Orbitofrontal cortex and dorsal striatum functional connectivity predicts incubation of opioid craving after voluntary abstinence

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We recently introduced a rat model of incubation of opioid craving after voluntary abstinence induced by negative consequences of drug seeking. Here, we used resting-state functional MRI to determine whether longitudinal functional connectivity changes in orbitofrontal cortex (OFC) circuits predict incubation of opioid craving after voluntary abstinence. We trained rats to self-administer for 14 d either intravenous oxycodone or palatable food. After 3 d, we introduced an electric barrier for 12 d that caused cessation of reward self-administration. We tested the rats for oxycodone or food seeking under extinction conditions immediately after self-administration training (early abstinence) and after electric barrier exposure (late abstinence). We imaged their brains before self-administration and during early and late abstinence. We analyzed changes in OFC functional connectivity induced by reward self-administration and electric barrier–induced abstinence. Oxycodone seeking was greater during late than early abstinence (incubation of oxycodone craving). Oxycodone self-administration experience increased OFC functional connectivity with dorsal striatum and related circuits that was positively correlated with incubated oxycodone seeking. In contrast, electric barrier–induced abstinence decreased OFC functional connectivity with dorsal striatum and related circuits that was negatively correlated with incubated oxycodone seeking. Food seeking was greater during early than late abstinence (abatement of food craving). Food self-administration experience and electric barrier–induced abstinence decreased or maintained functional connectivity in these circuits that were not correlated with abated food seeking. Opposing functional connectivity changes in OFC with dorsal striatum and related circuits induced by opioid self-administration versus voluntary abstinence predicted individual differences in incubation of opioid craving.

opioids | incubation of craving | functional MRI | relapse | voluntary abstinence

High rates of relapse perpetuate opioid addiction and are a major obstacle in addressing the US opioid crisis (1, 2). In humans, relapse and craving are often triggered by reexposure to cues and contexts previously associated with drug use (3, 4). In rats with a history of opioid (heroin or oxycodone) self-administration, opioid seeking progressively increases or incubates during homecage forced abstinence (5, 6). However, a main limitation of most current animal models of incubation of drug craving and relapse is that prior to relapse testing, abstinence is experimenter-imposed or forced (6, 7). This contrasts with the human condition where abstinence is often self-imposed due to adverse consequences of drug seeking (8).

Based on these considerations, we recently introduced a rat model of incubation of oxycodone craving after "voluntary abstinence" induced by adverse consequences of drug seeking (9). The model is based on the electric barrier conflict model (10) that was more recently adapted to study relapse to drug seeking (11). In our modified model, we induce abstinence by introducing an electric barrier near the drug-paired lever that rats must cross to gain access to oxycodone. As shock intensity increases over days, rats decrease their oxycodone intake and eventually stop self-administering oxycodone. We then assess relapse to drug seeking during early and late abstinence in the absence of oxycodone or shock. We found that oxycodone seeking in the relapse tests is greater after 15 and 30 abstinence days than after 1 d, demonstrating incubation of oxycodone craving after electric barrier–induced abstinence (9). Unexpectedly, in both sexes, the incubation effect was stronger after electric barrier–induced abstinence than after homecage forced abstinence (9).

In the present study, we determined whether functional connectivity changes that develop during oxycodone self-administration and subsequent electric barrier–induced abstinence would predict individual differences in incubation of oxycodone craving. We measured functional connectivity of orbitofrontal cortex (OFC)–related circuits using resting-state functional MRI (fMRI) (12, 13), a noninvasive brain imaging

Significance

We recently introduced a rat model of incubation (time-dependent increase) of oxycodone craving after voluntary abstinence induced by negative consequences of drug seeking. We used resting-state functional MRI to determine whether longitudinal functional connectivity changes in orbitofrontal cortex (OFC) circuits would predict incubation of oxycodone seeking after cessation of drug self-administration by introducing an electric barrier near the oxycodone-paired lever. We report that opposing functional connectivity changes in OFC with dorsal striatum and related circuits during oxycodone self-administration versus voluntary abstinence predicted incubation of opioid seeking. OFC functional connectivity changes did not predict food seeking, which abated after voluntary abstinence. We propose that functional connectivity changes in OFC-related circuits at different addiction phases can be used to predict opioid relapse vulnerability.

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The authors declare no competing interest.

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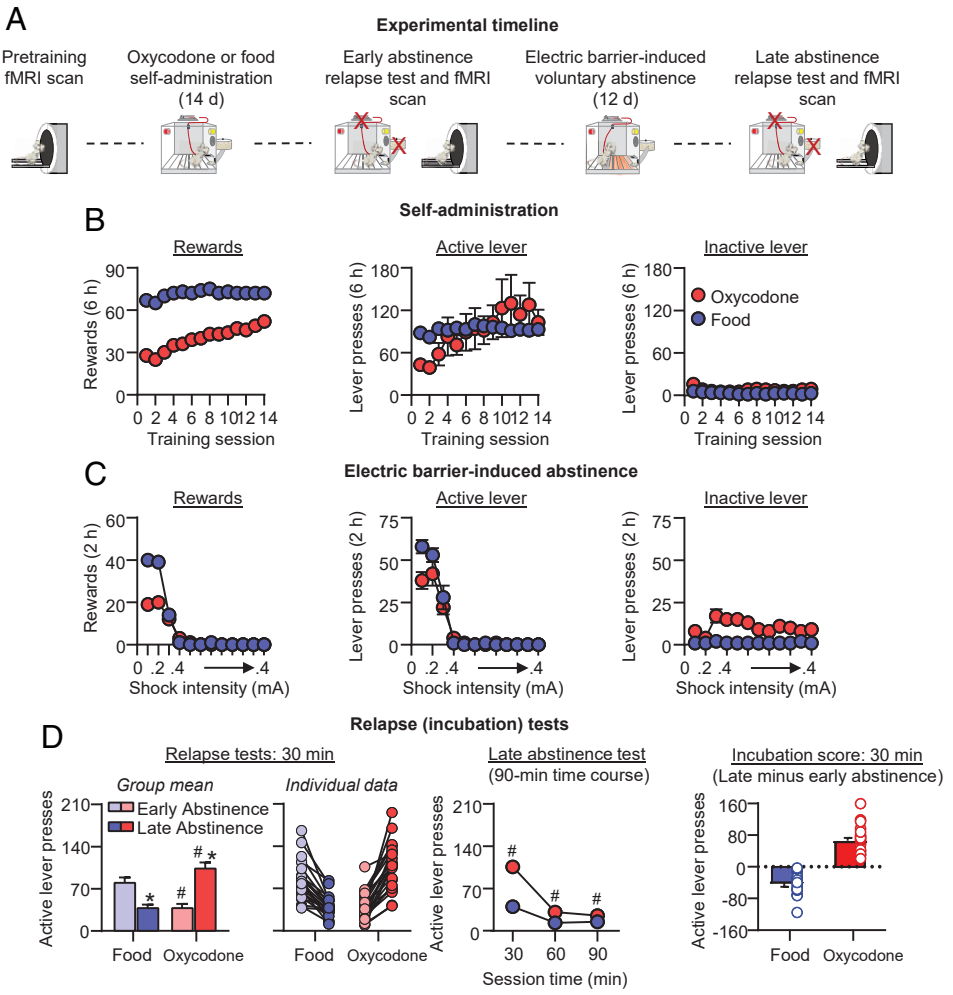

Fig. 1. Incubation of oxycodone versus food craving after electric barrier–induced abstinence. (*A*) Experimental timeline. (*B*) Food (5 pellets) and oxycodone (0.1 mg/kg/infusion) self-administration training: mean ± SEM number of rewards (food or oxycodone) and active and inactive lever presses during the 6-h sessions. (*C*) Electric barrier–induced abstinence: number of rewards and active and inactive lever presses during the 2-h sessions. (*D*) Relapse (incubation) tests: mean ± SEM number of active lever presses during the 30-min early abstinence (day 1) test and the first 30 min of the late abstinence test (day 15) test (*Left* and *Second to Left*), 30-min time course of active lever presses during the total 90-min session of late abstinence test (*Second to Right*), and mean ± SEM and individual data of the incubation score (active lever presses during the first 30 min of the late abstinence test minus active lever presses during the 30-min early abstinence test) (*Right*). During testing, active lever presses led to contingent presentation of the tone-light cue previously paired with food pellets or oxycodone infusions but not reward presentation (extinction conditions). *Different from early abstinence; #different from food group; $P < 0.05$. Data are mean ± SEM. Food group: $n = 22$; oxycodone group: $n = 22$.

technique that longitudinally measures synchronous activity between brain regions. Resting-state fMRI has been used to characterize the relationship between brain activity and behaviors in both humans (14, 15) and laboratory animals (16, 17). Our group developed a rat resting-state fMRI protocol (18) and used it to investigate neural mechanisms in rodent models of neurological and psychiatric disorders (17, 19). Here, we compared the fMRI measures of rats with a history of oxycodone self-administration to drug-naive rats trained to self-administer palatable food pellets that we use in our studies on relapse to drug seeking after food choice-induced abstinence (20).

We focused on the OFC as the seed region for assessing longitudinal functional connectivity changes because previous human imaging studies reported that craving induced by heroin cues is associated with increased OFC activity (21, 22). In rats, cue-induced reinstatement of heroin seeking after extinction (23), incubation of heroin seeking after forced abstinence (24, 25), and relapse to fentanyl seeking after food choice-induced abstinence (26) are associated with increased Fos expression and other immediate early genes in OFC. Additionally, reversible inactivation of OFC decreases incubated heroin and oxycodone seeking after forced abstinence (25, 27) and relapse to fentanyl seeking after food choice-induced abstinence (26). Finally, heroin self-administration causes long-lasting impairments of OFC-mediated decision-making processes (28).

Results

Overview of the Study Methods. The experiment consisted of the following general events (Fig. 1*A*): pretraining fMRI scan; 14 d of oxycodone or food self-administration training; early abstinence relapse test and fMRI scan; 12 d of electric barrier–induced abstinence; and late abstinence relapse test and fMRI scan. The goal of our study was to determine whether longitudinal OFC-related functional connectivity changes induced by oxycodone (or food) self-administration and subsequently electric barrier–induced abstinence would predict incubation of oxycodone (or food) seeking. We describe the flowchart of the image analysis strategy in *SI Appendix*, Fig. S1. In the following sections, we first describe the behavioral results (Fig. 1 and *SI Appendix*, Table S1) and then the imaging results (Figs. 2–8 and Table 1 and *SI Appendix*, Figs. S2–S7 and Table S2). We provide the dataset of the study in Dataset S1.

