Genetic background and epigenetic modifications in the core of the nucleus accumbens predict addiction-like behavior in a rat model

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This study provides a demonstration in the rat of a clear genetic difference in the propensity for addiction-related behaviors following prolonged cocaine self-administration. It relies on the use of selectively bred high-responder (bHR) and low-responder (bLR) rat lines that differ in several characteristics associated with "temperament," including novelty-induced locomotion and impulsivity. We show that bHR rats exhibit behaviors reminiscent of human addiction, including persistent cocaine-seeking and increased reinstatement of cocaine seeking. To uncover potential underlying mechanisms of this differential vulnerability, we focused on the core of the nucleus accumbens and examined expression and epigenetic regulation of two transcripts previously implicated in bHR/bLR differences: fibroblast growth factor (FGF2) and the dopamine D2 receptor (D2). Relative to bHRs, bLRs had lower FGF2 mRNA levels and increased association of a repressive mark on histones (H3K9me3) at the FGF2 promoter. These differences were apparent under basal conditions and persisted even following prolonged cocaine self-administration. In contrast, bHRs had lower D2 mRNA under basal conditions, with greater association of H3K9me3 at the D2 promoter and these differences were no longer apparent following prolonged cocaine self-administration. Correlational analyses indicate that the association of H3K9me3 at D2 may be a critical substrate underlying the propensity to relapse. These findings suggest that low D2 mRNA levels in the nucleus accumbens core, likely mediated via epigenetic modifications, may render individuals more susceptible to cocaine addiction. In contrast, low FGF2 levels, which appear immutable even following prolonged cocaine exposure, may serve as a protective factor.

addiction | dopamine | fibroblast growth factor | nucleus accumbens | reinstatement

Approximately 16% of adults in the United States report drug use within the past year (1). However, not everyone who experiments with drugs becomes an addict, as an estimated 8.5% of the population, or 25 million Americans, meet *Diagnostic and Statistical Manual of Mental Disorders IV* (2) criteria for substance abuse and dependence (1). Environmental and societal factors play a role in addiction liability (e.g., refs. 3–5), and there is ample evidence demonstrating a role for genetic factors (e.g., refs. 6–10). However, studying the interplay among these factors is difficult in human studies because of the inability to control for environmental factors and the challenge of parsing causes from consequences. Preclinical animal models are therefore essential for defining the complex interactions between genes and environment, and uncovering the neural mechanisms that might render an individual more susceptible to drug addiction.

The first animal model characterizing individual differences in the propensity to take drugs of abuse was introduced over two decades ago by Piazza et al. (11), who showed that, like humans, only some rats readily self-administer such drugs. Furthermore, this propensity to take drugs could be predicted by a behavioral trait, referred to as "sensation-seeking" (11). That is, high-responder (HR) rats, or those that exhibited increased locomotor activity when placed in a novel environment, were more likely to self-administer drugs of abuse [i.e., amphetamine, cocaine, morphine and ethanol (11–14)] relative to low-responder rats (LR), or those with low levels of activity in a novel environment. Subsequent studies showed that these rats differ in their stress response, with HRs exhibiting increased and prolonged corticosterone response to mild stress (15) and greater stress-induced elevations of dopamine activity (16) relative to LRs. Thus, these rats presented a model that captured both behavioral and neurobiological features associated with drug-taking behavior.

To better understand the genetic and neural underpinnings that might enhance the propensity to take and seek drugs, we started a colony of selectively bred HR (bHR) and LR (bLR) rat lines (17) that would provide an a priori way of predicting which rats were destined to become high- vs. low-responders. We could then exploit these selectively bred lines to examine the antecedent variables that determine the associated behavioral traits (18). Consistently, for over 40 generations, bHRs and bLRs have exhibited the key features of the outbred HR/LR rats, but there are additional characteristics that differ between the lines. In

Significance

Determining the factors that render an individual more susceptible or resilient to cocaine addiction has great implications for treatment. We exploit a unique model to demonstrate that genetic differences in vulnerability to cocaine addiction exist in the rat. We examined gene expression and the epigenetic regulation of two genes—fibroblast growth factor (FGF2) and the dopamine D2 receptor (D2)—in the nucleus accumbens core. Low levels of D2 mRNA, via epigenetic modifications, may play a role in susceptibility to cocaine addiction. Specifically, binding of a repressive mark on histones (H3K9me3) at the D2 promoter is associated with the propensity to relapse. In contrast, low levels of FGF2, which persist even following prolonged self-administration, may protect individuals from cocaine addiction.

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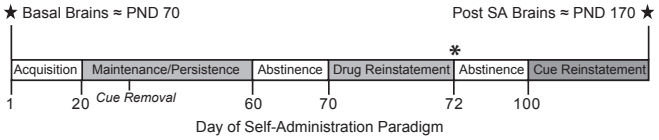

Fig. 1. Experimental timeline. A subset of bHR and bLR rats from the F24 generation was killed at baseline, around postnatal day (PND) 70. Other rats from the F24 and F26 generations underwent prolonged cocaine self-administration consisting of an acquisition phase (~days 1–20) and a maintenance phase (~days 20–60). Rats from the F24 generation were tested for the effects of cue removal (~day 30). Rats from the F26 generation were tested for drug-induced reinstatement test (~day 70), followed by 1 mo of abstinence and then a cue-induced reinstatement test (~day 100). Brains were collected ~1 wk later, when rats were ~170 d of age. The asterisk indicates the last day of cocaine exposure.

particular, bHR rats exhibit a constellation of traits relevant to addiction (18–20) that are not necessarily apparent in outbred HR rats (e.g., refs. 21–23). Relative to bLRs, bHRs are more impulsive (19), more aggressive (20), and more likely to sign-track to food- and drug-associated cues (19). Although we have previously shown that these selectively bred rats also differ in their initial propensity to take cocaine (24), the present study assesses individual differences in cocaine addiction liability by exposing bHR and bLR rats to a prolonged self-administration procedure (Fig. 1), and subsequently assessing some of the diagnostic criteria for addiction (25), including the persistence of drug-seeking behavior and, following abstinence, the propensity for drug-primed and cue-induced reinstatement, using a rat relapse model (for review, see ref. 26).

The selectively bred lines also allow us to examine the neurobiological antecedents and consequences of drug-taking and drug-seeking behaviors. We focused our analyses on the nucleus accumbens core (e.g., see Fig. S1), a brain region previously implicated in addiction-related behaviors (e.g., refs. 27 and 28) and examined gene expression and the epigenetic modification of two molecules: fibroblast growth factor (FGF2) and the dopamine D2 receptor (D2). These molecules are differentially expressed in the bHR and bLR rat lines (19, 29) and have been previously implicated in addiction-related behaviors (e.g., refs. 30–32). We have shown that administration of FGF2 early in life increases the acquisition of cocaine self-administration in adulthood (33) and renders bLRs more bHR-like (34), supporting a role for FGF2 as a neuromolecular antecedent of drug-taking behavior (18, 35). In addition, levels of association of a mark of transcriptional repression, H3K9me3, at the FGF2 promoter differs between bLR and bHR rats, and this basal epigenetic pattern is modifiable by early-life FGF2 administration (36). Furthermore, relative to bLRs, bHRs exhibit lower overall (i.e., not associated with a specific promoter) levels of H3K9me3 in the nucleus accumbens and others have reported cocaine-induced changes in this repressive mark on histones in the nucleus accumbens (37). In the present study, we further examined the role H3K9me3, and asked whether basal variations in the expression and epigenetic regulation of FGF2 or D2—that likely contribute to individual differences in the initial propensity to take drugs—are also apparent following prolonged cocaine self-administration. We also assessed which neurobiological consequences of the drug-taking experience were associated with addiction-related behaviors in our rat model.

Results

Acquisition of Self-Administration. Self-administration training was conducted in a manner that allowed us to control for the amount of drug intake and the number of drug–cue pairings (38). Imposing an infusion criterion during training also allowed us to minimize the potential impact of differences in locomotor activity on the acquisition of drug-taking behavior. Although more bLRs than bHRs failed to meet infusion criterion, there were no significant differences in the ability to learn to self-administer cocaine as measured by responses in the active and inactive nose-ports (Fig. 2*A*). That is, both phenotypes learned to distinguish between the nose-ports [Effect of Port, $F_{(1,\ 78)} = 363.2$, $P < 0.0001$] and both increased responding appropriately in the active port with increasing infusion criterion [Effect of Infusion Criterion, $F_{(3,\ 78)} = 21.1$, $P < 0.0001$]. Furthermore, as expected, pokes into the inactive port decreased or remained stable [Port × Infusion Criterion, $F_{(3,\ 78)} = 199.4$, $P < 0.0001$]. Thus, there was a significant difference in responding at the active vs. inactive port at infusion criterion 10, 20, and 45 ($P < 0.0001$).

Despite the fact that all rats were self-administering the same amount of drug, there were phenotypic differences in the rate at which they self-administered during the initial phases of training. There was a significant Effect of Phenotype [$F_{(1,\ 28)} = 13.4$, $P = 0.001$], an Effect of Infusion Criterion [$F_{(3,\ 38)} = 23.3$, $P < 0.0001$], and a significant Phenotype × Infusion Criterion interaction [$F_{(3,\ 38)} = 4.9$, $P = 0.006$] (Fig. 2*B*) for interinfusion interval. Relative to bLRs, bHRs took the drug more rapidly during the initial phases of training, at infusion criteria 5 ($P = 0.004$) and 10 ($P = 0.006$). However, after ~1 wk of training (i.e., once the rats moved up to infusion criterion 20), there were no significant differences in the rate of intake (Fig. 2*B*). Throughout the self-administration testing, a bHR was only removed from the testing chamber once a bLR had completed its session. Thus, the amount of time in the testing chamber was controlled for, as was the amount of cocaine intake and number of drug–cue pairings.

