Inhibition of a cortico-thalamic circuit attenuates cue-induced reinstatement of drug-seeking behavior in "relapse prone" rats

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Abstract

Relapse often occurs when individuals are exposed to stimuli or cues previously associated with the drug-taking experience. The ability of drug cues to trigger relapse is believed to be a consequence of incentive salience attribution, a process in which the incentive value of reward is transferred to the reward-paired cue. Sign-tracker (ST) rats that attribute enhanced incentive value to reward cues are more prone to relapse compared to goal-tracker (GT) rats that primarily attribute predictive value to such cues. The neurobiological mechanisms underlying this individual variation in relapse propensity remains largely unexplored. The paraventricular nucleus of the thalamus (PVT) has been identified as a critical node in the regulation of cue-elicited behaviors in STs and GTs, including cue-induced reinstatement of drug-seeking behavior. Here we used a chemogenetic approach to assess whether "top-down" cortical input from the prelimbic cortex (PrL) to the PVT plays a role in mediating individual differences in relapse propensity. Chemogenetic inhibition of the PrL-PVT pathway differentially affected neuronal activity (i.e. Fos) in the PVT in STs compared to GTs. This manipulation also selectively decreased cue-induced reinstatement of drug-seeking behavior in STs, without affecting behavior in GTs. In contrast, cocaine-primed drug-seeking behavior was unaffected in both phenotypes. These results highlight an important role for the PrL-PVT circuit in mediating individual differences in cue-induced reinstatement of drug-seeking behavior, or relapse propensity.

Introduction

Relapse remains one of the biggest challenges in the treatment of drug addiction [1], with relapse rates as high as 75% within one year of treatment for cocaine addiction [2]. Individuals with addiction often relapse upon exposure to cues (e.g. people, places, paraphernalia) associated with their prior drug-taking experience. Such environmental stimuli can acquire the ability to elicit craving [3, 4] and instigate drug-seeking behavior [5] through Pavlovian learning processes. When a cue reliably precedes the delivery of a reward, it is attributed with predictive value; but Pavlovian cues can also acquire excessive motivational value, or incentive salience [6]. The extent to which a reward cue is attributed with incentive value varies between individuals. Only for some individuals are reward cues transformed into incentive stimuli or

“motivational magnets” [7] and, when this occurs, such cues attain inordinate control and invigorate maladaptive behaviors, including drug seeking and drug taking.

Individual variation in cue-reward learning can be assessed using the sign-tracker/ goal-tracker animal model [8-12]. When rats are exposed to a Pavlovian conditioned approach procedure, wherein a discrete cue precedes the delivery of a food reward, different phenotypes emerge. Some rats, referred to as goal-trackers (GTs) [13], attribute only predictive value to the reward cue; others, termed sign-trackers (STs) [14], attribute both predictive and incentive value to the cue [8, 9, 12]. STs and GTs also differ on several addiction-related behaviors [15-25]. Specifically, relative to GTs, STs show greater drug-seeking behavior during tests for cocaine-primed [19] or cue-induced reinstatement [20, 21] following limited drug experience and abstinence. These phenotypic differences in relapse propensity support the long-standing belief that Pavlovian learning processes [6, 26-29], and more explicitly, incentive salience attribution [6], contribute to addiction. The ST/GT model, therefore, provides a means to elucidate the neurobiological mechanisms underlying the incentive motivational processes that contribute to relapse propensity.

Sign- and goal-tracking behaviors have been postulated to result from a neurobiological bias between “bottom-up” and “top-down” processes, respectively [23, 25, 30-33]. The paraventricular nucleus of the thalamus (PVT) may play a critical role in this regard, as it is ideally located to receive cortical and subcortical input and regulate motivated behaviors via output to the ventral striatum [34-37]. The PVT has long-been recognized as a node of the motive circuit [38, for review see 39], and more recently for its role in addiction-like behaviors, including drug seeking [40-43]. We have previously shown that inactivation of the PVT robustly increases cue-induced drug-seeking behavior in GTs, without affecting behavior in STs [44]. The PVT receives dense projections from the prelimbic (PrL) cortex, and both phenotypes engage this “top-down” pathway in response to a food cue, suggesting it mediates the predictive value of a Pavlovian reward cue [32]. Recently, however, we have found that activation of the PrL-PVT pathway in STs decreases the incentive value of a food cue and the tendency to sign-track, while inhibition of the same pathway in GTs permits the attribution of incentive value to a food cue and increases the tendency to sign-track [45]. These results suggest that the PrL-PVT pathway acts to suppress the incentive motivational value of a food cue.

In the current study we assessed the role of the PrL-PVT pathway in mediating individual variation in both cue-induced and cocaine-primed reinstatement of drug-seeking behavior. We used an inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) to selectively "turn off" the PrL-PVT pathway in STs and GTs. We hypothesized that the PrL-PVT pathway acts to suppress the incentive motivational value of the cocaine cue and that disinhibition of the pathway would, thereby, increase cue-induced reinstatement of drug-seeking behavior selectively in GTs. We also hypothesized that PrL-PVT inhibition would increase cocaine-primed reinstatement of drug-seeking behavior, as the PVT has been shown to play a role in this form of reinstatement as well [41].

Methods

Figure 1 shows the experimental timeline for the procedures described briefly in the following sections. For further details about the methods, see the Supplementary Materials and Methods.

Subjects. A total of 180 (6 cohorts of 30 rats each) male heterogeneous stock (N/NIH-HS) rats bred at the Medical College of Wisconsin were obtained for this study.

Viruses. The Cre-dependent adeno-associated virus (AAV) containing an inhibitory DREADD (pAAV-hSyn-DIO-hM4D(Gi)-mCherry, 1.9 x 10^{13} GC/ml, serotype 8) and the retrograde virus containing Cre (AAV retrograde pmSyn1-EBFP-Cre, 7.6 x 10^{12} GC/ml) were purchased from Addgene (Watertown, MA).

Drugs. Cocaine HCl (Mallinckrodt, St. Louis, MO) dissolved in 0.9% sterile saline was used in these studies. Clozapine-N-oxide (CNO; Log number 13626-76) was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program and used at a concentration of 5 mg/ml dissolved in 6% dimethyl sulfoxide (DMSO), which served as the vehicle treatment.

Surgical procedures. Rats underwent surgery to implant an indwelling jugular catheter for self-administration as previously described [46, 47], followed by stereotaxic surgery for viral infusions. The G_i DREADD was injected bilaterally into the PrL (relative to bregma: AP 3.0, ML: +/- 1.0, DV -4.0) over the course of 5 minutes at a rate of 200 nl/minute, for a total volume of 1 µL. The AAV-Cre was injected into the anterior (relative to bregma: AP -2, ML -1, DV -

5.4) and posterior PVT (relative to bregma: AP -3.0, ML -1.0, DV -5.5) over the course of 2 minutes at a rate of 50 nl/minute, for a total of 100 nl per injection site using a 1 µl syringe.

Locomotor test. After recovery from surgery, rats underwent a 60-min locomotor test in a novel environment as previously described [48]. Cumulative locomotor movements (vertical and horizontal movements) were used to classify rats as either high (HR) or low (LR) responders using a median split. The purpose of this test is described in the Supplemental Material and Methods.

Pavlovian conditioned approach (PavCA) training. The day following the locomotor test, rats started Pavlovian conditioned approach (PavCA) training as previously described [11]. Briefly, during training, an illuminated lever (conditioned stimulus, CS) entered the chamber for 8-sec, and immediately upon its retraction a food pellet (unconditioned stimulus, US) was delivered into an adjacent food magazine. Lever-CS/ food-US trials were on a variable interval 90-second schedule (range 30-150 seconds). Rats underwent five PavCA training sessions, with one session per day.

Based on behavior during training, the PavCA Index Score [11] was calculated. The PavCA index is a composite score that is based on response bias, latency and vigor of responding toward the lever-CS versus the food magazine. Scores range from -1 to 1, with a score of -1 representing individuals with a CR always directed solely towards the food magazine during lever-CS presentation (e.g. goal-tracker, GT), and a score of 1 represents individuals with a CR directed solely towards the lever-CS upon its presentation (e.g. sign-tracker, ST). The PavCA index for session 4 and 5 were averaged to characterize rats into their respective behavioral phenotypes. Rats were characterized as STs if they had a PavCA score between 0.3 and 1, GTs between -0.3 and -1, and intermediate rats (IN), individuals who vacillate between the two CRs, between -0.29 and 0.29.

Food self-administration. Rats were trained to perform an operant response for food delivery prior to drug self-administration. During these session**s,** a nose poke (fixed ratio (FR) 1) into the "active" port resulted in the delivery a single food pellet (same as PavCA training), presentation of a cue-light inside the port for 20 seconds and the house light turning off (i.e. time-out period). In order to minimize location bias, the "active" port was on the opposite side of the food

magazine as the lever-CS was during PCA training. Rats underwent four daily food self-administration sessions.

Cocaine self-administration. Cocaine self-administration sessions occurred as previously described [44]. Briefly, a nose poke into the active port (FR1) resulted in the intravenous delivery of cocaine and presentation of a cue light inside the port for 20-sec. Pokes into the inactive port, and the active port during the 20-sec cue light presentation, were without consequence. Sessions terminated after 3 hours, or once the rat met the infusion criterion (IC) for that session [19-21, 49]. An IC is the maximum number of infusions a rat can receive during a given session. Infusion criterion ensure that rats receive the same number of infusions each training session, as well as the same number of cocaine cue-light pairings. Rats underwent one cocaine self-administration training session per day for 15 consecutive days. Rats were not moved to the subsequent IC until all had met the IC for at least 2 consecutive days. This resulted in the following schedule: three days at IC5, four days at IC10, 3 days at IC20, and 5 days at IC45. At the conclusion of training, rats underwent a 28-day period of forced abstinence where they were left undisturbed in the colony room.

Extinction training 1 and 2. During extinction training, nose pokes into both the active and inactive ports were recorded, but without consequence. Sessions lasted for 2 hours, and rats underwent one session per day for a total of 13 sessions (Extinction 1) or 8 sessions (Extinction 2).

Cue-induced reinstatement test. The test for cue-induced reinstatement occurred the day following the last Extinction 1 training session. Twenty-five minutes prior to the test, rats received either an injection (i.p.) of CNO to activate the G_i DREADD, or vehicle. A conditioned reinforcement paradigm was used, such that pokes into the active port resulted in presentation of the drug-associated cue light inside the port and termination of the house light for 5-sec, but no cocaine infusion. The test lasted for 2 h, following which rats underwent five days of forced abstinence prior to Extinction 2 training.

Cocaine-induced reinstatement test. Following the last Extinction 2 training session, rats underwent a test for cocaine-primed reinstatement. As with the cue-induced reinstatement test, rats received the same treatment (CNO or vehicle) 25-min prior to the session. Before being put

into the testing chamber, all rats received a 15 mg/kg (i.p.) injection of cocaine. Pokes into the active and inactive ports were recorded, but without consequence.

Histology. Once testing was complete, all rats underwent transcardial perfusion, tissue fixation and immunohistochemistry as previously described [32]. Only rats with DREADD expression localized to the PrL and PVT were included in final analysis.

Fos protein expression was analyzed in a subset of rats to verify that the G_i DREADDs were in fact affecting the activity in the PrL-PVT pathway. Rats received either CNO or vehicle (same as test day treatment) eighty minutes prior to perfusions and remained in the colony room thereafter. This time point was chosen to capture peak Fos levels [50]. Co-localization of Fos positive cells and cells expressing the Gi DREADD in the PrL were manually quantified using ImageJ (National Institute of Health, Bethesda, MD) to determine selectivity of neuronal activation in response to CNO. To assess the effects of PrL-PVT inhibition on activity in the PVT, the density of Fos (cells per square mm) was determined using the ITCN (image-based tool for counting nuclei) plugin from ImageJ.

Statistical analyses

Behavioral analyses. Data from PavCA training, cocaine self-administration and extinction training sessions were analyzed using a linear mixed-effects model. These analyses were conducted using SPSS Statistics Program (Statistical Package for the Social Sciences), version 22 (IBM, Armok, NY).

An ANOVA was used to assess behavior during the locomotor test and the reinstatement test sessions. A repeated measures ANOVA was used to compare drug-seeking behavior (i.e. pokes into the active and inactive ports) between the last extinction training session and the reinstatement test sessions. All ANOVAs and correlational analyses were analyzed using StatView, version 5.0 (SAS Institute Inc., Cary, NC, USA). When significant main effects or interactions were detected, post-hoc analyses were conducted using Bonferroni tests to correct for multiple comparisons.

Histological analyses: PrL. Group differences in the number of Fos positive cells, DREADD positive cells, and cells expressing both markers were analyzed using an ANOVA. The

percentage of cells co-expressing DREADD and Fos relative to all cells expressing DREADD was analyzed using an ANOVA.

Histological analyses: PVT. The average density of Fos positive cells collapsed across the entire rostral-caudal axis of the PVT were assessed using an ANOVA. Rats in the vehicle group, regardless of phenotype, were collapsed into a single group as they did not statistically differ from one another. An unpaired t-test was used to assess differences between CNO-treated STs and the vehicle group, and CNO-treated GTs and the vehicle group. To further assess if PrL-PVT inhibition affected Fos density in the PVT of STs and GTs, the percent of Fos relative to all vehicle control rats (i.e. "baseline") was calculated for each phenotype. A single sample t-test with the hypothesized value set to 100% (i.e. baseline) was then performed. To determine if PrL-PVT inhibition differentially affected PVT Fos density relative to baseline in STs versus GTs, an unpaired t-test was performed.

For all tests, statistical significance was set at $p<0.05$. Outliers were identified using the boxplot method [51]. All significant results were subjected to a power analysis and exhibited adequate statistical power (the ability to reject the null hypothesis ($1-\beta$) >0.55).

Results

Only rats that successfully completed all training and exhibited accurate DREADD expression were included in final analysis (ST VEH, n=6; ST CNO, n=9; GT VEH, n=16, GT CNO, n=15). The sample size also varies as a function of the number of STs or GTs that we had in a given cohort. A subset of the rats underwent immunohistochemistry for Fos protein and DREADD expression in the PrL and PVT (GT VEH, n = 10, GT CNO, n = 8, ST VEH, n = 3 (n=2 for PrL quantification), ST CNO, n = 6). Due to the low number of IN rats in each group, these rats were excluded from the final analyses. Refer to the Supplementary materials and methods section for additional details regarding excluded rats.

Behavioral results: ST/GT model

PavCA behavior. As expected per their classification, STs and GTs differed in their PavCA index across training ($F_{4,48}=344.12$, $p<0.001$, Fig. 2a) and in their final index ($F_{1,42}=710.34$,

$p<0.001$, Fig. 2b). There were no significant differences in test day treatment between or within phenotypes on the final PavCA index (Fig. 2b).