Behavioral results. *Self-administration training.* We trained rats to self-administer oxycodone (0.1 mg/kg/infusion) or food (5 pellets/reward delivery) for 14 d (Fig. 1*B*). The rats demonstrated reliable oxycodone or food self-administration. The mixed ANOVA for number of rewards, which included the within-subjects factor of Training session (1–14) and the between-subjects factor of Reward (food, oxycodone), showed a main effect of Training session ($F_{13,546} = 15.9$, $P < 0.001$), Reward ($F_{1,42} = 130.7$, $P < 0.001$), and Reward × Training session ($F_{13,546} = 6.8$, $P < 0.001$). The main effect of Reward is due to the higher number of daily food rewards than oxycodone infusions. The significant Reward × Training session interaction is due to the daily increase in oxycodone infusions but not food rewards, which remained stable over time. The statistical results of active lever presses are described in *SI Appendix*, Table S1.

Table 1. OFC and dorsal striatum functional connectivity showing opposite correlations with the incubation score during self-administration versus voluntary abstinence phases

		Self-administration		Voluntary abstinence	
Seed area	Target area	Food	Oxycodone	Food	Oxycodone
Medial OFC	Dorsal striatum	r = −0.27	*r* = 0.57*	*r* = 0.39	r = −0.60**
Ventrolateral OFC	Posterior dorsal striatum	r = −0.28	*r* = 0.62**	*r* = 0.23	r = −0.79**
[Medial OFC] Dorsal striatum	Ventrolateral OFC	r = −0.29	*r* = 0.64**	*r* = 0.24	r = −0.58*

r, Pearson r correlation. *$P < 0.05$, **$P < 0.01$.

Electric barrier–induced voluntary abstinence. The rats voluntarily abstained from oxycodone or food self-administration when we introduced an electric barrier of increasing shock intensity, as indicated by a significant decrease in the number of rewards and active lever presses over time (Fig. 1*C*). The mixed ANOVA for number of rewards, which included the within-subjects factor of Electric barrier session (1–12) and the between-subjects factor of Reward, showed a main effect of Electric barrier session ($F_{11,462} = 227.1$, $P < 0.001$), Reward ($F_{1,42} = 26.3$, $P < 0.001$), and Reward × Electric barrier session ($F_{11,462} = 28.5$, $P < 0.001$). The main effect of Reward is due to the higher number of daily food rewards than oxycodone infusions at lower shock intensities. The significant Reward × Training session interaction is due to a steeper electric barrier–induced suppression of lever presses in the food versus the oxycodone group. A likely interpretation of this interaction effect is the higher baseline responding in the food group rather than group differences in shock sensitivity, which did not differ between the groups (Dataset S1). The statistical results for active lever presses are described in *SI Appendix*, Table S1.

Relapse (incubation) test. Oxycodone seeking (nonreinforced lever presses) in the relapse (incubation) tests was greater after 15 d than after 1 d of abstinence (Fig. 1*D*). In contrast, food seeking was greater after 1 d than after 15 d. The mixed analysis of covariance (ANCOVA) (covariate: inactive lever presses) for number of active lever presses, which included the within-subjects factor of Abstinence day (1, 15) and the between-subjects factors of Reward, showed significant effects of Abstinence day ($F_{1,40} = 5.7$, $P = 0.022$) and Abstinence day × Reward ($F_{1,40} = 114.9$, $P < 0.001$). We also ran a separate analysis for lever pressing during the total 90 min relapse test on day 15. The mixed ANCOVA (covariate: inactive lever presses) for number of active lever presses, which included the between-subjects factor of Reward and the within-subjects factor of Session time (30, 60, and 90 min), showed significant effects of Session time ($F_{2,82} = 61.2$, $P < 0.001$), Reward ($F_{1,41} = 29.0$, $P < 0.001$), and Session time × Reward interaction ($F_{2,82} = 21.5$, $P < 0.001$). Finally, as expected from the results described previously, the ANCOVA (covariate: inactive lever presses) of the "incubation score" (number of active lever presses in the first 30 min of the day 15 test minus the number of lever presses during the 30-min day 1 test) showed a significant effect of Reward ($F_{1,41} = 126.6$, $P < 0.001$). The incubation score of all rats in the oxycodone group was positive, while this score was negative for all rats in the food group.

In summary, our results demonstrate an unexpected opposite timecourse of oxycodone versus food seeking after electric barrier–induced abstinence. The results of the oxycodone group showing strong incubation of oxycodone seeking replicate those from our previous study using the present voluntary abstinence model (9). In contrast, the results of the food group provide an unexpected demonstration of "abatement" of food seeking during abstinence, which is opposite to results from previous studies showing robust incubation of food seeking after homecage forced abstinence (29, 30).

Imaging results. We conducted hypothesis-driven, whole-brain voxel-wise functional connectivity analysis using three OFC subdivisions (Fig. 2) as seeds. We assessed changes in OFC connectivity in the food and oxycodone groups 1) from before (pretraining scan) and after (early abstinence scan) self-administration (term herein "reward self-administration phase") and 2) from before (early abstinence scan) to after (late abstinence scan) electric barrier–induced abstinence (term herein "voluntary abstinence phase"). For brain regions showing effects of Reward × Time interaction, we examined the correlations between the changes in OFC connectivity for each phase and the "incubation score" (lever presses during the late abstinence relapse test minus lever presses in the early abstinence relapse test). We conducted post hoc voxel-wise *t* tests in the food and oxycodone groups to determine changes in functional connectivity during 1) the reward self-administration phase and 2) the voluntary abstinence phase. In *SI Appendix*, Fig. S1, we present a flowchart of the fMRI data analysis strategy.

Self-administration phase. *Reward self-administration–related changes in OFC functional connectivity: Medial OFC seed.* The ANOVA showed a significant Reward (food, oxycodone) × Time (pretraining, early abstinence) interaction in connectivity between medial OFC and dorsal striatum, posterior dorsal striatum, ventral striatum, thalamus, hippocampus, and primary sensory cortex (Fig. 3 *A* and *B*). Post hoc tests showed that the connectivity of medial OFC with the dorsal striatum, posterior dorsal striatum, and primary sensory cortex significantly increased in the oxycodone group. In contrast, the connectivity of medial OFC with dorsal and ventral striatum significantly decreased in the food group (Fig. 3 *A* and *B*). The changes in medial OFC connectivity with dorsal striatum and thalamus were positively correlated with the incubation score in the oxycodone but not food group (Fig. 3*C*).

Reward self-administration–related changes in OFC functional connectivity: Ventrolateral OFC seed. The ANOVA showed a significant Reward × Time interaction in connectivity between ventrolateral OFC and posterior dorsal striatum and primary sensory cortex (Fig. 3 *D* and *E*). Post hoc tests showed that the connectivity of ventrolateral OFC with posterior dorsal striatum and primary sensory cortex significantly increased in the oxycodone group. In contrast, the connectivity of ventrolateral OFC with posterior dorsal striatum and primary sensory cortex was not significantly decreased in the food group (Fig. 3*E*). The change of ventrolateral OFC connectivity with the posterior dorsal striatum was positively correlated with the incubation score in the oxycodone but not food group (Fig. 3*F*).

Reward self-administration–related changes in OFC functional connectivity: Dorsolateral OFC seed. There was no significant Reward × Time interaction in the dorsolateral OFC connectivity, using the same ANOVA model and correction criteria for multiple comparisons.

Taken together, oxycodone self-administration significantly increased functional connectivity in medial and ventrolateral OFC-related circuits. In contrast, food self-administration

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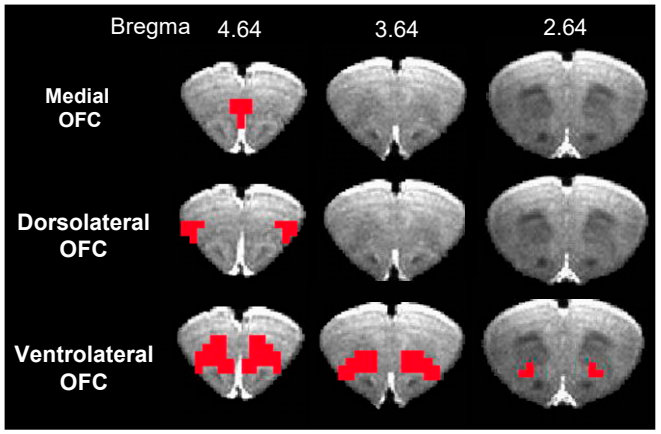

Fig. 2. Subdivisions of the OFC used as seeds for functional connectivity analyses. The three OFC subdivisions—medial OFC, ventrolateral OFC, and dorsolateral OFC—were defined based on a rat atlas (64). The Bregma range was from +2.64 to +6.64 mm. The underlay brain images are from a rat brain MRI template that was registered to a rat brain atlas (65).

significantly decreased functional connectivity in medial but not ventrolateral OFC-related circuits. Oxycodone and food self-administration did not result in statistically significant functional connectivity changes in dorsolateral OFC-related circuits, demonstrating anatomical specificity. Additionally, for the oxycodone group, the increased functional connectivity with dorsal striatum and thalamus (medial OFC seed) and posterior dorsal striatum (ventrolateral OFC seed) was positively correlated with the incubation score. In subsequent analyses, we follow up on these findings, as described in the following sections.

Reward self-administration follow-up analyses of medial OFC seed: dorsal striatum and thalamus. The statistical analyses of dorsal striatum and thalamus showed significant Reward × Time interaction (Fig. 3 *A* and *B*) and significant correlations with the incubation score (Fig. 3*C*) in the first-level analysis using medial OFC as a seed. We conducted follow-up analyses on these two brain regions using methods similar to the ones we used for the first-level analyses to assess changes in connectivity from pretraining-to-early abstinence (reward self-administration phase).

Dorsal striatum seed. The ANOVA showed a significant Reward × Time interaction in dorsal striatum connectivity with prelimbic cortex, ventrolateral OFC, piriform cortex, auditory cortex, dorsal and ventral striatum, and posterior hippocampus (Fig. 4 *A* and *B*). Post hoc tests showed that the dorsal striatum connectivity with ventrolateral OFC and prelimbic cortex significantly increased in the oxycodone group. In contrast, the dorsal striatum connectivity with all significant brain clusters significantly decreased in the food group (Fig. 4 *A* and *B*). The changes in dorsal striatum connectivity with itself and with ventrolateral OFC and auditory cortex were correlated with the incubation score in the oxycodone but not food group (Fig. 4*C*).

Thalamus seed. The ANOVA showed a significant Reward × Time interaction in thalamus connectivity with prelimbic cortex, ventrolateral OFC, and thalamus (Fig. 4 *D* and *E*). Post hoc tests showed that the thalamus connectivity with these significant brain clusters increased in the oxycodone group but modestly (nonsignificantly) decreased in the food group (Fig. 4*E*). There were no significant correlations between changes in thalamic connectivity and the incubation score.