Cue Removal. The ability of the discrete cocaine-associated cue to control drug-taking behavior was assessed after ~30 self-administration sessions. When the light in the active port that typically accompanied cocaine delivery was no longer illuminated (Fig. 3*B*), bHRs took half the number of cocaine infusions per minute, whereas bLRs maintained their "baseline" number of infusions [$t_{(15)} = 3.8$, $P = 0.002$] (Fig. 3*C*). In agreement, the mean interinfusion interval doubled for bHRs when the cue light was removed [$t_{(15)} = 2.5$, $P = 0.02$] (Fig. 3*D*). Thus, the discrete cue light associated with cocaine infusions had greater control over behavior for bHRs compared with bLRs.

Persistence of Drug-Seeking Behavior. To examine differences in the persistence of cocaine seeking, we assessed the number of nose pokes in the active ports during the "drug not available"

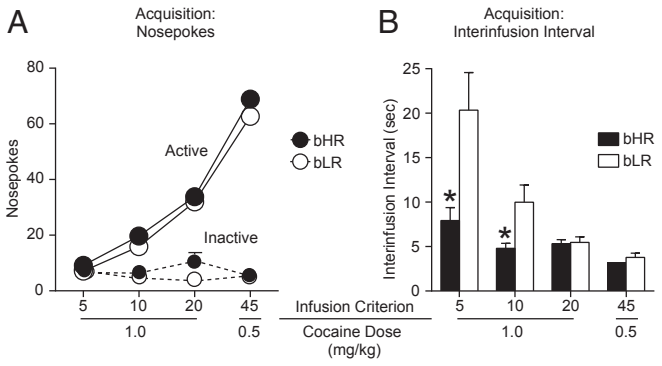

Fig. 2. Acquisition of self-administration. (*A*) Mean + SEM number of pokes in the active (solid lines) and inactive (dashed lines) ports during acquisition. Both bHRs ($n = 23$) and bLRs ($n = 17$) poke more in the active port relative to the inactive port at infusion criteria 10, 20, and 45 (Effect of Port, $P < 0.0001$). (*B*) Mean + SEM of the interinfusion interval (s) at each infusion criterion. bHRs self-administered cocaine at a faster rate at infusion criteria 5 and 10 (*$P < 0.006$).

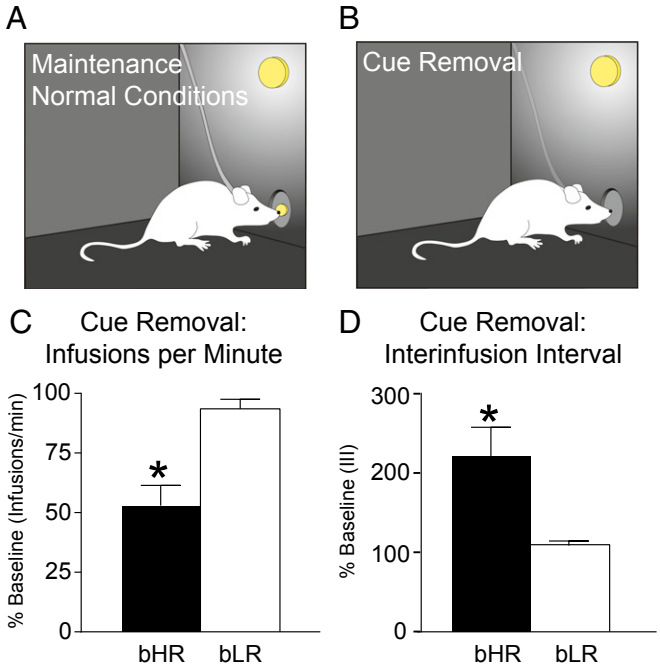

Fig. 3. Cue removal test. (*A*) Illumination of the discrete cue light upon a response in the active port under normal self-administration conditions. (*B*) The cue-removal test session, wherein the discrete cue light was no longer illuminated following a response in the active port. (*C*) Mean + SEM percent baseline responding for the number of infusions per minute for bHRs ($n = 10$) and bLRs ($n = 7$) from the F24 generation. bHRs self-administered half the number of cocaine infusions per minute when the discrete cue light was removed (bHR vs. bLR; $*P = 0.002$). (*D*) Mean + SEM percent baseline responding for interinfusion interval. bHRs took twice as long in between cocaine infusions when the discrete cue light was removed (bHR vs. bLR; $*P = 0.02$). (Illustrations adapted from ref. 79, with permission from AAAS.)

period (25), when the house light was turned off (Fig. 4*B*), and during the "drug available" period, when the house light was turned on (Fig. 4*A*). This behavior was examined across 40 self-administration sessions (after the initial training period of ~20 sessions). Following prolonged cocaine self-administration, bHRs showed enhanced cocaine-seeking behavior during the "drug not available" periods relative to bLRs (Fig. 4*E*). Importantly, these bHR/bLR differences were apparent only during the latter, and not the early, phases of cocaine self-administration. That is, bHRs begin to seek the drug when it is no longer available only after ~40 sessions [Phase × Phenotype interaction, $F_{(1,\ 25)} = 4.6$, $P = 0.04$; bHR vs. bLR at Late phase, $P = 0.03$] (Fig. 4*E*). The differences between phenotypes during the Late phase are driven by the tendency for bHRs to increase their drug-seeking behavior during the "drug not available" period over time (Early vs. Late phase, $P = 0.09$). There were no significant differences between phenotypes and no significant interactions for responding during the "drug available" phase (Fig. 4*D*). Thus, with prolonged drug experience, bHRs, but not bLRs, develop a tendency to seek drug when it is no longer available.

Cocaine Priming-Induced Reinstatement. Following varying periods of abstinence (39, 40) and extinction, priming injections of cocaine or re-exposure to a cocaine-paired cue can reinstate drug-seeking behavior, as measured by responses on the manipulandum (e.g., nose-port) that previously resulted in cocaine delivery (41, 42). This "reinstatement model" is commonly used in rodents to study relapse (for review, see refs. 26 and 43). One week after the prolonged cocaine self-administration experience, bHR and bLR rats were exposed to 11 extinction sessions conducted over 3 d, and then tested for cocaine priming-induced reinstatement. The last two extinction sessions occurred on the test day, immediately preceding the reinstatement test session. Although there were differences in behavior during extinction (Fig. 5*A*; Effect of Phenotype [$F_{(1,\ 14)} = 23.9$, $P < 0.001$], Port [$F_{(1,\ 294)} = 76.5$, $P < 0.001$], Session [$F_{(10,\ 294)} = 19.9$, $P < 0.001$, all interactions, $P < 0.05$]) these differences could be because of inherent differences in locomotor activity in the bred lines, rather than the rate of extinction per se (see *SI Results* for additional details). In fact, the significantly greater levels of responding upon initial placement in the testing chambers makes it difficult to determine if bHRs are slower to extinguish, as might be expected in an "addiction-prone" phenotype; or faster, because of the drastic decrease in responding across subsequent sessions.

Following extinction, cocaine priming-induced reinstatement was assessed using a within-session ascending dose–response procedure (similar to refs. 40 and 44). When a mixed linear-regression model was used to analyze responses in both nose-ports across cocaine doses (Fig. 5*C*), there was not a significant effect of Phenotype, and no significant interactions, but there was a significant Effect of Port [$F_{(1,\ 120)} = 5.7$, $P = 0.02$]. When the number of pokes into each port was analyzed separately, there was a significant effect of Dose for responses in the active port [$F_{(1,\ 3)} = 7.2$, $P = 0.001$]. For the inactive port, there was a significant effect of Dose [$F_{(3,\ 14)} = 5.4$, $P = 0.01$] and a significant Phenotype × Dose interaction [$F_{(3,\ 14)} = 5.7$, $P = 0.02$]. Thus, additional analyses to account for phenotypic differences in responding in the inactive port were warranted. We subsequently analyzed the number of pokes in the active port relative to those in the inactive port, and these data were square root-transformed to achieve homogeneity of variance across doses. Using the square root-transformed ratio of responding in the active/inactive port as the dependent variable, bHRs exhibited an increase in responding relative to bLRs (Fig. 5*D*) [Effect of Phenotype, $F_{(1,\ 12)} = 7.3$,

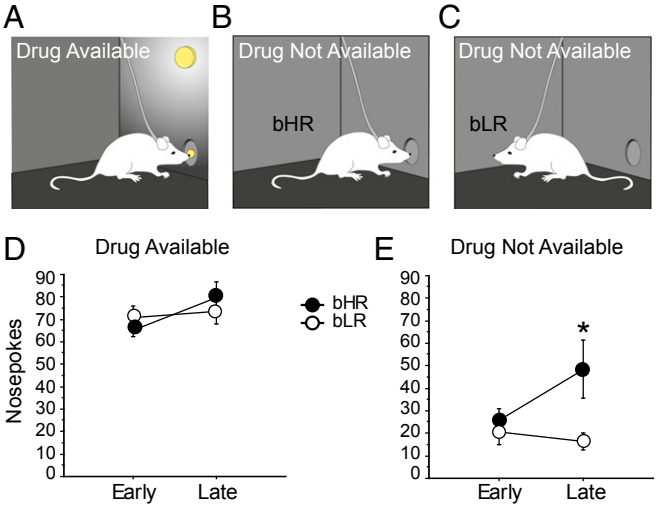

Fig. 4. Persistence of drug-seeking. (*A*) "Drug-available" signaled by illumination of the house light, during which pokes into the active port results in drug delivery accompanied by illumination of a discrete cue light. Prototypical responses during the "drug not available" period for (*B*) bHRs and (*C*) bLRs. Mean + SEM nosepokes into the active port for bHRs ($n = 14$) and bLRs ($n = 13$) from the F24 and F26 generations during (*D*) "drug-available" and (*E*) "drug not available" periods at "Early" (sessions ~15–20 of maintenance phase, ~40 sessions total) and "Late" (sessions ~35–40 of maintenance phase, ~60 sessions total) phases. With prolonged training, bHRs exhibited enhanced drug-seeking behavior relative to bLRs during the "drug not available" period (Effect of Phenotype at Late phase, $*P = 0.03$). (Illustrations adapted from ref. 79, with permission from AAAS.)

