

## **Supplemental Materials**

### **Supplemental Methods**

#### ***Subjects***

Adult male Long-Evans rats (Charles River Laboratories, Kingston, NY, USA) were group-housed (2-3/cage) on a 12 h/12 h light/dark cycle at the National Institute on Drug Abuse (NIDA) animal facilities in the Biomedical Research Center (Baltimore, MD, USA). Rats were implanted with catheters around 6 weeks of age (250-275 g) and underwent MRI scanning between 3 and 3.5 months of age. Standard rat chow and water were available *ad libitum* in home cages and throughout the self-administration experiments, but not during the other experimental procedures. The plurality of experimental procedures was performed entirely in the dark cycle, with few extending into the light cycle.

#### ***Drugs***

Heroin hydrochloride was obtained from NIDA and dissolved in 0.9% sterile saline for intravenous infusions (60 µg/kg/0.1 ml). Naloxone hydrochloride was obtained from Hospira (Lake Forest, IL, USA) and dissolved in 0.9% sterile saline for a subcutaneous injection in a volume of 1 ml/kg and concentration of 120 µg/kg.

#### ***Intravenous catheter surgery***

The rats were implanted with chronic indwelling intravenous catheters in the right external jugular vein under isoflurane anesthesia as previously described (9). The rats recovered for at least 5 days prior to behavioral testing.

#### ***Heroin self-administration***

*Apparatus.* Heroin self-administration (SA) sessions were conducted in standard operant boxes (30.5 cm × 24.1 cm × 21.0 cm; Med Associates, St. Albans, VT, USA) that were housed

inside light- and sound-attenuating chambers. At the start of each session, one active lever and one inactive lever extended into the box from the wall. Responses on the active lever resulted in 60 µg/kg/0.1 ml heroin infusions over 2.3 s. A stimulus light above the active lever turned on for 20 s at the onset of each infusion, during which time responses on the lever had no programmed consequences. Responses on the inactive were recorded but had no programmed consequences. The levers retracted at the end of the session.

*Training.* Intravenous self-administration procedures were conducted as previously reported (9). Briefly, the rats were trained to self-administer heroin under a fixed-ratio 1 schedule of reinforcement (i.e., each lever press resulted in one drug infusion) in 1 h sessions. Sessions occurred once per day during the dark phase, 3-5 times per week, for a total of 6-7 sessions. The number of heroin infusions per session was recorded.

*Escalation.* The rats self-administered heroin for 10 sessions. Short-access (ShA) rats underwent 1 h sessions. Long-access (LgA) rats underwent 12 h sessions (9). The rats were assigned to the ShA or LgA group, matched by responding during the training phase. Sessions occurred 3-4 times per week. The number of heroin infusions per session was recorded.

*Odor cue conditioning.* The rats underwent eight conditioning sessions. Thirty minutes into the self-administration session, the rats were injected with either saline (1 ml/kg, s.c.; i.e., 0 µg/kg naloxone) or naloxone (120 µg/kg, s.c.). The injection was paired with an odor cue (lemon- or vanilla-scented bedding) in the self-administration chamber for 30 min. During this time, access to the levers continued, and responses on the active lever resulted in infusions of heroin (60 µg/kg/0.1 ml). Following the 30 min treatment+odor cue pairing, LgA rats completed the remainder of their 12 h self-administration session with fresh, unscented bedding, whereas ShA rats were returned to their home cages. Treatment+odor cue pairings alternated each session

for a total of four sessions of naloxone+odor cue and four sessions of saline+odor cue.

Cue+treatment combinations were counterbalanced across subjects. Conditioning sessions occurred 3-4 times per week. The number of heroin infusions during the 30 min treatment+odor cue pairings was recorded. The data are presented as the mean number of heroin infusions for the four naloxone+odor cue pairings and four saline+odor cue pairings. Twenty-four hours after the last odor cue conditioning session, the rats continued with behavioral testing or were brought to the magnetic resonance scanner for brain imaging (see MRI procedures below).