For each sign-tracking, or lever-directed, measure, there was an effect of Session ($p<0.0001$), Phenotype ($p<0.0001$) and Session by Phenotype interaction ($p<0.0001$) (Fig. 2d-e). Compared to GTs, STs showed a greater probability to contact the lever-CS ($F_{1,42}=85.04$, $p<0.0001$), greater number of contacts with the lever-CS ($F_{1,42}=183.67$, $p<0.0001$), and lower latency to contact the lever-CS ($F_{1,42}=127.60$, $p<0.0001$). Post-hoc analyses revealed that STs differed from GTs on all three measures across the five sessions ($p<0.0001$ for all). There was also a significant effect of Session ($p<0.005$), Phenotype ($p<0.0001$) and a Session by Phenotype interaction ($p<0.0001$) for all measures of goal-tracking, or magazine-directed behaviors (Fig. 2f-h). Compared to STs, GTs showed a greater probability to contact the food magazine ($F_{1,45}=55.81$, $p<0.0001$), greater number of food contacts ($F_{1,45}=76.83$, $p<0.001$), and lower latency to approach the food magazine upon lever-CS presentation ($F_{1,48}=61.62$, $p<0.0001$). Post-hoc analyses showed that GTs differed from STs on all three measures on sessions two through five ($p<0.0001$). As expected, there were no effects of Treatment between or within phenotypes for lever- or magazine-directed behaviors, as PavCA training occurred prior to treatment and experimental groups were counterbalanced according to these metrics.

STs and GTs do not differ in acquisition of cocaine self-administration. Acquisition of drug self-administration was evident by an increase in nose pokes over the course of training (effect of IC, $F_{3,85}=84.88$, $p<0.001$), and discrimination between the active and inactive ports (effect of Port, $F_{1,85}=215.68$, $p<0.001$) (Fig. 3a). While nose pokes into the active port increased with training (IC x Port interaction, $F_{3,85}=63.32$, $p<0.0001$), nose pokes into the inactive port did not change ($p=0.25$). As expected, there was no effect of Phenotype ($F_{1,85}=0.60$, $p=0.44$) or Treatment ($F_{1,85}=0.80$, $p=0.78$). Additionally, all rats earned infusions faster as training progressed across IC (effect of IC, $F_{3,87}= 17.36$, $p<0.0001$), indicating an increase in the motivation to take cocaine (Fig. 3b). This measure was not impacted by phenotype ($F_{1,52}=0.53$, $p=0.47$) or test day treatment ($F_{1,52}=1.01$, $p=0.32$).

STs and GTs do not differ in extinction of drug-seeking behavior. There were no significant differences between STs and GTs in the extinction of nose-poking behavior (effect of Phenotype, $F_{1,84}=2.50$, $p=0.12$), and, as expected, test-day treatment did not affect extinction behavior (effect

of Treatment, $F_{1,84}$=0.12, p=0.73) (Fig. 4a). Rats made fewer responses into the ports as training progressed (effect of Session, $F_{12,84}$=24.04, p<0.0001), but continued to differentiate between the two ports (effect of Port, $F_{1,84}$=36.77, p<0.0001) until the last few sessions (Session x Port interaction, $F_{12,84}$=5.61, p<0.0001; Session 9, p=0.18; Session 10, p=0.81; Session 11, p=0.07) (Fig. 4a).

Inhibition of the PrL-PVT pathway selectively decreases cue-induced drug-seeking behavior in STs. It should be noted that two rats (ST VEH, ST CNO) were identified as statistical outliers, but they were not removed from analysis as results do not differ with their removal. All rats exhibited cue-induced reinstatement, as there was an increase in drug-seeking behavior (active-inactive NPs) during the test for cue-induced reinstatement compared to the last extinction training session (effect of Session, $F_{1,40}$=33.37, p<0.0001; Fig. 4b, d-g), regardless of treatment or phenotype. During the test for cue-induced reinstatements, more pokes were made into the active port compared to the inactive port (effect of Port, $F_{1,80}$=41.21, p<0.001) (Fig. 4c). As expected, STs showed enhanced cue-induced drug-seeking behavior relative to GTs (effect of Phenotype, $F_{1,80}$=4.18, p=0.04) [20, 21]. However, there was a significant effect of Treatment ($F_{1,80}$=4.36, p=0.04) and a Phenotype x Treatment x Port interaction ($F_{1,80}$=4.30, p=0.04). Inhibition of the PrL-PVT pathway rendered STs similar to GTs (vehicle group, p=0.72; CNO group, p=0.96). Thus, while STs in the VEH group showed greater responding into the active port compared to both VEH-treated (p=0.02) and CNO-treated GTs (p=0.01), STs treated with CNO did not significantly differ from GTs. CNO-treated STs also exhibited less cue-induced drug-seeking behavior relative to VEH-treated STs. That is, in STs, inhibition of the PrL-PVT pathway significantly reduced nose-poke responding into the active port (p=0.02), but not the inactive port (p=0.74; Fig. 4c). In contrast, in GTs, inhibition of the PrL-PVT pathway did not affect nose pokes into either the active or inactive port in GTs (GTs: CNO vs. VEH, active port, p=0.74; inactive port, p=0.74). Together, these data suggest that inhibition of the PrL-PVT pathway selectively affects the incentive motivational value of the cocaine-cue in STs.

Rats do not differ in extinction training after cue-induced reinstatement. Nose pokes into both ports decreased as training progressed (effect of Session, $F_{7,135}$=6.17, p<0.0001), and did not differ based on phenotype (effect of Phenotype, $F_{1,87}$=1.54, p=0.22) or test day treatment (effect of Treatment, $F_{1,87}$=0.26, p=0.61). However, rats continued to differentiate between the

ports throughout training (effect of Port, F1,87=8.26, p<0.001) (Fig. 4h). One rat (ST) was excluded from day 8 of analysis as a statistical outlier.

Inhibition of the PrL-PVT pathway does not affect cocaine-induced reinstatement of drug-seeking behavior. All rats showed greater drug-seeking behavior (active-inactive NPs) during the test for cocaine-induced reinstatement compared to the last extinction training session (effect of Session, $F_{1,40}$=34.48, p<0.0001; Fig. 4i). During the cocaine-primed reinstatement test, rats made more pokes into the active port compared to the inactive port (effect of Port, $F_{1,80}$=23.13, p<0.0001) (Fig. 4j). STs and GTs did not differ from one another during this reinstatement test (effect of Phenotype, $F_{1,80}$=0.64, p=0.43) and inhibition of the PrL-PVT pathway did not affect cocaine-primed reinstatement of drug-seeking behavior (effect of Treatment, $F_{1,80}$=1.39, p=0.24). Additionally, STs and GTs did not differ in the amount of time spent exhibiting cocaine-induced stereotyped behaviors, suggesting that sensitization to cocaine did not affect drug-seeking behavior (Figure S1). Two rats (GT VEH, ST CNO) were excluded from analyses as statistical outliers.

PrL Fos and DREADD expression. There were no significant effects of treatment, phenotype or interactions for the number of cells expressing DREADD (Treatment, $F_{1,22}$=1.37, p=0.26; Phenotype: $F_{1,22}$=3.25, p=0.09), Fos (Treatment: $F_{1,22}$=0.08, p=0.78; Phenotype: $F_{1,22}$=1.57, p=0.22), or co-expressing both Fos and DREADD (Treatment: $F_{1,22}$=0.54, p=0.47; Phenotype: $F_{1,22}$=2.65, p=0.19) in the PrL. As there was no effect of Phenotype in any analysis, STs and GTs were collapsed together for further analysis. Relative to vehicle, CNO administration resulted in a significant increase in the percent of cells co-expressing both Fos and DREADD relative to all cells expressing DREADD ($F_{1,22}$=5.8, p=0.02; Figure 5a). Thus, CNO administration increased neuronal activity in DREADD-expressing cells in the PrL.

PVT Fos expression. Density of Fos protein expression in the PVT was analyzed across PVT sub-regions (anterior, middle and posterior), but there was not a significant effect of Region ($F_{2,40}$=1.31, p=0.28) nor a significant Phenotype x Treatment x Region interaction ($F_{2,40}$=0.37, p=0.70). Therefore, all subsequent analyses focused on the average density of Fos across the rostral-caudal axis of the PVT, for which there was a significant Phenotype x Treatment interaction ($F_{1,23}$= 4.29, p<0.05). Post-hoc analyses showed that inhibition of the PrL-PVT circuit differentially affected average Fos density in STs relative to GTs (p=0.002), such that average

Fos density decreased in GTs treated with CNO relative to STs treated with CNO (Fig. S3). However, when compared to their respective vehicle-treated controls, PrL-PVT inhibition did not affect average Fos density in STs ($p=0.19$) or GTs ($p=0.10$). Because there was a not a significant difference in average Fos density between the vehicle-treated groups ($p=0.49$), additional analyses were conducted with a single vehicle group collapsed across phenotypes.

When the percent of average Fos density relative to baseline (i.e. collapsed vehicle control group) was analyzed, PrL-PVT inhibition resulted in greater PVT Fos density relative to baseline in STs compared to GTs ($t(12)=-4.10$, $p<0.002$; Figure 5c), which is also consistent with the results reported above using the raw data. Relative to baseline (i.e. 100%), inhibition of the PrL-PVT did not significantly affect Fos in the PVT in STs ($t(5)=1.36$, $p=0.23$), but did decrease Fos in the PVT in GTs ($t(7)=^{-}9.01$, $p<0.001$) (Fig. 5c). These results support the notion that the PrL-PVT pathway differentially mediates downstream activity STs and GTs.

Discussion

While the PrL-PVT pathway has been implicated in cue-reward learning [45, 52-54], the present study assessed the role of this pathway in mediating individual variation in cue-induced and cocaine-primed reinstatement of drug-seeking behavior using the ST/ GT animal model. As expected, STs and GTs did not differ in behavior during cocaine self-administration [19-21, 44] or extinction training prior to the reinstatement tests [19, 22, 44, 55, 56]. The latter finding suggests that inhibition of the PrL-PVT pathway during cue-induced reinstatement does not affect subsequent behavior during extinction training. Aligning with prior studies, STs showed greater cue-induced drug-seeking behavior relative to GTs [20, 21]. Notably, inhibition of the PrL-PVT pathway selectively decreased cue-induced drug-seeking behavior in STs to levels seen in GTs, eliminating individual differences in cue-induced reinstatement. Importantly, CNO administration in the absence of DREADD did not affect drug-seeking behavior in STs or GTs in either reinstatement test (see Fig. S2). Further, when the same rats were categorized as high- or low-responders, according to their locomotor response to a novel environment, there was no effect of PrL-PVT inhibition on drug-seeking behaviors (see Fig. S6c and f). Thus, the PrL-PVT pathway appears to play a selective role in individuals with a greater propensity to attribute incentive salience to drug-associated cues.

Contrary to our expectation [19], STs and GTs did not differ during cocaine-primed reinstatement despite the drug dose being the same as that given in previous experiments [19]. However, several methodological differences between the two studies [19] likely contribute to these differences. For example, in this study rats did not receive cocaine for approximately 2 months prior to cocaine-primed reinstatement, while in the study conducted by Saunders and Robinson (2011) rats were only abstinent for only 1 week [19]. It is possible that the presence of the forced abstinence period in our study, which is known to result in cellular adaptations following cocaine experience [for review see 57, 58], resulted in the differences observed between our results and previous findings. Regardless, the PrL-PVT pathway does not appear to mediate cocaine-induced drug-seeking behavior in either phenotype. These findings suggest that the PrL-PVT pathway specifically mediates the cue-reward relationship, but not the interoceptive effects of the drug itself.

Our results challenge our hypothesis that the PrL-PVT pathway inhibits the incentive value of the cocaine-cue during a test for reinstatement. However, this hypothesis was based on our previous work that assessed the role of this pathway during Pavlovian learning [45] , whereas the current study utilized an instrumental procedure. The neural circuitry mediating these two forms of associative learning are different [59-62], so it is possible that the PrL-PVT pathway has disparate functions in these two forms of learning. Additionally, Campus et al. used a natural reward (e.g. food) [45], whereas we used cocaine as the reward which can induce more substantial neuroplastic changes within the motive circuit [for review see 63, 64-66], especially during a period of prolonged abstinence [for review see 67]. Thus, it is possible that cocaine-induced neuroplastic changes uniquely alter the PrL-PVT pathway and its functional role. In support, cells in the PrL and posterior PVT become active shortly after the conclusion of cocaine self-administration training [54], and inhibiting this pathway at this time reduces cue-induced drug-seeking behavior [54]. Similar effects are reported in the current study, but only in individuals with an inherent tendency to attribute incentive value to reward cues. Thus, there appears to be a relationship between incentive salience attribution and cocaine-induced neuroplasticity within the PrL-PVT pathway. In support, we showed that PrL-PVT inhibition following cocaine exposure and abstinence differentially affects Fos protein levels in the PVT of STs and GTs. This pathway, therefore, appears to undergo different neuroplastic changes as a function of incentive salience attribution.

The PVT can elicit dopamine release within the nucleus accumbens (NAc) [34], where dopamine signaling contributes to greater cue-induced drug-seeking behavior in STs relative to GTs [21]. Inhibiting the PrL-PVT projection could attenuate NAc dopamine and, correspondingly, cue-induced drug-seeking behavior in STs. Our findings that CNO-induced Fos activity differs between STs and GTs in the PVT, and that inhibition of the PrL-PVT circuit increases neuronal activity in the PVT in STs relative to GTs likely reflects such downstream neuromodulation. We propose, therefore, that the role of the PrL-PVT pathway in incentive motivational processes and, thereby, cue-induced drug-seeking behavior, is ultimately mediated via a final common path between the PVT and the NAc.

In summary, these results highlight the complex role of the cortical projections from the PrL to the PVT in cue-reward learning. We report here that PrL-PVT inhibition selectively attenuates cue-induced drug-seeking behavior in rats with high relapse propensity (STs), but does not affect cocaine-primed reinstatement of drug-seeking behavior. Thus, this pathway plays a role in encoding the incentive motivational value of a cocaine-cue, but not the motivational value of the interoceptive effects of the drug. Further, our findings suggest that this pathway undergoes cocaine-induced neuroplastic changes, and these changes are a function of one's inherent tendency to attribute incentive salience to drug-associated cues. Future studies will further explore the molecular basis by which the reported effects occur, with a focus on cocaine-induced neuroplasticity within the PrL-PVT pathway.