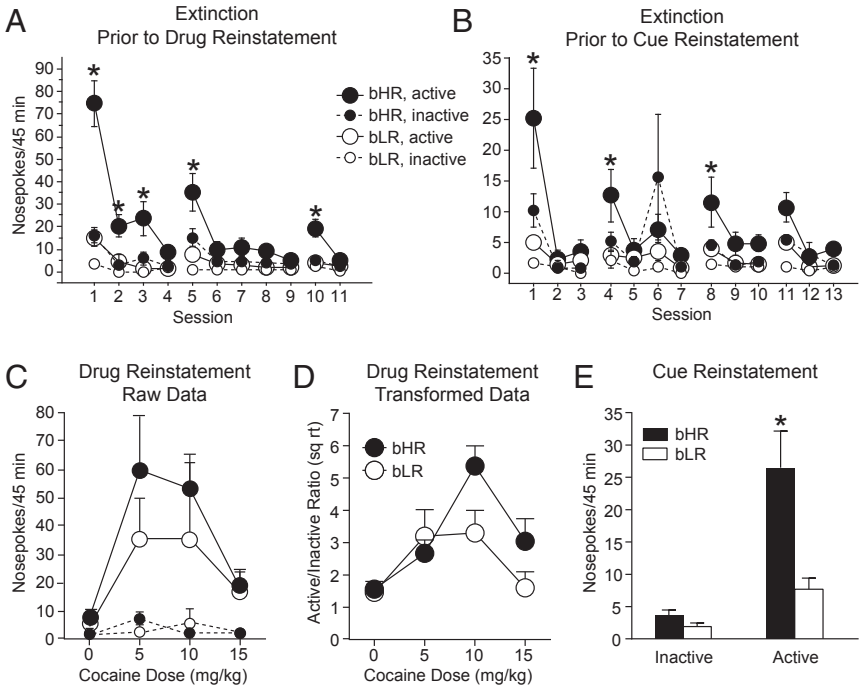

Fig. 5. Extinction and reinstatement. Mean + SEM nose pokes in the active and inactive ports for bHR ($n = 9$) and bLR ($n = 7$) rats from the F26 generation during extinction sessions conducted over a (*A*) 3-d period before the drug-induced reinstatement test, and (*B*) 4-d period before the cue-induced reinstatement test. Each day is separated by a break in the line. *$P < 0.05$, bHR vs. bLR responses in active port. (*C*) Mean + SEM number of pokes into the active and inactive ports (smaller circles, dashed lines) in response to 0, 5, 10, and 15 mg/kg cocaine during the reinstatement test. There was a significant effect of Dose for both ports ($P \leq 0.01$), but only for the inactive port was there a significant Phenotype × Dose interaction ($P = 0.02$). Pairwise comparisons revealed no significant differences. (*D*) Mean + SEM nose pokes in the ratio of responding (active/inactive port, square root-transformed) in response to vehicle (0) and 5, 10, and 15 mg/kg cocaine. There was a significant effect of Phenotype ($P = 0.02$) and Dose ($P = 0.02$), but no interaction. (*E*) Mean + SEM number of responses in the active and inactive nose-ports during cue-induced reinstatement. Both bHRs and bLRs respond more in the active vs. inactive ports ($P < 0.01$), but bHRs are much more responsive in the active port relative to bLRs (*$P = 0.01$).

$P = 0.02$], with an overall Effect of Dose [$F_{(2, 35)} = 4.3$, $P = 0.02$], but no significant Phenotype × Dose interaction. Thus, both phenotypes altered responding as a function of dose, but the difference between phenotypes was similar across doses. Importantly, when an alternative statistical approach was used in which the levels of responding in the inactive port were considered as a covariate, similar results emerged (Fig. S2). Taken together, when these data are analyzed in a manner that accounts for differences in responding at the inactive port, which may be reflective of inherent or cocaine-induced differences in activity levels (19), we find that bHRs appear to be more susceptible to drug-induced reinstatement relative to bLRs.

Cue-Induced Reinstatement. After ∼1 mo of abstinence following the cocaine priming-induced reinstatement test, rats were exposed to 13 extinction sessions conducted over the course of 4 d (Fig. 5*C*). The last three extinction sessions occurred on the test day, immediately preceding the reinstatement test. As above, there were significant differences in behavior during extinction (*SI Results*), but it is difficult to determine if these differences can be attributed to the rate of extinction per se, or inherent differences in activity.

During the cue-induced reinstatement test, responses in the active nose-port resulted in the presentation of the discrete cue light previously associated with cocaine delivery (45). Under these conditions, bHRs respond much more vigorously for presentation of the cue light relative to bLRs: Effect of Phenotype [$F_{(1, 28)} = 9.4$, $P = 0.005$]; Effect of Port [$F_{(1, 28)} = 18.3$, $P = 0.0002$]; Phenotype × Port interaction, $F_{(1, 28)} = 6.4$, $P = 0.017$] (Fig. 5*D*). Both phenotypes distinguish between the active and inactive ports ($P < 0.01$ for both); yet, there is a significant difference between bHRs and bLRs only for responses at the active port ($P = 0.01$). These data demonstrate that bHRs are more susceptible than bLRs to cue-induced reinstatement, and provide further evidence that the discrete cocaine-associated cue-light attains greater motivational value for bHRs.

Principal Components Analysis. To determine whether the behavioral outcomes described above could be reduced to fewer dimensions that might account for individual differences in addiction liability, principal components analysis was performed. When bHRs and bLRs are combined into a single population, the behavioral variables can be reduced to two factors that, together, account for 70% of the variance (Fig. 6 and Table 1). Factor 1, which accounts for 48% of the overall variance, has strong loadings (>0.7) from four behavioral variables: locomotor response to novelty, acquisition, persistence of drug-seeking behavior, and drug-induced reinstatement. Thus, in these bred lines this constellation of traits appear to be related and, when exhibited together, may contribute to addiction liability. Factor 2, which is orthogonal to Factor 1 and accounts for 22% of the total variance, is comprised largely of a single variable: cue-induced reinstatement (loading = 0.89). Cue-induced reinstatement may therefore represent a distinct behavioral variable that captures a separate dimension of addiction liability. When principal components analysis was conducted for each of the phenotypes separately, a different picture emerged, with some constructs similarly correlated within each phenotype, and others, such as the persistence of drug-seeking behavior, phenotype-dependent (Table S1).

Addiction Score: Selection of Brain Tissue. To assess the neurobiological consequences of "addiction-like" behavior, brains were

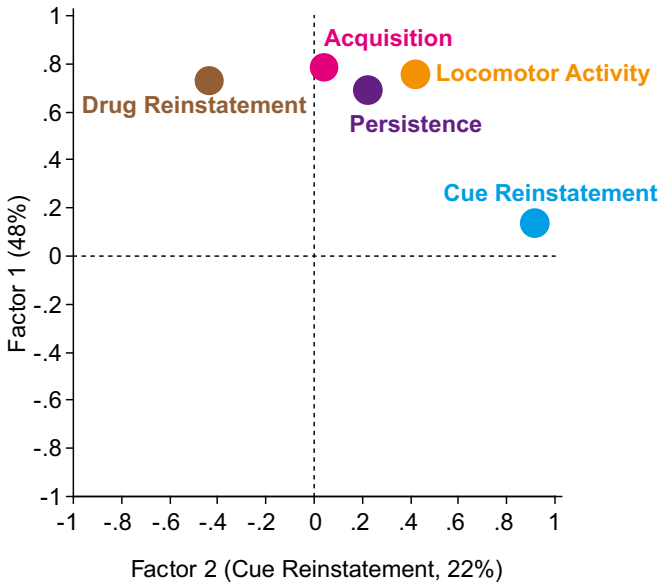

Fig. 6. Principal components analysis of the relationship between: locomotor response to novelty (orange), acquisition of cocaine self-administration (pink), persistence of drug-seeking behavior (purple), cocaine-priming induced reinstatement (brown), and cue-induced reinstatement (aqua). Two factors were revealed that, together, explained 70% of the variance in behavior. Four of the behavioral variables load heavily (>0.7) onto Factor 1, which accounts for 48% of the total variance. Factor 2, which accounts for 22% of the variance, is comprised largely of cue-induced reinstatement (loading = 0.89) (Table 1).

analyzed from six bLR rats and six bHR rats that were identified as the least or most "addiction-prone," respectively, based on a calculated Addiction Score (*SI Materials and Methods* and *SI Results*).