*Cue-induced heroin self-administration.* Twenty-four hours after the last treatment+cue conditioning session, a second cohort of rats ( $n=13$  ShA;  $n=10$  LgA) that underwent training, escalation, and treatment+cue conditioning as described above were tested for the motivational effects of conditioned withdrawal. Thirty-minutes into the self-administration session, the rats were injected with saline and presented with either the naloxone-paired or the saline-paired odor cue for 30 min. The number of heroin infusions during the cue presentation was recorded. Following cue presentation, LgA rats completed the remainder of their 12 h SA session with fresh, unscented bedding, whereas ShA rats were returned to their home cages. The test was repeated 1 day later with a saline injection and presentation of the odor cue that was opposite to the previous day. The order of odor cue presentations was counterbalanced across subjects.

*Cue-induced reinstatement of lever pressing.* A third cohort of rats underwent training, escalation, and treatment+cue conditioning as described above. Following the last treatment+cue conditioning session, rats were returned to the vivarium for two weeks of forced heroin abstinence. On the test day, rats were brought to the self-administration chambers for a 1-d extinction and reinstatement test protocol. Responses on the active lever resulted in a 0.1 ml saline infusion (i.e., unreinforced) over 2.3 s., instead of a heroin infusion, for all sessions. Each

extinction session lasted one hour and there were 10 min between sessions (rats remained in the chambers the entire time). Rats underwent 6 consecutive extinction sessions; or continued until the extinction criterion was met (less than 12 active lever presses in one hour). Following the 6<sup>th</sup> extinction session or once the criterion was met, the next session was the reinstatement test. Thirty minutes into the reinstatement test, rats were presented with either the saline or naloxone-paired odor cue for 30 min ( $n=8$  ShA/saline cue;  $n=9$  ShA/naloxone cue;  $n=7$  LgA/saline cue;  $n=9$  LgA/naloxone cue). Number of lever presses on the active lever was recorded.

*Behavioral data analysis and statistics.* The behavioral data are presented as mean  $\pm$  standard error of the mean (SEM). Statistical significance was set at  $\alpha = 0.05$ . The self-administration data (number of heroin infusions) were analyzed using two-way analysis of variance (ANOVA), with heroin access (ShA versus LgA) as a between-subjects factor and session or cue (saline- versus naloxone-paired) as the within-subjects factor. *Post hoc* comparisons were conducted when appropriate and *P* values were corrected for multiple comparisons using the Bonferroni method. The statistical analyses for behavioral experiments were performed using GraphPad Prism 7 software. One LgA rat was excluded from the MRI study for failed catheter patency. One ShA/naloxone cue rat was excluded from the cue-induced reinstatement of lever pressing experiment because it was an outlier (number of lever presses was greater than 5 standard deviations from the mean).

### ***Magnetic resonance imaging procedures***

Twenty-four hours after the last heroin self-administration conditioning session, rats ( $n=11$  ShA;  $n=10$  LgA) were anesthetized with a combination of isoflurane (Henry Schein) and dexmedetomidine (Domitor; Webster Veterinary) as previously described (32, 33). Anesthesia

was initially induced with 2.5% isoflurane, followed by a bolus intraperitoneal injection of dexmedetomidine (0.02 mg/kg). Following secure placement in an MRI cradle with a bite bar, dexmedetomidine was continuously infused subcutaneously at a rate of 0.02 mg/kg/h, and isoflurane was gradually decreased to 0.75% to maintain a respiration rate of ~65 breaths/min during fMRI scanning. Core body temperature was maintained at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  with a circulating water blanket. Heart rate, respiration, blood oxygenation and body temperature were continuously monitored. fMRI data were collected 90 min after the first bolus injection of dexmedetomidine. Scanning consisted of three sequential 6-min resting BOLD scans (data not shown), followed by odor cue presentation scans. A second bolus of dexmedetomidine (0.02 mg/kg) was infused over 1 min (0.2 ml/min) between resting and odor scans. The isoflurane line to the MRI cradle was modified such that the rats were exposed to intermittent presentations of air bubbled through odor cues (lemon or vanilla scents that had previously been paired with either the saline or naloxone injection) while undergoing BOLD acquisition. During the odor presentations, the rats were exposed to four ON blocks of air bubbled through a single odorant, lasting 40 s, followed by 80 s OFF blocks. The first ON block was preceded by a 40 s baseline period. This was followed by exposure to the second scent using the same exposure parameters. Scent exposure order was counterbalanced across subjects. Respiration rate was recorded every 5 seconds during odor cue presentation scans. Average respiration rate was calculated for each of the following: odor scan baseline, block 1, block 2, block 3, and block 4.

