of *Fos*-expressing neurons colabeled with *Vglut1* (Fig. 2C), whereas <10% colabeled with *Vgat* (Fig. 2D). The percentages of each cell type within the Fos-expressing ensembles were not significantly different among rats tested after 0, 2, or 7 previous days of extinction.

Experiment 2: effect of Daun02 inactivation of neurons in vmPFC activated during recall of cocaine self-administration or extinction memories

We used the Daun02 inactivation procedure (Koya et al., 2009a; Bossert et al., 2011; Cruz et al., 2014) to determine whether distinct Fos-expressing neuronal ensembles in vmPFC play a causal role in the recall of cocaine self-administration and extinction memories. For this purpose, we added a short 30 min induction session, identical to the subsequent test session 2 d later, to first recall the putative cocaine self-administration memories or extinction memories and then inactivate these memories using post-session Daun02 injections. We tested the hypothesis that inactivating extinction and cocaine self-administration memories during induction day would lead to increases and decreases in cocaine seeking during the test day, respectively.

The design of Experiment 2 is shown in Figure 3A. Figure 3B shows the mean $\pm$ SEM number of infusions earned and active

Figure 3. Experiment 2: Daun02 inactivation of activated vmPFC neurons during recall of cocaine self-administration or extinction of cocaine seeking. **A**, Experimental timeline for the Daun02 inactivation experiment. We trained rats to self-administer cocaine for 14 d. We divided them into three groups with varying number of extinction sessions: 0, 2, or 7 sessions. On induction day, we gave the rats a 30 min non-reinforced induction session before injecting them bilaterally with Daun02 or vehicle (VEH) 60 min later. On recall test day, 3 d later, we assessed cocaine seeking under extinction conditions. **B**, Number of active lever presses and cocaine infusions earned during self-administration and extinction training. **C**, Images showing placement of cannulas into vmPFC. **D**, Number of active lever presses over a 30 min session on induction day before Daun02 injections. **E**, Number of active lever presses over a 30 min extinction session on test day. Data are presented as mean $\pm$ SEM ($n = 9$ –13 per group). * $p < 0.05$ different from vehicle.

lever presses during the self-administration and extinction training phases. We performed two separate one-way ANOVAs with repeated measures to assess lever pressing across self-administration sessions and across extinction sessions. The rats learned to lever press for cocaine during the self-administration phase ($F_{(13,572)} = 42.2, p < 0.0001$) and decreased lever pressing when cocaine rewards were removed during the extinction phase

($F_{(6,114)} = 11.4, p < 0.0001$). Figure 3C shows cannula placement for these rats.

On induction day, non-reinforced lever pressing was assessed for 30 min before being placed in their home cages for 60 min and injected with Daun02 or vehicle into the vmPFC (Fig. 3D). We hypothesized that the cocaine self-administration memory is recalled following zero extinction sessions, while the extinction

memory is recalled following seven Prior-extinction sessions. The rats that have not experienced extinction maintain the lever-reward association from self-administration, whereas the rats that have experienced extinction are likely to recall the extinction memory during testing. On test day, we assessed the behavioral effects of prior Daun02 inactivation of the putative cocaine self-administration and extinction-associated neuronal ensembles (Fig. 3E).

Two-way ANOVA of induction-day lever presses before Daun02 injections indicated a significant main effect of Prior-extinction sessions ($F_{(2,61)} = 40.4, p < 0.0001$), but no difference between the injection groups that would later receive Daun02 or vehicle ($F_{(2,61)} = 0.62, p = 0.43$), nor a significant interaction between the two main factors ($F_{(2,61)} = 0.53, p = 0.59$). *Post hoc* analysis indicated the rats that received two and seven Prior-extinction sessions pressed the active lever less than rats that received zero extinction sessions ($p < 0.0001$), respectively.

We performed a two-way repeated measures ANCOVA of test day lever presses to determine whether Daun02 infusions influenced lever presses on test day across Drug (Daun02 or Vehicle) and Prior-extinction (0, 2, or 7 Prior-extinction sessions). We used lever pressing on induction day as the covariate to account for individual differences in non-reinforced lever pressing.