FGF2 in the Nucleus Accumbens Core. Consistent with our previous findings (34, 35), FGF2 mRNA levels in the core of the nucleus accumbens were higher in bHRs relative to bLRs under basal conditions [$t_{(9)} = 5.77$, $P = 0.0003$], and these differences were also apparent following prolonged cocaine self-administration [$t_{(9)} = 2.42$, $P = 0.038$] (Fig. 7*B*). In agreement, relative to bLRs, bHRs had lower levels of association of the repressive mark H3K9me3 at the FGF2 promoter under basal conditions [$t_{(9)} = 15.63$, $P < 0.0001$] and following prolonged cocaine exposure [$t_{(9)} = 9.97$, $P < 0.0001$] (Fig. 7*D*). Neither FGF2 mRNA levels nor the association of H3K9me3 at FGF2 correlated significantly with any of the "addiction-like" behaviors. Thus, the differences in FGF2 gene expression and its association with H3K9me3 represent a stable trait of the bHR and bLR phenotypes, which does not appear to account for the differences in addictive behavior per se. Nonetheless, in view of the fact that early life FGF2 administration promotes self-administration (33), we suggest that the low levels of FGF2 expression serve as a protective factor, enhancing resiliency to cocaine addiction.

Dopamine D2 Receptor in the Nucleus Accumbens Core. In agreement with our previous findings (19, 35), dopamine D2 receptor mRNA in the core of the nucleus accumbens was lower in bHRs relative to bLRs under basal conditions [$t_{(9)} = 3.55$, $P = 0.006$] (Fig. 7*F*). Following prolonged cocaine self-administration, however, these significant differences were no longer observed (Fig. 7*F*). Association of H3K9me3 at the D2 promoter was consistent with the gene-expression findings, showing greater association of this repressive mark in bHRs relative to bLRs under basal conditions [$t_{(9)} = 10.31$, $P < 0.0001$], and no significant differences following cocaine self-administration (Fig. 7*H*). Interestingly, there was a significant positive correlation between the binding ratio of H3K9me3 at D2 and the propensity for cue-induced reinstatement, as measured by the ratio of pokes in the active vs. inactive ports. This correlation is significant when the phenotypes are combined into a single population ($R^2 = 0.65$, $P = 0.001$) (Fig. 8), when they are analyzed separately in bHRs ($R^2 = 0.79$, $P = 0.02$) and bLRs ($R^2 = 0.70$, $P = 0.04$) (Fig. S3), and when the two apparent bHR outliers are removed from the combined dataset ($R^2 = 0.44$, $P = 0.04$). These analyses suggest that the greater the coupling of the histone H3K9me3 at the D2 promoter, the more likely one is to exhibit cue-induced cocaine-seeking behavior after extinction.

Discussion

The data presented here demonstrate the role of genetics in addictive behavior, as evidenced by the fact that rats selectively bred based on locomotor response to a novel environment differ on a number of behaviors reminiscent of human addiction. Relative to bLRs, bHR rats are more likely to seek cocaine when it is no longer available and more susceptible to control by cocaine itself and cocaine-associated cues. That is, following prolonged cocaine self-administration, bHR rats exhibit: (*i*) attenuated drug-taking behavior when the discrete drug-associated cue-light is removed; (*ii*) increased drug-seeking behavior when drug delivery is not available; and (*iii*) increased propensity for reinstatement of drug-seeking behavior.

The selective breeding of these rat lines, in conjunction with the fact that we controlled for the amount of cocaine intake throughout the self-administration period, provide a unique opportunity to examine the neurobiological antecedents and consequences of addiction liability and prolonged cocaine self-administration. We extended previous findings (36), showing that, relative to bHRs, bLRs have lower levels of FGF2 mRNA and greater levels of association of the H3K9me3 at the FGF2 promoter, specifically in the nucleus accumbens core, and these differences persist following prolonged cocaine exposure. Also in agreement with previous findings (19, 35), we show that, relative to bLRs, bHRs have lower levels of D2 mRNA in the nucleus accumbens core under basal conditions. Novel to the present study, we demonstrate that, relative to bLRs, bHRs showed greater levels of association of H3K9me3 at the D2 promoter in the nucleus accumbens core, and these phenotypic differences in gene expression and epigenetic regulation of D2 were no longer observed following prolonged cocaine self-administration. Perhaps most interestingly, however, we found that the association of H3K9me3 at the D2 promoter is positively correlated with cue-induced drug-seeking or the propensity to relapse following 1 mo of abstinence. These findings suggest that cocaine addiction liability may, in part, be driven by epigenetic modifications that result in low levels of the dopamine D2 receptor in the nucleus

Table 1. Principal components analysis, behavioral variables

Behavioral variables	Factor 1	Factor 2
Locomotor response to novelty	**0.79**	0.42
Acquisition	**0.80**	0.03
Persistence of drug-seeking	**0.72**	0.21
Drug-induced reinstatement	**0.73**	−0.43
Cue-induced reinstatement	0.12	**0.89**
Eigenvalue	2.41	1.12
Percent of variance (%)	48.15	22.45

Factor loadings from the rotated component matrix for the combined population of bHRs and bLRs. Loadings >±0.7 are shown in bold and plotted in Fig. 7. Seventy percent of the total variance in behavior is accounted for by the two factors.

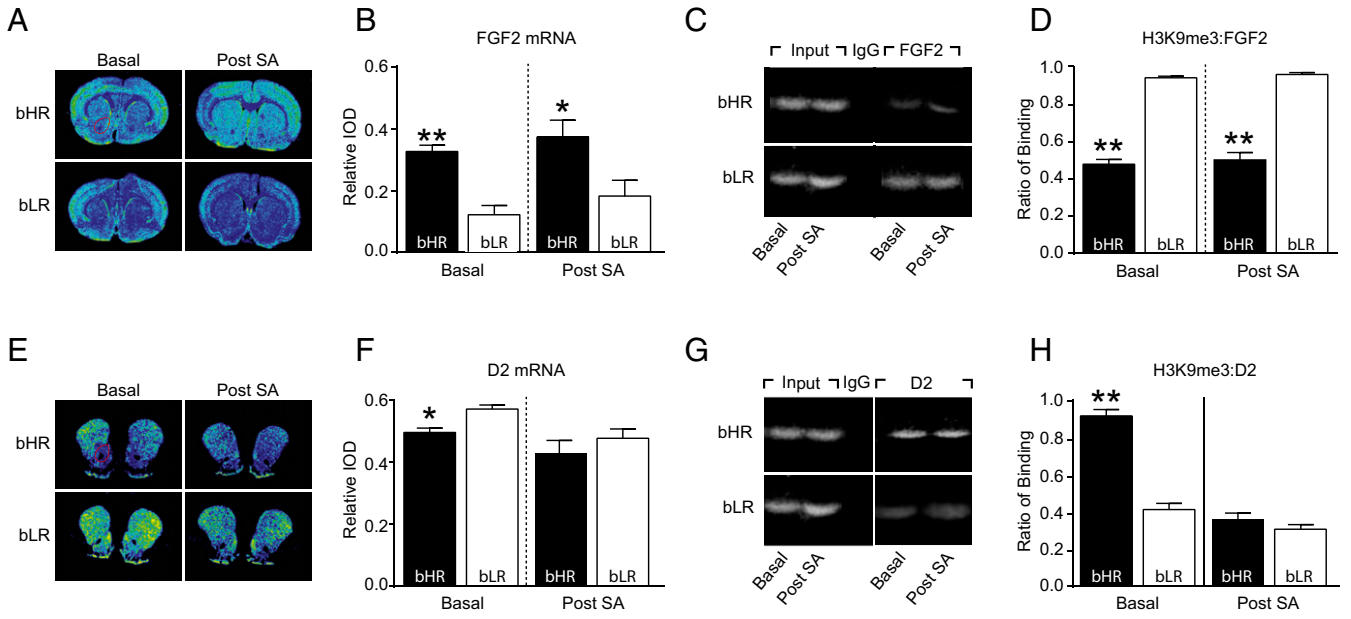

Fig. 7. FGF2 and D2 gene expression and epigenetic regulation in the nucleus accumbens core. (*A*) Color-enhanced in situ images of FGF2 mRNA expression in bHRs and bLRs under basal conditions and following prolonged self-administration (Post-SA). The nucleus accumbens core is outlined in red in the *Upper Left* image. (*B*) Mean + SEM relative integrated optical density (IOD) of FGF2 mRNA for bHRs and bLRs under basal conditions ($n = 6$ per phenotype) and following self-administration (bHRs, $n = 6$; bLRs, $n = 5$; $^{**}P < 0.001$; $^{*}P < 0.05$). (*C*) Representative gel image from ChIP assay showing the internal control (input) of extracted chromatin, the IgG (negative control), and the immunoprecipitated chromatin with the anti-H3K9me3 for FGF2. (*D*) Mean + SEM ratio of binding (normalized to input) for H3Kme3 at the FGF2 promoter for bHRs and bLRs under basal conditions ($n = 6$ per phenotype) and following prolonged cocaine self-administration ($n = 6$ per phenotype; $^{**}P < 0.001$). (*E*) Color-enhanced in situ images of D2 mRNA expression in bHRs and bLRs under basal conditions and Post-SA. The nucleus accumbens core is outlined in red in the *Upper Left* image. (*F*) Mean + SEM relative IOD of D2 mRNA for bHRs and bLRs under basal conditions ($n = 6$ per phenotype) and following self-administration ($n = 6$ per phenotype; $^{*}P < 0.01$). (*G*) Representative gel image from ChIP assay showing the internal control (input) of extracted chromatin, the IgG (negative control), and the immunoprecipitated chromatin with the anti-H3K9me3 for D2. Note that the input and IgG bands are the same as those in *C*, because all were run on the same gel to generate a sample image. (*H*) Mean + SEM ratio of binding (normalized to input) for H3K9me3 at the D2 promoter for bHRs and bLRs under basal conditions ($n = 6$ per phenotype) and following prolonged cocaine self-administration ($n = 6$/phenotype; $^{**}P < 0.001$).

accumbens core. In contrast, low FGF2 levels in the nucleus accumbens core may serve as a protective factor, decreasing the likelihood that an individual will "transition" to cocaine addiction.