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References

1. Sinha, R., *New findings on biological factors predicting addiction relapse vulnerability.* Curr Psychiatry Rep, 2011. **13**(5): p. 398-405.
2. Dodge, R., J. Sindelar, and R. Sinha, *The role of depression symptoms in predicting drug abstinence in outpatient substance abuse treatment.* J Subst Abuse Treat, 2005. **28**(2): p. 189-96.
3. Childress, A., et al., *Conditioned craving and arousal in cocaine addiction: a preliminary report.* NIDA Res Monogr, 1988. **81**: p. 74-80.
4. Childress, A., et al., *Classically conditioned factors in drug dependence*, in *Comprehensive Textbook of Substance Abuse*, J. Lowinson and R.P. Millman, Editors. 1993, Williams and Wilkins: Baltimore. p. 56-69.
5. Shaham, Y., et al., *The reinstatement model of drug relapse: history, methodology and major findings.* Psychopharmacology (Berl), 2003. **168**(1-2): p. 3-20.
6. Robinson, T.E. and K.C. Berridge, *The neural basis of drug craving: an incentive-sensitization theory of addiction.* Brain Res Brain Res Rev, 1993. **18**(3): p. 247-91.
7. Berridge, K.C., T.E. Robinson, and J.W. Aldridge, *Dissecting components of reward: 'liking', 'wanting', and learning.* Curr Opin Pharmacol, 2009. **9**(1): p. 65-73.
8. Flagel, S.B., et al., *Individual differences in the attribution of incentive salience to a reward-related cue: influence on cocaine sensitization.* Behav Brain Res, 2008. **186**(1): p. 48-56.
9. Flagel, S.B., et al., *Individual differences in the propensity to approach signals vs goals promote different adaptations in the dopamine system of rats.* Psychopharmacology (Berl), 2007. **191**(3): p. 599-607.
10. Flagel, S.B. and T.E. Robinson, *Neurobiological Basis of Individual Variation in Stimulus-Reward Learning.* Curr Opin Behav Sci, 2017. **13**: p. 178-185.
11. Meyer, P.J., et al., *Quantifying individual variation in the propensity to attribute incentive salience to reward cues.* PLoS One, 2012. **7**(6): p. e38987.
12. Robinson, T.E. and S.B. Flagel, *Dissociating the predictive and incentive motivational properties of reward-related cues through the study of individual differences.* Biol Psychiatry, 2009. **65**(10): p. 869-73.
13. Boakes, R.A., *Performance on learning to associate a stimulus with positive reinforcement*, in *Operant-Pavlovian interactions*, H. Davis and H. Hurwitz, Editors. 1977, Lawrence Erlbaum Associates: Hillsdale, NJ. p. 67-97.
14. Hearst, E. and H.M. Jenkins, *Sign-tracking: The stimulus-reinforcer relation and directed action*, in *Psychonomic Society*. 1974: Austin, TX.
15. Yager, L.M., et al., *Individual variation in the motivational and neurobiological effects of an opioid cue.* Neuropsychopharmacology, 2015. **40**(5): p. 1269-77.

16. Yager, L.M. and T.E. Robinson, *A classically conditioned cocaine cue acquires greater control over motivated behavior in rats prone to attribute incentive salience to a food cue.* Psychopharmacology (Berl), 2013. **226**(2): p. 217-28.
17. Flagel, S.B., et al., *An animal model of genetic vulnerability to behavioral disinhibition and responsiveness to reward-related cues: implications for addiction.* Neuropsychopharmacology, 2010. **35**(2): p. 388-400.
18. Lovic, V., et al., *Rats prone to attribute incentive salience to reward cues are also prone to impulsive action.* Behav Brain Res, 2011. **223**(2): p. 255-61.
19. Saunders, B.T. and T.E. Robinson, *Individual variation in the motivational properties of cocaine.* Neuropsychopharmacology, 2011. **36**(8): p. 1668-76.
20. Saunders, B.T. and T.E. Robinson, *A cocaine cue acts as an incentive stimulus in some but not others: implications for addiction.* Biol Psychiatry, 2010. **67**(8): p. 730-6.
21. Saunders, B.T., L.M. Yager, and T.E. Robinson, *Cue-evoked cocaine "craving": role of dopamine in the accumbens core.* J Neurosci, 2013. **33**(35): p. 13989-4000.
22. Saunders, B.T., et al., *A cocaine context renews drug seeking preferentially in a subset of individuals.* Neuropsychopharmacology, 2014. **39**(12): p. 2816-23.
23. Pitchers, K.K., et al., *'Hot' vs. 'cold' behavioural-cognitive styles: motivational-dopaminergic vs. cognitive-cholinergic processing of a Pavlovian cocaine cue in sign- and goal-tracking rats.* Eur J Neurosci, 2017b. **46**(11): p. 2768-2781.
24. Pitchers, K.K., et al., *Diverse Roads to Relapse: A Discriminative Cue Signaling Cocaine Availability Is More Effective in Renewing Cocaine Seeking in Goal Trackers Than Sign Trackers and Depends on Basal Forebrain Cholinergic Activity.* J Neurosci, 2017a. **37**(30): p. 7198-7208.
25. Paolone, G., et al., *Cholinergic control over attention in rats prone to attribute incentive salience to reward cues.* J Neurosci, 2013. **33**(19): p. 8321-35.
26. Bindra, D., *How adaptive behavior is produced: a perceptual motivation alternative to response reinforcement.* Behavior and Brain Sciences, 1978. **1**: p. 41-91.
27. Bolles, R., *Reinforcement, expectancy, and learning.* Psychological Review, 1972. **79**: p. 394-409.
28. Stewart, J., H. de Wit, and R. Eikelboom, *Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants.* Psychol Rev, 1984. **91**(2): p. 251-68.
29. Toates, F.M., *The control of ingestive behaviour by internal and external stimuli--a theoretical review.* Appetite, 1981. **2**(1): p. 35-50.
30. Flagel, S.B., et al., *A food predictive cue must be attributed with incentive salience for it to induce c-fos mRNA expression in cortico-striatal-thalamic brain regions.* Neuroscience, 2011a. **196**: p. 80-96.
31. Haight, J.L. and S.B. Flagel, *A potential role for the paraventricular nucleus of the thalamus in mediating individual variation in Pavlovian conditioned responses.* Front Behav Neurosci, 2014. **8**: p. 79.
32. Haight, J.L., et al., *A food-predictive cue attributed with incentive salience engages subcortical afferents and efferents of the paraventricular nucleus of the thalamus.* Neuroscience, 2017. **340**: p. 135-152.
33. Sarter, M. and K.B. Phillips, *The Neuroscience of Cognitive-Motivational Styles: Sign- and Goal-Trackers as Animal Models.* Behav Neurosci, 2018.

34. Parsons, M.P., S. Li, and G.J. Kirouac, *Functional and anatomical connection between the paraventricular nucleus of the thalamus and dopamine fibers of the nucleus accumbens.* J Comp Neurol, 2007. **500**(6): p. 1050-63.
35. Li, S. and G.J. Kirouac, *Projections from the paraventricular nucleus of the thalamus to the forebrain, with special emphasis on the extended amygdala.* J Comp Neurol, 2008. **506**(2): p. 263-87.
36. Li, S. and G.J. Kirouac, *Sources of inputs to the anterior and posterior aspects of the paraventricular nucleus of the thalamus.* Brain Struct Funct, 2012. **217**(2): p. 257-73.
37. Kirouac, G.J., *Placing the paraventricular nucleus of the thalamus within the brain circuits that control behavior.* Neurosci Biobehav Rev, 2015. **56**: p. 315-29.
38. Kelley, A.E., et al., *Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward.* Physiol Behav, 2005. **86**(5): p. 773-95.
39. Millan, E.Z., Z. Ong, and G.P. McNally, *Paraventricular thalamus: Gateway to feeding, appetitive motivation, and drug addiction.* Prog Brain Res, 2017. **235**: p. 113-137.
40. Hamlin, A.S., et al., *Paraventricular thalamus mediates context-induced reinstatement (renewal) of extinguished reward seeking.* Eur J Neurosci, 2009. **29**(4): p. 802-12.
41. James, M.H., et al., *Cocaine- and amphetamine-regulated transcript (CART) signaling within the paraventricular thalamus modulates cocaine-seeking behaviour.* PLoS One, 2010. **5**(9): p. e12980.
42. Matzeu, A., F. Weiss, and R. Martin-Fardon, *Transient inactivation of the posterior paraventricular nucleus of the thalamus blocks cocaine-seeking behavior.* Neurosci Lett, 2015. **608**: p. 34-9.
43. Matzeu, A., et al., *Orexin-A/Hypocretin-1 Mediates Cocaine-Seeking Behavior in the Posterior Paraventricular Nucleus of the Thalamus via Orexin/Hypocretin Receptor-2.* J Pharmacol Exp Ther, 2016. **359**(2): p. 273-279.
44. Kuhn, B.N., et al., *Transient inactivation of the paraventricular nucleus of the thalamus enhances cue-induced reinstatement in goal-trackers, but not sign-trackers.* Psychopharmacology (Berl), 2018.
45. Campus, P., et al., *The paraventricular thalamus is a critical mediator of top-down control of cue-motivated behavior in rats.* Elife, 2019. **8**.
46. Crombag, H.S., et al., *The role of contextual versus discrete drug-associated cues in promoting the induction of psychomotor sensitization to intravenous amphetamine.* Behav Brain Res, 2000. **116**(1): p. 1-22.
47. Flagel, S.B., D.M. Vazquez, and T.E. Robinson, *Manipulations during the second, but not the first, week of life increase susceptibility to cocaine self-administration in female rats.* Neuropsychopharmacology, 2003. **28**(10): p. 1741-51.
48. Garcia-Fuster, M.J., et al., *Adolescent cocaine exposure enhances goal-tracking behavior and impairs hippocampal cell genesis selectively in adult bred low-responder rats.* Psychopharmacology (Berl), 2017. **234**(8): p. 1293-1305.
49. Flagel, S.B., et al., *Genetic background and epigenetic modifications in the core of the nucleus accumbens predict addiction-like behavior in a rat model.* Proc Natl Acad Sci U S A, 2016. **113**(20): p. E2861-70.
50. Sonnenberg, J.L., et al., *Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure.* Neuron, 1989. **3**(3): p. 359-65.
51. Tukey, J.W., *Exploratory Data Anlysis.* 1977, Reading, MA: Addison-Wesley.

52. Otis, J.M., et al., *Prefrontal cortex output circuits guide reward seeking through divergent cue encoding.* Nature, 2017. **543**(7643): p. 103-107.
53. Do-Monte, F.H., K. Quinones-Laracuente, and G.J. Quirk, *A temporal shift in the circuits mediating retrieval of fear memory.* Nature, 2015. **519**(7544): p. 460-3.
54. Giannotti, G., et al., *Divergent Prelimbic Cortical Pathways Interact with BDNF to Regulate Cocaine-seeking.* J Neurosci, 2018. **38**(42): p. 8956-8966.
55. Yager, L.M. and T.E. Robinson, *Cue-induced reinstatement of food seeking in rats that differ in their propensity to attribute incentive salience to food cues.* Behav Brain Res, 2010. **214**(1): p. 30-4.
56. Kawa, A.B., B.S. Bentzley, and T.E. Robinson, *Less is more: prolonged intermittent access cocaine self-administration produces incentive-sensitization and addiction-like behavior.* Psychopharmacology (Berl), 2016. **233**(19-20): p. 3587-602.
57. Grimm, J.W., et al., *Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving.* J Neurosci, 2003. **23**(3): p. 742-7.
58. Robinson, T.E. and B. Kolb, *Structural plasticity associated with exposure to drugs of abuse.* Neuropharmacology, 2004. **47 Suppl 1**: p. 33-46.
59. Ostlund, S.B. and B.W. Balleine, *Orbitofrontal cortex mediates outcome encoding in Pavlovian but not instrumental conditioning.* J Neurosci, 2007. **27**(18): p. 4819-25.
60. Yin, H.H., S.B. Ostlund, and B.W. Balleine, *Reward-guided learning beyond dopamine in the nucleus accumbens: the integrative functions of cortico-basal ganglia networks.* Eur J Neurosci, 2008. **28**(8): p. 1437-48.
61. Wassum, K.M., et al., *Differential dependence of Pavlovian incentive motivation and instrumental incentive learning processes on dopamine signaling.* Learn Mem, 2011. **18**(7): p. 475-83.
62. Gruart, A., et al., *Functional basis of associative learning and its relationships with long-term potentiation evoked in the involved neural circuits: Lessons from studies in behaving mammals.* Neurobiol Learn Mem, 2015. **124**: p. 3-18.
63. Kalivas, P.W. and N.D. Volkow, *The neural basis of addiction: a pathology of motivation and choice.* Am J Psychiatry, 2005. **162**(8): p. 1403-13.
64. Bobadilla, A.C., et al., *Corticostriatal plasticity, neuronal ensembles, and regulation of drug-seeking behavior.* Prog Brain Res, 2017. **235**: p. 93-112.
65. Dong, Y., et al., *Circuit and Synaptic Plasticity Mechanisms of Drug Relapse.* J Neurosci, 2017. **37**(45): p. 10867-10876.
66. O'Brien, C.P., *Neuroplasticity in addictive disorders.* Dialogues Clin Neurosci, 2009. **11**(3): p. 350-3.
67. Kalivas, P.W. and C. O'Brien, *Drug addiction as a pathology of staged neuroplasticity.* Neuropsychopharmacology, 2008. **33**(1): p. 166-80.
68. Piazza, P.V., et al., *Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction.* J Neurosci, 2000. **20**(11): p. 4226-32.
69. Mantsch, J.R., et al., *Predictable individual differences in the initiation of cocaine self-administration by rats under extended-access conditions are dose-dependent.* Psychopharmacology (Berl), 2001. **157**(1): p. 31-9.
70. Ferris, M.J., et al., *Paradoxical tolerance to cocaine after initial supersensitivity in drug-use-prone animals.* Eur J Neurosci, 2013. **38**(4): p. 2628-36.

71. Piazza, P.V., et al., *Factors that predict individual vulnerability to amphetamine self-administration.* Science, 1989. **245**(4925): p. 1511-3.
72. Piazza, P.V., et al., *Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration.* Brain Res, 1990. **514**(1): p. 22-6.
73. Piazza, P.V., et al., *Corticosterone levels determine individual vulnerability to amphetamine self-administration.* Proc Natl Acad Sci U S A, 1991. **88**(6): p. 2088-92.
74. Piazza, P.V., et al., *Behavioral and biological factors associated with individual vulnerability to psychostimulant abuse.* NIDA Res Monogr, 1998. **169**: p. 105-33.
75. Klebaur, J.E., et al., *Individual differences in behavioral responses to novelty and amphetamine self-administration in male and female rats.* Behav Pharmacol, 2001. **12**(4): p. 267-75.
76. Cain, M.E., E.D. Denehy, and M.T. Bardo, *Individual differences in amphetamine self-administration: the role of the central nucleus of the amygdala.* Neuropsychopharmacology, 2008. **33**(5): p. 1149-61.
77. Suto, N., J.D. Austin, and P. Vezina, *Locomotor response to novelty predicts a rat's propensity to self-administer nicotine.* Psychopharmacology (Berl), 2001. **158**(2): p. 175-80.
78. Deroche-Gamonet, V., D. Belin, and P.V. Piazza, *Evidence for addiction-like behavior in the rat.* Science, 2004. **305**(5686): p. 1014-7.

