Behavioral/Systems/Cognitive

Role of Orbitofrontal Cortex Neuronal Ensembles in the Expression of Incubation of Heroin Craving

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In humans, exposure to cues previously associated with heroin use often provokes relapse after prolonged withdrawal periods. In rats, cue-induced heroin seeking progressively increases after withdrawal (incubation of heroin craving). Here, we examined the role of orbitofrontal cortex (OFC) neuronal ensembles in the enhanced response to heroin cues after prolonged withdrawal or the expression of incubation of heroin craving. We trained rats to self-administer heroin (6 h/d for 10 d) and assessed cue-induced heroin seeking in extinction tests after 1 or 14 withdrawal days. Cue-induced heroin seeking increased from 1 to 14 d and was accompanied by increased Fos expression in ~12% of OFC neurons. Nonselective inactivation of OFC neurons with the GABA agonists baclofen + muscimol decreased cue-induced heroin seeking on withdrawal day 14 but not day 1. We then used the Daun02 inactivation procedure to assess a causal role of the minority of selectively activated Fos-expressing OFC neurons (that presumably form cue-encoding neuronal ensembles) in cue-induced heroin seeking after 14 withdrawal days. We trained *c-fos–lacZ* transgenic rats to self-administer heroin and 11 d later reexposed them to heroin-associated cues or novel cues for 15 min (induction day), followed by OFC Daun02 or vehicle injections 90 min later; we then tested the rats in extinction tests 3 d later. Daun02 selectively decreased cue-induced heroin seeking in rats previously reexposed to the heroin-associated cues on induction day but not in rats exposed previously to novel cues. Results suggest that heroin-cue-activated OFC neuronal ensembles contribute to the expression of incubation of heroin craving.

Introduction

In humans, relapse to heroin use can occur after prolonged abstinence periods and is often precipitated by exposure to cues previously associated with heroin (Wikler, 1973; O'Brien et al., 1992). In rats, cue-induced relapse to heroin seeking, as assessed in extinction and reinstatement procedures (Self and Nestler, 1998; Shaham et al., 2003), progressively increases after withdrawal from heroin (Shalev et al., 2001; Zhou et al., 2009) and other drugs (Neisewander et al., 2000; Bienkowski et al., 2004; Shepard et al., 2004; Abdolahi et al., 2010), a phenomenon termed incubation of drug craving (Grimm et al., 2001). Studies on neural mechanisms of incubation of drug craving have focused on cocaine, whereas mechanisms of incubation of heroin craving are unknown (Wolf and Ferrario, 2010; Pickens et al., 2011).

Human imaging studies demonstrated that heroin cues activate the orbitofrontal cortex (OFC) and that this activation correlates with drug craving (Sell et al., 2000; Langleben et al., 2008).

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In rats, cue-induced heroin seeking is associated with the induction of several immediate early genes in OFC (Koya et al., 2006; Kuntz et al., 2008b), including the neuronal activity marker *c-fos* (Morgan and Curran, 1991). Reversible inactivation of the OFC decreases discrete-cue- and context-induced reinstatement of cocaine seeking in rats (Fuchs et al., 2004; Lasseter et al., 2009); to date, a role of OFC in cue-induced heroin seeking has not been similarly investigated.

Not all OFC neurons may be equally involved in drug seeking. We recently used a context-induced reinstatement procedure (Crombag et al., 2008) and found that only a small number of sparsely distributed ventral medial prefrontal cortex (mPFC) neurons are activated after exposure to the heroin-associated context (Bossert et al., 2011). We then used the pharmacogenetic Daun02 inactivation procedure (Koya et al., 2009b) to demonstrate that selective inactivation of these context-activated neurons decreases context-induced reinstatement. We proposed that a small subset of ventral mPFC neurons forms neuronal ensembles that encode learned associations between heroin reward and heroin-associated contexts and that reactivation of these neuronal ensembles by drug-associated contexts during abstinence provokes relapse (Bossert et al., 2011).

Here, we used the Daun02 inactivation procedure to determine whether OFC neuronal ensembles contribute to enhanced cue-induced heroin seeking after prolonged withdrawal or the expression of incubation of heroin craving. We use the term "cues" to encompass all external cues, including contextual and discrete cues, associated with heroin self-administration. The term "neuronal ensembles" refers to a small subset of selectively

activated OFC neurons that encodes the learned associations between heroin reward and heroin cues that provoke drug seeking.

We first used Fos immunohistochemistry to assess activation of OFC neurons during cue-induced heroin seeking in extinction tests after 1 or 14 withdrawal days. We then assessed the effect of nonselective inactivation of OFC neurons with the GABA agonists baclofen + muscimol (McFarland and Kalivas, 2001) on cue-induced heroin seeking after 1 or 14 withdrawal days. Finally, we used the Daun02 inactivation procedure (Koya et al., 2009b) to selectively inactivate the small number of OFC neurons that express Fos in response to heroin cues to determine the role of cue-activated neuronal ensembles in enhanced cue-induced heroin seeking after 14 withdrawal days or the expression of incubation of heroin craving.

Materials and Methods

Animals. Male Sprague Dawley rats (350–400 g; total $n = 170$; Charles River Laboratories) were maintained under a reverse 12 h light/dark cycle, with food and water available *ad libitum* in home cages. Rats were allowed to habituate to their home cages for at least 7 d before surgery. On the day of surgery, rats were anesthetized with intraperitoneal injections of Equithesin (60 mg/kg sodium pentobarbital + 25 mg/kg chloral hydrate), and intravenous catheters were implanted as described previously (Bossert et al., 2009; Koya et al., 2009a; Lu et al., 2009). The opiate analgesic buprenorphine (0.1 mg/kg, s.c.; National Institute on Drug Abuse) was injected after surgery to decrease postsurgical pain. During recovery (7–10 d) and training, catheters were flushed every 24–48 h with sterile saline and the antibiotic gentamicin (4.25 mg/ml) to prevent infections. All procedures followed the guidelines outlined in the NIH *Guide for the Care and Use of Laboratory Animals*.

Heroin self-administration and extinction tests. For all experiments, rats underwent three experimental phases: heroin self-administration training, withdrawal period, and extinction tests for cue-induced heroin seeking. During the training phase, rats were trained to self-administer heroin once daily for 10 d while chronically housed in self-administration chambers that were located inside sound-attenuating cabinets and controlled by a MED Associates system. Each day, the rats were trained to self-administer heroin (0.075 mg/kg per infusion over 3.2 s) during six 1-h sessions separated by 5 min using a fixed ratio 1 with 20 s timeout reinforcement schedule (Airavaara et al., 2011). We find that including a 5 min off period between each 1 h session facilitates the acquisition of heroin self-administration training. At the beginning of the training sessions, catheters were connected via a modified cannula (Plastics One) to liquid swivels (Instech) with polyethylene 50 tubing. Sessions started at the onset of the dark cycle and began with insertion of the active lever and illumination of a red house light that remained on during the sessions. Active lever presses activated the infusion pump and produced a 5 s light cue. At the end of each 1 h session, the house light was turned off and the active lever was retracted. Lever presses were recorded from both the active lever and a nonretractable inactive lever that did not activate the infusion pump. Food and water was available *ad libitum* for all days of training. During the withdrawal phase, rats were removed from the self-administration chambers, kept in new home cages in the animal facility for 1 or 13–15 d (referred to as 14 d withdrawal), and handled three times per week.