Individual Differences in Cocaine Addiction Liability. Over the years, preclinical studies have shifted the focus from the initiation of drug-taking behavior to compulsive drug use (25, 46–49). One prominent example is a study demonstrating that the diagnostic criteria for addiction could be modeled in rats (25). With the emergence of this animal model of "addiction," it was reported that locomotor response to novelty—or "sensation-seeking" as measured in the rat—is not associated with addiction liability per se (21, 25, 50), but does remain a good predictor of the initial propensity to take drugs (22, 51). Although the design of the present study did not permit phenotypic differences to emerge in the acquisition of drug-taking behavior as it is typically assessed (i.e. in number of infusions), we did find that bHRs initially self-administered cocaine at a faster rate than bLRs, and more bLRs than bHRs failed to meet infusion criteria early in training. These results support the notion that locomotor response to novelty is associated with the initial propensity to take psychostimulant drugs (11).

Although willingness to experiment with illicit drugs is a critical element contributing to vulnerability to substance abuse (e.g., ref. 50), elucidating the factors contributing to the conversion from initial drug-taking behavior to addiction is of great value for potential therapeutic interventions. To assess the "transition" to cocaine addiction, we examined the persistence of cocaine-seeking behavior, or the inability to refrain from drug-seeking. In support of previous studies (25, 52), we found that only after prolonged self-administration experience did rats begin to exhibit addiction-like behaviors: seeking cocaine when it was not available. However, only bHRs showed evidence for this "switch" to persistent cocaine use. Thus, although the behavior of bLRs was relatively stable throughout the maintenance phase, with prolonged experience bHRs showed an enhanced tendency to increase drug-seeking behavior.

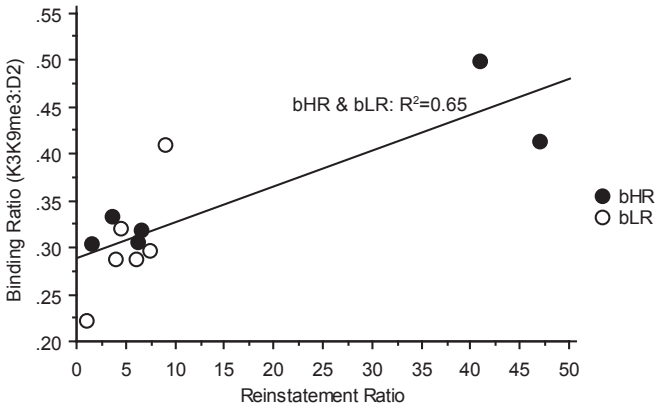

Fig. 8. Correlation between cue-induced reinstatement of cocaine seeking and epigenetic regulation of the dopamine D2 receptor. There is a significant positive correlation between the association of H3K9me3 at the D2 promoter and the ratio of active/inactive nose pokes (i.e., reinstatement ratio) during the cue-induced reinstatement test ($n = 12$, $P = 0.001$). This correlation is also significant when the two apparent bHR outliers are removed from the combined dataset ($n = 10$, $P = 0.04$).

Because relapse is one of the biggest problems in the treatment of addiction (53), and most often triggered by environmental cues that have previously been associated with the drug-taking experience (54–56), we examined the ability of the drug-associated cue to control behavior. Even during relatively early phases of self-administration training, the drug-associated cue acquired the ability to control cocaine-taking behavior of bHRs, but not bLRs (similar to refs. 38 and 57). bHRs were also more susceptible to cue-induced reinstatement after 1 mo of forced abstinence. During the cue-induced reinstatement test, bHRs responded ∼four times more than bLRs for presentation of the discrete cue light in the absence of cocaine reward. These findings are in agreement with those showing that bHRs have a greater propensity to attribute incentive salience to drug-associated cues (19). Taken together, these data suggest that the ability of the discrete drug-associated cue to control behavior contributes, at least in part, to the addictive behaviors exhibited by bHRs.

The findings reported here are in contrast to those in outbred rats, for which there is no association between addiction liability and locomotor response to novelty (21, 22, 25, 50). It is important to reiterate, however, that the bred lines exhibit differences in other traits that have been associated with addiction liability, including impulsivity (19), and these differences are not necessarily apparent in outbred LR/HR rats (22, 23; see also ref. 18). Furthermore, the principal components analysis conducted here revealed a relationship between locomotor response to novelty, acquisition of drug-taking behavior, the persistence of drug-seeking behavior, and drug-induced reinstatement, all of which loaded onto a single factor accounting for close to 50% of the variance in behavior. Thus, at least in these bred lines, this constellation of traits, when expressed together, may contribute to addiction liability.

Neurobiological Antecedents and Consequences of Cocaine Addiction Liability. To examine neurobiological antecedents that might contribute to individual differences in the initial propensity to take drugs, we examined brains from a subset of bLR and bHR rats that were killed under basal conditions at ∼70 d old, the same time that selectively bred counterparts began self-administration. Because the consequences of the prolonged cocaine exposure were assessed in the brains of rats that were ∼170 d old, we cannot completely rule out the possibility that aging might have contributed to phenotypic differences that were apparent at the latter time point and not under basal conditions. However, the pattern of differences we found does not support this explanation. Furthermore, the aim of the study was not to compare across time points, but to instead examine what phenotypic differences exist before and then following the cocaine-taking experience.

The role of FGF2 in cocaine addiction liability. Basic fibroblast growth factor (bFGF or FGF2) is a neurotrophic factor that is necessary for the survival, growth and differentiation of neurons (58, 59). Several studies have implicated FGF2 in psychomotor sensitization and drug-induced neuronal plasticity (60–64); and our previous findings suggest that FGF2 is a neuromolecular antecedent of drug-taking behavior (18). That is, early-life FGF2 administration increases the acquisition of cocaine self-administration (33) and renders bLRs more bHR-like, increasing their response to repeated cocaine exposure (35). Furthermore, we recently found that the long-term effects of early life FGF2 treatment in the bred lines might be mediated by H3K9me3 (36). In agreement, the present findings suggest that low levels of FGF2 in the nucleus accumbens core, accompanied by greater association of H3K9me3 at the FGF2 promoter, plays a protective role, preventing the transition from initial cocaine use to addiction. Indeed, this was the neuromolecular profile of bLRs, both before and after prolonged cocaine exposure.

The role of the dopamine D2 receptor in cocaine addiction liability. The dopamine D2 receptor has been heavily implicated in addiction and related disorders (65–67). Specifically, low availability of striatal D2 receptors has been reported in human cocaine abusers (68–70) and associated with increased rates of cocaine self-administration in nonhuman primates (71, 72). Although these studies report levels of D2 availability as a consequence of cocaine exposure, Dalley et al. found lower levels of D2/D3 receptor availability in impulsive rodents that were never exposed to cocaine, but known to have an increased propensity for cocaine self-administration (73), suggesting that low levels of D2 availability may also be an antecedent of drug-taking behavior. In support, here we show that bHRs have lower baseline levels of D2 mRNA relative to bLRs, with accompanying differences in the association of H3K9me3 at the D2 promoter. These differences are consistent with our previous findings showing that bHRs and bLRs differ in their baseline dopaminergic profile (19). Interestingly, however, the phenotypic differences in D2 mRNA and the association of H3K9me3 were no longer apparent following prolonged cocaine self-administration. Although these findings may seem incongruent with reports of low D2 receptor availability observed in human cocaine addicts during or after cocaine exposure (68, 69), the relationship between mRNA expression levels and the index of D2 receptor availability used in imaging studies is unknown.

Given the magnitude of the phenotypic differences in the association of H3K9me3 at D2 under basal conditions, it is surprising that there are no observed differences following prolonged cocaine self-administration. On the other hand, although our study was not designed to compare across time points, these findings suggest that, in individuals prone to cocaine addiction, the drug may affect the epigenetic modification of the dopamine D2 receptor in a manner that facilitates compulsive use. In agreement, the epigenetic regulation of D2 was the only neuromolecular correlate of addictive behavior, as it was strongly correlated with cue-induced reinstatement. That is, the higher the levels of binding of H3K9me3 at the D2 promoter, the more robust drug-seeking behavior was. This finding is especially intriguing because cue-induced reinstatement represented a distinct dimension of the principal components analysis. Thus, the association of H3K9me3 at the D2 promoter might be a key factor in cocaine addiction liability, and especially in the propensity to relapse; and should therefore be considered as a potential neuromolecular target for the treatment of cocaine addiction.

Summary

In sum, using rats that are selectively bred based on locomotor response to novelty, we demonstrated differences in the genetic predisposition for cocaine addiction. bHR rats exhibit greater addictive behavior relative to bLR rats, as measured by the ability of a cocaine-associated cue to control behavior, the persistence of cocaine-seeking behavior, and an increased propensity for reinstatement of drug-seeking behavior. Furthermore, differential patterns of FGF2 expression and its epigenetic regulation in the nucleus accumbens core exist before and following prolonged cocaine self-administration, whereas differential patterns in expression and epigenetic regulation of the dopamine D2 receptor were only evident before the cocaine-taking experience. Interestingly, however, the epigenetic regulation of D2 seems to be predictive of the propensity for cue-induced reinstatement or relapse. Taken together, these findings suggest that low D2 mRNA levels resulting from epigenetic modifications in the nucleus accumbens core is associated with greater susceptibility, whereas low levels of FGF2 and its epigenetic regulation may protect individuals from cocaine addiction.