Reward self-administration follow-up analyses of ventrolateral OFC seed: posterior dorsal striatum. The statistical analyses of posterior dorsal striatum showed significant Reward × Time

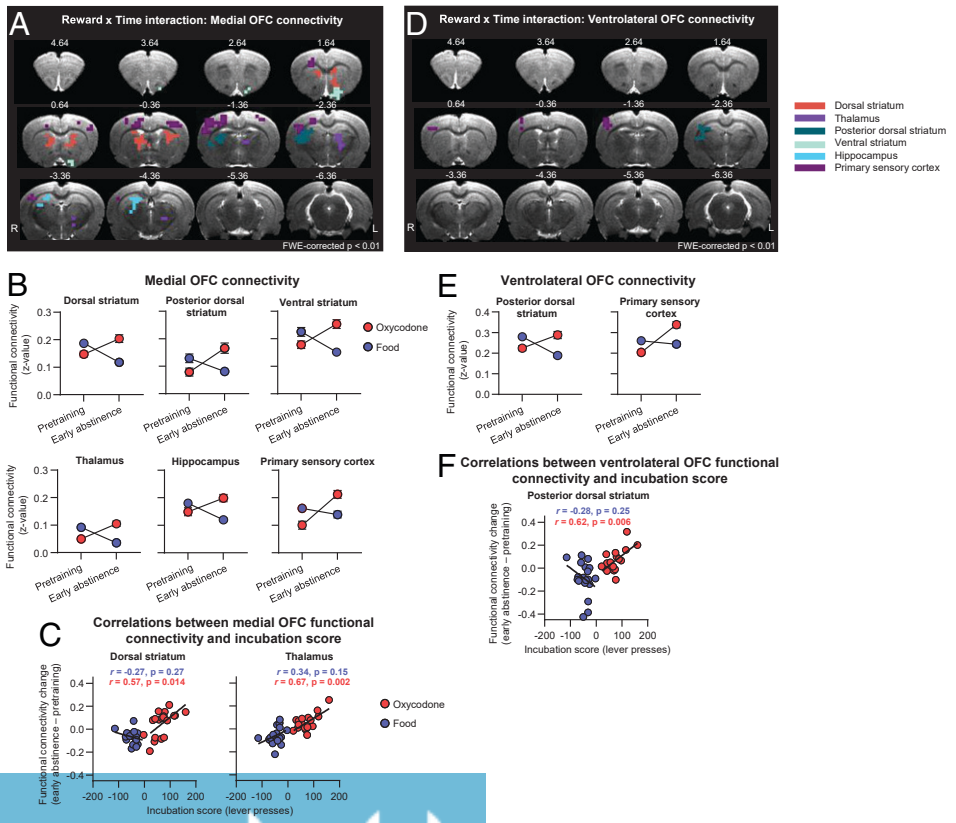

Fig. 3. Reward self-administration–related changes in OFC functional connectivity. (*A* and *B*) Medial OFC seed: Significant Reward (food, oxycodone) × Time (pretraining, early abstinence) interactions in functional connectivity of medial OFC with dorsal striatum, thalamus, posterior dorsal striatum, ventral striatum, hippocampus, and primary sensory cortex (interaction regions in *A* and interaction patterns in *B*). (*C*) Medial OFC seed: correlations between the changes in medial OFC-dorsal striatum and medial OFC-thalamus functional connectivity and the incubation score (lever presses during the late abstinence relapse test minus lever presses during the early abstinence relapse test). (*D* and *E*) Ventrolateral OFC seed: Significant Reward × Time interactions in functional connectivity of ventrolateral OFC with posterior dorsal striatum and primarily sensory cortex (interaction regions in *D* and interaction patterns in *E*). (*F*) Ventral OFC seed: correlations between the change in ventrolateral OFC-posterior dorsal striatum functional connectivity and the incubation score. Imaging results were corrected for whole-brain multiple comparison at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

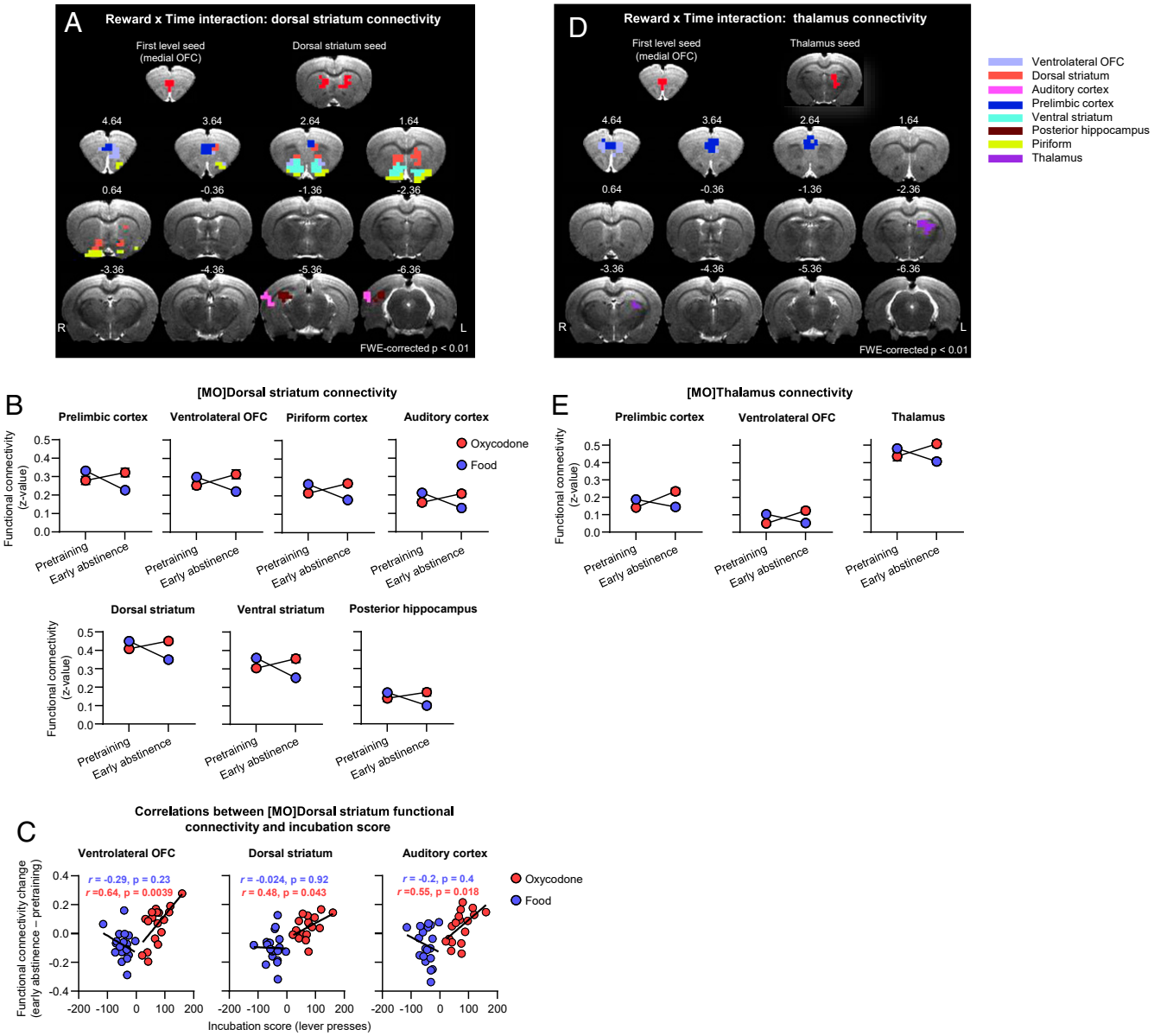

Fig. 4. Reward self-administration–related follow-up analyses of medial OFC (MO) seed: dorsal striatum and thalamus. (*A* and *B*) Dorsal striatum seed: Significant Reward × Time (pretraining, early abstinence) interactions in functional connectivity of dorsal striatum with ventrolateral OFC, dorsal striatum, auditory cortex, prelimbic cortex, ventral striatum, posterior hippocampus, and piriform (interaction regions in *A* and interaction patterns in *B*). (*C*) Dorsal striatum seed: correlations between the change in dorsal striatum functional connectivity and the incubation score. (*D* and *E*) Thalamus seed: Significant Reward × Time interactions in functional connectivity of thalamus with ventrolateral OFC, prelimbic cortex, and thalamus (interaction regions in *D* and interaction patterns in *E*). Imaging results were corrected for whole-brain multiple comparisons at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

interaction (Fig. 3 *D* and *E*) and a significant correlation with the oxycodone incubation score (Fig. 3*F*) in the first-level analysis using ventrolateral OFC as a seed. The follow-up ANOVA showed a significant Reward × Time interaction for prelimbic cortex, ventrolateral OFC, piriform cortex, auditory cortex, primary sensory cortex, dorsal and ventral striatum, hippocampus, and thalamus (Fig. 5 *A* and *B*). Post hoc tests showed that in the oxycodone group, except for ventral striatum, posterior dorsal striatum connectivity significantly increased with the brain clusters showing significant interaction effects. In contrast, in the food group, with the exception of auditory cortex, posterior dorsal striatum-related connectivity significantly decreased (Fig. 5 *A* and *B*). The changes in posterior dorsal striatum connectivity with piriform, primary sensory, and auditory cortex and with ventral and dorsal striatum were significantly correlated with the incubation score in the oxycodone but not food group (Fig. 5*C*).

Summary. The fMRI results of functional connectivity changes induced by oxycodone self-administration showed that functional connectivity in OFC with dorsal striatum and follow-up dorsal striatum-related circuits increased over time in the oxycodone group and was positively correlated with incubation of oxycodone seeking. In contrast, functional connectivity of OFC-related circuits decreased or maintained after palatable food self-administration and was not correlated with abatement of food seeking.