On test days, some of the rats returned to the self-administration chambers for the extinction tests, while the other rats remained in their home cages and were not exposed to the extinction tests (no test). For the extinction test groups, 90 min extinction tests were conducted under the same experimental conditions as in training, except that active lever presses were not reinforced with heroin. During the extinction tests, the rats were exposed to the heroin context, and lever presses led to contingent presentations of the discrete light cue previously paired with heroin infusions during training; this discrete cue serves as a conditioned reinforcer during the tests. Tests started at the onset of the dark cycle and began with the insertion of the active lever and illumination of the red house light that remained on for the duration of the session. Active lever presses during testing resulted in contingent presentations of the light cue that was previously paired with heroin infusions during training but not heroin.

Experiment 1: cue-induced heroin seeking and OFC neuronal activation after withdrawal. A total of 44 rats were used. The rats in the extinction test groups were killed immediately after the 90 min extinction tests (performed after 1 or 14 withdrawal days, $n = 12$ and $n = 9$, respectively); rats in the no-test groups were killed after 1 or 14 withdrawal days ($n = 13$ and $n = 10$, respectively) at the same time as their respective extinction test groups. The rats were deeply anesthetized with isoflurane and perfused with 100 ml of PBS, followed by 400 ml of 4% paraformaldehyde. The brains were postfixed in paraformaldehyde for 90 min and transferred to 30% sucrose in PBS solution at 4°C for 2–3 d. Brains were frozen in powdered dry ice and kept at −80°C until sectioning.

Coronal sections were cut 40 μm thick between bregma +3.7 and +2.7 mm (Paxinos and Watson, 2005). Free-floating sections were washed three times 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 (sc-52; Santa Cruz Biotechnology) diluted 1:4000 in blocking solution. Sections were washed again with PBS and incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (1:400; Vector Laboratories) in PBS-Tx and 1% NGS. After washing in PBS, sections were incubated for 1 h in avidin–biotin–peroxidase complex (ABC Elite kit, PK-6100; Vector Laboratories) in PBS containing 0.5% Triton X-100. Finally, sections were washed in PBS and developed in 3,3′-diaminobenzidine for ∼3 min, transferred into PBS, and mounted onto chromalum–gelatin-coated slides. Once dry, the slides were dehydrated through a graded series of alcohol and cleared with Citrasolv (Thermo Fisher Scientific) before coverslipping with Permount (Sigma).

Bright-field images of Fos immunoreactivity in the OFC were captured using a CCD camera (Photometrics Coolsnap; Roper Scientific) and Qimaging Exi Aqua attached to a Carl Zeiss Axioskop 2 microscope. Images for counting labeled cells were captured at 50× magnification. Labeled cells from two to four hemispheres per rat were automatically counted using IPLab software for Macintosh, version 3.9.4 r5 (Scanalytics) and iVision for Macintosh, version 4.0.15 (BioVision). The sampled areas of the OFC from each hemisphere were ∼1.25 mm^2. Counts from all images from each rat were averaged, so that each rat was an *n* of 1.

Experiment 2: characterization of activated OFC neurons using fluorescent double-labeling immunohistochemistry. We used double-label fluorescent immunohistochemistry to characterize OFC neurons activated during the extinction tests. For these experiments, one group of seven rats underwent the same three experimental phases as those described above. All rats were perfused with paraformaldehyde immediately after the 90-min day 14 extinction test, and their brains were processed as described above and kept at −80°C until sectioning. Coronal sections were cut between bregma +3.7 and +2.7 mm (Paxinos and Watson, 2005).

We determined the proportion of all OFC neurons expressing Fos during extinction testing by double labeling for Fos and the neuron-specific protein NeuN (Mullen et al., 1992). We also assessed the phenotype of Fos-expressing neurons by double labeling for Fos and calcium/calmodulin-dependent protein kinase II (CaMKII), a marker of cortical glutamatergic pyramidal projection neurons (Liu and Jones, 1996), and glutamic acid decarboxylase 67 (GAD67), a marker of GABAergic neurons (Kaufman et al., 1986, 1991).

For Fos + CaMKII and Fos + GAD67 labeling, 30-μm-thick sections were first washed three times in PBS. Sections were incubated for 1 h in a blocking solution (5% NGS and 2.5% bovine serum albumin in PBS with 0.2% Triton X-100) and then incubated for 48 h with the anti-Fos primary antibody (rabbit, 1:400 dilution, sc-52; Santa Cruz Biotechnology) and either anti-CaMKII primary antibody (mouse, 1:100 dilution, MA1-048; Pierce Biotechnology) or anti-GAD67 primary antibody (mouse, 1:1000 dilution, MAB5406; Millipore) in blocking solution. After washing, sections were incubated for 2 h in blocking solution with secondary antibodies Alexa Fluor 488-labeled donkey anti-rabbit (1:200 dilution, A-21206; Invitrogen) and Alexa Fluor 568-labeled goat anti-mouse antibody (1:200 dilution, A-11004; Invitrogen). Sections were then washed,

mounted on chromalum–gelatin-coated slides, air dried, and coverslipped with Vectashield fluorescent mounting medium.

For Fos + NeuN labeling, 40 μm sections were washed three times in Tris-buffered saline (TBS) and permeabilized for 30 min in TBS with 0.2% Triton X-100. Sections were incubated in primary antibodies diluted in TBS with 0.2% Triton X-100 for 24 h on a shaker at 4°C. Primary antibodies were anti-Fos (rabbit, 1:400 dilution, sc-52; Santa Cruz Biotechnology) and biotinylated anti-NeuN (mouse, 1:2000 dilution). Sections were washed three times in TBS and incubated in secondary fluorescent labels diluted in TBS with 0.2% Triton X-100 for 2 h on a shaker at room temperature. Secondary antibodies were Alexa Fluor 488-labeled donkey anti-rabbit (1:200 dilution, A-10042; Invitrogen) and Alexa Fluor 350-conjugated streptavidin (1:2000 dilution, S-11249; Invitrogen) to label NeuN. After labeling, sections were washed in TBS, mounted onto chromalum–gelatin-coated slides, and coverslipped with Vectashield hard-set mounting media.

All fluorescent images of OFC (3.2 mm anterior to bregma) were captured using a CCD camera (Photometrics Coolsnap; Roper Scientific) attached to a Carl Zeiss Axioskop 2 microscope. Images for colocalization of Fos and NeuN were captured at 200× magnification, whereas colocalization of other proteins was captured at 400× magnification. The number of Fos-labeled and double-labeled cells from the OFC of one section per rat were counted using iVision for Macintosh, version 4.0.15 (BioVision).