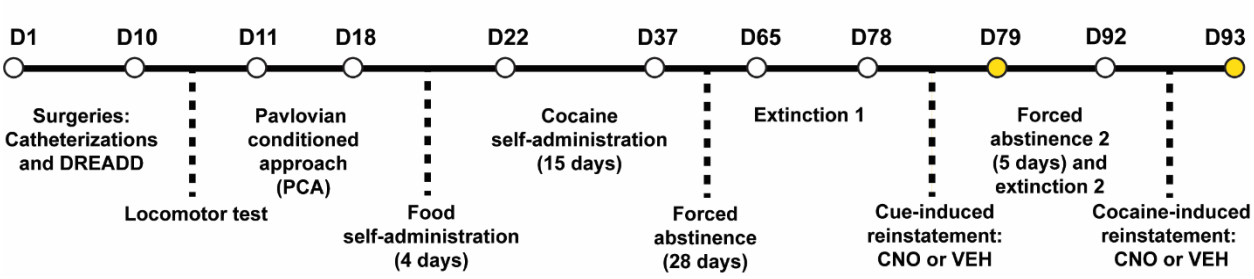
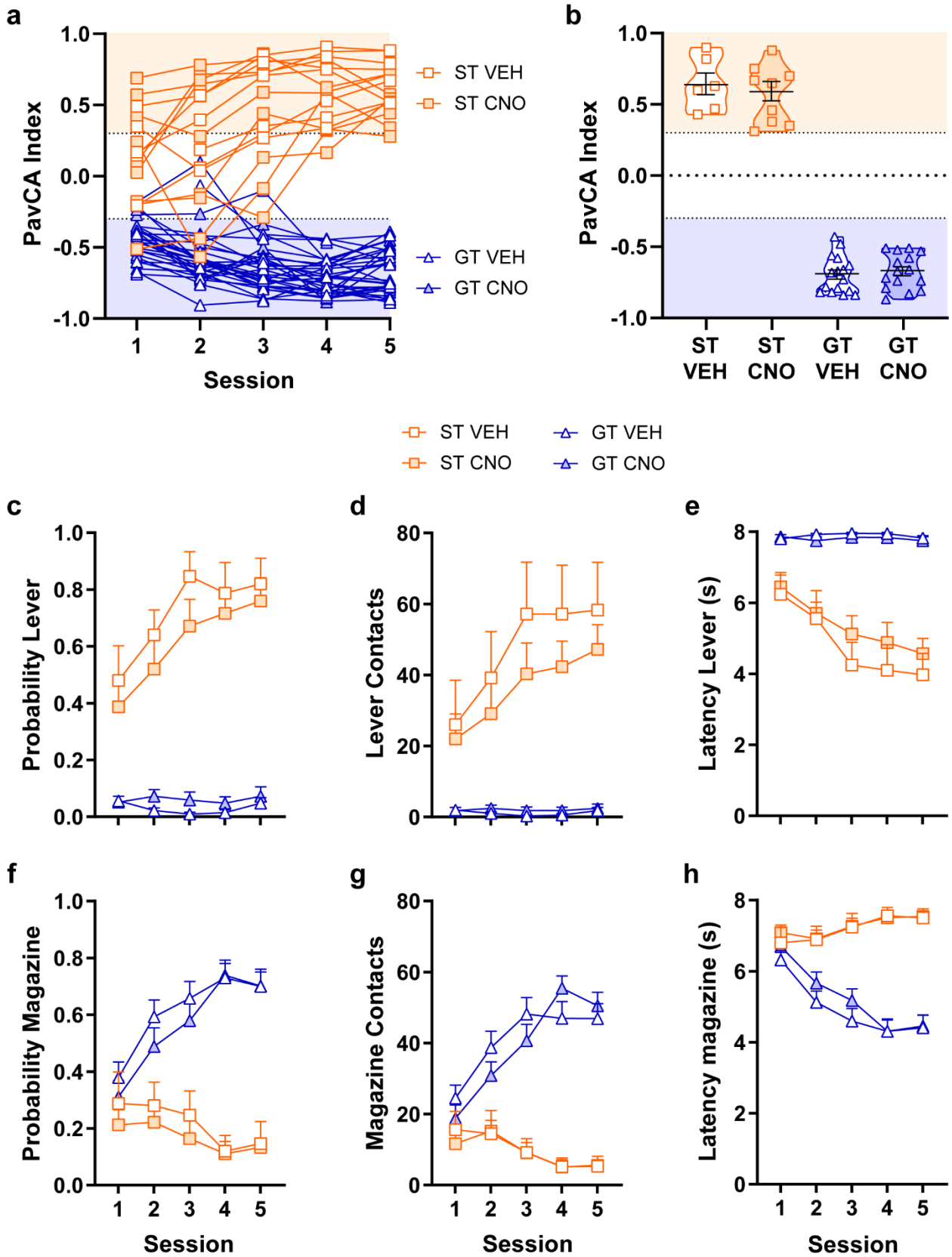


Fig. 1 Experimental timeline. Rats underwent surgeries for indwelling jugular catheter implantation and viral infusions into the PrL, aPVT and pPVT for G_i DREADD expression in the PrL-PVT pathway. Rats were then characterized based on their locomotor response to a novel environment and PavCA training. Food self-administration (4 days) and cocaine self-administration (15 days) followed, with a subsequent 28-day forced abstinence period. Daily extinction sessions occurred for 13 consecutive days prior to the cue-induced reinstatement test. Rats were given an injection (i.p.) of either vehicle (VEH) or 5 mg/kg clozapine-N-oxide (CNO) to activate the G_i DREADD prior to the reinstatement test. Following cue-induced reinstatement, rats underwent 5 days of forced abstinence followed by extinction training for 8 consecutive days before cocaine-primed reinstatement. Prior to the reinstatement test, rats were given the same treatment (vehicle or CNO) as that prior to the cue-induced reinstatement, as well as a 15 mg/kg injection of cocaine (i.p.) immediately before being placed into the testing chamber.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e
Latency Lever (s)
f
Probability Magazine
g
Magazine Contacts
h
Latency magazine (s)

Fig. 2 Individual differences in Pavlovian conditioned approach (PavCA) training. a) PavCA scores across 5 days of training, and **b)** the average PavCA score from session 4 and 5 of training. Scores between 0.3 and 1 indicates a conditioned response directed toward the lever-CS (i.e. ST), whereas a score between -0.3 and -1 corresponds to a conditioned response directed toward the food magazine (i.e. GT). Mean + SEM for **c)** the probability to approach the lever-CS during its presentation, **d)** the number of lever-CS contacts made, **e)** latency to approach the lever-CS, **f)** probability to contact the food magazine during lever-CS presentation, **g)** number of food magazine contacts, and **h)** latency to contact the food magazine during lever-CS presentation. There was a significant effect of Phenotype, Session, and Phenotype x Session interaction for all measures ($p<0.01$). Sign-trackers (VEH, n=6; CNO, n=9) acquired lever-CS directed behavior, and goal-trackers (VEH, n=15; CNO, n=15) acquired food-magazine directed behavior. Rats did not receive any treatment prior to PavCA training but data are illustrated according to the treatment (i.e. VEH or CNO) rats will receive prior to the reinstatement tests.

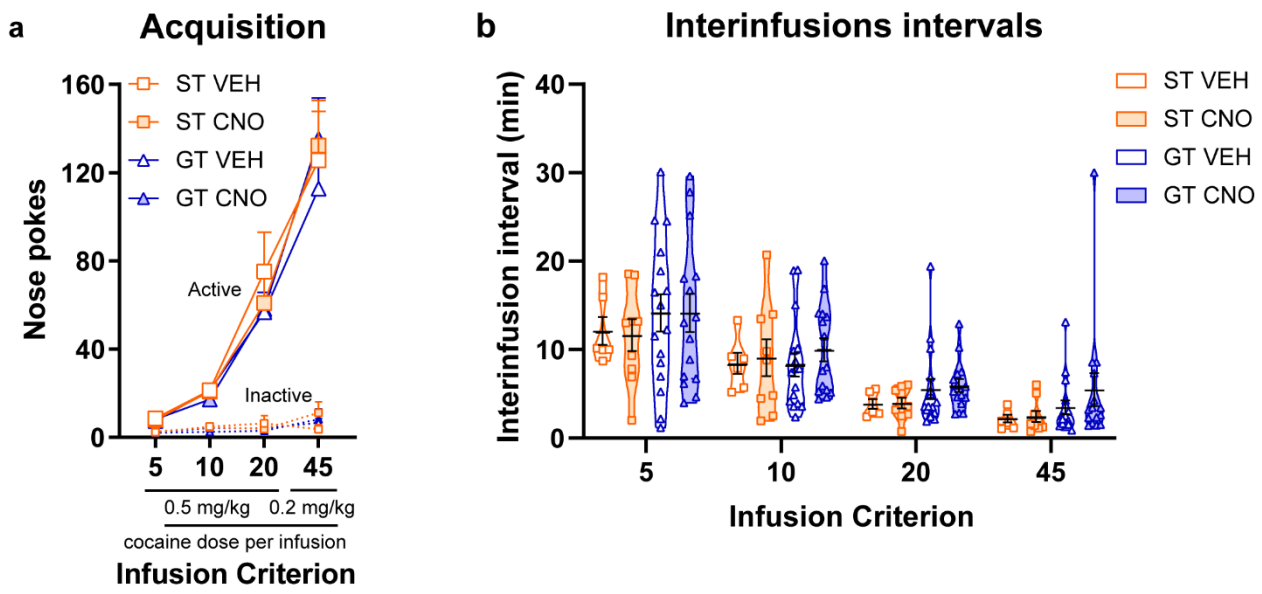

Fig. 3 Acquisition of cocaine self-administration in STs and GTs. Mean + SEM for **a)** nose pokes into the active and inactive port for STs and GTs during acquisition of cocaine self-administration across infusion criterion (IC). Rats made more pokes into the active port ($p<0.001$) across infusion criterion ($p<0.001$). **b)** Interinfusion intervals (III) for STs and GTs at each IC. III decreased across all groups as training progressed ($p<0.001$). (ST VEH, n=6, ST CNO, n=9, GT VEH, n=16, GT CNO, n=15)

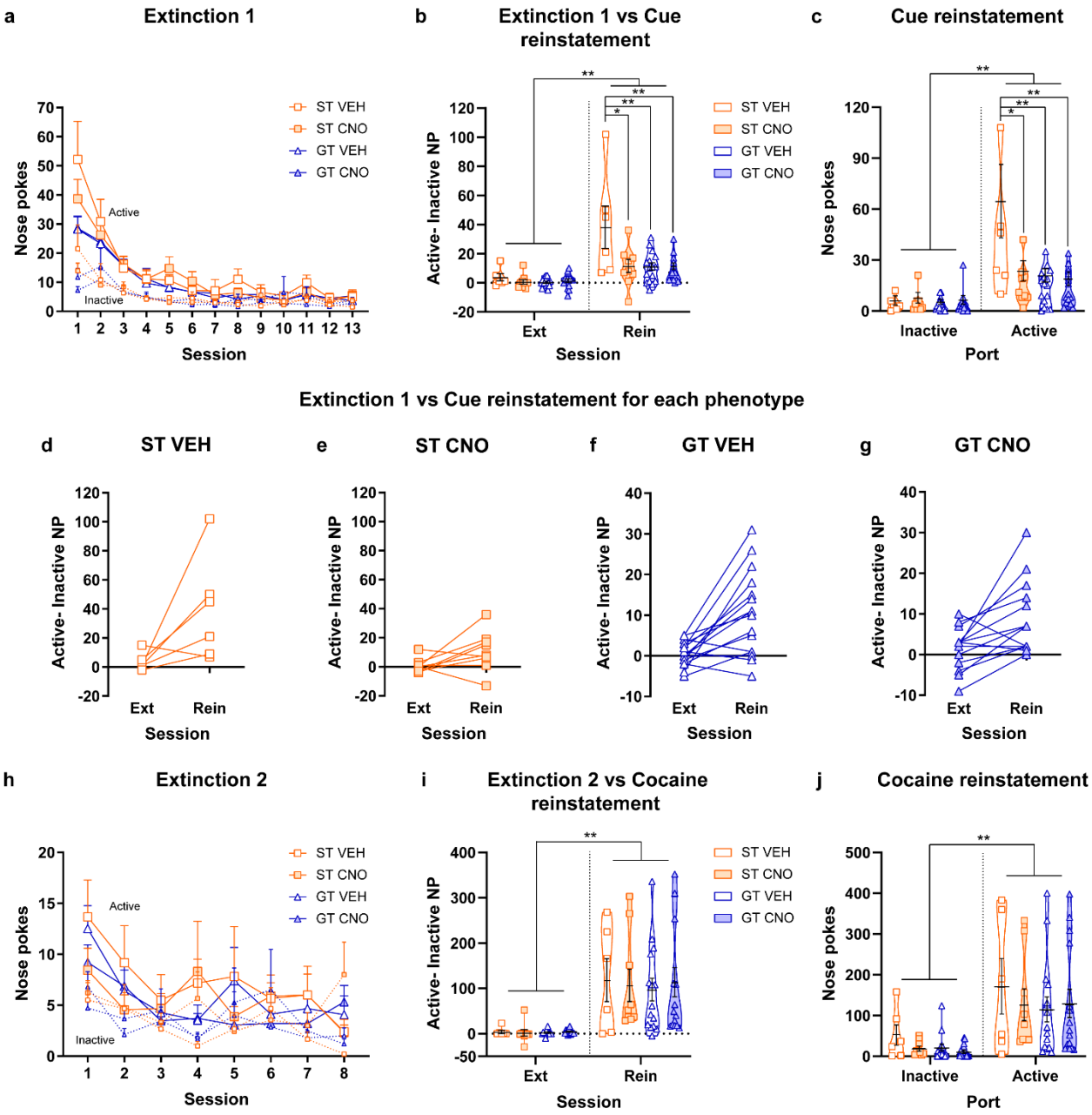

Fig. 4 Extinction training and drug-seeking behavior during the tests for cue-induced and cocaine-primed reinstatement in STs and GTs. Mean + SEM for **a)** Nose pokes (NP) made into the active (solid lines) and inactive (dashed lines) ports across 13 sessions of extinction training in STs and GTs. Rats decreased drug-seeking behavior across training sessions ($p<0.0001$). **b)** Active-inactive NPs made during the last extinction training session compared to the test for cue-induced reinstatement. All rats showed an increase in drug-seeking behavior during the test compared to extinction ($p<0.0001$). c**)** NPs into the inactive and active port during

the test for cue-induced reinstatement. The ST VEH group made more pokes into the active port compared to the GT VEH ($p=0.02$) and GT CNO ($p= 0.01$) groups. Inhibition of the PrL-PVT pathway selectively decreased drug-seeking behavior in STs ($p=0.02$) compared to the ST VEH group. Active-inactive NPs made during the last extinction training session (Ext) and the test for reinstatement (Rein) for each rat in the **d)** ST VEH, **e)** ST CNO, **f)** GT VEH, and **g)** GT CNO groups. **h)** Nose pokes made into the active (solid lines) and inactive (dashed lines) ports across 8 sessions of Extinction 2 training in STs and GTs. Rats decreased drug-seeking behavior across training sessions ($p<0.0001$). **i)** Active-inactive nose pokes (NP) made during the last extinction training session compared to the test for cocaine-primed reinstatement. All rats showed an increase in drug-seeking behavior during the test compared to extinction ($p<0.0001$). **j)** Nose pokes into the inactive and active port during a test for cocaine-induced reinstatement. *$p<0.05$, **$p<0.01$ (ST VEH, n=6, ST CNO, n=8, GT VEH, n=16 (n=15 for cocaine reinstatement), GT CNO, n=15)