Electric barrier–induced voluntary abstinence phase. *Voluntary abstinence-related changes in OFC functional connectivity: Medial and ventrolateral OFC seeds.* The ANOVAs showed

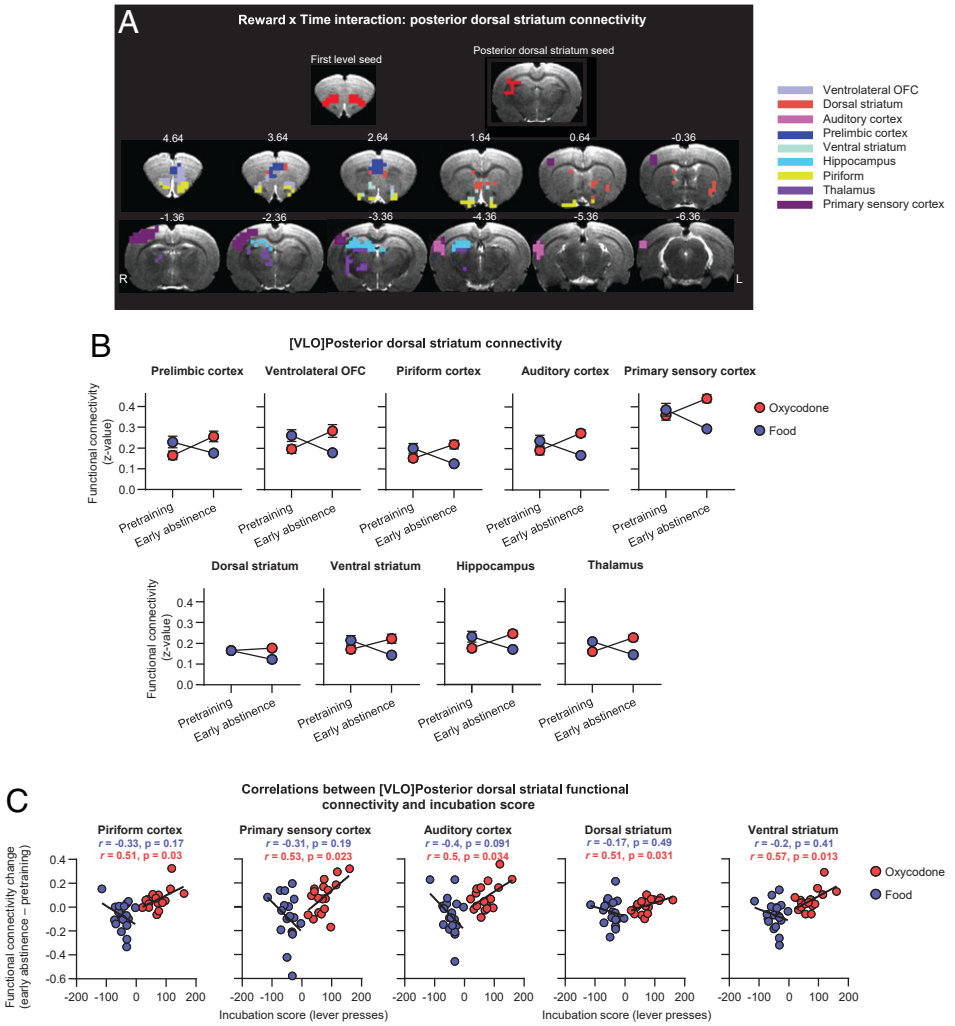

Fig. 5. Reward self-administration–related follow-up analyses of ventrolateral OFC (VLO) analyses: posterior dorsal striatum. (*A* and *B*) Significant Reward × Time (pretraining, early abstinence) interactions in functional connectivity of posterior dorsal striatum with dorsal striatum, ventral striatum, piriform cortex, primary sensory cortex, auditory cortex, ventrolateral OFC, prelimbic cortex, thalamus, and hippocampus (interaction regions in *A* and interaction patterns in *B*). (*C*) Correlations between the change in posterior dorsal striatum functional connectivity and the incubation score. Imaging results were corrected for whole-brain multiple comparisons at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

significant Reward (food, oxycodone) × Time (early abstinence, late abstinence day) interactions in connectivity with dorsal striatum and posterior dorsal striatum (Fig. 6 *A*, *B*, *D*, and *E*). For the oxycodone group, post hoc tests showed that for the medial OFC seed, connectivity was significantly decreased with dorsal striatum and posterior dorsal striatum. Additionally, for the ventrolateral OFC seed, connectivity significantly decreased with dorsal striatum. For the food group, post hoc tests showed that for both OFC subregions connectivity modestly (nonsignificantly) increased with dorsal striatum and posterior dorsal striatum (Fig. 6 *B* and *E*). The changes in medial and ventrolateral OFC connectivity with dorsal striatum and posterior dorsal striatum were negatively correlated with the incubation score in the oxycodone but not food group (Fig. 6 *C* and *F*).

Voluntary abstinence-related changes in OFC functional connectivity: Dorsolateral OFC seed. There were no significant Reward × Time interactions in dorsolateral OFC connectivity.

Taken together, in rats with a history of oxycodone self-administration, electric barrier–induced abstinence significantly decreased functional connectivity in medial and ventrolateral OFC-related circuits. In contrast, in rats with a history of food self-administration, electric barrier–induced abstinence modestly increased functional connectivity in medial and ventrolateral OFC-related circuits. Oxycodone and food self-administration did not result in statistically significant functional connectivity changes in dorsolateral OFC-related circuits, demonstrating anatomical specificity. Additionally, for the oxycodone group, the decreased functional connectivity with dorsal striatum and posterior dorsal striatum was positively correlated with the incubation score. In subsequent analyses, we follow up on these findings, as described in the following sections.

Voluntary abstinence follow-up analyses of medial OFC seed: dorsal striatum and posterior dorsal striatum. The statistical analyses of dorsal striatum and posterior dorsal striatum showed significant Reward × Time interactions (Fig. 6 *A* and *B*) and significant correlations with the oxycodone incubation score (Fig. 6*C*) in the first-level analysis using medial OFC as a seed.

Dorsal striatum seed. The ANOVA showed a significant Reward × Time interaction in dorsal striatum connectivity with prelimbic, ventrolateral, piriform, auditory, primary sensory, secondary motor, and posterior parietal cortices and ventral and dorsal striatum (Fig. 7 *A* and *B*). Post hoc tests showed that connectivity of dorsal striatum with these significant brain clusters significantly decreased in the oxycodone but not the food group (Fig. 7 *A* and *B*). The changes in dorsal striatum connectivity with ventrolateral OFC significantly negatively correlated with the incubation score in the oxycodone but not food group (Fig. 7*C*).

Posterior dorsal striatum seed. The ANOVA showed a significant Reward × Time interaction in connectivity of posterior dorsal striatum with auditory and primary sensory cortices, dorsal striatum, hippocampus, and thalamus (Fig. 7 *D* and *E*). Post

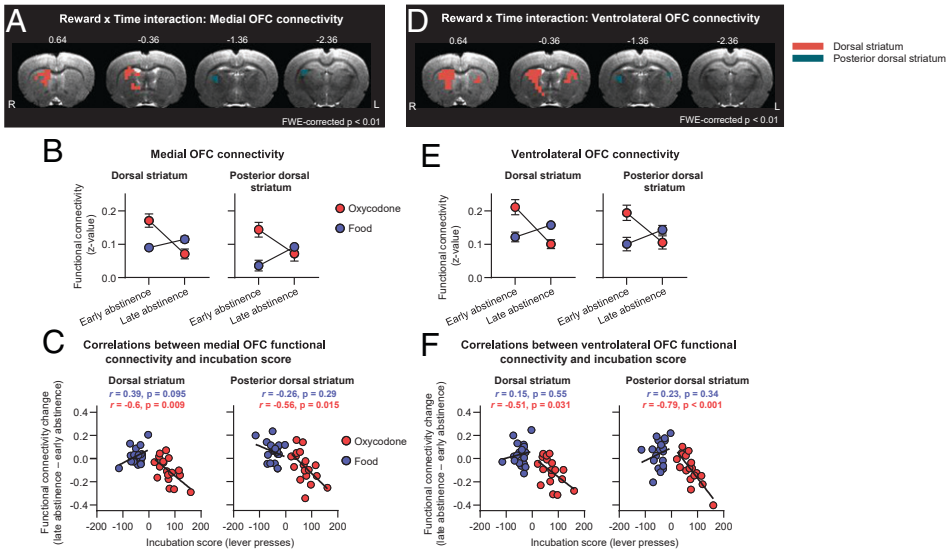

Fig. 6. Voluntary abstinence-related changes in OFC functional connectivity. (*A* and *B*) Medial OFC seed: Significant Reward × Time (early abstinence, late abstinence) interactions in functional connectivity of medial OFC with dorsal striatum and posterior dorsal striatum (interaction regions in *A* and interaction patterns in *B*). (*C*) Medial OFC seed: correlations between the changes in medial OFC-dorsal striatum and medial OFC-posterior dorsal striatum functional connectivity and the incubation score. (*D* and *E*) Ventrolateral OFC seed: Significant Reward × Time interactions in functional connectivity of ventrolateral OFC with dorsal striatum and posterior dorsal striatum (interaction regions in *D* and interaction patterns in *E*). (*F*) Ventrolateral OFC seed: correlations of the changes in ventrolateral OFC-dorsal striatum and ventrolateral OFC-posterior dorsal striatum functional connectivity and the incubation score. Imaging results were corrected for whole-brain multiple comparisons at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

hoc tests showed that the connectivity of posterior dorsal striatum with these significant brain clusters significantly decreased in the oxycodone but not the food group (Fig. 7 *D* and *E*). The changes in posterior dorsal striatum connectivity with auditory and primary sensory cortices and hippocampus significantly negatively correlated with the incubation score in the oxycodone but not the food group (Fig. 7*F*).

Voluntary abstinence follow-up analyses of ventrolateral OFC seed: dorsal striatum and posterior dorsal striatum. The statistical analyses of dorsal striatum and posterior dorsal striatum showed significant Reward × Time interaction (Fig. 6 *D* and *E*) and significant correlations with the oxycodone incubation score (Fig. 6*F*) in the first-level analysis using ventrolateral OFC as a seed.

Dorsal striatum seed. The ANOVA showed a significant Reward × Time interaction in prelimbic cortex, ventrolateral OFC, piriform, auditory, primary sensory, and posterior parietal cortices and dorsal and ventral striatum (Fig. 8 *A* and *B*). Post hoc tests showed that dorsal striatum connectivity with these significant brain clusters significantly decreased in the oxycodone group (Fig. 8 *A* and *B*). In contrast, dorsal striatum connectivity with these significant brain clusters modestly (nonsignificantly) increased in the food group (Fig. 8 *A* and *B*). The changes in dorsal striatum connectivity with ventrolateral OFC (but not other regions) significantly negatively correlated with the incubation score in the oxycodone but not food group (Fig. 8*C*).

Posterior dorsal striatum seed. The ANOVA showed significant Reward × Time interactions in posterior dorsal striatum connectivity with primary and secondary sensory cortices, thalamus, dorsal striatum, and posterior dorsal striatum (Fig. 8*D*). Post hoc tests showed that posterior dorsal striatum connectivity with these significant brain clusters significantly decreased in the oxycodone but not the food group (Fig. 8*E*). The changes in posterior dorsal striatum connectivity with thalamus significantly negatively correlated with the incubation score in the oxycodone but not food group (Fig. 8*F*).

Summary. The fMRI results of functional connectivity changes from early abstinence to late abstinence (electric barrier–induced voluntary abstinence-related changes) showed that functional connectivity of OFC with dorsal striatum and follow-up dorsal striatum-related circuits decreased over time in the oxycodone group and was negatively correlated with incubated oxycodone seeking. In contrast, functional connectivity of OFC-related circuits modestly (nonsignificantly) increased in the food group and was not correlated with abated food seeking. This pattern of results for the effect of voluntary abstinence on OFC-related functional connectivity changes and their correlations with the incubation score are opposite to the pattern of results we observed for the effect of oxycodone self-administration. Indeed, as shown in Table 1, for several specific connections (medial OFC–dorsal striatum, ventrolateral OFC–posterior dorsal striatum, and dorsal striatum–ventrolateral OFC), the functional connectivity changes induced by oxycodone self-administration positively correlated with the incubation score, while the functional connectivity changes induced by voluntary abstinence were negatively correlated with this score (see *SI Appendix*, Table S2 for a complete reporting of the correlations between functional connectivity changes induced by reward self-administration and voluntary abstinence and the incubation score). Finally, we further illustrate the opposite effects of reward self-administration and voluntary abstinence for the oxycodone versus food groups in *SI Appendix*, Figs. S2–S7, which show longitudinal functional connectivity changes from pretraining to late abstinence.