Experiment 3: effect of pharmacological inactivation of OFC on cue-induced heroin seeking. Forty-eight Male Sprague Dawley rats were anesthetized as described above and implanted with permanent bilateral guide cannulae (23 gauge; Plastics One) aimed 1 mm above the OFC. The stereotaxic coordinates were +3.2 mm anteroposterior, ±2.6 mm mediolateral, −4.0 mm dorsoventral (10° angle). After cannulae implantation, rats received intravenous catheters and underwent recovery as described above. Rats then underwent heroin self-administration training as described above, followed by 1 or 14 withdrawal days. On withdrawal day 1 or 14, rats received bilateral injections of either muscimol (0.03 nmol/0.5 μl/side) + baclofen (0.3 nmol/0.5 μl/side) (Tocris Bioscience) dissolved in sterile saline or its vehicle 5 min before the 90 min extinction test. Doses were based on previous studies (McFarland and Kalivas, 2001; Koya et al., 2009a; Bossert et al., 2011).

Intracranial injections were administered using a syringe pump (Harvard Apparatus) and 10 μl Hamilton syringes attached via polyethylene-50 tubing to 30 gauge injectors (Plastics One). Baclofen + muscimol and its vehicle were injected over 1 min, and the injectors were left in place for 1 min. The number of rats per group included the following: vehicle day 1, $n = 7$; baclofen + muscimol day 14, $n = 9$; vehicle day 14, $n = 20$; and baclofen + muscimol day 14, $n = 12$).

To rule out the possibility that the effect of baclofen + muscimol on day 14 extinction responding (see Results) was attributable to motor deficits, seven rats were trained after completion of this experiment to lever press for 45 mg food pellets (catalog #1811155; Test Diet) under a fixed ratio 1 and 20 s timeout reinforcement schedule (Pickens et al., 2012) for 12 60-min sessions. Subsequently, we assessed the effect of vehicle or baclofen + muscimol injections into OFC on food-maintained responding in two different 60 min sessions separated by 48 h. Test sessions were counterbalanced, and rats were run in food self-administration sessions on the day between the tests.

Experiment 4: effect of Daun02 inactivation of OFC activated neurons on cue-induced heroin seeking. We used the Daun02 inactivation procedure (Koya et al., 2009b) to determine a functional role of OFC neurons that were activated during the extinction tests. The mechanism of Daun02 inactivation is depicted in Figure 5*A*. This procedure uses *c-fos–lacZ* transgenic rats that have a transgene containing a *c-fos* promoter that induces *lacZ* transcription and the protein product β-galactosidase (βgal) in activated neurons similar to that for *c-fos* transcription and Fos protein from the endogenous *c-fos* gene (Kasof et al., 1995). βgal metabolizes the prodrug Daun02 to its active form daunorubicin, which inactivates only the previously activated βgal-expressing neurons (Farquhar et al., 2002).

Forty-two transgenic *c-fos–lacZ* rats bred for 45–50 generations on a Sprague Dawley background were anesthetized and implanted with permanent bilateral guide cannulae (23 gauge; Plastics One) aimed 1 mm above the OFC. Daun02 was dissolved in 5% dimethylsulfoxide, 6% Tween 80, and 89% 10 mM PBS. Intracranial injections were administered as described above for baclofen + muscimol.

The experimental timeline is shown in Figure 4*A*. Briefly, four groups of rats ($n = 8$–13 per group) were trained first to self-administer heroin. During the withdrawal phase, rats were removed from the self-administration chambers and kept in new home cages in the animal facility for 11 d. On induction day (day 11), half of the rats were exposed to the heroin-paired training context and cues during a short 15 min extinction session (termed heroin context) to induce βgal, whereas the other half were exposed to a novel empty cage with clean bedding (termed novel context), and then all rats were returned to their home cages for 75 min. Ninety minutes after the start of the extinction test, the rats were bilaterally injected with Daun02 (2 μg/0.5 μl/side) or vehicle into the OFC and returned to their home cages. The Daun02 dose was based on our previous studies (Koya et al., 2009b; Bossert et al., 2011). Three days after the "induction" day, all rats underwent a 90 min drug-free extinction test as described above. At the end of the test session, the rats were anesthetized and perfused with paraformaldehyde as described above. Brains were removed for βgal and Fos immunohistochemistry. Rats in the vehicle and Daun02 groups were matched for their heroin intake and number of active lever presses during training and induction day (induction day extinction session: vehicle, 71.5 ± 21.3 active lever presses/15 min; Daun02, 60.0 ± 8.2; $p = 0.6$).

We used X-gal histochemistry to visualize βgal as an indicator of neuronal activation. Thirty-micrometer free-floating sections were washed three times for 10 min each in PBS and incubated in reaction buffer (2.4 mM X-gal, 100 mM sodium phosphate, 100 mM sodium chloride, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% Triton X-100, 5 mM K_3FeCN_6, and 5 mMK_4FeCN_6) for 4.5 h at 37°C with gentle shaking. Sections were washed three times for 10 min each in PBS, mounted onto chromalum–gelatin-coated slides, and air dried. Slides were dehydrated through a graded series of ethanol (30, 60, 90, 95, 100, 100% ethanol), cleared with Citrasolv, and coverslipped with Permount. Bright-field images of OFC βgal activity were captured using a Qimaging Exi Aqua camera attached to a Carl Zeiss Axioskop 2 microscope. Images for counting labeled cells were captured at 50× magnification. βgal-expressing nuclei, characterized by blue nuclear staining, were counted using iVision MacOS 10.62 (version 4.0.15). We counted labeled nuclei in sampling areas (~1.09 mm^2) around the OFC injection site (left and right hemispheres) in two coronal sections per rat. Counts from all images from each rat were averaged, so that each rat was represented as a single observation in the statistical analyses and figures.

To assess coexpression, we double-labeled βgal and Fos using fluorescent immunohistochemistry in four *c-fos–lacZ* rats. Experimental procedures were similar to those described for Fos + NeuN labeling, with the exception that 30 μm sections were used. Primary antibodies were rabbit anti-Fos primary antibody (1:500 dilution, sc-52; Santa Cruz Biotechnology) and goat anti-βgal primary antibody (1:1000 dilution, 4600-1409; Biogenesis), and secondary antibodies were Alexa Fluor 488-labeled donkey anti-rabbit and Alexa Fluor 568-labeled donkey anti-goat (both 1:200 dilution).

Statistical analyses. The behavioral and molecular data were analyzed by ANOVAs using the statistical program SPSS (GLM procedure); significant effects ($p < 0.05$) were followed by Fisher's PLSD *post hoc* tests. The dependent measures and the factors used in the statistical analyses are described in Results. Inactive lever responding, a potential measure of nonspecific activity and/or response generalization (Shalev et al., 2002), was used as a covariate in the statistical analysis, and the data for this measure are described in Table 1.