The two-way ANCOVA indicated no significant main effects of Daun02 injections ($F_{(1,60)} = 1.7, p = 0.679$) or Prior-extinction sessions ($F_{(2,60)} = 2.2, p = 0.124$), but there was a significant interaction between the two factors ($F_{(2,60)} = 19.7, p < 0.001$). *Post hoc* analysis indicated that Daun02 injections reduced active lever presses ~50% in rats with 0 Prior-extinction sessions ($p = 0.006$), but increased lever presses more than three-fold in rats that underwent 7 Prior-extinction sessions compared with vehicle-treated controls ($p = 0.007$). Following testing, we perfused rats and assessed β -gal expression in vmPFC. Two-way ANOVA indicated that β -gal expression did not vary as a function of the number of Prior-extinction sessions ($F_{(2,49)} = 2.1, p = 0.13$), Daun02 injection ($F_{(2,49)} = 0.01, p = 0.99$), nor was there a significant interaction between the two factors ($F_{(1,49)} = 0.17, p = 0.68$). In Vehicle-treated rats, 0 Prior-extinction sessions induced 373 ± 104 β -gal-positive nuclei/mm², 2 Prior-extinction sessions induced 442 ± 68 β -gal-positive nuclei/mm², and 7 Prior-extinction sessions induced 251 ± 68 β -gal-positive nuclei/mm². In Daun02-treated rats, 0 Prior-extinction sessions induced 395 ± 103 β -gal-positive nuclei/mm², 2 Prior-extinction sessions induced 466 ± 100 β -gal-positive nuclei/mm², and 7 Prior-extinction sessions induced 296 ± 60 β -gal-positive nuclei/mm².

Because Daun02 interacts exclusively with β -gal in Fos-expressing ensembles (Cruz et al., 2013), the results of bidirectional modulation of cocaine seeking after Daun02 injections suggest that distinct neuronal ensembles within the vmPFC encode cocaine self-administration and extinction memories.

Experiment 3: neuronal ensembles associated with cocaine self-administration or extinction project to different NAc subregions

To determine whether Fos-expressing self-administration or extinction ensembles in the vmPFC project preferentially to different NAc subregions, we used retrograde CTb conjugates to label these projections.

Figure 4A shows CTb infusion placements in the NAc core and shell subregions. CTb spread for shell injections were limited to the medial shell (but not to the dorsal horn), whereas CTb spread for core injections were limited to the dorsomedial core. Figure 4B shows representative CTb and Fos labeling in the

vmPFC following recall of the Extinction or Self-administration memories. Figure 4C shows the number of neurons within the vmPFC showing CTb labeling from the Core. Two-way ANOVA indicates no significant main effect of Test-day exposure ($F_{(1,17)} = 0.005, p = 0.94$), Prior-extinction sessions ($F_{(1,17)} = 0.005, p = 0.94$) and no significant interaction between the two factors ($F_{(1,17)} = 0.95, p = 0.34$). Figure 4D shows the number of neurons within the vmPFC expressing CTb labeling from the Shell. Two-way ANOVA indicates no significant main effect of Test-day exposure ($F_{(1,17)} = 0.12, p = 0.73$) or Prior-extinction sessions ($F_{(1,18)} = 0.46, p = 0.51$) with no significant interaction between the two factors ($F_{(1,17)} = 3.8, p = 0.08$).

Figure 4E shows Fos immunoreactivity 90 min after the start of the test. Two-way ANOVA indicates a significant main effect of Test-day exposure ($F_{(1,17)} = 18.36, p = 0.0005$), but not of Prior-extinction sessions ($F_{(1,17)} = 0.02, p = 0.88$) and no significant interaction between the two factors ($F_{(1,17)} = 0.65, p = 0.43$). *Post hoc* analysis indicated that Test-day exposure increased Fos expression in both the 0 ($p = 0.029$) and 7 ($p = 0.036$) Prior days of extinction groups compared with No-Test controls. In Figure 4F, we compared percentage of Fos-positive cells in the vmPFC that projected to either NAc shell or NAc core. Two-way ANOVA indicated no significant main effects of projection (core, shell; $F_{(1,18)} = 0.04, p = 0.85$) or Prior-extinction sessions ($F_{(1,18)} = 0.3, p = 0.59$), but there was a significant interaction between the two factors ($F_{(1,18)} = 14.0, p = 0.001$). *Post hoc* analyses indicated that Fos-expressing neurons associated with 7 d of extinction had significantly less colabeling with CTb coming from the NAc core ($p = 0.03$), but significantly more colabeling with CTb coming from the NAc shell ($p = 0.01$) compared with rats in the 0 d of extinction group.

Together, the results of Experiments 1–3 suggest that the vmPFC is activated by recall of both self-administration and extinction memories. Furthermore, neuronal ensembles associated with recall of cocaine self-administration preferentially project to NAc core, whereas ensembles associated with recall of extinction of cocaine seeking preferentially project to NAc shell.