Discussion

We combined a rat model of incubation of drug craving after voluntary abstinence due to negative consequences of drug seeking with a rat fMRI method to study longitudinal brain changes associated with incubation of oxycodone craving. We report three main findings. First, oxycodone seeking during the relapse (incubation) tests was greater during late abstinence than during early abstinence, replicating our recent finding on incubation of oxycodone craving after electric barrier–induced abstinence (9). In contrast, food seeking in the relapse tests was greater during early abstinence than during late abstinence, demonstrating abatement of food craving after electric barrier–induced abstinence. Second, during the self-administration phase, functional connectivity in OFC–dorsal striatum and follow-up dorsal striatum circuits increased in the oxycodone group, and the functional connectivity changes were positively correlated with incubated oxycodone seeking. In contrast, functional connectivity of these circuits either decreased or

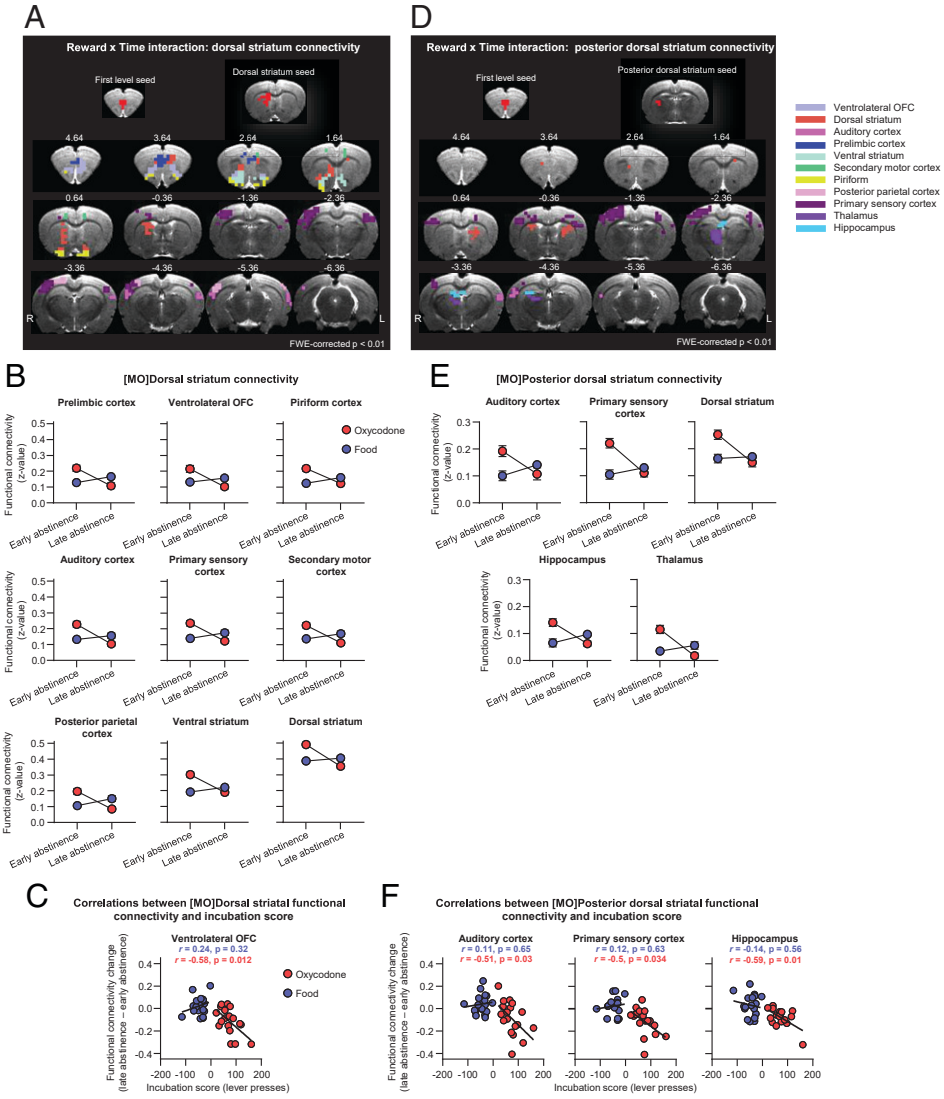

Fig. 7. Voluntary abstinence-related follow-up analyses of medial OFC (MO) seed: dorsal striatum and posterior dorsal striatum. (*A* and *B*) Dorsal striatum seed: Significant Reward × Time (early abstinence, late abstinence) interactions in functional connectivity of dorsal striatum with ventrolateral OFC, dorsal striatum, prelimbic, ventral striatum, secondary motor cortex, posterior parietal cortex, piriform cortex, primary sensory cortex, and auditory cortex (interaction regions in *A* and interaction patterns in *B*). (*C*) Dorsal striatum seed: correlations of the change in dorsal striatum-ventrolateral OFC functional connectivity and the incubation score. (*D* and *E*) Posterior dorsal striatum seed: Significant Reward × Time interactions in functional connectivity of posterior dorsal striatum with hippocampus, auditory cortex, primary sensory cortex, dorsal striatum, and thalamus (interaction regions in *D* and interaction patterns in *E*). (*F*) Posterior dorsal striatum seed: correlations of the change in dorsal striatum functional connectivity and the incubation score. Imaging results were corrected for whole-brain multiple comparisons at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

did not significantly change in the food group and was not correlated with abatement of food seeking. Third, during the voluntary abstinence phase, functional connectivity in OFC–dorsal striatum and follow-up dorsal striatum circuits decreased in the oxycodone group, and the functional connectivity changes were negatively correlated with incubated oxycodone seeking. In contrast, functional connectivity of these circuits either increased or did not significantly change in the food group and was not correlated with abatement of food seeking.

Opposing Time-Dependent Changes in Oxycodone versus Food Seeking after Voluntary Abstinence. In previous studies, we and others reported reliable incubation of opioid (heroin or oxycodone) and food (sucrose solution or different food pellets) craving after homecage forced abstinence (30, 31). More recently, we reported that incubation of oxycodone craving also occurs after electric barrier–induced abstinence (9). Here, we determined the generality of this incubation phenomenon to a nondrug reward: high-carbohydrate food pellets that rats strongly prefer over addictive drugs in an operant choice setting (6, 20, 26). Based on previous findings on incubation of food craving after forced abstinence, we predicted that incubation of palatable food craving would be observed after electric barrier–induced abstinence. Unexpectedly, we found strong time-dependent decreases in food seeking during the relapse tests. It is unlikely that higher sensitivity to shock in the food-trained rats can account for these results because we did not observe group differences in either electric barrier–induced suppression of operant responding (Fig. 1*C* and Dataset S1) or footshock threshold sensitivity (oxycodone group: 0.166 ± 0.051 mA; food group: 0.164 ± 0.049 mA, mean ± SEM, $P > 0.1$).

The reasons for the opposing time-dependent effects of electric barrier–induced abstinence on oxycodone versus food seeking are unknown. Of note, our data extend results from previous studies on dissociable effects of stress exposure on reinstatement of drug versus food seeking after extinction of operant responding (32): identical parameters of intermittent footshock reinstate drug but not food seeking (33, 34) (see ref. 35 for a discussion of potential reasons for this differential effect). More speculatively, the differential effects of intermittent footshock and electric barrier exposure on drug versus food seeking may reflect drug-induced brain neuroadaptations that may increase stress-induced relapse vulnerability (32, 35).

Opposing OFC-Related Functional Connectivity Changes after Oxycodone Self-Administration versus Voluntary Abstinence. Early human imaging studies reported that cocaine exposure induces