Results

The rats demonstrated reliable heroin self-administration in all experiments (Fig. 1*A*, final total $n = 141$). ANOVA indicated a main effect of training session ($F_{(1,117)} = 23.7, p < 0.01$) but not experiment ($p > 0.05$). Mean ± SEM 10-day daily heroin intake (infusions per 6 h) was 30.0 ± 2.8, 26.0 ± 4.5, 28.0 ± 1.8, and

Table 1. Mean ± SEM of inactive lever presses in experiments 1– 4

Experiment 1	Experiment 2	Experiment 3	Experiment 4
Day 1: 1.8 ± 1.0 Day 14: 5.9 ± 1.7	6.9 ± 0.6	Day 1 vehicle: 3.0 ± 2.2 Day 1 baclofen + muscimol: 1.0 ± 0.5 Day 14 vehicle: 12.0 ± 2.7 Day 14 baclofen + muscimol: 8.0 ± 2.2	Novel context + vehicle: 29 ± 8.5 Novel context + Daun02: 39 ± 15.1 Heroin context + vehicle: 22 ± 5.7 Heroin context + Daun02: 30 ± 6.0

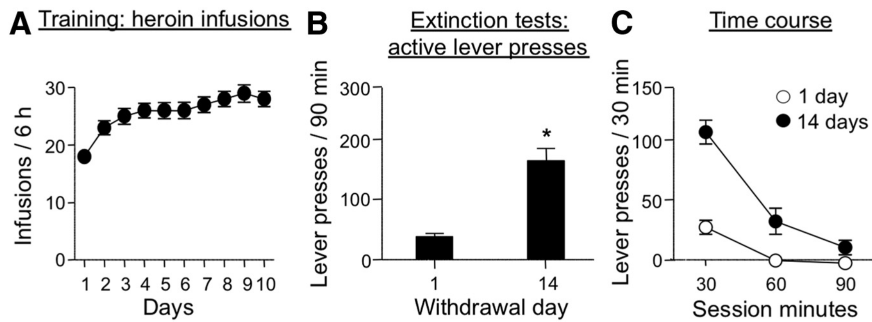

Figure 1. Rats reliably self-administer heroin and demonstrate time-dependent increases in cue-induced heroin seeking in the extinction tests after withdrawal (incubation of heroin craving). ***A***, Rats were trained to self-administer heroin over 10 d. Data are pooled across experiments 1– 4 and represent mean ± SEM heroin infusions per day (total $n = 141$). ***B***, ***C***, Cue-induced heroin seeking after 1 or 14 withdrawal days ($n = 9$–13 per group). During the extinction tests, active lever presses resulted in delivery of a light cue paired previously with heroin infusions but not heroin. Data are mean ± SEM active lever presses from experiment 1; $*p < 0.05$, different from day 1.

27.0 ± 2.3 for experiments 1 ($n = 44$), 2 ($n = 7$), 3 ($n = 48$), and 4 ($n = 42$), respectively. From all experiments, 29 rats were excluded: four for inadequate training (<10 infusions/d), 24 for misplaced cannulae (experiments 3 and 4; rostral to +4.5 mm or caudal to +2.5 mm from bregma; see also additional text below for experiment 3), and one because lever presses in the extinction test were >2 SDs from the group mean (experiment 3).

Cue-induced heroin seeking and OFC neuronal activation after withdrawal from heroin

Extinction tests

Cue-induced heroin seeking (operationally defined as the number of non-reinforced responses on the previously active lever during extinction tests) was significantly higher after 14 withdrawal days ($n = 9$) than after 1 d ($n = 13$; Fig. 1*B*), confirming previous reports on incubation of heroin craving (Shalev et al., 2001; Kuntz et al., 2008a; Airavaara et al., 2011). The ANOVA of total responding (90 min) on the active lever, which included the between-subjects factor of withdrawal day and the covariate of inactive lever responding, demonstrated a significant effect of withdrawal day ($F_{(1,19)} = 31.9$, $p < 0.01$). Time course (in 30 min intervals) of active lever presses during the extinction tests on withdrawal days 1 and 14 is shown in Figure 1*C*.

Fos

Exposure to the heroin context and cues during the extinction tests increased Fos expression in OFC, an effect that was somewhat more pronounced after 14 withdrawal days than after 1 d (Fig. 2*A*). The ANOVA, which included the between-subjects factors of group (day 1 extinction test, day 1 no test, day 14 extinction test, and day 14 no test), demonstrated a main effect of group ($F_{(1,39)} = 5.0$, $p < 0.01$). *Post hoc* analysis (Fisher's PLSD test) indicated that Fos immunoreactivity was significantly increased in the extinction test group versus the no-test group on withdrawal day 14 ($n = 10$; $p < 0.05$) but not day 1 ($n = 12$; $p > 0.05$).

Characterization of OFC activated neurons on withdrawal day 14

We used double-labeling immunohistochemistry to further characterize the OFC neurons in seven rats that were trained to self-administer heroin for 10 d and given a 90 min extinction test after 14 withdrawal days (mean ± SEM of active and inactive lever responding during the test was 130.0 ± 38.0 and 7.0 ± 0.06, respectively). Using NeuN and Fos colocalization, we found that 12.1 ± 1.4% of all OFC neurons in the sections were activated during the extinction test (Fig. 2*C*--*E*). Of these activated neurons, 55.2 ± 6.7% was colocalized with CaMKII and 10.2 ± 4.9% with GAD67.

Reversible inactivation of the majority of OFC neurons decreased cue-induced heroin seeking after prolonged but not early withdrawal

Injections of baclofen + muscimol into the OFC just before the extinction tests on withdrawal day 14 but not day 1 decreased cue-induced heroin seeking in these tests (Fig. 3*A*,*B*). The ANOVA of total (90 min) active lever presses, which included the between-subjects factors of drug condition (baclofen + muscimol, vehicle) and withdrawal day, and the covariate of inactive lever responding demonstrated a significant main effect of withdrawal day ($F_{(1,43)} = 15.0$, $p < 0.01$). Initial analysis did not reveal a main effect of drug condition ($p > 0.05$) or an interaction between withdrawal day and drug condition ($p > 0.05$) but subsequent one-way ANOVA across the four groups (day 1 vehicle, $n = 7$; day 14 vehicle, $n = 20$; day 1 baclofen + muscimol, $n = 9$; day 14 baclofen + muscimol, $n = 12$) demonstrated a significant effect of group ($F_{(3,43)} = 7.3$, $p < 0.01$; covariate of inactive lever responding). *Post hoc* analyses demonstrated significant differences between day 14 vehicle group versus day 1 vehicle ($p < 0.01$) and day 14 baclofen + muscimol ($p < 0.05$) groups. Time course of active lever presses in 30 min blocks is shown in Figure 3*B*.