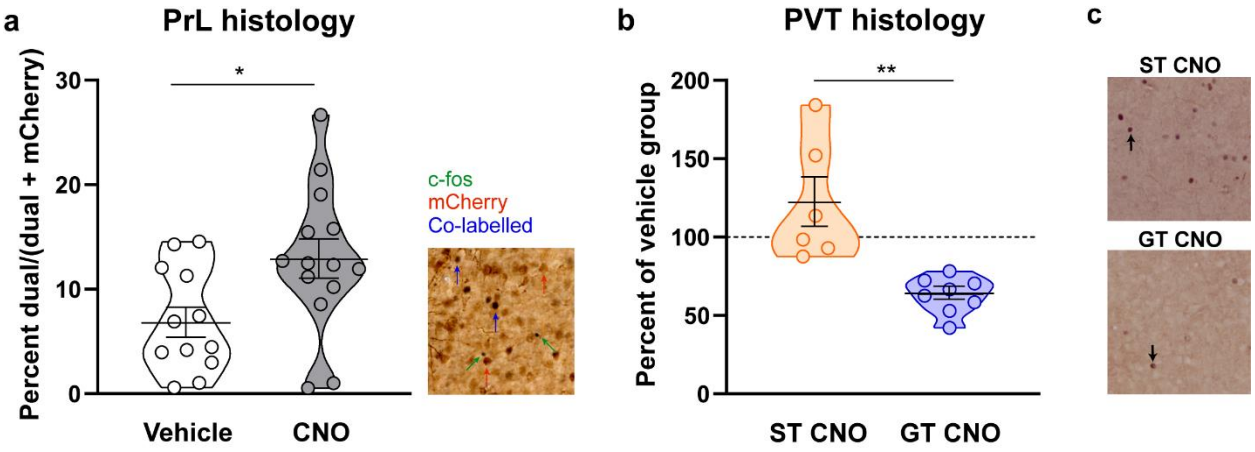

Fig. 5 Histology of cells in the PrL and PVT. a) Percent of cells across the PrL (anterior, middle, posterior) co-expressing both Fos and mCherry (DREADD tag) in relation to all cells expressing mCherry. CNO administration increases this percentage compared to vehicle treated rats (p=0.02) (VEH group, n=12 (ST, n=2; GT, n=10); CNO group, n=14 (ST, n=6; GT, n=8). Adjacent is a representative image of the PrL with green arrows indicating Fos expression, red mCherry expression and blue are cells co-labelled with both Fos and mCherry. **b)** Mean + SEM for percent Fos density throughout the entire PVT (anterior, middle and posterior) relative to the vehicle-treated group (i.e. baseline). Inhibition of the PrL-PVT pathway results in a decrease in Fos density in GTs relative to vehicle-treated rats (p<0.001), and no effect in STs relative to vehicle-treated rats (p=0.23). Inhibition of the PrL-PVT pathway differentially affected PVT Fos density (% vehicle) STs relative to GTs (p=0.002; **p<0.01). **c)** Representative images of Fos expression in the PVT of a ST and GT following PrL-PVT inhibition (i.e. CNO administration). Black arrows indicate Fos protein. (VEH group, n=13 (ST, n=3; GT, n=10); ST CNO, n=6; GT CNO, n=8)

Supplementary materials and methods

High-responder (HR)/ Low-responder (LR) model. In addition to analyzing data within the scope of the ST/GT model, we also analyzed the data within the context of the high-responder (HR)/ low-responder (LR) animal model, which captures individual variation in the acquisition of drug self-administration. When characterized based on lomocotor response to an inescapable novel environment, high-responders (HRs) self-administer cocaine [68-70], amphetamine [71-76], and nicotine [77] at a faster rate compared to low-responders (LRs). After prolonged cocaine self-administration, however, outbred animals characterized as HRs and LRs do not differ in other-addiction related behaviors [78]. Thus, this outbred animal model appears to solely capture individual variation in the initiation of drug-taking behavior. In the current study, all rats underwent a locomotor test prior to being characterized as a ST or GT. The objective of this analysis was to assess whether PrL-PVT inhibition similarly affects cue-induced and cocaine-primed drug-seeking behavior in rats using two different models of addiction vulnerability: one that captures individual variation in reinstatement propensity (ST/ GT model); and one that captures individual variation in the acquisition of cocaine self-administration (HR/LR model).

Methods

Subjects. All experimental procedures used were approved by the University of Michigan Institutional Animal Care and Use Committee and abided by the standards set in *The Guide for the Care and Use of Laboratory Animals: Eighth Edition*, published by the National Academy of Sciences and revised in 2011. Throughout the study, rats had ad libitum access to food and water and were housed in a climate-controlled room with a 12-hour light: dark cycle (lights came on at either 06:00 h or 07:00 h depending upon daylight savings time). Upon arrival, rats were pair-housed until surgeries, after which they were single-housed for the remainder of the study to protect the catheters from possible damage from a cage mate. All behavioral training occurred during the light cycle, between 08:00 h and 18:00 h, with specific testing times for each procedure included below.

Surgical procedures. After arrival in our housing facilities, rats were allowed to acclimate to the housing room for a minimum of 5 days prior to surgery. Rats were anesthetized using ketamine (90 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and implanted with the jugular vein catheter. After

the surgery was completed, rats were given a 5 ml injection (s.c.) of saline to minimize dehydration and facilitate recovery. To ensure sufficient recovery, rats underwent stereotaxic surgery 24 h to 48 h after catheterization surgery.

For stereotaxic surgery, rats were anesthetized using 5% isoflurane, and then maintained under 2% isoflurane for the duration of the surgery. Each stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) was outfitted with two digital manipulator arms (Stoelting, Wood Dale, IL). Rats were fitted into the ear bars, and then the scalp was cleaned with a series of Betadine solution (Purdue Products, Stamford, CT) and ethanol wipes prior to an incision being made to expose the skull. Bregma was determined, and the skull was then leveled such that lambda was within +/- 0.1 mm of the bregma coordinates. The G_i DREADD was injected bilaterally into the PrL using a standard infusion pump (Pump 11 Elite, Harvard Apparatus) to depress a 5 µl Hamilton syringe with P50 tubing connecting it to a 31-gauge injector. The AAV-Cre was injected into the anterior and posterior PVT using a 1 µl Hamilton Microsyringe. Injections into the PVT were made at a 10° angle to the midline to avoid puncturing the superior sagittal sinus and causing unnecessary bleeding. At the completion of each injection, the injector was kept in place for an additional five minutes to allow the virus to diffuse away from the injector and into the tissue. At the conclusion of the surgery, surgical staples were used to bring the scalp back together.

Prior to the start of each surgery, and 24 h following surgery, an injection of 5 mg/ml of Carprofen was given as an analgesic. Additionally, rats received daily infusions i.v. of heparin (100 units/ml, 0.05 ml) and gentamicin sulfate (1 mg/ml, 0.05 ml) in order to decrease the chance of infection and maintain catheter patency until the conclusion of cocaine self-administration training. Following surgeries, rats were given a minimum of five days of recovery prior to the start of behavioral training. All surgical staples and sutures were removed within ten days of surgeries. Prior to the start of cocaine self-administration, catheter patency was checked using methohexital sodium diluted in sterile saline (10 mg/ml i.v., 0.1 ml). Rats were removed from the study due to lack of patency if the rat did not become ataxic within 10 seconds of infusion. Additionally, if rats stopped self-administering cocaine in the midst of the study, catheter patency was checked, and rats removed from the study accordingly.

Locomotor test. After the surgical recovery period, rats underwent a 60-min locomotor test. Testing chambers (43 x 21.5 cm floor area, 25.5 cm high) were outfitted with infrared beams to track both lateral (beams 2.3 cm above grid floor) and rearing (beams 6.5 cm from grid floor) movements. All testing occurred between 10:00 h and 16:00 h under red light. Lateral and rearing movements were recorded in 5-min increments. At the conclusion of the test, rats were returned to their home cages in the colony room. Cumulative locomotor movements (i.e. rearing and lateral movements) were used to classify rats into their respective phenotypes. Rats for this study were run in six separate cohorts and classified as HRs and LRs within each cohort using a median split based on the cumulative locomotor movements.

Pavlovian conditioned approach (PCA) training. Two days prior to the start of Pavlovian conditioned approach (PCA) training rats were given 45-mg banana-flavored grain pellets (about 25 pellets per rat; Bio-Serv, Flemington, NJ, USA) in their home cage to habituate them to the food reward used during training. Rats started PCA training the day following the locomotor test. Standard behavioral testing chambers (MED Associates, St. Albans, VT, USA; 20.5 x 24.1 cm floor area, 29.2 cm high) were used for PCA training. Chambers were situated in a sound-attenuating boxes fitted with a fan to provide constant background noise and air circulation. Within each chamber, a food magazine was situated 6-cm above the flooring and attached to a pellet dispenser. When the photo beam within the food magazine was broken a “contact” to the magazine was recorded. A retractable lever that illuminated upon presentation was situated to either the right or left of the food magazine and at the same height. In order for a lever “contact” to be registered and recorded, a minimum of 10-g of force had to be exerted. A white house light in the center of the opposite wall (1-cm from the top of the chamber) remained illuminated for the duration of each session.

Rats underwent two days of pre-training, with one session a day. During pre-training, the food magazine was baited with two banana-flavored grain pellets to direct the rats’ attention to the location of reward delivery. After a 5-min acclimation period, the house light turned on and the session started, lasting approximately 12.5 minutes. During pre-training sessions, the lever remained retracted, and pellets were delivered to the food magazine on a variable interval 30-second schedule (range 0-30 seconds). Sessions consisted of 25 trials, with one pellet being delivered per trial. At the conclusion of the session, the house light turned off. The number of

food magazine entries made and pellets remaining in the food magazine were recorded at the end of each session.

Following pre-training, rats underwent five PCA training sessions, with one session per day. As with pre-training, the start of each session was signaled with the house light turning on, and the conclusion by the house light turning off. Each training session consisted of 25 lever-CS/ food-US trials on a variable interval 90-second schedule (range 30-150 seconds). Each session lasted approximately 40-min, and all training occurred between 10:00 h and 16:00 h.

For each PCA training sessions, the Med Associates software recorded the following information: (1) number of magazine contacts made during the 8-sec lever-CS presentation, (2) latency to the first magazine contact during lever-CS presentation, (3) number of lever-CS contacts, (4) latency to the first lever-CS contact, (5) the number of magazine entries made during the inter-trial interval (i.e. contacts made between lever-CS presentations). Using these measures, the PCA index was calculated for each rat and used to characterize rats into their behavioral phenotypes based on their conditioned response (CR) made during training. The following formula is used to calculate the PCA index: [Probability Difference Score + Response Bias Score + (-Latency Difference Score)/3] [11].

Food self-administration. Following PCA training, rats had a 24-hour break during which they remained in the colony room undisturbed before starting food self-administration training. Food self-administration took place in the same testing chambers as PCA training, however the chambers were reconfigured such that the lever was removed, and two nose ports were put on either side of the food magazine. The house light and a discrete cue light within the active port turned on one minute after the program was started. The cue light remained on until the first poke was made into the active port in order to direct the rat's attention to the port. Pokes into the active port resulted in the delivery of one 45-mg banana flavored grain pellet (FR1 schedule of reinforcement), the same reward used during PCA training, as well as presentation of the cue-light inside the port for 20 seconds. Additional pokes made into the active port during the 20-sec cue-light presentation period were recorded, but were without consequence. During cue-light presentation, the house light also turned off. Pokes into the inactive port were recorded, but without consequence. Rats underwent four days of food self-administration testing, with one

session per day. Sessions were terminated after rats had acquired 25 pellets, or after 30-min. All training occurred between 10:00 h and 14:00 h.

Cocaine self-administration. At the conclusion of food self-administration, rats were left undisturbed in the colony room for 24 hours prior to the start of cocaine self-administration. Testing chambers remained in the same configuration as food self-administration, with the exception that the food magazine and pellet dispenser were removed. The house light and cue light in the active port turned on one minute after the session started. In contrast to food self-administration, the cue light remained on for 20-sec at the start of the session, during which time a poke into the active port could result in an infusion of cocaine. Throughout the session, pokes into the active port (FR1 schedule of reinforcement) resulted in the presentation of the cue light for 20-sec and a 0.5 mg/kg infusion of cocaine (Mallinckrodt, St. Louis, MO) diluted in 0.9% sterile saline, delivered in 25 µl over 1.6 seconds. However, during the IC45 sessions, the cocaine infusion dose was decreased to 0.2 mg/kg/infusion to enhance drug-seeking behavior. Additional pokes into the active port during the 20-sec cue light presentation were recorded, but without consequence. Pokes made into the inactive port were recorded, but also without consequence. Rats underwent one cocaine self-administration training session per day for 15 consecutive days between 8:00 h and 18:00 h. In order to move to the next IC and remain in the study, rats had to meet the IC for two consecutive sessions and maintain catheter patency. At the conclusion of training, rats underwent a 28-day period of forced abstinence where they were left undisturbed in the colony room.

Extinction training 1 and 2. Testing chambers remained in the same configuration as that during cocaine self-administration, however pokes into both the active and inactive nose port were recorded, but without consequence (i.e. pokes into the active port did not result in cue-light presentation or drug delivery). Sessions lasted for 2 hours, and rats underwent one session per day for a total of 13 sessions (Extinction 1) or 8 sessions (Extinction 2). Rats had to make less than ten pokes into the active port for two consecutive sessions in order to be eligible to move to the subsequent reinstatement test, or they were eliminated from the study. All training took place between 09:00 h and 18:00 h. After the last training session, rats were moved to an adjacent testing room and given an i.p. injection of 6% DMSO in 0.9% sterile saline (CNO vehicle) to habituate them to the injections that would occur prior to the reinstatement test.