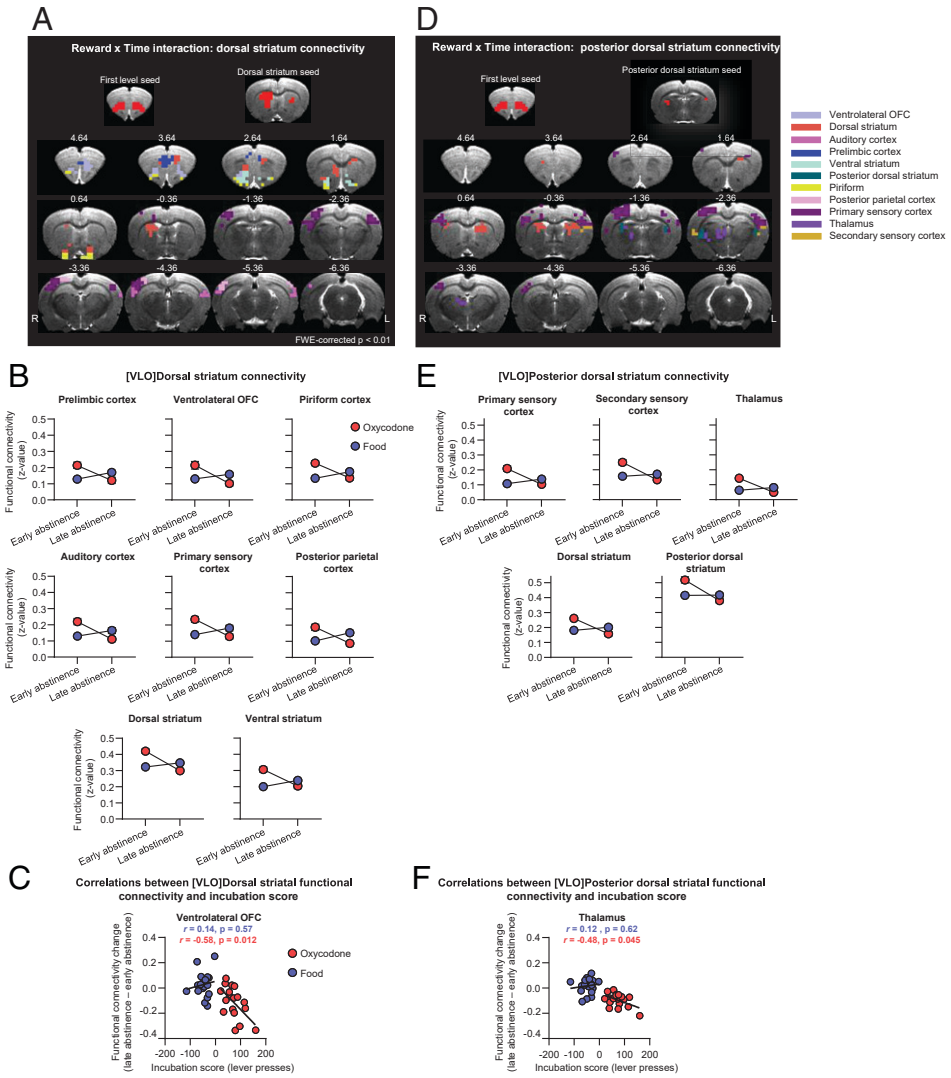

Fig. 8. Voluntary abstinence-related follow-up analyses of ventrolateral OFC (VLO) seed: dorsal striatum and posterior dorsal striatum. (*A* and *B*) Dorsal striatum seed: Significant Reward × Time (early abstinence, late abstinence) interactions in functional connectivity of dorsal striatum with ventrolateral OFC, prelimbic cortex, dorsal striatum, ventral striatum, posterior parietal cortex, piriform cortex, primary sensory cortex, and auditory cortex (interaction regions in *A* and interaction patterns in *B*). (*C*) Dorsal striatum seed: correlations of the change in dorsal striatum-ventrolateral OFC functional connectivity and the incubation score. (*D* and *E*) Posterior dorsal striatum seed: Significant Reward × Time interactions in functional connectivity of posterior dorsal striatum with thalamus, dorsal striatum, posterior dorsal striatum, and primary/secondary sensory cortices (interaction regions in *D* and interaction patterns in *E*). (*F*) Posterior dorsal striatum seed: correlations of the change in posterior dorsal striatum-thalamus functional connectivity and the incubation score. Imaging results were corrected for whole-brain multiple comparisons at $p_{corr} < 0.01$. Data are mean ± SEM. Food group: $n = 19$; oxycodone group: $n = 18$. The underlay brain images in *A* and *D* are from a rat brain MRI template that was registered to a rat brain atlas (65).

changes in OFC function: hypoactivity during protracted withdrawal and hyperactivity after drug exposure that correlates with drug craving (36). Subsequent studies showed that human opioid and cocaine use is associated with lower gray matter density and thinner cortical thickness in OFC (37, 38) and that these structural changes are associated with high risk taking in a gambling task (39) and compulsivity measures (40). More recent studies showed that altered functional connectivity in OFC–striatal circuits is associated with severity of human cocaine or alcohol dependence (15, 41) and punishment-resistant methamphetamine self-administration in rats (16). However, other rat studies in which relapse to cocaine seeking was determined after punishment-induced abstinence showed that OFC is not selectively activated (indexed by Fos expression) during the relapse test and that pretraining permanent OFC lesions had no effect on relapse (42, 43). On the other hand, cue-induced heroin craving in humans is associated with increased OFC activity (21, 22, 44), and OFC activity is critical to incubated heroin and oxycodone seeking after forced abstinence and relapse to fentanyl seeking after food choice-induced abstinence (25–27).

Based on these studies, we used a longitudinal rat fMRI method (18) to determine whether functional connectivity changes in OFC-related circuits induced by oxycodone self-administration experience or electric barrier–induced abstinence experience would predict incubation of oxycodone craving, operationally assessed using the "incubation score" (see *Materials and Methods* and *Results*). We found that oxycodone self-administration and electric barrier–induced abstinence caused opposing functional connectivity changes in OFC-related circuits—increased connectivity by self-administration experience versus decreased connectivity by electric barrier–induced abstinence experience. Some of these opposing functional connectivity changes were strongly correlated with the incubation score. Additionally, in follow-up analyses, we found a similar pattern of results for functional connectivity of dorsal striatum-related circuits for both the self-administration and voluntary abstinence phases. In this regard, the role of different dorsal striatum subregions in incubation of opioid craving is unknown, but previous studies reported a role of both dorsolateral and dorsomedial striatum in incubation of methamphetamine craving after forced abstinence (45) and dorsomedial striatum in incubation of methamphetamine craving after food choice–induced abstinence (46).

The reasons for the opposing functional connectivity changes induced by oxycodone self-administration experience versus electric barrier–induced voluntary abstinence experience are unknown. However, while the qualitative changes in connectivity appear surprising, they agree with a large literature on different drug-induced neuroadaptations that emerge during early

versus protracted withdrawal (abstinence), including time-dependent changes in glutamate and dopamine transmission, and other synaptic and structural changes in mesocorticolimbic areas (47–49). More recently, researchers used longitudinal positron emission tomography (PET) (50) and fMRI (51) methods and reported abstinence duration-dependent changes in brain function in cortical and subcortical areas in rats trained to self-administer cocaine. Additionally, Groman et al. (52, 53) used a longitudinal PET method and reported that cocaine-induced alterations in midbrain dopamine D3 receptor availability and cortical mGluR5 availability predict escalation of cocaine self-administration and cocaine-induced decision-making impairments. Finally, Beveridge et al. (54) combined a rhesus monkey cocaine self-administration procedure with receptor autoradiography procedures and reported abstinence duration-dependent changes in the expression of dopamine receptors and dopamine transporter in ventral and dorsal striatum. Using similar methods, Smith et al. (55) reported abstinence-dependent changes in the expression of norepinephrine transporter and alpha-2 adrenoreceptors in terminals of the dorsal and ventral noradrenergic bundle.

A methodological issue to consider is the cause of the putative self-administration–related and voluntary abstinence-related functional connectivity changes. An issue here is that because of potential carryover performance-disrupting effects of the imaging procedure on operant responding 1 d after the imaging session (see *Materials and Methods*), we performed the early and late abstinence scans 1 d after the relapse tests. Thus, an alternative interpretation of the data is that the functional connectivity changes are due to acute relapse test experience rather than self-administration or voluntary abstinence experience. This possibility is unlikely for two reasons. First, when we performed the correlations depicted in Figs. 3 *C* and *F*, 4*C*, and 5*C* with early abstinence lever presses instead of the incubation score, we found that only 1 correlation out of the 11 positive correlations for the oxycodone group was significant ($P = 0.024$). Second, if the functional connectivity changes were due to operant responding during the relapse tests, we would expect significant correlations between the connectivity changes and the incubation score in both the oxycodone and food groups. As described in the *Results* section and figures, the correlations were specific to oxycodone.

Another methodological issue to consider is that post hoc voxel-wise *t* tests showed that a small number (2 out of 27) of pretraining connectivity data were significantly higher in the food group (Figs. 3–5); the majority (25 out of 27) of the pretraining connectivity data were nonsignificantly higher. The reasons for the somewhat higher pretraining connectivity in the food group are unknown. Additionally, this result is unexpected because we randomly assigned drug-naive rats to the food and oxycodone groups. However, it is unlikely that this methodological issue confounds data interpretation. This is because our two-way mixed ANOVAs were designed to detect differences in the slope of the time-dependent changes in the food versus oxycodone groups (interaction effects), which, unlike *t* tests at a single timepoint, are largely independent of the pretraining score.

Finally, our functional connectivity analyses identified several brain areas that have not been traditionally considered in studies on circuit mechanisms of drug relapse, including medial OFC, lateral thalamic areas, and piriform, auditory, and sensory cortices. The role of these brain areas in relapse-related behaviors is a subject for future research. Additionally, our analyses were limited to OFC subregions seeds. Thus, the present imaging dataset can be used by imaging researchers to identify other novel relapse-related brain circuits, as assessed in the electric barrier voluntary abstinence relapse model.

Conclusions

We studied longitudinal functional connectivity changes induced by oxycodone self-administration and voluntary abstinence using a rat model that mimics features of the human condition of relapse after self-imposed abstinence due to adverse consequences of drug seeking (8). At the behavioral level, the main finding was the opposite time course of oxycodone (incubation) versus palatable food (abatement) seeking after electric barrier–induced abstinence. At the brain imaging level, the main findings were that oxycodone self-administration and electric barrier–induced abstinence caused opposing functional connectivity changes in OFC- and dorsal striatum-related circuits and that these opposing functional connectivity changes correlated with incubation of oxycodone seeking. To the extent that findings from rat models of voluntary abstinence and relapse translate to the human condition (56, 57), we propose that longitudinal changes in the functional connectivity of OFC- and dorsal striatum-related circuits at different addiction phases can be used to predict individual differences in relapse vulnerability.

Materials and Methods

Subjects. We used male ($n = 46$) Sprague-Dawley rats (Charles River) weighing 260 to 360 g prior to surgery. We maintained the rats under a reverse 12:12 h light/dark cycle (lights off at 8:00 AM) with food and water freely available. We housed two rats/cage prior to surgery and individually after surgery. We performed the experiment in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (eighth edition) (58) under a protocol approved by the Animal Care and Use Committee. We excluded two rats from both the behavior and image analyses due to poor health or death and seven additional rats from the image analysis due to head movement that exceeded our criteria or poor physiology during fMRI scans.

In our previous study (9), we found no evidence for sex differences in oxycodone self-administration, electric barrier–induced suppression of drug self-administration, or incubation of oxycodone craving. In the present study, we used only males because of methodological considerations of the fMRI procedure related to sex differences in brain anatomy and functional connectivity in rats (59, 60) and recent evidence from human studies on sex differences and ovarian hormones' cycle-dependent fluctuations in functional connectivity (61, 62). These two factors—sex differences in functional connectivity and hormonal-dependent fluctuations in functional connectivity—would significantly increase variability of fMRI data and would require at a minimum tripling the number of subjects (to measure functional connectivity during estrus and nonestrus) to this very labor-intensive study for proper data interpretation if females are included in the study.

Drugs. We obtained oxycodone hydrochloride from the National Institute on Drug Abuse (NIDA) pharmacy and dissolved it in sterile saline. We chose a unit dose of 0.1 mg/kg for self-administration training based on our previous study (9).

Intravenous Surgery. We anesthetized the rats with isoflurane (5% induction; 2% to 3% maintenance, Covetrus). We attached silastic catheters to a modified 22-gauge cannula cemented to polypropylene mesh (Amazon), inserted the catheter into the jugular vein, and fixed the mesh to the midscapular region of the rat (20). We injected the rats with ketoprofen (2.5 mg/kg, subcutaneous [s.c.], Covetrus) after surgery and the following day to relieve pain and decrease inflammation. We allowed the rats to recover for 5 d before the first fMRI scan and an additional 2 d before the start of oxycodone or food self-administration training. During all experimental phases, we flushed the catheters daily with gentamicin (4.25 mg/mL, Fresenius Kabi) dissolved in sterile saline.

Apparatus. We used standard Med Associates self-administration chambers. Each chamber had two levers located 7.5 to 8 cm above the grid floor on opposing walls. Lever presses on the active retractable lever activated the infusion pump or the pellet dispenser; the 45 mg palatable food pellets (TestDiet, catalog # 1811155, 12.7% fat, 66.7% carbohydrate, and 20.6% protein) were delivered to a pellet dispenser located near the food-paired lever. Lever presses on the inactive nonretractable lever had no programmed consequences. We equipped each chamber with a stainless-steel grid floor connected to a shocker (Med Associates ENV-410B).