Histological analysis indicated that baclofen + muscimol injections had no effect on extinction responding after 14 withdrawal days if the injector tip was located exclusively within the ventral OFC (medial to ±2.2 mm from the midline). Thus, cases in which injector tips were located in the ventral OFC ($n = 4$) were not included in the baclofen + muscimol analysis; mean ± SEM of active lever presses during the 90 min extinction test for these rats was 153.0 ± 36.0, which was higher than the mean of the day 14 vehicle group described in Figure 3 (108.0 ± 14.0). Five additional rats were excluded, because one injector was placed in the lateral OFC and another in the ventral OFC; the mean ± SEM of active lever press during the 90 min extinction test for these rats was 74.0 ± 45.0, which was similar to the mean for the day 14 baclofen + muscimol group described in Figure 3

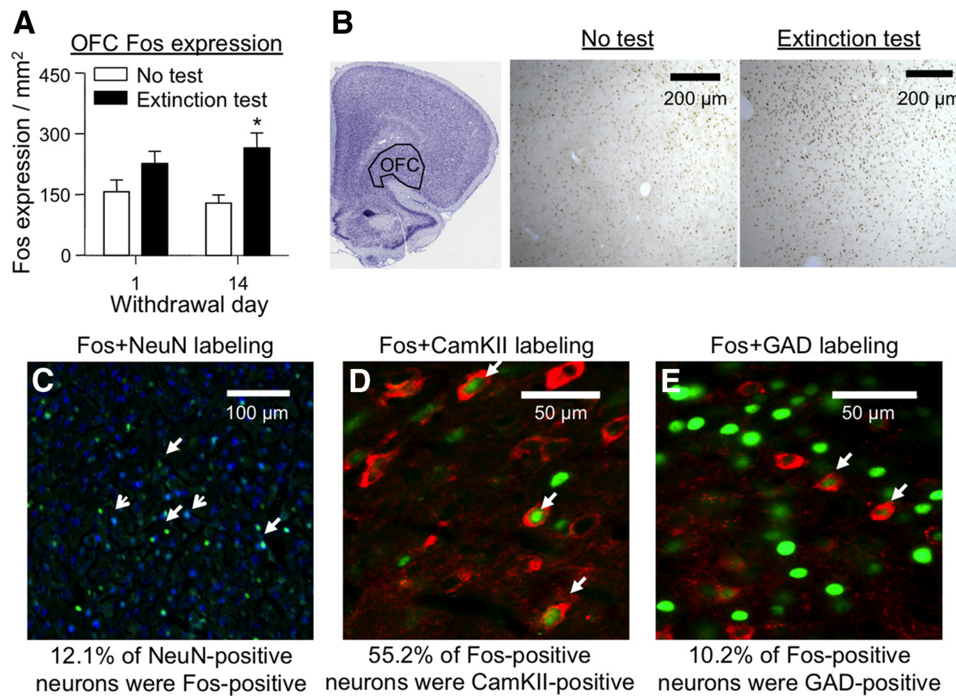

Figure 2. Effect of cue-induced heroin seeking on OFC Fos expression and characterization of activated OFC neurons. All rats were trained to self-administer heroin for 10 d, followed by 14 withdrawal days; rats were then tested (Extinction test) or not tested (No test) for cue-induced heroin seeking. ***A***, Fos expression in OFC in extinction test versus no-test rats; $*p < 0.01$, different from the no-test group; $n = 9$–13 per experimental condition. ***B***, Representative OFC images for extinction test and no-test rats on day 14. Scale bar, 200 μm. ***C–E***, Characterization of Fos-expressing neurons in OFC of extinction test rats after 14 withdrawal days. ***C***, Fos + NeuN double labeling in OFC. Scale bar, 100 μm. NeuN labeling in blue, Fos labeling in green, and double-labeled neurons in blue and green (indicated by white arrows). ***D***, Fos + CaMKII double labeling in OFC. Scale bar, 50 μm. CaMKII labeling in red, Fos labeling in green, and double-labeled neurons in red and green (indicated by white arrows). ***E***, Fos + GAD67 double labeling in OFC. Scale bar, 50 μm. GAD67 labeling in red, Fos labeling in green, and double-labeled neurons in red and green (indicated by white arrow).

(69.0 ± 14.0). Because extinction responding was not affected by cannula placement medial to ±2.2 mm in the day 14 vehicle group, rats in the vehicle groups who had at least one cannula lateral to ±2.2 mm were included in the behavioral analyses ($n = 5$ of 20 rats in the day 14 vehicle group).

Baclofen + muscimol OFC injections had no effect on high-rate food-reinforced responding: mean ± SEM number of active lever presses per 60 min was 166.0 ± 36.0 after vehicle injections and 180.0 ± 44.0 after baclofen + muscimol injections ($p > 0.1$). These data indicate that the effects of OFC baclofen + muscimol injections on cue-induced heroin seeking in the extinction test on withdrawal day 14 are not attributable to motor deficits.

Selective inactivation OFC neuronal ensembles decreased cue-induced heroin seeking

Based on our anatomical results above with baclofen + muscimol, we injected Daun02 and vehicle into the lateral OFC. Injections of Daun02 on induction day, 3 d before the withdrawal day 14 extinction test, decreased enhanced cue-induced heroin seeking (or the expression of incubation of heroin craving) and βgal expression