Experiment 4: effect of disconnecting activated vmPFC ensembles to NAc shell on extinction of cocaine seeking

The goal of Experiment 4 was to determine whether neuronal ensemble-specific interactions with the NAc shell, indirectly or directly, are necessary for extinction of cocaine seeking. Combined with the Daun02 inactivation results of Experiment 2, we hypothesized that the vmPFC neurons that are activated by extinction of cocaine seeking inhibit cocaine seeking through projections to the shell. From this, we predicted that contralateral inactivation of extinction-associated ensembles in vmPFC with Daun02 and ensemble nonspecific inhibition of NAc shell neurons with SCH would increase lever pressing relative to the ipsilateral manipulation; this was the key comparison. The ipsilateral manipulation preserves one functional hemisphere to mediate behavior, whereas the contralateral manipulation interferes with the function of both hemispheres in behavior. We chose the Drd1 antagonist SCH to inhibit neurons in the NAc shell or core subregions because we wanted to independently confirm our CTb findings about whether the vmPFC ensembles have separate interactions with the different NAc subregions. The No-Daun02 control groups received vehicle infusions into the vmPFC and SCH injections into the NAc shell or core. The No-Daun02 control was used only to compare with the ipsilateral test group to assess whether unilateral injections of Daun02 have an effect on behavior.

Figure 4. Experiment 3: vmPFC neuronal ensembles associated with cocaine self-administration or extinction recall project to different subregions of the NAC. **A**, Placement of CTb infusions into nucleus accumbens. **B**, Representative images showing CTb-AlexaFluor 594 or CTb-AlexaFluor 488 colabeling with Fos expression in the vmPFC of rats that underwent 0 (Self-administration) or 7 (Extinction) Prior-extinction sessions. Core-projecting neurons are labeled red, whereas shell-projecting neurons are labeled green; the white arrows indicate neurons double-labeled for CTb and Fos. **C**, Number of core CTb-labeled nuclei per square millimeter in vmPFC. **D**, Number of shell CTb-labeled nuclei per square millimeter in vmPFC. **E**, Number of Fos-labeled nuclei per mm² in vmPFC. * $p < 0.05$ different from the No-Test group for each extinction session. **F**, Percentage of CTb-labeled cells that colabel with Fos in the vmPFC. Data are presented as mean $\pm$ SEM ($n = 5$ –6 per group). * $p < 0.05$ different from corresponding projection group in 0 Extinction session group.

The timeline of Experiment 4 is shown in Figure 5A. We performed two separate one-way ANOVAs with repeated measures to assess lever pressing across self-administration sessions and across extinction sessions. The rats learned to lever press for infusions of cocaine during the self-administration phase ($F_{(13,390)} = 18.2, p < 0.0001$) and decreased their lever presses during the 7 d of extinction

training ($F_{(6,168)} = 17.7, p < 0.0001$; Fig. 5B). Cannula placements in vmPFC and NAC Shell are shown in Figure 5C.

On Test day, we assessed the behavioral effects of inactivating extinction-associated neuronal ensembles in vmPFC with ipsilateral or contralateral inactivation of the shell. All rats received SCH injections into the NAC Shell. One-way ANOVA indicated

Figure 5. Experiment 4: Pharmacological disconnection of vmPFC neuronal ensembles and NAc shell and core during recall of extinction of cocaine seeking. **A**, We trained rats to self-administer cocaine for 14 d and subsequently exposed them to 7 extinction sessions. On induction day, we exposed them to a 30 min non-reinforced recall session and infused Daun02 or vehicle (VEH) into one hemisphere of the vmPFC 90 min after the start of the session. Two days later, on Test day, we infused SCH (10 min pretreatment time) into the ipsilateral or contralateral NAc shell and tested for extinction memory recall during a non-reinforced 30 min test session. **B**, Number of active lever presses and cocaine infusions earned during self-administration and extinction. **C**, Images showing placement of cannulas into vmPFC (left) and NAc shell (right). **D**, Number of active lever presses during the 30 min non-reinforced test session after unilateral SCH injections into the NAc shell. Data are presented as mean $\pm$ SEM ($n = 7$ – 10 per group). **E**, Number of active lever presses during the 30 min non-reinforced test session after unilateral SCH injections into the NAc core. Data are presented as mean $\pm$ SEM ($n = 8$ per group). * $p < 0.05$ different from ipsilateral group.