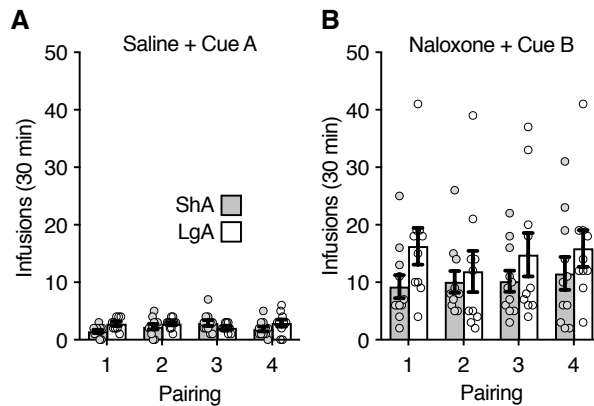
*Image Acquisition.* The MRI experiments were performed using a Bruker Biospin 9.4T scanner (Bruker Medizintechnik). High-resolution, T2-weighted anatomical images were acquired using a rapid acquisition with relaxation enhancement (RARE) sequence (repetition time [TR] = 2200, effective echo time [TE] = 37.33, 31 slices of 0.6 mm thickness). Functional

scans were acquired using a four-shot gradient-recalled echo-planar imaging (EPI) sequence. The scan parameters for the odor scans were the following: field of view (FOV) = 35 mm × 35 mm, matrix size = 80 × 80, TE = 10.5 ms, TR = 4.0 s, slice number = 25, slice thickness = 0.75 mm, to acquire a total of 130 time points.

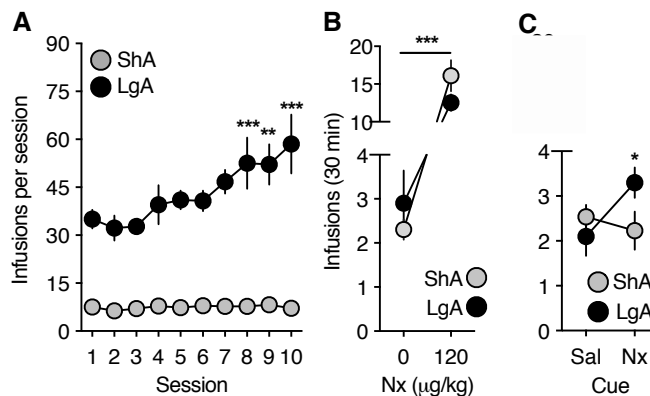
*Data preprocessing.* The data were preprocessed using AFNI (34). T2-weighted anatomical scans were skull stripped, aligned to a common space using a published method of anatomical tagging in rats (35), and co-registered to their corresponding functional scans. For the odor scans, data preprocessing included (i) first order detrending, (ii) spatial averaging of 0.35 mm, and (iii) calculating the percent signal change during the first two blocks (blocks 1 and 2) and last 2 blocks (blocks 3 and 4) of odor ON/OFF.

*fMRI data analysis and statistics.* The averaged signal from white matter and the ventricles was removed by multiple regression analysis. We conducted a whole brain 2 (heroin access: ShA versus LgA) × 2 (cue: saline- versus naloxone-paired odor) × 2 (cue presentation block: blocks 1 and 2 versus blocks 3 and 4) mixed-design ANCOVA of the percent BOLD signal change, using average respiration rate during the block as a covariate. Respiration rates fluctuated from baseline in response to cue presentation and were greater during blocks 3 and 4 than during blocks 1 and 2. Therefore, we included cue presentation block as a within-subjects factor and respiration rate as a covariate in our fMRI data analysis. No *a priori* regional hypotheses were tested. The 3dClustSim program in AFNI was used to estimate the probability of false positive clusters, which was used to estimate the cluster size threshold for a given voxel-wise  $p$  value threshold to correct for multiple comparisons. A cluster size of 13 with a corrected  $P < 0.01$  (uncorrected  $P < 0.01$ ) was considered significant. The relationship between behavior (withdrawal severity as the number of heroin infusions during naloxone conditioning) and BOLD

signal (in response to odor cues in each of the 19 ROIs that were extracted from the activation cluster, defined by a significant cue  $\times$  heroin access interaction) was evaluated using the Pearson correlation method. The  $P$  values were corrected for multiple comparisons using False Discovery Rate ( $q=0.05$ ).