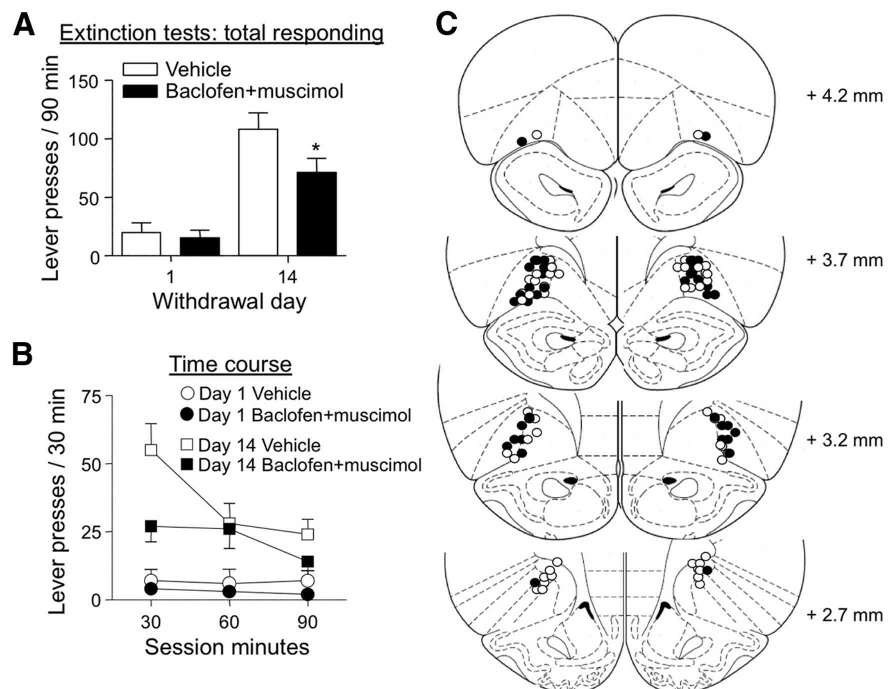

Figure 3. Lateral OFC baclofen + muscimol injections decreased incubated cue-induced heroin seeking after 14 withdrawal days. ***A***, Total lever responding during 90 min extinction test. Baclofen + muscimol (0.3 nmol and 0.03 nmol/0.5 μl/side) or vehicle was injected into the lateral OFC 5–10 min before 90 min extinction tests after 1 or 14 withdrawal days ($n = 7$–20 per experimental condition). ***B***, Time course of active lever presses in 30 min bins. ***C***, Cannulae placement: approximate placement of injector tips (Paxinos and Watson, 2005); white and black dots indicate locations of vehicle and baclofen + muscimol injections, respectively. $*p < 0.05$, different from vehicle.

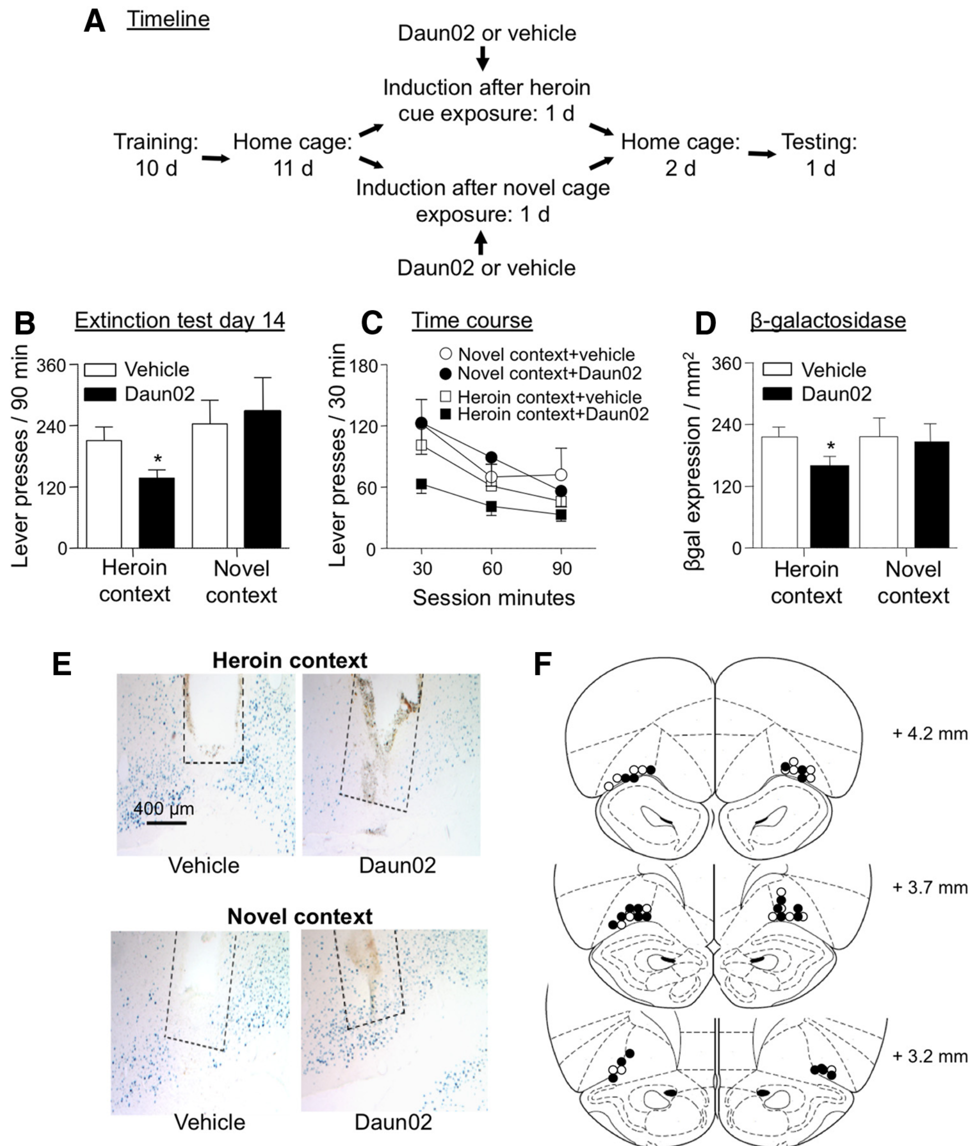

Figure 4. OFC Daun02 injections after exposure to the heroin cues in a short extinction test during induction day decreased subsequent incubated cue-induced heroin seeking and neuronal activation. ***A***, Timeline of experimental procedure: during induction day, Daun02 (2 μg/side) or vehicle was injected into the OFC 90 min after a short 15 min exposure to heroin cues in an extinction session or a novel context. ***B***, ***C***, Daun02 injections after exposure to the heroin cues during a short extinction session, but not to a novel environment, on induction day reduced cue-induced heroin seeking (active lever presses) and heroin cue-induced activation of OFC neurons (βgal expression) 3 days later (***D***) ($n = 8$–13 per group). Data are mean ± SEM total active lever presses. ***E***, Images depict βgal-labeled nuclei in OFC for each behavioral group; dotted lines indicate approximate area of injector tip. Scale bar, 400 μm. ***F***, Cannulae placement: approximate placement of injector tips; white and black dots indicate locations of vehicle and Daun02 injections, respectively. $^*p < 0.05$, different from vehicle.

when Daun02 was injected after brief exposure to a short extinction session (15 min) in the heroin self-administration context but not when Daun02 was injected after exposure to a novel context (Fig. 4*B*,*C*). We used a short 15 min extinction session on induction day to activate neurons sufficiently to induce βgal while minimizing a carryover effect of extinction responding from induction day (day 11) to day 14 extinction test. We kept the rats for an additional 75 min in clean home cages to ensure sufficient production of βgal in the Fos-activated neurons.

Extinction test

Because the novel context and heroin context groups had different extinction experiences before the 90 min extinction test on withdrawal day 14 [no extinction experience in the novel context vs brief (15 min) extinction experience in the heroin chambers on induction day], extinction test data from these groups were analyzed separately. The ANOVA of total (90 min) active lever presses for the novel context groups ($n = 9$, vehicle; $n = 8$, Daun02), which included the between-subjects factor of drug condition (Daun02, vehicle) and the covariate of inactive lever responding, indicated no significant group difference ($p > 0.1$). The ANOVA of total (90 min) active lever presses for the heroin context groups ($n = 12$, vehicle; $n = 13$, Daun02), which included the between-subjects factors of drug condition (Daun02, vehicle) and the covariates of inactive lever test responding and active lever responses on induction day, indicated a significant effect of drug condition ($F_{(1,25)} = 6.8$, $p < 0.05$). Time course of active lever responding in 30 min blocks is shown in Figure 4*C*.