Cue-induced reinstatement test. Rats underwent a test for cue-induced reinstatement the day following the last extinction training 1 session (i.e. session 13). Rats were counterbalanced into treatment groups based on behavior during PCA training (lever- and magazine-directed behavior, PCA index), locomotor index (cumulative locomotor movements), and behavior during cocaine self-administration and extinction training (nose pokes made into the active and inactive ports). Twenty-five minutes prior to the test, rats received either an injection of CNO (5 mg/kg i.p.) to activate the G_i DREADDs or vehicle (6% DMSO in 0.9% sterile saline) in an adjacent testing room. After the rats were placed in the testing chambers, one minute elapsed prior to illumination of the house light. The cue light in the active port also came on at the session start, and remained on for 20 seconds, as it did during the cocaine self-administration procedure. Pokes into the active port were recorded and resulted in presentation of the cue light for 5-sec, but no cocaine infusion, while pokes into the inactive port were recorded but without consequence. The test lasted for 2 h and occurred between 10:00 h and 17:00 h.

Cocaine-induced reinstatement test. Rats underwent 5 days of forced abstinence followed by extinction training 2 sessions (8 sessions) prior to the cocaine-induced reinstatement test. As with the cue-induced reinstatement test, rats received the same treatment (i.e. CNO or vehicle) 25-min prior to the session in an adjacent testing room as. Before being put into the testing chamber, all rats received a 15 mg/kg (i.p.) injection of cocaine. This dose of cocaine was chosen as it has previously been shown to result in ST/GT differences in drug-seeking behavior during a test for cocaine-induced reinstatement [17]. The house light came on one minute after the session was initiated, but in contrast to the cue-induced reinstatement test the cue-light did not come on at any point during the test session. Pokes into the active and inactive port were recorded, but without consequence (i.e. no cue-light presentation when pokes were made into the active port). The session lasted for 2 h and occurred between 10:00 h and 17:00 h.

Histology. Once testing was complete, all rats were anesthetized with ketamine (90 mg/kg. i.p.) and xylazine (10 mg/kg, i.p.) and underwent transcardial perfusion. Rats were first perfused with 0.9% saline followed by 4% formaldehyde (pH= 4.7) at 4°C. After extraction, brains remained in formaldehyde for a 24 h period. Brains were then moved to graduated sucrose solutions (10%, 20% then 30% sucrose in phosphate buffer, pH= 7.4, 4°C) every 24 hours for 3 days for

cryoprotection. Following cryoprotection, brains were encased in Tissue-Plus O.C.T. cryoprotectant (Fisher HealthCare, Houston, TX) and frozen using dry ice. They were then sliced at a thickness of 30 µm on a cryostat and underwent free-floating immunohistochemistry (IHC) for mCherry (protein fused to DREADD) to assess hM4D(G_i) DREADD expression, and a subset of brains underwent an additional IHC for Fos protein expression.

All procedures occurred at room temperature on a lab shaker on a low speed. Slices were washed in 0.1 M phosphate-buffered saline (PBS, pH= 7.3-7.4) five times (5-min each wash) and then blocked in 1% hydrogen peroxide in PBS solution for 10-min. Slices were then washed in PBS (4 washes) and blocked in 0.4% Triton-X and 2.5% Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA) diluted in PBS for one hour, and then incubated overnight in a PBS solution containing 0.4% Triton-X and 1% Normal Donkey Serum with rabbit anti-mCherry primary antibody (Abcam, Cambridge, MA) at a 1:30,000 dilution. The next day, slices were washed three times in PBS and incubated for one hour in a PBS solution containing biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:500 dilution, 0.4% Triton-X, and 1% Normal Donkey Serum. After another series of three PBS washes, slices were incubated for one hour in ABC-elite (Vector Laboratories, Burlingame, CA) diluted at 1:1000 in PBS, and then underwent three PBS washes. In order to view DREADD expression, slices were then incubated in a 0.1M sodium phosphate-buffered solution containing 0.02% 3, 3’ diaminobenzidine tetrahyrochloride (DAB; Sigma Aldrich, St. Louis, MO) and 0.012% hydrogen peroxide. Incubation lasted for 8-min, and then slices were rinsed three times in PBS and stored at 4°C until being mounted. DAB staining creates a brown residue at the location of DREADD expression, allowing for subsequent visualization.

Fos protein expression in response to test day treatment (CNO or vehicle) was analyzed in a subset of rats (GT VEH: 10, GT CNO: 8, ST VEH: 3, ST CNO: 6) to verify that the G_i DREADDs were in fact affecting the activity in the PrL-PVT pathway. Eighty minutes prior to perfusions, rats were injected (i.p.) with either 5.0 mg/kg CNO or vehicle (same as test day treatment) and left in the colony room. This time period was chosen as Fos protein expression has been shown to peak 60-90-min after cellular activation [50]. Brains from rats with good DREADD expression underwent free-floating IHC for co-expression of Fos protein and DREADD expression. The IHC for Fos protein was performed first, and all procedures are the same as previously discussed above with the following exceptions: a goat anti-Fos primary

antibody (Santa Cruz Biotechnology, Dallas, TX) diluted at 1:1000 and a biotinylated donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted at 1:500 were used. Additionally, nickel sulfate (0.08%) was added to the DAB solution so that Fos protein expression would have a black, and not brown residue, allowing for contrast between the two stains. Immediately following the IHC for Fos protein, the IHC for DREADD expression was completed as described above.

Brains were then mounted and dehydrated in ethanol solution, washed in xylene and coverslipped with Permount (Fisher Scientific, Fair Lawns, NJ). Using a Leica DM1000 light microscope (Buffalo Grove, IL), DREADD expression in the PrL, aPVT and pPVT were qualitatively scored by two researchers who were blind to group assignments. DREADD expression scores ranged from 0 to 3 and were based on localization and strength of the expression. In order to be included in final analysis (scores of 1-3), DREADD expression had to be localized to the PrL (relative to bregma: AP 5-2.50), aPVT (relative to bregma: AP ¯1.20-¯2.50) and pPVT (relative to bregma: AP ¯2.51-¯3.50), and had to be easily visible in the microscope. A score of 1 indicated weak expression, whereas a score of 3 indicated strong expression. Rats with a score of zero (e.g. inaccurate injection, lack of expression in a region, etc.) were not included in final analyses.

To assess the effects of PrL-PVT inhibition on activity in the PVT, Fos protein expression was quantified in the PVT. One PVT section in the anterior (relative to Bregma: ¯2.00), middle (relative to Bregma: AP ¯2.50), and posterior (relative to Bregma: AP ¯3.00) subregions was analyzed using ImageJ (National Institute of Health, Bethesda, MD). The number of Fos positive cells and density of expression (cells per square mm) were determined using the ITCN (image-based tool for counting nuclei) plugin from ImageJ.

Video analysis: Cocaine-primed reinstatement test. Both stereotyped and non-stereotyped behaviors during the cocaine-primed reinstatement test for each rat was quantified. Quantified behavior was selected due its high presence shown by several rats when videos were randomly sampled for screening. Stereotyped behaviors include: circling, head movements but no port entry, head movements and port entry. Non-stereotyped behaviors include: immobility, orientation toward the active port without movement toward it, approach to the active port without port entry. A more detailed description of the criteria for each behavior can be found in

Table S1. Behavior was analyzed for 30-sec every ten minutes for the first hour of the reinstatement test, as this is when peak drug-seeking behavior occurred. The percentage of time (min) rats spent exhibiting each behavior across the six bins was quantified.

Statistical analyses. To assess if there was a relationship present between rate of extinction (i.e. decrease in active nose pokes) and the reinstatement tests, a quadratic regression model was fit to each rat's extinction training 1 and 2 curve. The quadratic term was then regressed onto the number of nose pokes into the active port during the reinstatement test that followed each extinction training. This analysis allows us to account for differences in drug-seeking behavior during the reinstatement tests as a function of rate of extinction. SPSS version 22 was used for these analyses and statistical significance was set at $p<0.05$.

All significant results for the tests for cue-induced and cocaine-primed reinstatement were subjected to a power analysis and exhibited adequate statistical power ($1-\beta>0.55$).

Results

Subjects. Seven rats died during catheterization or stereotaxic surgery, prior to training. Three rats were eliminated from the study following PCA training as they did not acquire the task (i.e. did not approach the lever-CS or food magazine during training). One rat was sacrificed at the conclusion of food self-administration training due to health complications. Additional rats were removed from the study if they did not meet the IC during self-administration training (n= 51 (ST: 4/36; GT: 29/96; IN: 17/38) or due to loss of catheter patency, n= 1 (ST)).

Behavioral results: ST/GT model

More STs acquire cocaine self-administration than GTs. Unlike previous experiments, more GTs (30%) were excluded from the study due to not meeting IC requirements compared to STs (11%). This suggests that while behavior did not differ between rats that did acquire cocaine self-administration, STs are more likely to acquire self-administration compared to GTs, at least in this study.

All rats exhibited cue-induced reinstatement and inhibition of the PrL-PVT pathway selectively attenuates drug-seeking behavior in STs. To compare responding during the cue-

induced reinstatement test to that during the last extinction session, we examined the effects of Phenotype (ST or GT) and Treatment (VEH or CNO) on the number of NPs in the active port minus those in the inactive port (i.e. active – inactive nose pokes; dependent variable). All rats showed an increase in drug-seeking behavior during the test for cue-induced reinstatement compared to the last extinction training session (effect of Session, $F_{1,40}$=33.37, p<0.0001). There was a significant effect of Phenotype ($F_{1,40}$=4.59, p=0.04), effect of Treatment ($F_{1,42}$=7.51, p=0.009) and a Treatment x Session interaction ($F_{1,40}$=5.52, p=0.02), indicating that responding was differentially affected in a treatment-dependent manner between the two sessions. Lastly, it should be noted that there were no significant correlations between nose pokes made into the active port during the test for cue-induced reinstatement and PCA index score (ST VEH, r^2=0.74, p= 0.06; ST CNO, r^2=0.003, p= 0.91; GT VEH, r^2=0.02, p=0.62; GT CNO, r^2=0.05, p= 0.43). Thus, the degree to which one sign- or goal-tracks does not appear to predict cue-induced reinstatement of drug-seeking behavior.

Removal of outliers for cue-induced reinstatement test does not affect results. As indicated in the main text, there were two outliers in the test for cue-induced reinstatement that were not removed from final analysis (ST VEH, ST CNO). As described below, the results remained consistent even with their removal. Compared to the last extinction training session, all rats exhibited greater drug-seeking behavior during the test for cue-induced reinstatement (effect of Session, $F_{1,40}$=33.37, p<0.001). During the reinstatement test, all rats made more pokes into the active port compared to the inactive port (effect of Port, effect of Phenotype, $F_{1,80}$=41.21, p<0.001, and STs showed great drug-seeking behavior compared to GTs (effect of Phenotype, $F_{1,80}$=4.18, p=0.44). Treatment administered affected drug-seeking behavior (effect of Treatment, effect of Phenotype, $F_{1,80}$=4.36, p=0.04), and a Phenotype x Treatment x Port interaction was present (effect of Phenotype, $F_{1,80}$=4.30, p=0.04). Post-hoc analyses revealed that STs made more pokes into the active port compared to GTs (p=0.02), and inhibition of the PrL-PVT pathways decreased drug-seeking behavior in ST (p=0.02). Thus, our main findings are not affected by excluding these rats from final analyses.

All rats exhibited cocaine-primed reinstatement. Drug-seeking behavior (active-inactive nose pokes) between the last extinction session and the test for cocaine-induced reinstatement was analyzed as a means to directly compare responses between the two sessions. All rats showed

greater drug-seeking behavior during the test for cocaine-induced reinstatement compared to the last extinction training session (effect of Session, $F_{1,40}$=34.48, p<0.0001). However, there was not a significant effect of Treatment (effect of Treatment, $F_{1,40}$=0.06, p=0.81), effect of Phenotype (effect of Phenotype, $F_{1,40}$=0.01, p=0.93), nor any significant interactions present.

In an attempt to account for the variance in drug-seeking behavior seen during this test, correlations between the number of nose pokes made into the active port and other behaviors throughout the study were assessed. There were no significant correlations between nose pokes made into the active port during the tests for cue-induced reinstatement and cocaine-primed reinstatement (ST VEH, r^2=0.12, p= 0.57; ST CNO, r^2=0.03, p= 0.72; GT VEH, r^2=0.23, p=0.07; GT CNO, r^2<0.001, p= 0.96); nor was there a significant correlation between PCA index score and cocaine-primed drug-seeking behavior (ST VEH, r^2=0.51, p= 0.11; ST CNO, r^2=0.02, p= 0.76; GT VEH, r^2=0.11, p=0.23; GT CNO, r^2=0.10, p= 0.26).

STs and GTs do not differ in cocaine-induced stereotyped behaviors during cocaine primed-reinstatement. Stereotyped behaviors during cocaine-primed reinstatement were assessed in a subset of rats (ST VEH, n=4; ST CNO, n=3; GT VEH, n=10; GT CNO, n=9). Rats spent more time exhibiting "non-stereotyped behaviors" (e.g. active port orientation/ approach, immobility) compared to "stereotyped behaviors" (e.g. head movements, circling) (effect of Category, $F_{1,22}$=8.13, p<0.001) (Fig. S1). On average, rats spent ~5-10% percent of the time exhibiting stereotyped behaviors. STs and GTs did not differ on this measure (effect of Phenotype, $F_{1,22}$<0.0001, p=0.99) and treatment did not affect cocaine-induced stereotypy (effect of Treatment, $F_{1,22}$=0.04, p=0.84) (Fig. S1). These data suggest that at least some animals exhibited stereotypy, which may have interfered with cocaine-induced reinstatement measures; but the small sample size and lack of significant treatment and phenotype effects warrants further investigation in this regard.

Rate of extinction does not predict reinstatement of drug-seeking behavior in STs or GTs. There was not a significant relationship present between the rate of extinction (extinction 1; i.e., the change in responding in the active nose port across sessions) and the number of nose pokes made into the active port during the test for cue-induced reinstatement (ST VEH, r^2=0.20, p=0.45; ST CNO, r^2=0.12, p=0.41; GT VEH, r^2=0.01, p=0.79; GT CNO, r^2=0.05, p=0.45). This suggests that the rate by which rats decreased nose pokes made into the active port during

extinction training did not correlate with cue-induced reinstatement of drug-seeking behavior. There was also no relationship present between the rate of extinction (extinction 2) and the number of nose pokes made into the active port during cocaine-primed reinstatement (ST VEH, r^2=0.41, p=0.17; ST CNO, r^2=0.03, p=0.67; GT VEH, r^2=0.02, p=0.67; GT CNO, r^2=0.20, p=0.10), suggesting that variation in drug-seeking behavior seen during this test is not due to differences in extinction rate.