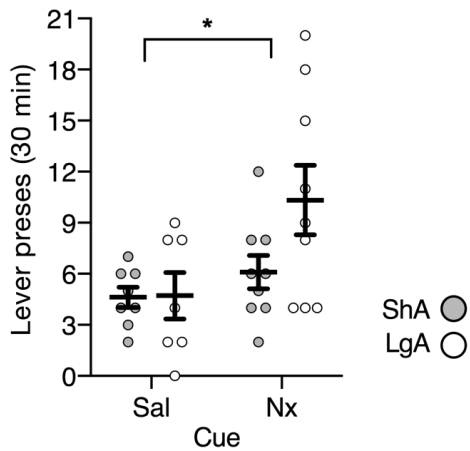


**Supplemental Figure S1. Heroin intake was stable over the course of saline and naloxone conditioning for rats in the MRI experiment.** Heroin intake in ShA ( $n=11$ ) and LgA ( $n=10$ ) rats during the four saline+cue pairings and four naloxone ( $120 \mu\text{g/kg}$ )+cue pairings. Dot plots represent individual values for each condition. For saline or naloxone treatments, there was no effect of pairing session (i.e., heroin intake in response to the treatments did not change over time). Data represent means  $\pm$  SEM.

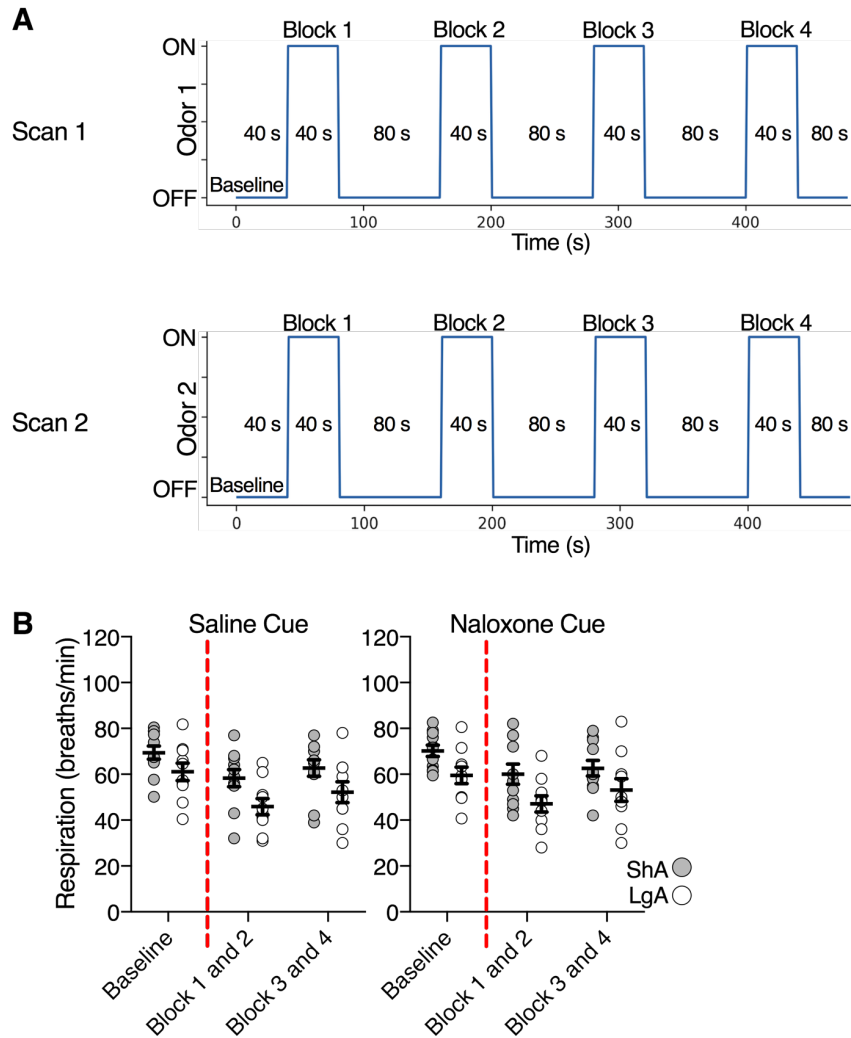


**Supplemental Figure S2. Naloxone-paired cue motivates heroin intake in LgA rats.** (A) LgA rats ( $n=10$ ) but not ShA rats ( $n=13$ ) in the Cue-induced Heroin Self-Administration experiment escalated heroin intake. A significant heroin access  $\times$  session interaction was observed ( $F_{9,189}=4.30$ ,  $P<0.0001$ , two-way repeated-measures ANOVA). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , significantly different from session 1 (corrected for multiple comparisons). (B) Heroin intake following saline ( $0 \mu\text{g/kg}$ ) or naloxone ( $120 \mu\text{g/kg}$ , Nx) treatment+cue pairings, presented as the average of the four pairings per treatment. A significant main effect of treatment ( $F_{1,21}=87.9$ ,  $P<0.0001$ ) was found (two-way repeated-measures ANOVA). (C) Heroin intake during cue only presentation (i.e., no naloxone treatment). A significant heroin access  $\times$  cue interaction was observed ( $F_{1,21}=6.2$ ,  $P=0.02$ ; two-way repeated-measures ANOVA). The naloxone-paired cue increased heroin intake relative to the saline-paired cue in LgA rats only. Data represent means  $\pm$  SEM.