βgal labeling

The ANOVA, which included the between-subjects factor of group (heroin context + vehicle, heroin context + Daun02,

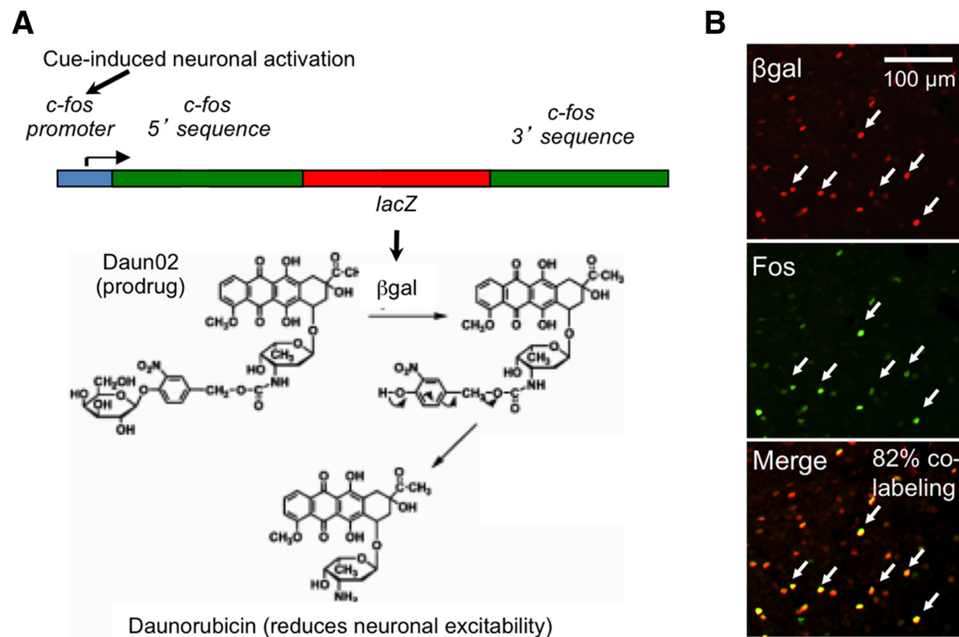

Figure 5. Daun02 mechanism and colocalization with Fos. ***A***, Schematic mechanism for Daun02 inactivation in *c-fos–lacZ* rats (adapted from Koya et al., 2009b). The *c-fos–lacZ* transgene contains a *c-fos* promoter that drives transcription of *lacZ* that encodes the bacterial protein βgal. βgal catalyzes conversion of the prodrug Daun02 into daunorubicin, which decreases cellular excitability. ***B***, Double labeling of βgal and Fos in OFC; βgal labeling in red, Fos labeling in green, and double-labeled neurons in red and green (indicated by white arrows). Scale bar, 400 μm.

novel context + vehicle, novel context + Daun02), demonstrated a significant effect of group ($F_{(3,37)} = 3.5$, $p < 0.05$; Fig. 4*D*). *Post hoc* analysis (Fisher's PLSD test) indicated that βgal labeling was significantly lower in the heroin context + Daun02 group than in the other three groups (p values <0.05).

Finally, we quantified the degree of colabeling for βgal and Fos. We found that 82 ± 1% of βgal-expressing neurons in OFC were also Fos labeled (Fig. 5*B*). This value is similar to what has been observed previously in the mPFC (Bossert et al., 2011).

Discussion

We studied the role of cue-activated OFC neuronal ensembles in the expression of incubation of heroin craving after withdrawal. Cue-induced heroin seeking in the extinction tests was higher after 14 withdrawal days than after 1 d, confirming previous reports on incubation of heroin craving (Shalev et al., 2001; Kuntz et al., 2008a). Cue-induced heroin seeking also increased Fos expression on withdrawal day 14 (but not day 1), similar to previous reports (Koya et al., 2006; Kuntz et al., 2008b). Double-labeled immunohistochemistry indicated that these Fos-positive neurons were both glutamatergic and GABAergic neurons and comprised ~12% of all OFC neurons in a 30-μm-thick slice, which translates to ~4% in a three-dimensional volume. General inactivation of the majority of neurons in the lateral OFC with baclofen + muscimol decreased incubated cue-induced heroin seeking on withdrawal day 14 but had no effect on non-incubated responding on day 1. Selective inactivation of only the Fos- and βgal-positive neurons that were activated previously during cue-induced heroin seeking decreased subsequent heroin seeking on withdrawal day 14. Based on these data, we suggest that OFC neuronal ensembles encode learned associations between environmental cues and heroin effects that contribute to the expression of incubation of cue-induced heroin craving after withdrawal.

Neuronal ensembles have been traditionally studied with *in vivo* electrophysiology using multielectrode recordings, which provide temporal information on neuronal activity patterns (i.e., "when" the neurons are activated during behavior) (Eichenbaum, 1993; Pennartz et al., 1994) or with histochemical detection of immediate-early genes such as *c-fos* or *arc* that provide information about the spatial expression patterns of neurons activated during behavior (i.e., "where" the neuronal ensembles are in the brain) (Guzowski et al., 1999, 2001). However, data from studies using these methods are correlational and do not establish causal roles of the activated neurons in behavior.

We developed the Daun02 inactivation procedure to study causal roles of putative neuronal ensembles in conditioned drug effects and relapse (Koya et al., 2009b). With this method, selective inactivation of behaviorally activated neurons is performed by injecting the prodrug Daun02 into specific brain areas of *c-fos–lacZ* transgenic rats (Kasof et al., 1996) that express βgal (the *lacZ* gene protein product) in neurons strongly activated during behavior (Koya et al., 2009b). βgal within the behaviorally activated neurons converts Daun02 into daunorubicin, which disrupts the normal function of these neurons (Koya et al., 2009b). We used the Daun02 procedure in two previous studies to demonstrate causal roles of putative neuronal ensembles in nucleus accumbens and ventral mPFC in context-specific locomotor sensitization (Koya et al., 2009b) and context-induced reinstatement of heroin seeking (Bossert et al., 2011).

In the current study, we found that cue-induced heroin seeking on test day was attenuated by previous Daun02 inactivation of only OFC neurons that were selectively activated by heroin cues and drug seeking to induce Fos and βgal on induction day (Fig. 4*B–D*). An unlikely interpretation of our data is that Daun02 injections on induction day decreased cue-induced heroin seeking during testing 3 d later by nonspecifically inactivating neurons regardless of their activation state or by inactivating a random set of Fos-activated neurons. We previously demonstrated that Daun02 injections had no effect on context-

dependent cocaine psychomotor sensitization or context-induced reinstatement of heroin seeking when injections were made after exposure to alternate environments associated with saline injections or extinction training (Koya et al., 2009b; Bossert et al., 2011). In the current study, Daun02 did not attenuate subsequent cue-induced heroin seeking when infused into OFC of rats exposed to a novel context on induction day; it is well established that novel context exposure causes strong Fos induction in cortical areas (Badiani et al., 1998; Badiani and Robinson, 2004; Paolone et al., 2007). Based on these data, we postulate that specific heroin-cue-activated neuronal ensembles in OFC mediate at least part of the learned associations between heroin effect and the environment of drug intake, which drives heroin seeking after prolonged abstinence.