CNO in the absence of DREADD does not affect reinstatement of drug-seeking behavior. A separate cohort of rats (ST, n= 10; GT, n=15) underwent all experimental procedures as previously described in this manuscript, with the exception of undergoing stereotaxic surgery for DREADD virus expressions. During the reinstatement tests, rats in this group were administered either CNO or vehicle and acted as a control for the effects of CNO on behavior independent of circuit manipulation. All rats exhibited reinstatement of drug-seeking behavior relative to the last extinction training session during the test for cue-induced reinstatement (effect of Session, $F_{1,21}$=15.97, p=0.001) and cocaine-prime reinstatement (effect of Session, $F_{1,19}$=58.81, p<0.0001). Furthermore, rats made more pokes into the active port compared to the inactive port during both reinstatement tests (Cue: effect of Port, $F_{1,42}$=15.17, p<0.001, Fig. S2a; Prime: effect of Port, $F_{1,38}$=42.26, p<0.0001, Fig. S2b). STs and GTs did not differ in either cue-induced ($F_{1,42}$=0.003, p=0.95) or cocaine-primed ($F_{1,42}$=0.003, p=0.95) reinstatement of drug-seeking behavior. Importantly, administration of CNO prior to the test session in the absence of DREADDs did not affect drug-seeking behavior (Cue: effect of Treatment, $F_{1,42}$=0.53, p=0.47, Fig. S2a; Prime: effect of Treatment, $F_{1,38}$=0.07, p=.80, Fig. S2b).

Behavioral results: HR/LR model

HRs show greater locomotor activity in a novel environment compared to LRs. HRs showed greater locomotor activity in response to a novel environment compared to LRs (effect of Phenotype, $F_{1,42}$=63.02, p<0.0001; Figure S4), and locomotor activity of rats did not differ between cohorts (effect of Cohort, $F_{1,38}$=1.08, p=0.31). Additionally, in agreement with prior reports using outbred populations [12], there was not a significant correlation between locomotor activity and PavCA index for STs (r^2=0.10, p=0.25) or GTs (r^2=0.01, p=0.69), indicating that

these two traits – "sensation-seeking" and the propensity to attribute incentive salience to reward cues – are independent from one another.

HRs ad LRs differ in the acquisition of cocaine self-administration. All rats acquired cocaine self-administration (effect of IC, $F_{3,85}$=103.23, p<0.0001) and discriminated between the two ports (effect of Port, $F_{1,85}$=251.14, p<0.0001; Fig. S5a). Responses in the ports were dependent upon the IC (IC x Port interaction, $F_{3,85}$=76.64, p<0.0001), such that rats increased nose pokes made into the active port as training progressed (p<0.0001), but not the inactive port. Behavior in HRs and LRs differed with each successive IC (Phenotype x IC interaction, $F_{3,85}$=4.07, p=0.01), as HRs showed greater nose-poking behavior during IC10 (p=0.01) and IC20 (p=0.02) compared to LRs (Fig. S5a). A treatment by IC interaction (F3,85=3.35, p=0.02) was also present revealing that rats in the vehicle treatment group showed greater nose-poking behavior at IC10 (p=0.04) than rats in the CNO treatment group. However, the two treatment groups did not differ at IC5 (p=0.67), IC20 (p=0.32) or IC45 (p=0.26).

To assess individual variation in the acquisition of cocaine self-administration, the interinfusion interval (III), or time (min) between pokes into the active port that resulted in an infusion of cocaine, was analyzed for each IC. Rats decreased their III as training progressed (effect of IC, $F_{3,74}$=30.30, p<0.0001), but HRs showed lower III across training compared to LRs (effect of Phenotype, $F_{1,50}$=6.78, p=0.01; Fig. S5b). A decrease in III is a function of both phenotype and the IC (Phenotype x IC interaction, $F_{3,74}$=4.16, p=0.01). Post-hoc analyses showed that HRs had lower III compared to LRs during IC5 (p=0.01) but did not differ during IC10 (p=0.06), IC20 (p=0.07) and IC45 (p=0.31) (Fig. S5b). Session length was also analyzed across cocaine self-administration training. Session length increased across IC (effect of IC, $F_{3,80}$=5.14, p=0.003) as would be expected, however HRs finished sessions faster than LRs across training (effect of Phenotype, $F_{1,83}$=14.82, p<0.0001). This is to be expected as HRs showed shorter III than LRs. Thus, despite the fact that this paradigm controlled for the maximum number of infusions received, we were able to capture individual differences in the acquisition of drug-taking behavior in outbred HR/LR rats.

Extinction 1 training. All rats decreased nose pokes into both ports (effect of Session, $F_{12,84}$=26.67, p<0.001) and differentiated between the two ports across sessions (effect of Port, $F_{1,84}$=41.94, p<0.0001; Fig. S6a). However, as training progressed rats discriminated less

between the two ports (Session x Port interaction, $F_{12,84}$=6.19, p<0.0001), specifically during session 9 (p=0.12), 10 (p=0.93) and 11 (p=0.07). Behavior differed within and between HRs and LRs depending upon impending test day treatment (Phenotype x Treatment interaction, $F_{1,84}$=11.54, p<0.001), such that rats in the vehicle groups made more pokes into both ports compared to rats in the CNO groups (p=0.001; Fig. S6a). Test day treatment also affected pokes into the active and inactive port within each phenotype (HR, p=0.01; LR, p=0.04; Fig. S6a). Despite these differences, there were no differences between HRs and LRs (effect of Phenotype, $F_{1,84}$=2.20, p=0.14) or test day treatment (effect of treatment, $F_{1,84}$=1.97, p=0.16) on nose pokes made into either port during the last extinction training session. Thus, by the conclusion of extinction training, all rats were behaving the same.

All rats exhibited cue-induced reinstatement. Compared to the last extinction training sessions, all rats showed greater drug-seeking behavior during the cue-induced reinstatement test compared to the last extinction training session (effect of Session, $F_{1,40}$=31.89, p<0.0001; Fig. S6b). There was a significant effect of Phenotype ($F_{1,40}$=5.30, p=0.03), but no significant effect of Treatment ($F_{1,40}$=3.52, p=0.07) or any interactions.

PrL-PVT inhibition does not affect cue-induced drug-seeking behavior in LRs or HRs. During the test, rats made more pokes into the active port compared to the inactive port (effect of Port, $F_{1,80}$=38.84, p<0.0001; Fig. S6c). HRs and LRs also differed in pokes into the active versus inactive port (Port x Phenotype interaction, $F_{1,80}$=3.99, p<0.05), as well as overall behavior in response to either CNO or vehicle treatment (Phenotype x Treatment interaction, $F_{1,84}$=6.35, p=0.01). However, there were no significant post-hoc analyses or a Phenotype x Treatment x Port interaction ($F_{1,80}$=0.16, p=0.69). Two rats (HR VEH, HR CNO) were excluded from analyses as statistical outliers.

Nose pokes made into the active port during the reinstatement test did not correlate with locomotor score for rats in any of the groups (HR VEH, r^2=0.46, p=0.09; HR CNO, r^2=0.26, p= 0.11; LR VEH, r^2=0.001, p=0.92; LR CNO, r^2=0.13 p= 0.26). Therefore, it appears locomotor activity in a novel environment does not predict levels of cue-induced reinstatement of drug-seeking behavior in HRs or LRs.

No differences in extinction training after cue-induced reinstatement. Pokes into both ports decreased (effect of Session, $F_{7,84}$=9.82, p<0.0001) and rats discriminated between both ports

across training (effect of Port, $F_{1,84}$=6.13, p=0.02). HRs and LRs did not differ from one another (effect of Phenotype, $F_{1,84}$=0.76, p=0.39), and test day treatment did not affect behavior (effect of Treatment, $F_{1,84}$=0.12, p=0.73; Fig. S6d).

All rats exhibited cocaine-primed reinstatement. All rats exhibited greater drug-seeking behavior during the cocaine-prime reinstatement test compared to the last extinction training session (effect of Session, $F_{1,40}$=39.78, p<0.0001; Fig. S6e). Behavior did not differ in either session between HRs and LRs (effect of Phenotype, $F_{1,40}$=0.25, p=0.62) or treatment given (effect of Treatment, $F_{1,40}$=0.01, p=0.94), and there were not significant interactions.

PrL-PVT inhibition does not affect cocaine-primed drug-seeking behavior in LRs or HRs. Both HRs and LRs preferred the active port relative to the inactive port (effect of Port, $F_{1,80}$=26.24, p<0.0001; Fig. S6f), with no difference between phenotypes (effect of Phenotype, $F_{1,80}$=0.13, p=0.72). Inhibition of the PrL-PVT pathway did not affect drug-seeking behavior (effect of Treatment, $F_{1,80}$=0.83, p=0.37). Furthermore, cumulative locomotor movements made during the locomotor test did not correlate with drug-seeking behavior (HR VEH, r^2=0.01, p= 0.83; HR CNO, r^2=0.09, p= 0.38.; LR VEH, r^2=0.18, p= 0.15; LR CNO, r^2=0.004, p= 0.85).

HRs and LRs do not differ in cocaine-induced stereotyped behaviors during cocaine primed-reinstatement. Stereotyped behaviors during cocaine-primed reinstatement were assessed in a subset of rats (HR VEH, n=5; HR CNO, n=8; LR VEH, n=9; LR CNO, n=4). Rats spent less time exhibiting "stereotyped behaviors" (~5-15% of time) compared to "non-stereotyped behaviors" (effect of Category, F1,22=9.33, p<0.01; Fig. S7). HRs and LRs did not differ on this measure (effect of Phenotype, F1,22=0.22, p=0.65) and treatment did not affect behavior (effect of Treatment, F1,22=0.29, p=0.59; Figure S5). Thus, in parallel to what was observed analyzing data using the ST/GT model, it appears some animals exhibited stereotypy, which may have interfered with cocaine-induced reinstatement measures. However, the small sample size and lack of significant treatment and phenotype effects warrants further investigation in this regard.

Rate of extinction does not predict reinstatement of drug-seeking behavior in HRs or LRs. There was not a significant relationship present between rate of extinction (extinction 1) and cue-induced drug-seeking behavior (HR VEH, r^2=0.04, p=0.68; HR CNO, r^2=0.02, p=0.68; LR VEH, r^2<0.001, p=0.99; LR CNO, r^2=0.13, p=0.26), or extinction rate (extinction 2) and cocaine-

primed drug-seeking behavior (HR VEH, r^2=0.11, p=0.41; HR CNO, r^2=0.08, p=0.41; LR VEH, r^2=0.06, p=0.43; LR CNO, r^2=0.15, p=0.22). Thus, the variation in drug-seeking behavior during the test for cocaine-primed reinstatement does not appear to be due to differences in extinction rate.

Behavior	Definition
Stereotyped behaviors	
Circling (i.e. "spinning")	Four consecutive 90° turns occurring within close succession of one another
Head movements	Lateral head movements made when the back paws remain on the flooring and in the same position for a minimum of 2-sec
Non-stereotyped behaviors	
Active port orientation	Oriented toward the active port, but not advancing toward the port
Active port approach	Approached the active port, but made no pokes into the port
Immobility	Time during which all 4 paws remain on the flooring in the same position for a minimum of 2-sec, which no other behaviors co-occurring

Table S1 Table of behaviors quantified by a blind observer during video analysis of the test for cocaine-primed reinstatement. Behaviors were scored in 30-sec bins every 10-min for the first 60-min of the session. Time spent doing each behavior was recorded per bin.

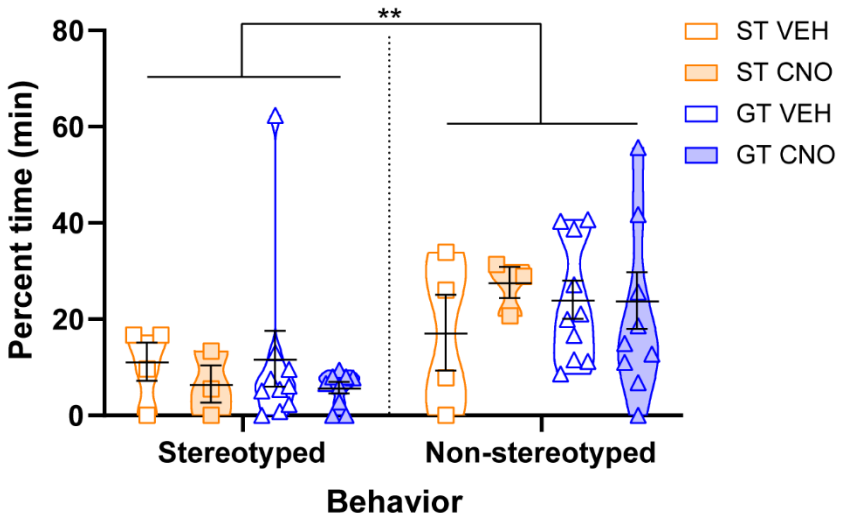

Fig. S1 Stereotyped behavior and non-stereotyped behavior during cocaine-primed reinstatement. Mean + SEM for percent time exhibiting stereotyped behavior during the first hour of the test for cocaine-primed reinstatement. All rats exhibited more non-stereotyped than stereotyped behavior ($p<0.001$), and behavior did not differ based on phenotype ($p=0.99$) or treatment ($p=0.84$). (ST VEH, n=4; ST CNO, n=3; GT VEH, n=10; GT CNO, n=9) **$p<0.01$

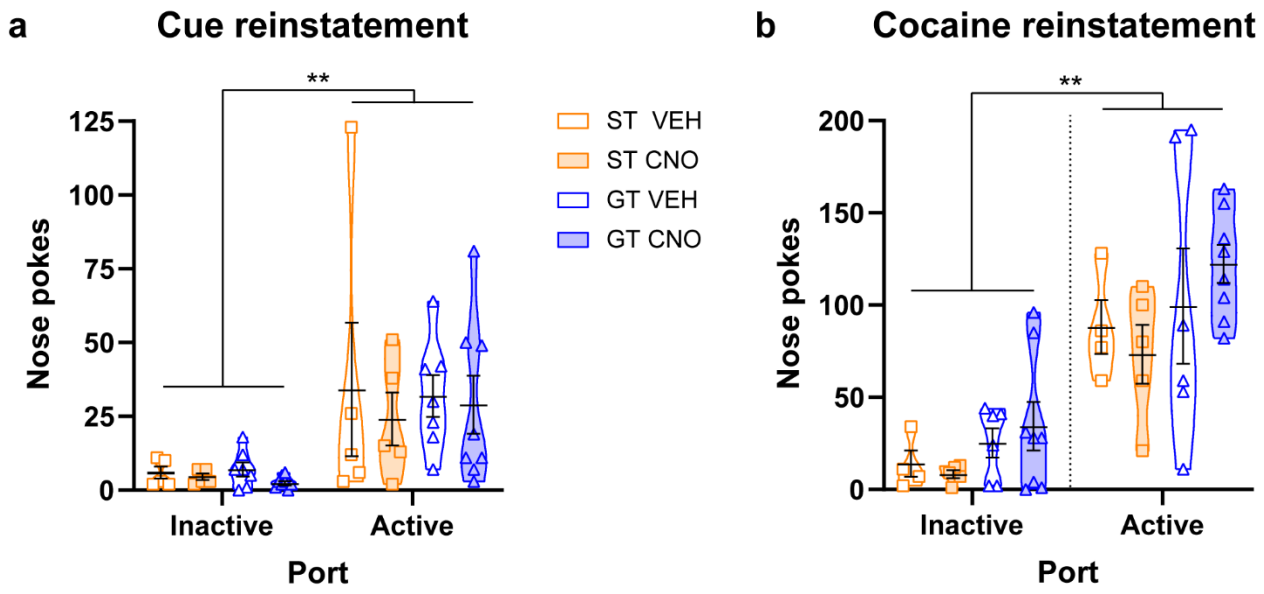

Fig. S2 CNO administration in the absence of DREADDs did not affect drug-seeking behavior during reinstatement. Mean + SEM for **a)** nose pokes into the inactive and active port during a test for cue-induced reinstatement. **b)** Nose pokes into the inactive and active port during a test for cocaine-primed reinstatement. CNO administration in the absence of DREADDs did not affect drug-seeking behavior during either reinstatement test in STs or GTs compared to their respective vehicle group. (Cued reinstatement: ST VEH, n=5; ST CNO, n=5; GT VEH, n=7; GT CNO, n=8; Cocaine primed reinstatement: ST VEH, n=4; ST CNO, n=5; GT VEH, n=6; GT CNO, n=8)