Materials and Methods

A timeline of the experimental design can be found in Fig. 1. See *SI Materials and Methods* for details. All procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Selectively Bred Rats (bHR and bLR). bHR and bLR male rats from the 24th and 26th generations of our in-house breeding colony (74) were used.

Prolonged Cocaine Self-Administration Procedures.

Acquisition. The start of a session was signaled by illumination of the house light. A nose poke into the port designated "active" resulted in an intravenous infusion of cocaine hydrochloride dissolved in 0.9% sterile saline (1.0 mg/kg per infusion in 25 μL delivered over 1.6 s) on a fixed ratio (FR) 1 schedule. Concurrent with the delivery of cocaine was illumination of a discrete cue light located inside the active nose-port. This light remained on for an additional 18.4 s, resulting in a 20-s timeout period during which nose pokes were recorded, but without consequence.

To control for the number of infusions and the number of drug–cue (i.e., active nose-port light) pairings, we imposed an infusion criterion (similar to refs. 38 and 57) such that session length was determined by how long it took the rats to reach a given criterion. Rats remained at each infusion criterion for at least two sessions, or until they reached the criterion for two consecutive sessions. All rats were initially allowed to take five infusions, and the infusion criterion was then increased to 10, 20, and 45. At 45 the cocaine unit dose was lowered to 0.5 mg/kg (75), which was then used for the remainder of the self-administration procedure.

Cue removal test. Rats from the 24th generation underwent the cue-removal test. After rats showed stable behavior during the "drug-available" vs. "drug-not-available" sessions, they were switched back to the original infusion criterion 45 schedule for four sessions, during which drug was available the entire session. Thus, the cue removal test occurred after ∼30 self-administration sessions. The test session consisted of removal of the discrete cue light located inside the active port, which previously signaled drug delivery (Fig. 3). The session terminated when the rats self-administered 45 infusions, similar to the baseline sessions, keeping the total number of infusions constant. The rate at which cocaine was self-administered during the test session relative to baseline (the session immediately preceding the test session) was used to assess the ability of the discrete cue to control drug-taking behavior.

Persistence of drug-taking behavior. The prolonged self-administration procedure was adapted from ref. 25. Following the acquisition phase (i.e., after ∼20 d), daily sessions continued for ∼40 additional days. Each session during this maintenance period consisted of three "drug-available" periods that were signaled by house light illumination. Once rats received 15 infusions during the drug-available period, the house light was turned off and a "drug not available" period commenced. There were three "drug not available" periods in a given session, and each was 15 min in duration. The total length of the sessions was dependent on how long it took the rats to self-administer the 45 infusions (i.e., 15 infusions per each "drug-available" period).

Cocaine priming-induced reinstatement. After ∼60 self-administration sessions, rats were exposed to 1 wk of abstinence and then underwent extinction, during which the house light was illuminated, but responses in both active and inactive nose-ports were without consequence. Extinction sessions were conducted until all rats were consistently responding at "baseline" levels, (i.e., <10 responses per session for at least two consecutive sessions). This resulted in 11 45-min extinction sessions over a 3-d period (Fig. 5*A*). Rats were tested for reinstatement induced by cocaine-priming injections during four 45-min sessions, similar to Lu et al. (40). All rats received 0, 5, 10, and 15 mg/kg cocaine in escalating order before each 45-min session. The reinstatement test sessions were identical to the extinction sessions such that responding in the nose-ports was without consequence. Reinstatement was measured as the number of pokes in the active vs. inactive nose-port (i.e., active/inactive ratio) following each dose of cocaine.

Cue-induced reinstatement. After ∼1 mo of abstinence following prolonged self-administration, the rats were exposed to another 13 45-min extinction sessions conducted over a 4-d period (Fig. 5*C*). The cue-induced reinstatement test was similar to that described by Grimm et al. (39), and began with illumination of the house light and presentation of the discrete cue light in the active nose-port that had previously accompanied cocaine infusion. Following the first nose poke in the active port, each subsequent cue presentation was contingent upon responses (FR1) into this port. Thus, rats were responding for presentation of the discrete cue light, similar to a conditioned reinforcement procedure. The cue-induced reinstatement session was 45 min in duration and during this time nose pokes into the active and inactive ports were recorded.

Brain Tissue Processing. Details of tissue processing are provided in the *SI Materials and Methods*.

Laser Capture Microdissection for Chromatin Immunoprecipitation. For laser-capture microdissection (LCM) for chromatin immunoprecipitation (ChIP), 10-μm-thick coronal sections were cross-linked in 1% paraformaldehyde for 15 min at 25 °C and after proper washes, LCM was performed as described in *SI Materials and Methods*. For each rat, ∼seven slides were used, resulting in the bilateral collection of 40–50 nucleus accumbens cores per rat for the ChIP assays.

ChIP Assays. Chromatin was extracted and sheared (Branson) from the LCM-captured tissue. The amount of DNA was quantified and one-fifth of the lysate was aliquoted for "input" before immunoprecipitation. Chromatin from each sample was then subjected to immunoprecipitation using anti-H3K9me3 (Abcam). Protein-DNA-antibody complexes were precipitated with Dynabeads@protein A (Invitrogen) for 2 h at 4 °C, then eluted and reverse cross-linked with 0.3 M NaCl at 65 °C overnight. Proteins were digested with proteinase K for 1 h at 45 °C. The DNA was extracted and purified (Qiagen). Specific primers (*SI Materials and Methods* and Table S2) directed to the gene promoters for FGF2 or D2 were used for amplification. Following PCR, the input and immunoprecipitated DNA for each sample were run on 1% agarose gels and quantified using ImageJ (National Institutes of Health Image software). The amount of immunoprecpitated DNA was normalized to the input for each sample.

In Situ Hybridization Histochemistry. In situ hybridization was performed as previously described (76, 77) using brain sections adjacent to those used for LCM. Postfixed sections were hybridized with a ^{35}S-labeled cRNA probe directed against rat FGF2 mRNA or D2 mRNA. Following posthybridization rinses and dehydration, slides were opposed to Kodak Biomax MR film (Eastman Kodak). Autoradiograms were captured and digitized and the magnitude of the signal from the hybridized ^{35}S-cRNA probe was determined using ImageJ (National Institutes of Health Image software) (*SI Materials and Methods*). One bLR sample from the post self-administration group had to be eliminated from the FGF2 mRNA analysis because of poor tissue quality.

Statistical Analyses.

Behavioral data. Linear mixed-effects models (78) were used to examine significant main effects (e.g., Phenotype, Session) and interactions for behavioral data with repeated longitudinal measures. The covariance structure was explored and modeled appropriately for each dependent variable and when significant interactions were detected Bonferroni post hoc comparisons were conducted. Independent *t* tests were used to examine phenotypic differences on the cue-removal test. A repeated measures two-way (Phenotype × Phase) ANOVA was used to examine drug-seeking behavior during the different periods ("drug available" and "drug not-available") of the maintenance phase, and a two-way (Phenotype × Nose-Port) ANOVA was used to assess differences in nose poke responding between bHRs and bLRs during the cue-induced reinstatement test.

Principal components analysis was used to reduce the behavioral data to fewer dimensions and to identify underlying constructs. The behavioral variables analyzed were: (*i*) locomotor response to novelty, (*ii*) acquisition, (*iii*) persistence of drug-seeking, (*iv*) drug-induced reinstatement, and (*v*) cue-induced reinstatement. The formulas used to generate each of these measures are described in *SI Materials and Methods*.

Neurobiological data. For data generated by ChIP assays and in situ hybridization, phenotypes were compared at baseline or following prolonged self-administration using independent *t* tests.

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Supporting Information

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SI Materials and Methods

Selectively Bred Rats (bHR and bLR). bHR and bLR male rats from the 24th (bHR, $n = 11$; bLR, $n = 11$) and 26th (bHR, $n = 19$; bLR, $n = 18$) generations of our in-house breeding colony (74) were used for these studies. These rats are bred based on locomotor response to an inescapable novel environment, as previously described (17). The founding population of these lines was comprised of Sprague–Dawley rats purchased from three different breeding colonies at Charles River Laboratories and a number of measures were taken to maximize initial genetic variation and to minimize inbreeding in these lines (17). Males and females that fall within the top and bottom 20% of locomotor scores are selected for breeding, allowing us to predict with almost 100% certainty that bLR offspring will be generated from a bLR/bLR breeding pair and bHR offspring from a bHR/bHR breeding pair (17, 18). Nonetheless, before the start of these studies, around 55 d of age, the bHR/bLR phenotype was confirmed by testing rats for locomotor response to a novel environment, as previously described (17, 19). Rats were housed in pairs of the same phenotype and kept on a 12-h–light/12-h–dark cycle with controlled temperature and humidity.

Prolonged Self-Administration Procedures. Self-administration training began around 80 d of age, following recovery from surgery (see below). For the duration of the self-administration studies, rats were singly housed and a controlled feeding procedure was implemented such that ∼25 g of chow was provided after each self-administration session. This feeding procedure allowed rats to maintain bodyweight throughout the prolonged self-administration studies and prevented loss of catheter patency because of excessive growth. The controlled feeding schedule also helped ensure that the rats were motivated to self-administer cocaine. All procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Jugular catheterization. Rats underwent jugular catheterization surgery around 70 d of age, as previously described (80, 81). Catheters and backports were made in-house. Briefly, one end of silicone catheter was inserted into the external jugular vein and the other was passed subcutaneously to exit the back of the rat, where it was connected to a pedestal constructed from a 22-gauge cannula and connected to a piece of polyethylene mesh using dental cement. Rats were administered 0.1 mL of a mixture containing gentamicin (10 mg/mL) and heparin (20 USP units) in bacteriostatic saline the day of surgery and for 14 d after surgery. Every day thereafter, 0.1 mL of the heparin solution (20 USP units) was administered before and immediately following each self-administration session.