One methodological consideration in our study is the anatomical specificity of baclofen + muscimol or Daun02 injections into the lateral OFC. At issue here is that drugs injected into specific brain sites can change behavior by diffusing away from the injection site into nearby sites (Wise and Hoffman, 1992). We cannot rule out a potential role of the nearby claustrum or the anterior insular cortex. However, our results demonstrate an anatomical-specific effect for the lateral versus ventral OFC, because ventral OFC baclofen + muscimol injections were ineffective (see Results). This pattern of results is similar to that of Fuchs et al. (2004) who reported that lateral but not ventral (termed medial in the authors' paper) OFC inactivation decreased cue-induced reinstatement of cocaine seeking. Future experiments are necessary to demonstrate conclusively that baclofen + muscimol inactivation of ventral OFC has no effect on heroin seeking under our experimental conditions. Finally, baclofen + muscimol or Daun02 inactivation of the lateral OFC only partially attenuated heroin-seeking behavior on test day (~35%). These data suggest that other brain areas also contribute to the expression of incubation of heroin craving.

Theoretical considerations

One issue within the neuronal ensemble framework is that we do not know what specific component of heroin-seeking behavior was disrupted by Daun02 inactivation of OFC neuronal ensembles. Lever presses during the short extinction sessions on induction day (Fig. 4) are controlled by distinct learning processes that are likely encoded by different neuronal ensembles: these different learning processes include operant responding previously reinforced by heroin, the conditioned reinforcing effects of the discrete cue previously paired with heroin injections (Feltenstein and See, 2008), and reexposure to contextual cues in the chambers that induce drug seeking (Crombag et al., 2008). Different neural circuitry has been shown to mediate contextual cue versus discrete cue-induced drug seeking (Bossert et al., 2007; Crombag et al., 2008). Future experiments are required to dissect which components of heroin seeking are mediated by OFC neuronal ensembles in our study.

Another issue is whether the OFC plays a unique role in incubation of cue-induced heroin craving versus a more general role in cue-induced drug seeking independent of the withdrawal period. Support for a unique role in incubation is the pattern of results for the Fos data (significant extinction test effect on withdrawal day 14 but not day 1; Fig. 2) and the baclofen + muscimol data (significant drug effect on extinction responding on withdrawal day 14 but not day 1; Fig. 3). However, we cannot rule out the latter possibility—a general role of lateral OFC in cue-induced drug seeking—because we did not observe statistically significant interactions of withdrawal day × extinction test condition (test, no test) for Fos induction or withdrawal day × drug condition (baclofen + muscimol, vehicle) for lever presses (data not shown). Additionally, the nonsignificant effect of baclofen + muscimol on day 1 may reflect a floor effect attributable to low responding.

Finally, we do not know which OFC-dependent psychological processes are disrupted by baclofen + muscimol or Daun02 inactivation to attenuate cue-induced heroin seeking. Imaging studies showed that cue-induced OFC activation correlates with subjective craving (Sell et al., 1999, 2000; Langleben et al., 2008), which supports the idea that lateral OFC neuronal ensembles in our study encode cue-induced motivational effects that promote drug seeking. This idea is consistent with evidence that OFC neurons respond to motivationally significant cues to mediate outcome-guided behavior (Schoenbaum and Eichenbaum, 1995; Gallagher et al., 1999; Noonan et al., 2010). Because the OFC has been implicated in behaviors reflecting current values of reward outcomes (Schoenbaum et al., 2009), the involvement of OFC here suggests that incubation of heroin craving could reflect a time-dependent growth of the current value of heroin.

Drug exposure also produces general impairments of OFC-dependent psychological processes (Lucantonio et al., 2012). Such impairments have been suggested to underlie impulsive decision-making and compulsive drug use despite adverse consequences (Jentsch and Taylor, 1999; Volkow and Fowler, 2000; Schoenbaum and Shaham, 2008). Cocaine exposure mimics the deleterious effects of OFC lesions on reversal learning (Jentsch et al., 2002; Schoenbaum et al., 2004) and pavlovian over-expectation (Lucantonio et al., 2012). These learning deficits, however, do not seem to underlie the current findings, because Daun02 or baclofen + muscimol decreased cue-induced heroin seeking at the beginning of the extinction test session before any opportunity for impaired learning to occur. Furthermore, evidence for OFC-dependent aberrant learning processes in addiction comes primarily from studies using psychostimulants (Jentsch and Taylor, 1999; Lucantonio et al., 2012), which often have different effects on brain function than opiates (Badiani et al., 2011). Indeed, morphine self-administration had no effect on pavlovian over-expectation (F. Lucantonio and G. Schoenbaum, unpublished observations), an OFC-dependent task impaired by cocaine exposure (Lucantonio et al., 2012).

Concluding remarks

We used classical behavioral, immunohistochemistry, and reversible inactivation procedures in combination with the pharmacogenetic Daun02-selective inactivation procedure to demonstrate a role of putative OFC neuronal ensembles in cue-induced heroin seeking after withdrawal. The present study and previous studies (Koya et al., 2009b; Bossert et al., 2011) are consistent with the "cell assembly" hypothesis proposed by Hebb (1949) that specific patterns of neurons activated by external and internal cues during learning form neuronal ensembles that encode and control learned behaviors. Similar *c-fos* or CREB promoter-based procedures have been used recently to demonstrate causal roles of neuronal ensembles in conditioned fear using transgenic mice (Han et al., 2009; Garner et al., 2012; Liu et al., 2012). In these studies, Fos-expressing neurons had to have high integrated levels of activity during the first 15–30 min of exposure to environmental cues to induce Fos. Thus, average levels of activation of these neurons had to have been synchronized over this 15–30 min period, which may be necessary for psychological processes that persist over these timescales. Schoenbaum,

Setlow, and colleagues have documented correlations between OFC neuronal activity and behavior using *in vivo* electrophysiology (Schoenbaum and Setlow, 2001; Schoenbaum et al., 2003). Future studies will have to examine the relationships between these electrophysiology data and activated Fos and βgal-expressing neurons in our study and whether the activated neurons demonstrate unique drug-induced synaptic alterations that are different from the majority of the surrounding non-activated neurons. Finally, our data extend recent reports on the important role of different subregions of the prefrontal cortex in relapse to heroin seeking (LaLumiere and Kalivas, 2008; Rogers et al., 2008; Van den Oever et al., 2008, 2010; Bossert et al., 2012).

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