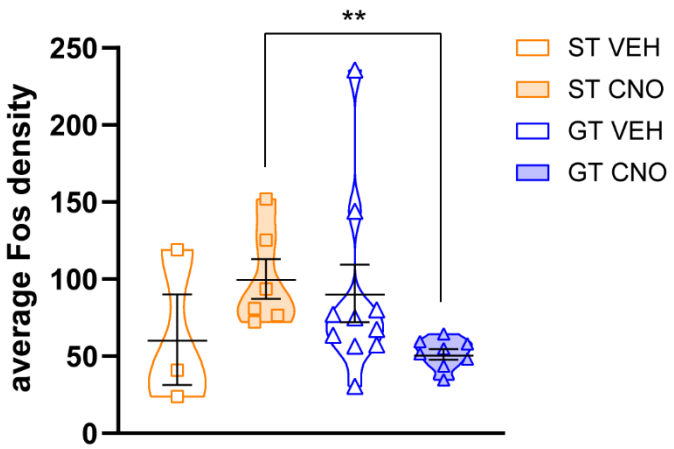

Fig. S3 Average Fos density of in the PVT of STs and GTs. Mean + SEM for average Fos density throughout the entire PVT (anterior, middle and posterior). Inhibition of the PrL-PVT pathway results in a decrease in average Fos density in GTs relative to STs (p=0.002). However, inhibition of the Pr-PVT pathway does not affect average Fos density in either phenotype with respect to their vehicle-treated controls (ST: p= 0.19; GT, p= 0.10), and rats in the vehicle groups did not differ from one another (ST VEH relative to GT VEH, p=0.49). (ST VEH, n=3; ST CNO, n=6; GT VEH, n=10; GT CNO, n=8) **p<0.01

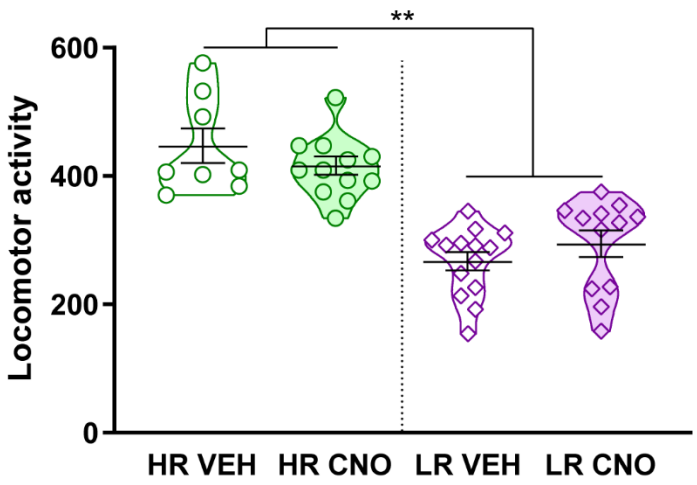

Fig. S4 Locomotor response in a novel environment in HRs and LRs. Mean + SEM for cumulative locomotor activity in a novel environment. HRs show greater locomotor activity compared to LRs (effect of Phenotype, $p<0.0001$). Rats did not receive treatment prior to the locomotor test. (HR VEH, n=8; HR CNO, n=12; LR VEH, n=14; LR CNO, n=12) **$p<0.01$

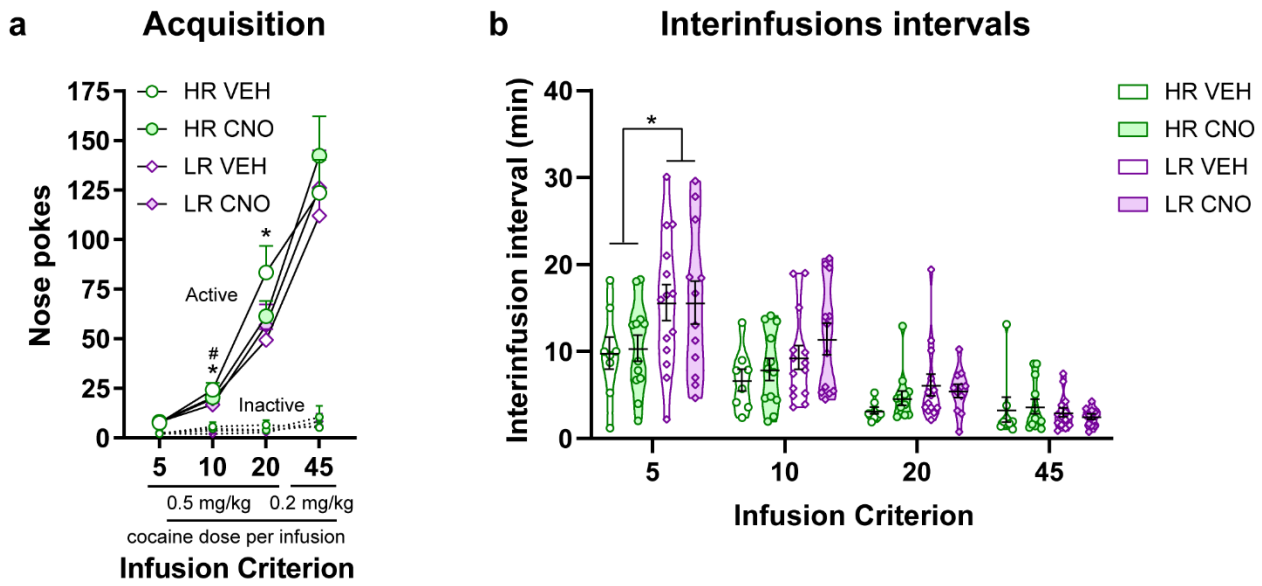

Fig. S5 Acquisition of cocaine self-administration in HRs and LRs. Mean + SEM for **a)** nose pokes made into the inactive and active port across infusion criterion (IC). Rats made more pokes into the active port ($p<0.0001$) across infusion criterion ($p<0.0001$). HRs showed more drug-seeking behavior during IC10 ($p=0.01$) and IC20 than LRs ($p=0.02$). Rats in the vehicle-treated group also showed more drug-seeking behavior during IC10 ($p=0.04$) compared to rats treated with CNO. B) Interinfusion intervals (III) for HRs and LRs at each IC. III decreased across all groups as training progressed ($p<0.001$). However, HRs earned infusions faster than LRs at IC5 ($p=0.01$), and there was a trend toward HRs continuing to earn infusions faster compared to LRs at IC 10 ($p=0.06$) and IC20 ($p=0.07$). (HR VEH, n=8; HR CNO, n=12; LR VEH, n=14; LR CNO, n=12) *$p<0.05$ HR compared to LR; #$p<0.05$ VEH vs CNO

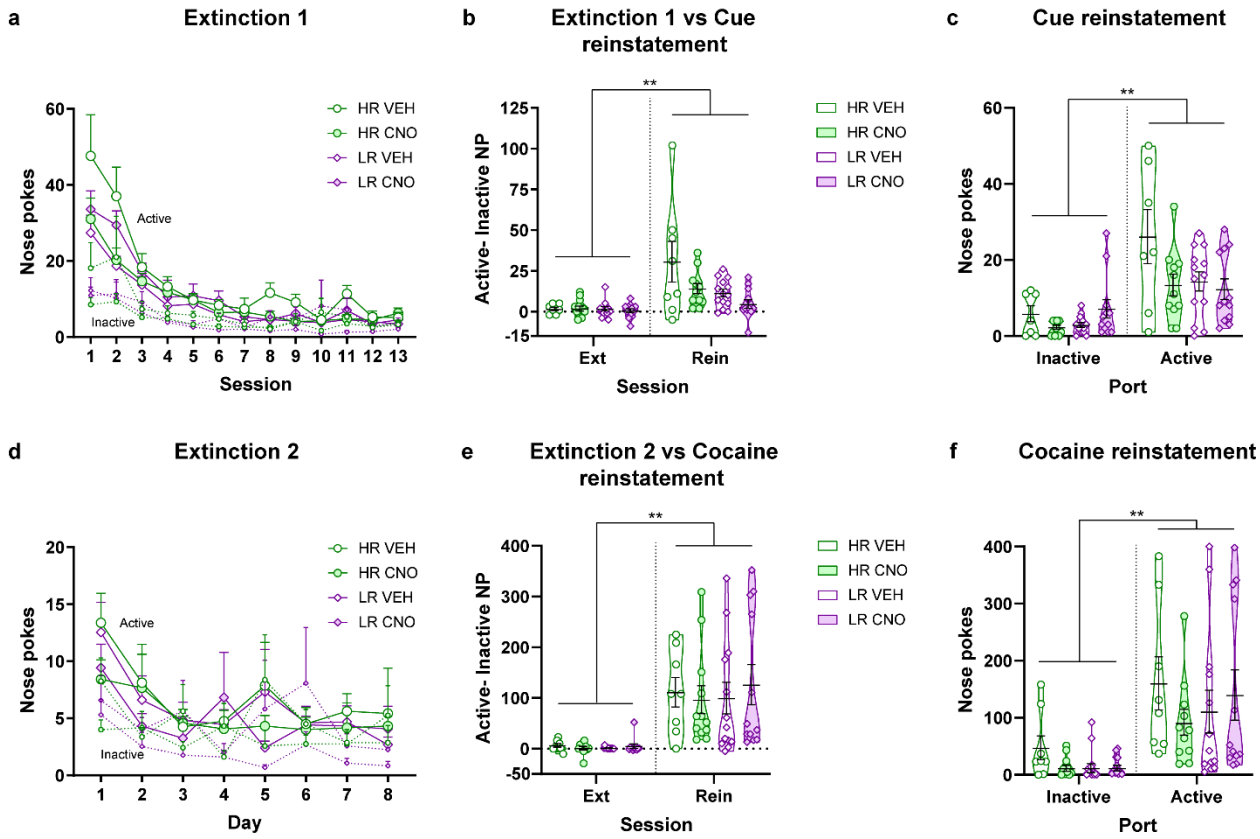

Fig S6 Extinction training and drug-seeking behavior during the tests for cue-induced and cocaine-primed reinstatement in HRs and LRs. Mean + SEM for **a)** Nose pokes (NP) made into the active (solid lines) and inactive (dashed lines) ports across 13 sessions of extinction training in HRs and LRs. Rats decreased drug-seeking behavior across training sessions ($p<0.0001$). Rats in the HR vehicle group showed greater drug-seeking behavior throughout training compared to rats in the LR vehicle group ($p=0.001$) and HR CNO group ($p=0.01$). LR rats in the CNO group also showed greater drug-seeking behavior compared to rats in the LR vehicle group ($p=0.04$). **b)** Active-inactive NPs made during the last extinction training session compared to the test for cue-induced reinstatement. All rats showed an increase in drug-seeking behavior during the test compared to extinction ($p<0.0001$). **c)** Nose pokes made into the inactive and active port during the test for cue-induced reinstatement. **d)** Nose pokes made into the inactive and active port across 8 sessions of extinction training. All rats decreased drug-seeking behavior across training sessions ($p<0.0001$). **e)** Nose pokes made into the active (solid lines) and inactive (dashed lines) ports across 8 sessions of Extinction 2 training in HRs and LRs. Rats decreased drug-seeking behavior across training sessions ($p<0.0001$). **f)** Nose pokes made into

the inactive and active port during the test for cocaine-primed reinstatement. (HR VEH, n=7; HR CNO, n=11 (n=10 for cocaine-prime reinstatement); LR VEH, n= 13, LR CNO, n= 12) *$p<0.05$, **$p<0.01$

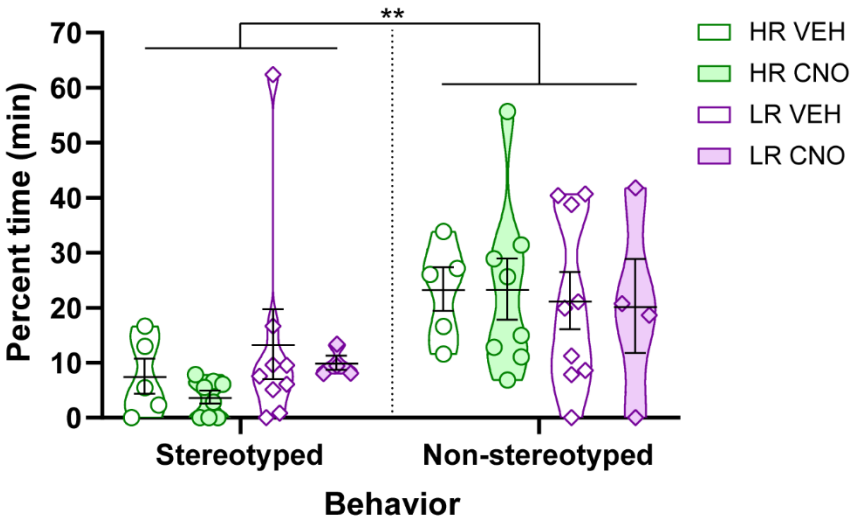

Fig. S7 Cocaine-induced stereotyped behavior during cocaine-primed reinstatement. Mean + SEM for percent time exhibiting stereotyped behavior during the first hour of the test for cocaine-primed reinstatement. Behavior did not differ based on phenotype (p=0.59) or treatment (p=0.76). (HR VEH, n=5; HR CNO, n=8; LR VEH, n=9; LR CNO, n=4)