Catheter patency. Catheter patency was checked on a weekly basis by injecting 0.1 mL of the short-acting barbiturate, sodium thiopentothal (intravenously, 20 mg/mL in sterile water). Rats that became ataxic within 5 s were considered to have patent catheters. If behavioral changes suggested loss of patency, the catheter was immediately checked and, under some circumstances, the rat would undergo additional surgery to repair or replace the catheter. Most often, however, rats were eliminated from the study if catheter patency was lost. In total, 10 bHRs and 6 bLRs were eliminated because of loss of catheter patency. Data were excluded from these rats in the week that preceded the failed patency test and thereafter. Thus, the number of rats per phenotype differed throughout the self-administration paradigm depending on whether or not their catheters were considered patent.

Apparatus. Self-administration training and testing occurred in standard (21.6 × 17.8 × 12.7 cm) test chambers (Med Associates) located inside sound-attenuating cabinets equipped with a fan to mask background noise. Two nose-poke ports were located 3 cm above the stainless steel grid floor on a wall opposite the house light. The cocaine infusion was delivered by an external pump connected to a tube that attached to the back port located in the midscapular region of the rat. The infusion tube was suspended in the chamber via a swivel mechanism, allowing the rat to move freely. Behavioral measures were recorded using Med Associates software. Nose pokes into the port designated "inactive" were recorded, but without consequence.

Addiction Score: Selection of Brain Tissue. The Addiction Score was calculated based on the sum of the following measures: (*i*) Inverse Acquisition Score = (1/average intertrial interval at infusion criteria 5 and 10); Persistence of Drug Seeking = [(average of pokes in active port during "drug not available"/pokes in active port during "drug available" for sessions ∼35–40 of maintenance phase) × 100]; Drug-induced reinstatement = [(average [pokes in active port/pokes in inactive port in response to 5, 10, or 15 mg/kg cocaine]/(pokes in active port/pokes in inactive port in response to vehicle)]; Cue-induced reinstatement = (average pokes in active port/pokes in inactive port). Thus, higher values represented rats with the most pronounced drug-taking and drug-seeking behaviors.

Brain Tissue Processing. Rats from the 26th generation that underwent the prolonged self-administration procedures were killed ∼1 wk following the cue-induced reinstatement test. Thus, ∼1 mo elapsed between the last drug experience (i.e., cocaine priming-induced reinstatement) and the time of tissue collection. After self-administration, tissue was collected and analyzed from the six rats per phenotype, chosen based on their Addiction Score.

At the time of killing, brains were removed, rapidly frozen in isopentane and then stored at −80 °C until further processing. Brains were cut on a cryostat into 10-μm-thick coronal sections and mounted onto slides in a serial manner. The serial method we used resulted in multiple sets of slides with adjacent sections that could be used for cresyl-staining, LCM, and in situ hybridization, as described below. Brain sections were obtained throughout the rostral-caudal gradient of the brain, but the focus of the current studies was on the nucleus accumbens core (between bregma 1.7 and 1.0 mm).

ChIP Assays. Following LCM, the extracted tissue for each rat was pooled and suspended in 500 μL of nuclear lysis buffer. The nuclear extract was quantified using Qubit Fluorometric Quantitation and an equal amount of nuclear extract was added for the ChIP assays. The putative promoter regions were identified from the National Center for Botechnology Information gene bank and the primers were selected using software from Thermo Fisher Scientific (tools.thermofisher.com/content.cfm?pageid=9716). For FGF2 and D2, the promoter sequences were obtained from GenBank ID-U78079.1 and GenBank ID-U79717.1 and the primers directed to the gene promoters listed in Table S2 (which were further BLAST with the promoter sequence) were used for amplification. For PCR, Taq MeanGreen Master Mix (Syzygy Biotech) was used. For the thermal cycler reaction, the mix was first subjected to 94 °C for 3 min, followed by denaturation at 94 °C for 30 s Annealing was done at 58 °C for 40 s and extension at 72 °C for 2 min. Cycles were repeated 34 times for FGF2 and 31 times for D2, followed by final extension at 72 °C for 7 min.

The amplified products were mixed with loading dye and run in 1% agarose gel containing ethidium bromide. The gels were imaged with the Spectroline UV Transilluminator 2.5 Amp, Model UC-312R attached to a camera (Kodak DC 290: Kodak Electrophoresis Documentation and Analysis System 120), and the software used to capture the image before analysis was Kodak 1D Limited Edition v3.6.1. The total DNA (input) and immunoprecipitated DNA was quantified using ImageJ (National Institutes of Health Image software). We report the binding ratio of H3K9me3 normalized to input for each target of interest.

In Situ Hybridization Histochemistry. The D2 receptor probe was a 495-bp fragment directed against the rat D2 mRNA, and the FGF2 probe was a 278-bp fragment directed against rat FGF mRNA. The specificity of the hybridization signal was previously confirmed by control experiments using sense probes (19). Each probe was diluted in hybridization buffer, and brain sections were coverslipped and incubated overnight at 55 °C. Slides were rinsed and dehydrated and sections were exposed to film for 7 d for detection of D2 mRNA and for 13 wk for FGF2 mRNA. Autoradiograms were captured and digitized using Microtek ScanMaker 1000XL and the scanner was driven by Lasersoft Imaging (SilverFast) software.

A macro (Serge Campeau, University of Colorado, Boulder, CO) was integrated into the ImageJ software, which enabled signal above background to be automatically determined. The relative IOD of these signal pixels was obtained by multiplying the size of the area quantified by the signal intensity. The signal for each probe was quantified in the core of the nucleus accumbens (between Bregma levels 1.7 and 1.0). Optical density measurements were taken from the left and right sides of at least two brain sections per animal. A mean IOD value was then generated for each region of interest to yield one data point per animal. Independent *t* tests were used to examine phenotypic differences for D2 and FGF mRNA at baseline and following prolonged self-administration.

Statistical Analysis. As an alternative approach for accounting for differences in responding in the inactive port during the cocaine priming-induced reinstatement test, we ran an ANCOVA using SPSS (v22) that incorporated the number of pokes into the inactive port as a continuous covariate, with pokes into the active port as the dependent variable. Error terms within a rat were allowed to have a general (unstructured) covariance structure, with different error variances for different doses and general covariances of the repeated measures. The existence of negative covariances between some of the errors precluded the use of mixed-effects (or multilevel) models with random rat effects.

SI Results

Acquisition of Self-Administration. Three bLRs were excluded from the study for never reaching five infusions, and one bHR and three bLRs were excluded after they consistently failed to reach 10 infusions at IC10.

Cocaine Priming-Induced Reinstatement.

Extinction. During extinction, responses in the nose-ports were recorded, but without consequence. When the total number of responses across all extinction sessions was examined, there was a significant effect of Phenotype [$F_{(1,\ 28)} = 32.6$, $P < 0.001$], Port [$F_{(1,\ 28)} = 22.2$, $P < 0.001$], and a Phenotype × Port interaction [$F_{(1,\ 28)} = 9.3$, $P = 0.005$]. Post hoc analyses revealed that bHRs responded significantly more in both the active and inactive ports ($P < 0.01$), but both phenotypes responded more in the active port relative to the inactive port ($P < 0.05$).

When responding was considered with Session as the repeated variable, the most pronounced and significant differences in responding in the active port between phenotypes occurred during the first three sessions ($P \leq 0.002$) and then on sessions 5 ($P < 0.001$) and 10 ($P = 0.003$), which were the first sessions conducted on a given day. Furthermore, there were also significant differences between bHRs and bLRs in responding in the inactive port on sessions 1 ($P = 0.01$) and 5 ($P = 0.006$). The greater activity for bHRs upon initial placement into the testing chamber on a given day is not surprising given their general hyperaroused state and delayed patterns of locomotor habituation to a given environment (19). Thus, it is difficult to parse differences in locomotor activity from differences in the rate of extinction.

Test. It should be noted that the raw data (Fig. 5*D*) indicate a decrease in responding in the active port at higher doses of cocaine, which is not necessarily consistent with what one might expect. However, we suspect that this decrease in responding is because of some rats going into stereotypy at locations distal from the port, as a result of the repeated escalating doses of cocaine. Unfortunately, the behavior of the rats was not videotaped, so this is merely speculation based on real-time observation of a few rats.

Using an ANCOVA as an alternative approach to account for differences in responding at the inactive port, with Inactive Port as a continuous covariate and Pokes into the Active Port as the dependent variable, there was a significant effect of Pokes into the Inactive Port [$F_{(1,\ 9.9)} = 14.8$, $P = 0.003$]; a significant effect of Dose [$F_{(3,\ 17.1)} = 3.7$, $P = 0.03$]; a significant interaction between Phenotype and Pokes into the Inactive Port [$F_{(1,\ 16.7)} = 5.1$, $P = 0.04$]; and a significant interaction between Dose and Pokes into the Inactive Port [$F_{(3,\ 17.6)} = 6.2$, $P = 0.005$]. Thus, the more active the rats are in general (i.e., at the inactive port), the larger the phenotypic differences are in the number of pokes into the active port, and this is true regardless of dose (Fig. S2*A*). Furthermore, the dose effects become larger as the number of pokes into the inactive port becomes greater, independent of phenotype (Fig. S2*B*). Post hoc comparisons of means across levels of dose and phenotype were conducted using five hypothesized values (0, 2, 4, 6, 8) of pokes into the inactive port, and the results demonstrate that: (*i*) as the number of pokes into the inactive port increases, the effect of phenotype increases; and (*ii*) as the number of pokes into the inactive port increases, the dose-effect differences in responding at the active port becomes more apparent (Fig. S2 *C* and *D*). These data support the phenotypic differences reported in the primary text (Fig. 5*D*), and further highlight the importance of considering responses in the inactive port.