that active lever presses varied as a function of the Group condition ($F_{(2,23)} = 3.6$, $p = 0.04$). *Post hoc* analysis using Dunnett's test indicated that contralateral Daun02 + SCH injections increased active lever presses compared with ipsilateral injections ($p = 0.0505$; Fig. 5D). Lever pressing in the No-Daun02 control was not significantly different from the ipsilateral test group ($p = 0.987$). These data suggest that extinction-associated neuronal ensembles within vmPFC play a role in cocaine seeking via interactions with the NAc shell. The behavioral effect is specific to vmPFC-shell interactions, because a similar experiment targeting

extinction-ensemble projections to NAc core found no effect ($F_{(2,21)} = 0.21$, $p = 0.81$ for a main effect of Group; Fig. 5E).

Together, the results of Experiment 4 suggest that vmPFC neuronal ensembles that interact with the NAc Shell play a role in inhibition of lever pressing after extinction.

Experiment 5: disconnection of activated vmPFC ensembles to NAc core on cocaine seeking before extinction

The goal of Experiment 5 was to determine whether neuronal ensemble-specific interactions with the NAc core, directly or in-

Figure 6. Experiment 5: Pharmacological disconnection of vmPFC neuronal ensembles and NAc core during recall of cocaine self-administration. **A**, We trained rats to self-administer cocaine for 14 d followed by seven No-Lever sessions in the chamber. On induction day, we exposed them to a 30 min non-reinforced recall test session and infused Daun02 or vehicle (VEH) into the vmPFC of one hemisphere 90 min after the start of the session. Two days later, on Test day, we infused SCH into the NAc core of the ipsilateral or contralateral hemisphere and tested self-administration memory recall during a non-reinforced 30 min test session. We infused SCH-39166 unilaterally in all rats, either ipsilaterally or contralaterally to prior vehicle or Daun02 injections into the vmPFC. **B**, Number of active lever presses and cocaine infusions earned during self-administration. **C**, Images showing placement of cannulas into vmPFC (left) and NAc core (right). **D**, Number of active lever presses during the 30 min non-reinforced test session after unilateral SCH injections into the NAc shell. Data are presented as mean $\pm$ SEM ($n = 10$ –14 per group). * $p < 0.05$ different from ipsilateral.

directly, are necessary for cocaine seeking before extinction. Based on the results of Experiments 2 and 3, we hypothesized that cocaine self-administration-encoding vmPFC ensembles drive cocaine seeking through projections to the core. From this, we predicted that contralateral inactivation of self-administration-associated ensembles in vmPFC with Daun02 and ensemble non-specific inhibition of NAc core with SCH would decrease lever pressing relative to ipsilateral inactivation; this was the key comparison. The No-Daun02 control groups received vehicle infusions into the vmPFC and SCH injections into the NAc core. The

No-Daun02 control was used only to compare with the ipsilateral test group to assess whether unilateral injections of Daun02 and SCH have an effect on behavior.

The timeline of Experiment 5 is shown in Figure 6A. We performed two separate one-way ANOVAs with repeated measures to assess lever pressing across self-administration sessions and across extinction sessions. The rats learned to lever press for infusions of cocaine during the self-administration phase ($F_{(13,468)} = 11.4$, $p < 0.0001$; Fig. 6B). Cannula placements in vmPFC and NAc core are shown in Figure 6C.

On test day, we assessed the behavioral effects of inactivating cocaine-associated neuronal ensembles in vmPFC with ipsilateral or contralateral inactivation of the core. One-way ANOVA indicated that active lever presses varied as a function of the Group condition ($F_{(2,35)} = 3.6$, $p = 0.04$). *Post hoc* analysis using Dunnett's test indicated that contralateral Daun02 + SCH injections decreased active lever presses compared with ipsilateral Daun02 + SCH injections ($p = 0.022$; Fig. 6D). Lever pressing in the No-Daun02 control was not significantly different from the ipsilateral test group ($p = 0.603$). Together, the results of Experiment 5 suggest that vmPFC neuronal ensembles interact with the NAc core to drive cocaine seeking.