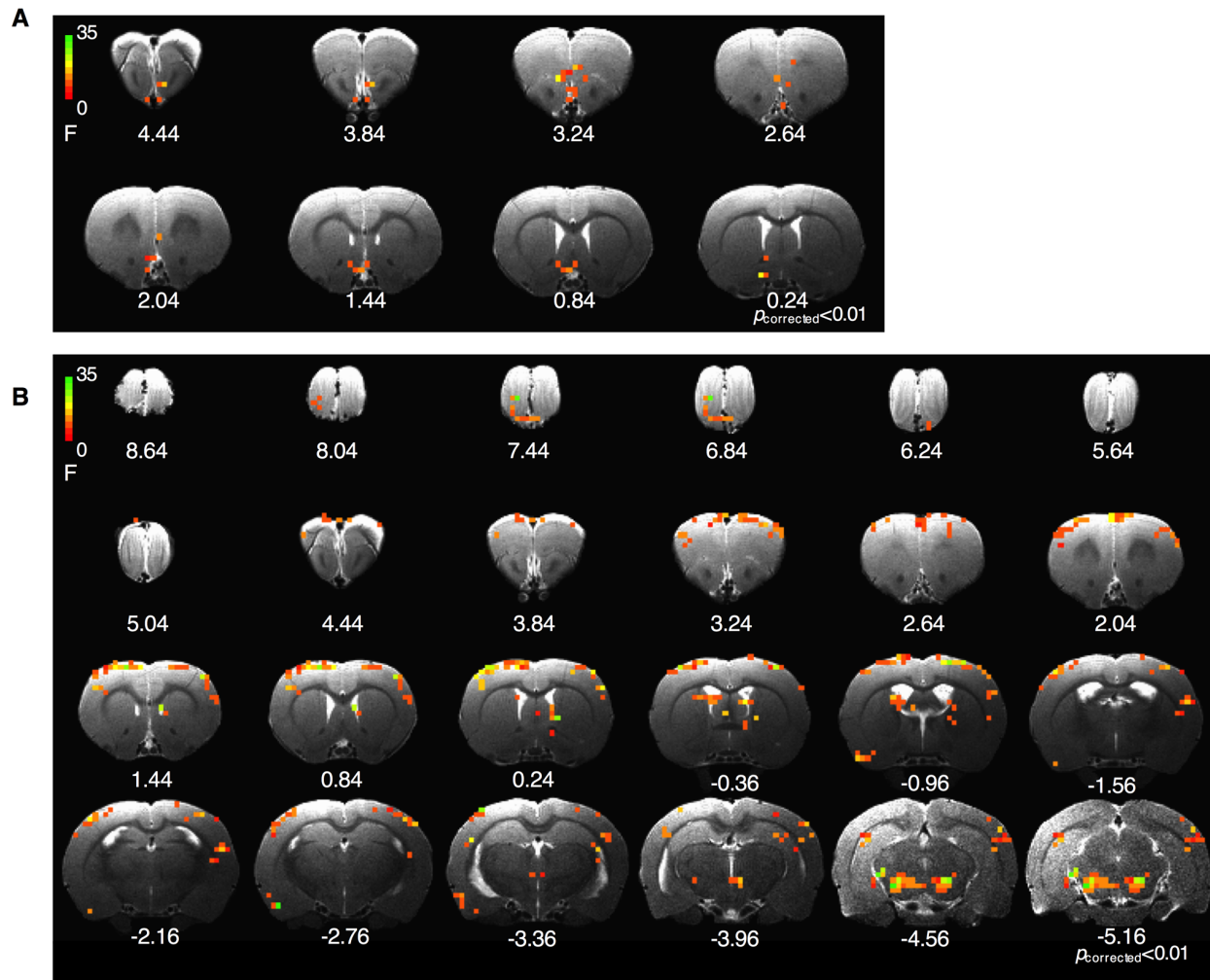




**Supplemental Figure S3. Naloxone-paired cue reinstates lever pressing following extinction.** Rats in the Cue-induced Reinstatement of Lever Pressing experiment underwent training, escalation, and treatment+cue conditioning. Following two weeks of forced heroin abstinence, lever pressing was extinguished in a 1 d protocol. Once the extinction criterion was met (less than 12 active lever presses in 1 h), rats were given a reinstatement test. After 30 min, the saline (Sal) or naloxone (Nx)-paired odor cue was presented for 30 min. A two-way ANOVA yielded a significant main effect of cue ( $F_{1,29}=6.38$ ,  $P=0.02$ ). Presentation of the naloxone-paired cue led to greater reinstatement of lever pressing in both self-administration groups compared with the saline-paired cue. Dot plot displays individual values for each condition. Data represent means  $\pm$  SEM.  $n=8$  for ShA/Sal cue;  $n=9$  for ShA/Nx cue;  $n=7$  for LgA/Sal cue;  $n=9$  for LgA/Nx cue.  $*P<0.05$ .



**Supplemental Figure S4. Experimental design for fMRI procedures.** (A) Experimental design for the olfactory cue presentation during fMRI. Cue and scent presentation order were counterbalanced across subjects. (B) Mean ( $\pm$  SEM) respiration rates for ShA and LgA rats during saline-paired (*left*) or naloxone-paired (*right*) cue presentations in the scanner. Dot plots display individual observations for each condition. Dotted lines (*red*) separate baseline respiration rates from respiration rates in response to cue presentations, shown as the average of presentation blocks 1 and 2, and blocks 3 and 4. A three-way ANOVA with heroin access (LgA versus ShA) as the between-subjects factor and cue (saline-paired versus naloxone-paired) and