MRI Scanning. We conducted structural and resting-state fMRI scans on the rats using a Bruker Biospin 9.4 T MRI scanner (Bruker). We used a volume coil (model: MT0381) for radiofrequency excitation and a surface coil (model: MT0105-20) for magnetic resonance signal reception. We anesthetized the rats with a combination of dexmedetomidine (0.015 mg/kg/h, s.c.) and isoflurane (from initially 2.5% reducing to the range between 0.5% and 0.75%) (18), and collected high-resolution T2-weighted anatomical images (repetition time = 2,200 ms, field of view = 35 × 35 mm^2, matrix size = 256 × 256, slice thickness = 1 mm, and 23 slices per volume), and 2 to 3 runs of resting-state fMRI (echo time = 13 ms, repetition time = 1,000 ms, field of view = 35 × 35 mm^2, matrix size = 64 × 64, slice thickness = 1 mm, 17 slices per volume, and 600 volumes per run) for each rat at each scanning session. During scanning, we placed the rats in a customized cradle in the prone position, secured their heads with a custom-made bite bar and ear bars, and continuously delivered isoflurane in oxygen-enriched air with a nose cone. We monitored respiration, heart rate, core body temperature, and oxygen saturation throughout scanning. We maintained their respiration at ∼60 breaths/min to preserve neurovascular coupling and spontaneous brain oscillation (18).

Experimental Procedure. The experiment consisted of seven phases: pretraining fMRI scan, oxycodone or food self-administration training (14 d), early abstinence relapse test (abstinence day 1), early abstinence fMRI scan (abstinence day 2), electric barrier–induced voluntary abstinence (12 d), late abstinence relapse test (abstinence day 15), and late abstinence fMRI scan (abstinence day 16). We provide details of the different phases as follows, and the timeline for the experiment is shown in Fig. 1*A*. (Note: we considered switching the order of early and late abstinence scans with early and late relapse tests so the longitudinal imaging would be "truly" prospectively predicting incubation of oxycodone seeking. We decided to not do this because we were concerned that the intensive imaging procedure, which requires hours of anesthesia and causes significant stress to the rats, would interfere with the rats' behavior if we performed the relapse tests 24 h after the imaging sessions.)

First fMRI scan (pretraining). At 5 d after the intravenous surgery, we imaged all rats using fMRI to obtain baseline measurements of brain activity.

Self-administration training. At 2 d after the first fMRI scan, we trained the rats to self-administer oxycodone (n = 22) or palatable food pellets (n = 22) for 6 h/d (six 1-h sessions separated by 10 min) for 14 d. Oxycodone (0.1 mg/kg/infusion) was infused at a volume of 100 μL over 3.5 s, and five palatable food pellets were delivered per reward separated by 1 s. Each session began with illumination of a red houselight (Med Associates ENV-215M) that remained on for the entire session, followed 10 s later by the insertion of the active lever. Active lever presses led to oxycodone infusions or delivery of palatable food pellets that were paired with a 20-s tone-light cue (ENV-223 AM; ENV-221M, white lens) under a fixed-ratio 1 reinforcement schedule 20-s timeout (fixed interval 20 schedule). Responses on the inactive lever were recorded but had no programmed consequences. At the end of each 1-h session, the houselight was turned off and the active lever was retracted. We limited the number of rewards to 15 infusions or rewards/hour.

Early abstinence relapse test. We tested the rats for relapse to oxycodone or food seeking under extinction conditions on abstinence day 1. The test sessions on day 1 were 30 min to minimize extinction learning/experience, which may decrease incubated drug seeking on day 15. We gave all rats a 30-min habituation period in the self-administration chamber before the start of the test session. Lever presses resulted in the delivery of the reward-paired tone-light cue but no delivery of oxycodone or food.

Early abstinence fMRI scan. On abstinence day 2, we performed the second fMRI scan.

Electric barrier–induced voluntary abstinence. During this phase (12 d), oxycodone or food was available for 2 h/d. We used the same parameters (oxycodone dose, number of food pellets, reinforcement schedule, tone-light cues, etc.) we used during the training phase. We achieved abstinence by introducing an electric barrier in front of the reward lever, creating a "shock zone." We used a plastic demarcation (McMaster-Carr, catalog # 9852K61) to separate the "shock zone" (two-thirds of the chamber) from the "safe zone" (one-third of the chamber). If the rats approached the reward-paired lever, they received a continuous mild footshock (0.1 to 0.4 mA) through the grid floor. On the first day, we set the current at 0.0 mA and gradually increased current intensity by 0.1 mA increments every day until 0.3 mA. If the rats did not suppress oxycodone or food self-administration (<3 rewards/day), we increased the intensity to 0.4 mA the next day. Before the electric barrier phase, we tested the rats' sensitivity to footshock (operationally defined as the minimal shock level that causes the withdrawal of the front paw). There were no group differences in shock sensitivity, starting at 0.05 mA and assessed at 0.05 mA increments, and the values for individual rats' sensitivity ranged from 0.15 to 0.2 mA.

Late abstinence relapse test. We tested the rats for relapse to oxycodone or food seeking under extinction conditions on abstinence day 15. The test parameters were the same as for the relapse test on abstinence day 1, except we tested the rats in 90-min sessions. We used a longer test session on day 15 to obtain more information on the behavioral effect on "incubated" oxycodone versus food seeking.

Late abstinence fMRI scan. On abstinence day 16, we performed the third fMRI scan.

Statistical Analyses.

Behavioral data. We analyzed the behavioral data with repeated-measures or mixed-factorial ANOVAs or ANCOVA (inactive lever as a covariate) using SPSS (Version 24, GLM procedure). We followed significant main effects and interactions ($P < 0.05$) with post hoc tests (univariate ANOVAs or Fisher's protected least significant differences). We describe the different between- and within-subjects factors for the different statistical analyses in the *Results* section. Because the multifactorial ANOVAs yielded multiple main and interaction effects, we only report significant effects that are critical for data interpretation. Additionally, for clarity, we indicate the results of post hoc analyses by asterisks in the figures but do not describe them in the *Results* section (see *SI Appendix*, Table S1 for a complete reporting of the statistical analyses of the behavioral data).

fMRI data. We preprocessed all functional images with a standard pipeline, including skull stripping, motion correction, coregistration to a template, noise component removal, band-pass filtering (0.01 to 0.1 Hz), spatial smoothing (full width at half maximum = 0.6 mm), nuisance covariance regression for respiration, and motion and body temperature (17). We excluded 7 of the 44 rats from the analyses because their framewise displacements of motion were greater than 0.2 mm (63). Then, we conducted a seed-based connectivity analysis on functional images (16, 17). We selected seed regions from three OFC subdivisions (Fig. 2, medial, ventrolateral, and dorsolateral) based on a rat atlas (64) or clusters showing significant Reward × Time interaction in the first-level analyses. We computed voxel-wise Pearson's correlation coefficients between the time course of the seed and all brain voxels and converted them to z scores (achieving an approximate normal distribution) to evaluate functional connectivity between brain regions. We conducted a voxel-wise Reward (food [n = 19], oxycodone [n = 18]) × Time (pretraining, early abstinence) ANOVA to assess differential changes of functional connectivity associated with oxycodone or food self-administration (self-administration–related connectivity changes). We then conducted a voxel-wise Reward (food [n = 19], oxycodone [n = 18]) × Time (early abstinence, late abstinence) ANOVA to examine the effect of electric barrier–induced abstinence on functional connectivity in the two groups (voluntary abstinence-related connectivity changes). In the voxel-wise ANOVA, we corrected for multiple comparisons and used corrected $p_{corr} < 0.01$ (with uncorrected $P < 0.005$ and cluster size > 10 based on Monte Carlo simulation in AFNI) as a threshold to determine voxels that are significant in the analyses. For brain regions showing significant effects of interaction in the aforementioned ANOVAs, we extracted functional connectivity signals and correlated them with the change in active lever presses during the relapse tests (lever presses during the late abstinence test after electric barrier exposure minus lever presses during the early abstinence test before electric barrier exposure, termed herein "incubation score"). We performed post hoc voxel-wise t tests to assess changes in the functional connectivity during the reward self-administration and voluntary abstinence phases in the food and oxycodone groups. A flowchart of fMRI data analyses is shown in *SI Appendix*, Fig. S1.

Data Availability. The individual data of the behavioral part of the study and the mean ± SEM of the imaging data are provided in Dataset S1. The fMRI brain images and the individual data used for the data analyses and reported in Figs. 3–8 are available at Figshare, https://doi.org/10.6084/m9.figshare.16733185. (For technical questions regarding the web link and its content, contact Y.Y. at yihongyang@intra.nida.nih.gov.)

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1. R. A. Rudd, N. Aleshire, J. E. Zibbell, R. M. Gladden, Increases in drug and opioid overdose deaths–United States, 2000-2014. *MMWR Morb. Mortal. Wkly. Rep.* **64**, 1378–1382 (2016).

2. C. Nunes, R. Carvalho, L. Ribeiro, L. Lencastre, When acrocyanosis in intensive care unit is not due to vasopressor support. *Intensive Care Med.* **44**, 244–245 (2018).