Discussion

We found that separate neuronal ensembles in vmPFC contribute to recall of cocaine self-administration and extinction memories. Self-administration and extinction recall induced similar levels of Fos expression in similar proportions of glutamate and GABAergic neurons in vmPFC. Fos-expressing neurons in the self-administration ensemble projected preferentially to the NAc core, whereas Fos-expressing neurons in the extinction ensemble projected preferentially to the NAc shell. Daun02 ablation of the self-administration ensemble (0 Prior-extinction sessions) reduced lever pressing in a test for cocaine seeking, whereas Daun02 ablation of the extinction ensemble (7 Prior-extinction sessions) increased lever pressing in the same test. Subsequent anatomical disconnection experiments indicated that the self-administration ensemble drives cocaine seeking via interactions with the NAc core, whereas the extinction ensemble inhibits cocaine seeking via interactions with the NAc shell. These data indicate that self-administration and extinction memories can be encoded within neuronal ensembles that intermingle within the same brain area but exert their behavioral effects through distinct outputs to different downstream brain regions.

Role of vmPFC in drug and non-drug reward seeking

The mPFC plays a critical role in learning and memory for both drug and natural rewards (Kalivas and Volkow, 2005; Phillips et al., 2008; Floresco, 2013). It has been hypothesized that the mPFC mediates reward seeking and inhibition of reward seeking after extinction via distinct subregions: the dmPFC drives reward seeking, whereas the vmPFC inhibits reward seeking (Peters et al., 2008b, 2009; Peters and De Vries, 2013). Inactivation experiments seem to support this hypothesis. Pharmacological or optogenetic inhibition of the dmPFC decreases food and drug seeking after extinction (McFarland and Kalivas, 2001; Capriles et al., 2003; Nair et al., 2011; Stefanik et al., 2013). Conversely, lesions or inactivation of vmPFC induce reinstatement and potentiate spontaneous recovery of drug or food rewards (Rhodes and Killcross, 2004, 2007; Peters et al., 2009). However, results from other studies (Jonkman et al., 2009; Koya et al., 2009b; Bossert et al., 2011, 2012; Moorman et al., 2015; McGlinchey et al., 2016), do not support the hypothesis that dmPFC and vmPFC have opposing roles in reward seeking.

We used the Daun02 inactivation procedure to selectively inactivate neuronal ensembles previously activated by recall of cocaine self-administration memories or extinction memories. Inactivation of neurons associated with recall of cocaine self-administration memories decreased lever pressing in a subsequent test for cocaine seeking. This finding is consistent with our previous findings showing that inactivation of neuronal ensembles associated with food or drug-paired cues and contexts decreased food or drug seeking, respectively (Bossert et al., 2011;

Cruz et al., 2014; Warren et al., 2016; Caprioli et al., 2017). Conversely, we showed that inactivation of neurons previously activated by recall of extinction of cocaine seeking increased lever pressing in the subsequent cocaine-seeking test. This finding is also consistent with our previous findings using food as a reward (Warren et al., 2016), suggesting that neuronal ensembles in vmPFC also mediate extinction learning. This finding adds to a growing literature suggesting that neuronal ensembles within vmPFC are capable of encoding different and even opposing learned behaviors (Suto et al., 2016; Warren et al., 2016). Further research would be necessary to determine whether similar self-administration and extinction ensembles were used for both food and cocaine seeking. It is possible that some Daun02 may have diffused to the dmPFC region; however, this does not affect the hypothesis that different intermingling ensembles contribute to self-administration versus extinction.

Our findings with Daun02 inactivation bring up an interesting question about the recall of self-administration versus extinction memories. The same set of cues and conditions are present during recall of both memories. However, it appears that the most recent operant learning experience with these cues determines which of these memories and their associated neuronal ensembles are activated. If the last lever pressing experience was with cocaine, then the self-administration memory and associated ensemble was recalled. If the last lever pressing experience was without reinforcer, then the extinction memory and associated ensemble was recalled. An interesting possibility is that activation of the extinction ensemble inhibits the self-administration ensemble, and that Daun02 ablation of the extinction ensembles removes inhibition of the self-administration ensemble. We speculate that the lack of behavioral effect after Daun02 ablation of neuronal ensembles following two Prior-extinction sessions was because at this time point, the same cues activated both self-administration and extinction memories and associated ensembles. However, the extinction ensemble was not fully formed after just 2 d of extinction and had not yet developed enough to repress the self-administration ensemble.

