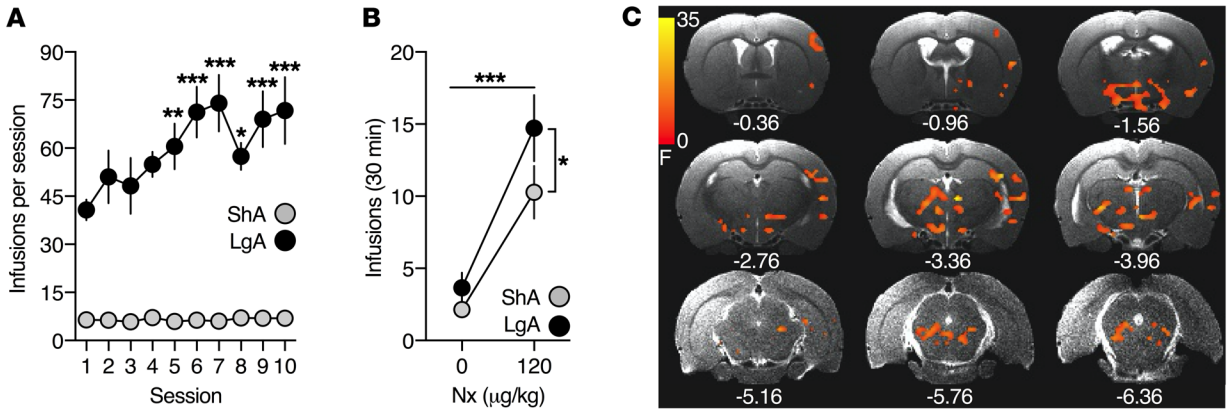

Figure 1. Conditioned heroin withdrawal engages negative emotional learning neurocircuits. (**A**) Heroin intake during ShA and LgA self-administration sessions. A significant heroin-access × session interaction was observed ($F_{9,171}$ = 4.25, P = 0.002; 2-way repeated-measures ANOVA). (**B**) Heroin intake following saline (0 μg/kg, s.c.) or naloxone (Nx) (120 μg/kg, s.c.) treatments during cue pairings, presented as the average of the 4 cue pairings per treatment. Significant main effects of treatment ($F_{1,19}$ = 35.5, P < 0.0001) and heroin access ($F_{1,19}$ = 4.215, P = 0.05) were found (2-way repeated-measures ANOVA). (**C**) Statistical map (F values) of the cue × heroin-access BOLD signal interaction following whole-brain 3-way ANCOVA, with respiration as the covariate (P < 0.01; 233 voxels, corrected for multiple comparisons). The upsampled (to anatomical images) statistical map is superimposed on anatomical coronal images from a representative subject. Below each section is the anterior-posterior distance from bregma (in mm). Data represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 (different from session 1 and corrected for multiple comparisons in **A**). n = 11 ShA rats; n = 10 LgA rats.

in LgA rats produced greater reinstatement of heroin seeking after extinction compared with presentation of an odor previously paired with saline (Supplemental Figure 3).

To explore the brain circuits that underlie conditioned withdrawal, we presented the olfactory cues alone to lightly anesthetized rats that were subjected to spontaneous heroin withdrawal during functional magnetic resonance imaging (fMRI) blood oxygen level–dependent (BOLD) signal acquisition 24 hours after their last cue conditioning self-administration session (Supplemental Figure 4). The light anesthetic regimen used is known to maintain neurovascular coupling and preserved odor-specific sensory processing (12, 13) (Supplemental Figures 4 and 5). The cues were presented in a counterbalanced, blocked design in sequential scans, and respiration rates fluctuated in response to cue presentation (Supplemental Figure 4).

Using the percentage change in the BOLD signal from baseline in response to cue-only presentation as the dependent measure and respiration as the covariate, a whole brain 2 (cues, saline versus naloxone paired) × 2 (heroin access, ShA versus LgA) × 2 (cue presentation block, blocks 1 and 2 versus blocks 3 and 4) ANCOVA yielded a significant cue × heroin-access interaction (Figure 1C and Supplemental Figure 4). The large cluster was segregated into 19 anatomically defined regions of interest (ROIs) using a spatially aligned rat atlas (14) (Table 1).

We then evaluated the relationship between activation in each ROI and the number of heroin infusions during naloxone plus cue conditioning sessions (shown in Figure 1B); the latter was used as an index of withdrawal severity. After correcting for multiple comparisons, BOLD activation was associated with withdrawal severity in 2 ROIs: (a) a hypothalamic cluster that made up the paraventricular nucleus of the hypothalamus (PVN) and ventromedial hypothalamus (VM) (Figure 2A) and (b) amygdala nuclei that made up the medial amygdala, central nucleus of the amygdala, and extended amygdala (Figure 2D). In both regions, the naloxone-paired cue increased the BOLD signal in LgA rats and decreased the BOLD signal in ShA rats (Figure 2, B and E). Heroin intake during naloxone plus cue conditioning (Figure 1B) was correlated with both the hypothalamic cluster (Figure 2C) and the amygdala (Figure 2F) BOLD response to the naloxone-paired cue in both self-administration groups. Greater withdrawal severity during conditioning was associated with greater activation in these regions during conditioned withdrawal.

Opioid withdrawal activates extrahypothalamic (e.g., extended amygdala) emotional systems in opioid-dependent rats (11, 15), and opioid dependence alters amygdala connectivity in humans (16). Opioid withdrawal also is known to potently activate the hypothalamic-pituitary-adrenal (HPA) arousal/stress axis in opioid-dependent humans and rats (17, 18). Activation of the PVN during opioid withdrawal and subsequent driving of the HPA axis may be an early dysregulation that is associated with excessive opioid intake. We hypothesize that dysregulation of the HPA axis and sensitization of the extended amygdala maintain negative emotional states via glucocorticoid signaling (19, 20). These results suggest that previously neutral stimuli gain motivational value when paired with opioid withdrawal, first by a hormonal stress-like response, which in turn activates extrahypothalamic brain stress systems in the extended amygdala, forming a pathway for negative emotional states that drive craving and relapse in humans (19–21).

Several other regions exhibited activation patterns that were similar to those seen in the hypothalamic cluster and amygdala (Table 1). Many of these regions have been implicated in emotional learning and are hypothesized to be dysregulated in addiction (2, 22, 23). These include the lateral hypothalamus, dorsomedial nucleus of the thalamus, ventrolateral thalamus, and dorsal striatum. Along with the amygdala, these regions are activated by heroin cues in individuals with OUD (22, 23). The extended amygdala promotes both positive and negative emotional states via its downstream connections to such areas as the lateral hypothalamus (24).