presentation block (blocks 1 and 2 versus blocks 3 and 4) as the within-subjects factors, yielded main effects of heroin access ( $F_{1,19}=5.0$ ;  $P<0.05$ ) and block ( $F_{1,19}=17.8$ ;  $P<0.001$ ). ShA rats had greater respiration rates during the cue presentations compared with LgA rats. Respiration rates were greater during blocks 3 and 4 compared with blocks 1 and 2. As respiration can affect  $p\text{CO}_2$ , which may influence vasodilation and the BOLD signal independent of neuronal activity, we included cue presentation block as a within-subjects factor and respiration rate as a covariate in the fMRI data analysis.  $n = 11$  for ShA and  $n = 10$  for LgA rats.



**Supplemental Figure S5. Effects of olfactory stimuli on percent change in BOLD signal from baseline.** The panels show statistical maps superimposed on anatomical coronal images from a representative subject. These same representative coronal images are used in **SF5A**, **SF5B**, and Figure 1C. Below each section is the anterior-posterior distance from bregma in millimeters. The data were analyzed using a 2 (scent: lemon versus vanilla)  $\times$  2 (heroin access: ShA versus LgA)  $\times$  2 (cue presentation block: blocks 1 and 2 versus blocks 3 and 4) ANCOVA, with respiration during the cue presentation as a covariate. Lemon and vanilla scents differentially activate the olfactory bulb and several cortical regions, indicating that the rats sensed olfactory stimuli in the lightly anesthetized state. **(A)** Main effect of scent (30 voxels,  $P < 0.01$ , corrected for multiple comparisons). **(B)** Heroin access  $\times$  cue interaction (337 voxels,  $P < 0.01$ , corrected for multiple comparisons). Color scale indicates  $F$  values.  $n = 11$  for ShA and  $n = 10$  for LgA rats.