The composition of the current Fos-expressing neuronal ensembles is consistent with previous findings (Bossert et al., 2011; Fanous et al., 2012; Cruz et al., 2013, 2014, 2015). Only a small percentage (~2%) of neurons in vmPFC of our 2-dimensional histochemical slices were Fos-positive. This translates ~to 0.3% in a 3-dimensional volume, which is the more realistic percentage of neurons within these ensembles. Fos neurons are sparsely distributed and rarely in clusters. Thus, Fos-expressing ensembles cannot be considered merely smaller homotypic divisions, especially since these ensembles intermingle in the same brain area. Consistent with previous findings, we found that both pyramidal glutamatergic projection neurons and GABAergic neurons were activated during the test sessions using *in situ* hybridization. Ninety percent of the Fos-expressing neurons in vmPFC were *Vglut1* (glutamatergic) cells. Less than 10% of Fos-expressing neurons were GABAergic (*Vgat*). No differences in cell type were seen between activated neurons after 0, 2, or 7 Prior-extinction sessions. Therefore, comparable proportions of projection and interneurons are activated by the recall of cocaine self-administration memories and extinction memories. Because recall of cocaine self-administration and extinction of cocaine seeking involve heterogeneous populations of cells, this finding emphasizes that specific memories are encoded by precise patterns of neuronal activation that are generally cell-type independent (Bossert et al., 2011; Fanous et al., 2012; Cruz et al., 2014; Li et al., 2015; Rubio et al., 2015; Caprioli et al., 2017). These results

also emphasize that brain region or cell-type-specific manipulations do not mimic or model endogenous activity in learned behaviors.

Role of vmPFC outputs to NAc core and shell in drug seeking

We found that distinct neuronal ensembles for self-administration and extinction preferentially project to different NAc subregions. The self-administration ensemble preferentially projects to the NAc core, while the extinction ensemble preferentially projects to the NAc shell. These separate projections support the hypothesis that these two ensembles are largely non-overlapping and physically distinct from each other. Furthermore, when we contralaterally inactivated neuronal ensembles associated with recall of the extinction memory, lever pressing increased compared with ipsilateral controls. Conversely, when we inactivated neuronal ensembles associated with recall of the self-administration memory, lever pressing decreased compared with ipsilateral controls. These findings suggest that the self-administration and extinction ensembles exert their effects on behavior through distinct downstream connections.

An issue to consider, however, is that the results from our anatomical disconnection experiments showed relatively small effects. These relatively small effects may be due to projections coming from the still intact contralateral vmPFC extinction ensembles that may convey extinction-related information (James et al., 2018). These results mirror findings from chemogenetic activation studies suggesting opposing roles of the mPFC to NAc core and shell pathways in driving and inhibiting drug seeking after extinction (Augur et al., 2016), as well as supporting earlier studies showing that NAc neuronal ensembles mediate drug-related learning (Koya et al., 2009a; Cruz et al., 2014). On the other hand, McGlinchey et al. (2016) did not find that activated Fos-expressing vmPFC neurons project preferentially to NAc core versus shell during tests of reinstatement or extinction recall. The critical difference here may be that McGlinchey et al. (2016) used DAB amplification to identify Fos-expressing neurons, while we used immunofluorescence. DAB amplification is more sensitive than immunofluorescence and the latter method may have lowered the detection threshold to include weakly activated neurons, while the higher detection threshold with immunofluorescence likely identified only the most strongly activated neurons. It is possible that the additional neurons detected in the McGlinchey et al. (2016) study masked the subpopulation of neurons identified here.

An important consideration is that our vmPFC ensemble neurons are primarily glutamatergic pyramidal neurons with efferents to many brain areas in addition to the NAc (Chang et al., 1997; Marchant et al., 2016; Venniro et al., 2017; Bloodgood et al., 2018). The retrograde transport of CTb detects only the direct projections from vmPFC to NAc and does not assess the alternative efferents from the vmPFC ensemble neurons. In addition, the anatomical disconnection strategy does not require a direct monosynaptic projection from vmPFC to NAc to be effective. Thus, it is possible that the vmPFC ensembles control self-administration and extinction behaviors via indirect projections from vmPFC to NAc rather than via direct projections from the vmPFC ensembles to the NAc.

Concluding remarks

In conclusion, we showed causal roles for intermingling neuronal ensembles in vmPFC that contribute to self-administration and extinction of cocaine seeking through distinct interactions with

NAc core and shell. Our experiments support the hypothesis that neuronal ensembles mediating opposing behaviors can coexist and compete within one brain area. More generally, these data emphasize that when studying the neural circuitry of learned behaviors, we have to consider that the nodes of communication are not necessarily brain areas or cell types but likely intermingling neuronal ensembles made up of small numbers of sparsely distributed neurons of heterogeneous cell types.

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