3. C. P. O'Brien, A. R. Childress, A. T. McLellan, R. Ehrman, Classical conditioning in drug-dependent humans. *Ann. N. Y. Acad. Sci.* **654**, 400–415 (1992).
4. R. Sinha, New findings on biological factors predicting addiction relapse vulnerability. *Curr. Psychiatry Rep.* **13**, 398–405 (2011).
5. U. Shalev, M. Morales, B. Hope, J. Yap, Y. Shaham, Time-dependent changes in extinction behavior and stress-induced reinstatement of drug seeking following withdrawal from heroin in rats. *Psychopharmacology (Berl.)* **156**, 98–107 (2001).
6. I. Fredriksson *et al.*, Animal models of drug relapse and craving after voluntary abstinence: A review. *Pharmacol. Rev.* **73**, 1050–1083 (2021).
7. M. Venniro, M. L. Banks, M. Heilig, D. H. Epstein, Y. Shaham, Improving translation of animal models of addiction and relapse by reverse translation. *Nat. Rev. Neurosci.* **21**, 625–643 (2020).
8. D. H. Epstein, K. L. Preston, The reinstatement model and relapse prevention: A clinical perspective. *Psychopharmacology (Berl.)* **168**, 31–41 (2003).
9. I. Fredriksson *et al.*, Effect of the dopamine stabilizer (-)-OSU6162 on potentiated incubation of opioid craving after electric barrier-induced voluntary abstinence. *Neuropsychopharmacology* **45**, 770–779 (2020).
10. T. N. Jenkins, L. H. Warner, C. J. Warden, Standard apparatus for the study of animal motivation. *J. Comp. Psychol.* **6**, 361–382 (1926).
11. A. Cooper, N. Barnea-Ygael, D. Levy, Y. Shaham, A. Zangen, A conflict rat model of cue-induced relapse to cocaine seeking. *Psychopharmacology (Berl.)* **194**, 117–125 (2007).
12. B. B. Biswal *et al.*, Toward discovery science of human brain function. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4734–4739 (2010).
13. M. E. Raichle *et al.*, A default mode of brain function. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 676–682 (2001).
14. A. Zilverstand, A. S. Huang, N. Alia-Klein, R. Z. Goldstein, Neuroimaging impaired response inhibition and salience attribution in human drug addiction: A systematic review. *Neuron* **98**, 886–903 (2018).
15. Y. Hu, B. J. Salmeron, H. Gu, E. A. Stein, Y. Yang, Impaired functional connectivity within and between frontostriatal circuits and its association with compulsive drug use and trait impulsivity in cocaine addiction. *JAMA Psychiatry* **72**, 584–592 (2015).
16. Y. Hu *et al.*, Compulsive drug use is associated with imbalance of orbitofrontal- and prelimbic-striatal circuits in punishment-resistant individuals. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 9066–9071 (2019).
17. P. J. Tsai *et al.*, Converging structural and functional evidence for a rat salience network. *Biol. Psychiatry* **88**, 867–878 (2020).
18. H. Lu *et al.*, Rat brains also have a default mode network. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 3979–3984 (2012).
19. D. Liu *et al.*, Brain regional synchronous activity predicts tauopathy in 3×TgAD mice. *Neurobiol. Aging* **70**, 160–169 (2018).
20. D. Caprioli *et al.*, Effect of the novel positive allosteric modulator of metabotropic glutamate receptor 2 AZD8529 on incubation of methamphetamine craving after prolonged voluntary abstinence in a rat model. *Biol. Psychiatry* **78**, 463–473 (2015).
21. L. A. Sell *et al.*, Neural responses associated with cue evoked emotional states and heroin in opiate addicts. *Drug Alcohol Depend.* **60**, 207–216 (2000).
22. D. D. Langleben *et al.*, Acute effect of methadone maintenance dose on brain FMRI response to heroin-related cues. *Am. J. Psychiatry* **165**, 390–394 (2008).
23. E. Koya *et al.*, Enhanced cortical and accumbal molecular reactivity associated with conditioned heroin, but not sucrose-seeking behaviour. *J. Neurochem.* **98**, 905–915 (2006).
24. K. L. Kuntz, K. M. Patel, P. S. Grigson, W. M. Freeman, K. E. Vrana, Heroin self-administration: II. CNS gene expression following withdrawal and cue-induced drug-seeking behavior. *Pharmacol. Biochem. Behav.* **90**, 349–356 (2008).
25. S. Fanous *et al.*, Role of orbitofrontal cortex neuronal ensembles in the expression of incubation of heroin craving. *J. Neurosci.* **32**, 11600–11609 (2012).
26. D. J. Reiner *et al.*, Role of projections between piriform cortex and orbitofrontal cortex in relapse to fentanyl seeking after palatable food choice-induced voluntary abstinence. *J. Neurosci.* **40**, 2485–2497 (2020).
27. R. D. Altshuler *et al.*, Role of orbitofrontal cortex in incubation of oxycodone craving in male rats. *Addict. Biol.* **26**, e12927 (2021).
28. F. Lucantonio, T. A. Stalnaker, Y. Shaham, Y. Niv, G. Schoenbaum, The impact of orbitofrontal dysfunction on cocaine addiction. *Nat. Neurosci.* **15**, 358–366 (2012).
29. J. W. Grimm, Y. Shaham, B. T. Hope, Effect of cocaine and sucrose withdrawal period on extinction behavior, cue-induced reinstatement, and protein levels of the dopamine transporter and tyrosine hydroxylase in limbic and cortical areas in rats. *Behav. Pharmacol.* **13**, 379–388 (2002).
30. J. W. Grimm, Incubation of food craving in rats: A review. *J. Exp. Anal. Behav.* **113**, 37–47 (2020).
31. D. J. Reiner, I. Fredriksson, O. M. Lofaro, J. M. Bossert, Y. Shaham, Relapse to opioid seeking in rat models: Behavior, pharmacology and circuits. *Neuropsychopharmacology* **44**, 465–477 (2019).
32. J. R. Mantsch, D. A. Baker, D. Funk, A. D. Lê, Y. Shaham, Stress-induced reinstatement of drug seeking: 20 years of progress. *Neuropsychopharmacology* **41**, 335–356 (2016).
33. S. H. Ahmed, G. F. Koob, Cocaine- but not food-seeking behavior is reinstated by stress after extinction. *Psychopharmacology (Berl.)* **132**, 289–295 (1997).
34. Y. Buczek, A. D. Lê, A. Wang, J. Stewart, Y. Shaham, Stress reinstates nicotine seeking but not sucrose solution seeking in rats. *Psychopharmacology (Berl.)* **144**, 183–188 (1999).
35. Y. Shaham, S. Erb, J. Stewart, Stress-induced relapse to heroin and cocaine seeking in rats: A review. *Brain Res. Brain Res. Rev.* **33**, 13–33 (2000).
36. N. D. Volkow, J. S. Fowler, Addiction, a disease of compulsion and drive: Involvement of the orbitofrontal cortex. *Cereb. Cortex* **10**, 318–325 (2000).
37. I. K. Lyoo *et al.*, Prefrontal and temporal gray matter density decreases in opiate dependence. *Psychopharmacology (Berl.)* **184**, 139–144 (2006).
38. T. R. Franklin *et al.*, Decreased gray matter concentration in the insular, orbitofrontal, cingulate, and temporal cortices of cocaine patients. *Biol. Psychiatry* **51**, 134–142 (2002).
39. J. Tanabe *et al.*, Medial orbitofrontal cortex gray matter is reduced in abstinent substance-dependent individuals. *Biol. Psychiatry* **65**, 160–164 (2009).
40. K. D. Ersche *et al.*, Abnormal structure of frontostriatal brain systems is associated with aspects of impulsivity and compulsivity in cocaine dependence. *Brain* **134**, 2013–2024 (2011).
41. K. E. Courtney, D. G. Ghahremani, L. A. Ray, Fronto-striatal functional connectivity during response inhibition in alcohol dependence. *Addict. Biol.* **18**, 593–604 (2013).
42. Y. Pelloux *et al.*, Context-induced relapse to cocaine seeking after punishment-imposed abstinence is associated with activation of cortical and subcortical brain regions. *Addict. Biol.* **23**, 699–712 (2018).
43. Y. Pelloux, J. E. Murray, B. J. Everitt, Differential roles of the prefrontal cortical subregions and basolateral amygdala in compulsive cocaine seeking and relapse after voluntary abstinence in rats. *Eur. J. Neurosci.* **38**, 3018–3026 (2013).
44. Z. Shi *et al.*, The role of withdrawal in mesocorticolimbic drug cue reactivity in opioid use disorder. *Addict. Biol.* **26**, e12977 (2020).
45. X. Li *et al.*, Incubation of methamphetamine craving is associated with selective increases in expression of Bdnf and trkb, glutamate receptors, and epigenetic enzymes in cue-activated fos-expressing dorsal striatal neurons. *J. Neurosci.* **35**, 8232–8244 (2015).
46. D. Caprioli *et al.*, Role of dorsomedial striatum neuronal ensembles in incubation of methamphetamine craving after voluntary abstinence. *J. Neurosci.* **37**, 1014–1027 (2017).
47. P. W. Kalivas, The glutamate homeostasis hypothesis of addiction. *Nat. Rev. Neurosci.* **10**, 561–572 (2009).
48. M. E. Wolf, Synaptic mechanisms underlying persistent cocaine craving. *Nat. Rev. Neurosci.* **17**, 351–365 (2016).
49. Y. Dong, J. R. Taylor, M. E. Wolf, Y. Shaham, Circuit and synaptic plasticity mechanisms of drug relapse. *J. Neurosci.* **37**, 10867–10876 (2017).
50. C. Nicolas *et al.*, Longitudinal changes in brain metabolic activity after withdrawal from escalation of cocaine self-administration. *Neuropsychopharmacology* **42**, 1981–1990 (2017).
51. C. A. Orsini, L. M. Colon-Perez, S. C. Heshmati, B. Setlow, M. Febo, Functional connectivity of chronic cocaine use reveals progressive neuroadaptations in neocortical, striatal, and limbic networks. *eNeuro* **5**, ENEURO.0081-18.2018 (2018).
52. S. M. Groman *et al.*, Midbrain D3 receptor availability predicts escalation in cocaine self-administration. *Biol. Psychiatry* **88**, 767–776 (2020).
53. S. M. Groman *et al.*, Dysregulation of decision making related to metabotropic glutamate 5, but Not midbrain D3, receptor availability following cocaine self-administration in rats. *Biol. Psychiatry* **88**, 777–787 (2020).
54. T. J. Beveridge, H. R. Smith, M. A. Nader, L. J. Porrino, Abstinence from chronic cocaine self-administration alters striatal dopamine systems in rhesus monkeys. *Neuropsychopharmacology* **34**, 1162–1171 (2009).
55. H. R. Smith, T. J. Beveridge, M. A. Nader, L. J. Porrino, Effects of abstinence from chronic cocaine self-administration on nonhuman primate dorsal and ventral noradrenergic bundle terminal field structures. *Brain Struct. Funct.* **221**, 2703–2715 (2016).
56. I. Fredriksson *et al.*, Role of ventral subiculum neuronal ensembles in incubation of oxycodone craving after electric barrier-induced voluntary abstinence. bioRxiv [Preprint] (2021). https://doi.org/10.1101/2021.03.24.436801 (Accessed 1 March 2021).
57. M. Venniro, D. Caprioli, Y. Shaham, Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence. *Prog. Brain Res.* **224**, 25–52 (2016).
58. National Research Council, *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC, ed. 8, 2011).
59. A. Sumiyoshi, H. Nonaka, R. Kawashima, Sexual differentiation of the adolescent rat brain: A longitudinal voxel-based morphometry study. *Neurosci. Lett.* **642**, 168–173 (2017).
60. Z. Wang, Y. Guo, E. A. Mayer, D. P. Holschneider, Sex differences in insular functional connectivity in response to noxious visceral stimulation in rats. *Brain Res.* **1717**, 15–26 (2019).
61. L. Pritschet *et al.*, Functional reorganization of brain networks across the human menstrual cycle. *Neuroimage* **220**, 117091 (2020).
62. M. Filippi *et al.*, The organization of intrinsic brain activity differs between genders: A resting-state fMRI study in a large cohort of young healthy subjects. *Hum. Brain Mapp.* **34**, 1330–1343 (2013).
63. Y. Liu *et al.*, An open database of resting-state fMRI in awake rats. *Neuroimage* **220**, 117094 (2020).
64. G. Paxinos, C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, San Diego, CA, ed. 6, 2008).
65. H. Lu *et al.*, Registering and analyzing rat fMRI data in the stereotaxic framework by exploiting intrinsic anatomical features. *Magn. Reson. Imaging* **28**, 146–152 (2010).