Cue-Induced Reinstatement: Extinction. When the total number of responses across all extinction sessions was examined, there was a significant effect of Phenotype [$F_{(1,\ 28)} = 17.2$, $P = 0.0003$] and Port [$F_{(1,\ 28)} = 6.2$, $P = 0.02$], but no significant interaction, as the differences in responding between the phenotypes was similar for both the active and inactive ports. When responding was considered with Session as the repeated variable there was a significant effect of Phenotype [$F_{(1,\ 14)} = 19.2$, $P = 0.001$], Port [$F_{(1,\ 350)} = 12.1$, $P = 0.001$], and Session [$F_{(12,\ 350)} = 5.1$, $P < 0.001$], and a significant Phenotype × Session interaction [$F_{(12,\ 350)} = 5.1$, $P = 0.002$] for nose pokes during the extinction sessions. We conducted pairwise comparisons based on the patterns of responding we saw during the previous extinction sessions and, as shown in Fig. 5*B*, the significant differences in responding in the active port were again restricted to the first session on each day of testing (i.e., session 1, $P < 0.001$; session 4, $P = 0.007$; session 8, $P = 0.03$) with no significant differences after session 8. Significant differences in responding in the inactive port were only apparent on session 1 ($P = 0.02$) and session 6 ($P < 0.001$), with the latter being because of a single rat exhibiting aberrant behavior that session. Similar to above, we do not believe these data support differences in the rate of extinction as currently measured.

Principal Components Analysis. As shown in Table S1, when the principal components analysis was conducted for each of the phenotypes separately, three factors were identified for bHRs that together accounted for 91% of the variance in their behavior, and two factors were identified for bLRs that together accounted for 81% of the variance. For bHRs, Factor 1 accounted for 37% of the variance and was largely defined by locomotor response to novelty and cue-induced reinstatement. Factor 2 accounted for 29% of the variance and was largely defined by behavior during the acquisition phase of cocaine self-administration and drug-induced reinstatement; and Factor 3 accounted for 23% of the variance and had a single significant loading associated with the persistence of drug-seeking behavior. For bLRs, Factor 1 accounted for 51% of the variance in behavior and was largely defined by locomotor response to novelty, persistence of drug-seeking behavior, and cue-induced reinstatement. Factor 2 for bLRs was very similar to Factor 2 for bHRs, accounting for 30% of the variance in behavior and defined primarily by behavior during the acquisition phase and drug-induced reinstatement (in agreement with ref. 82). Thus, although there appear to be similar constructs underlying some of the addiction-related behaviors exhibited by bHRs and bLRs, others are phenotype-dependent; notably, the persistence of drug-seeking behavior.

Addiction Score. The resulting Addiction Scores ranged between 22 and 190, and brains were analyzed from the "extremes" of the population, resulting in six bLRs within the range of 22–44 and six bHRs within 53–190. It is important to reiterate, however, that despite the range in Addiction Scores, all rats received the same amount of cocaine throughout the study.

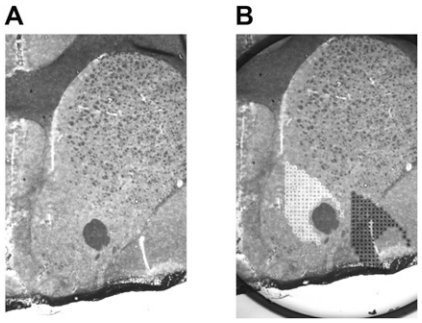

Fig. S1. Representative images of the nucleus accumbens core (*A*) before and (*B*) after LCM. The captured core can be seen in *B* in the "shadow" to the lower right of the lighter captured area. (Magnification: 3×.)

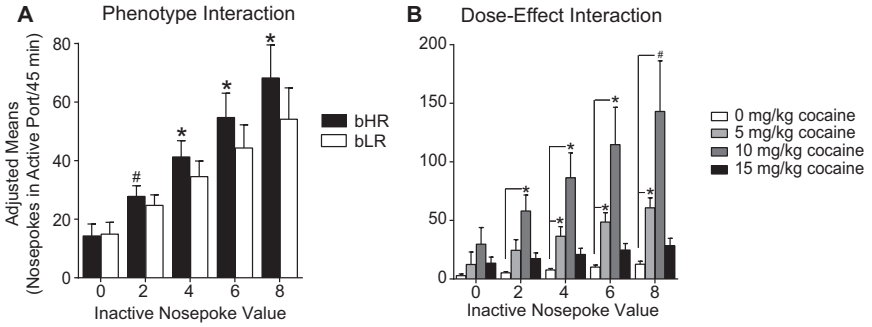

Fig. S2. Covariate-adjusted means for nose poke responding during the cocaine priming-induced reinstatement test. The mean + SEM covariate adjusted values for responses in the active port when considering responses in the inactive port as a continuous covariate are illustrated across increasing values of responses in the inactive port for (*A*) bHRs and bLRs collapsed across doses, and (*B*) each dose collapsed across phenotypes. Significant differences emerge between the phenotypes with increasing values of responses in the inactive port (*A*: $^{*}P < 0.05$, bHR vs. bLR,). The dose-effects also become more prominent with increasing values of responses in the inactive port (*B*: $^{*}P < 0.05$ vs. 0; $^{\#}P = 0.08$ vs. 0).

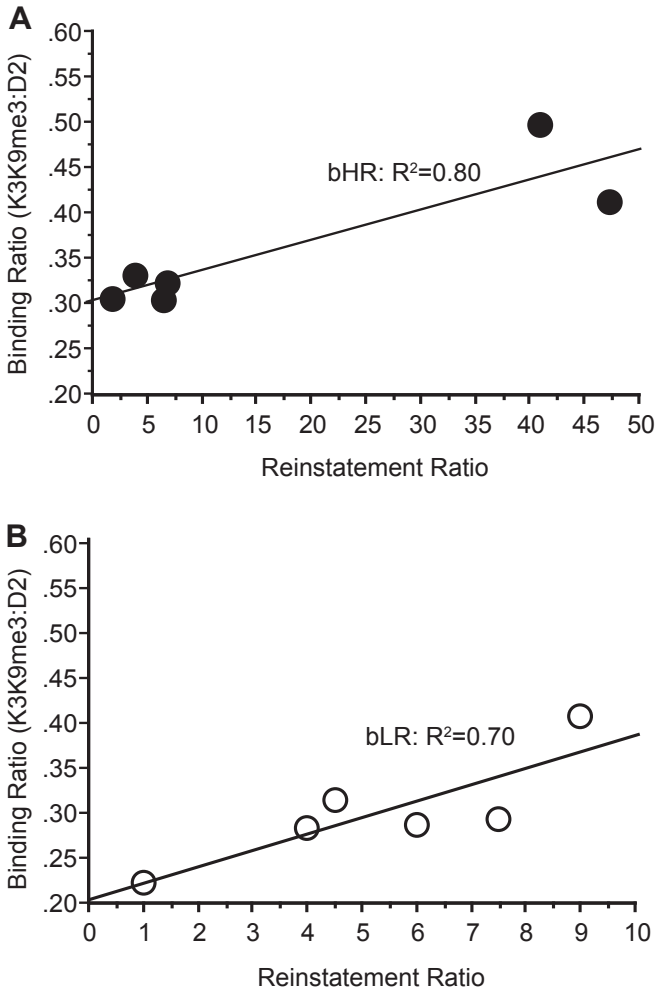

Fig. S3. Bivariate scatterplots illustrating the relationship between the propensity for cue-induced reinstatement of cocaine-seeking and epigenetic regulation of the dopamine D2 receptor. There is a significant positive correlation between the association of H3K9me3 at D2 and the ratio of active/inactive nose pokes (i.e., reinstatement ratio) during the cue-induced reinstatement test for both (*A*) bHRs ($n = 6$, $P = 0.02$) and (*B*) bLRs ($n = 6$, $P = 0.04$).

Table S1. Principal components analysis, behavioral variables

	bHRs			bLRs	
Behavioral variables	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2
Locomotor response to novelty	**0.92**	−0.05	−0.30	**0.80**	−0.41
Acquisition	−0.23	**0.94**	−0.22	−0.09	**0.96**
Persistence of drug-seeking	−0.62	−0.04	**0.98**	**0.85**	0.08
Drug-induced reinstatement	0.23	**0.74**	0.47	−0.08	**0.89**
Cue-induced reinstatement	**−0.88**	−0.18	−0.25	**−0.89**	0.09
Eigenvalue	1.89	1.47	1.19	2.55	1.52
Percent of variance (%)	37.72	29.40	23.70	50.95	30.45

Factor loadings from the rotated component matrix are shown separately for bHRs and bLRs. Loadings >±0.7 are shown in bold. For bHRs, the total variance accounted for by all three factors is 91%. For bLRs, the total variance accounted for is for by 2 factors is 81%.

Table S2. Primers for the ChIP experiment

Genes	Primers for ChIP	Source (accession ID)
FGF2	Forward 5′-ACACGCAGGGAGAGAAGCTA-3′ Reverse 3′-GGGAAGATGAAAACCGTTGA-5′	U78079
D2	Forward 5′-GTCCAGGGCACATAGGAAAA-3′ Reverse 5′-AACCAGCAGTGGAACAGTCC-3′	U79717

The primers directed to the promoter regions for FGF2 and D2 are listed, as is the accession ID for each of the genes analyzed using ChIP.