



## ARTICLE



# Inactivation of the infralimbic cortex decreases discriminative stimulus-controlled relapse to cocaine seeking in rats

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Persistent susceptibility to cue-induced relapse is a cardinal feature of addiction. Discriminative stimuli (DSs) are one type of drug-associated cue that signal drug availability (DS+) or unavailability (DS−) and control drug seeking *prior* to relapse. We previously established a trial-based procedure in rats to isolate DSs from context, conditioned stimuli, and other drug-associated cues during cocaine self-administration and demonstrated DS-controlled cocaine seeking up to 300 abstinence days. The behavioral and neural mechanisms underlying trial-based DS-control of drug seeking have rarely been investigated. Here we show that following discrimination training in our trial-based procedure, the DS+ and DS− independently control the expression and suppression of cocaine seeking during abstinence. Using microinjections of GABA<sub>A</sub> + GABA<sub>B</sub> receptor agonists (muscimol + baclofen) in medial prefrontal cortex, we report that infralimbic, but not prelimbic, subregion of medial prefrontal cortex is critical to persistent DS-controlled relapse to cocaine seeking after prolonged abstinence, but not DS-guided discriminated cocaine seeking or DS-controlled cocaine self-administration. Finally, using *ex vivo* whole-cell recordings from pyramidal neurons in the medial prefrontal cortex, we demonstrate that the disruption of DS-controlled cocaine seeking following infralimbic cortex microinjections of muscimol+baclofen is likely a result of suppression of synaptic transmission in the region via a presynaptic mechanism of action.

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## INTRODUCTION

Relapse is a cardinal feature of addiction [1, 2]. In both human and rodent models, environmental stimuli previously paired with drug self-administration can elicit drug seeking even after prolonged abstinence [3–9]. Discriminative stimuli (DSs) are one type of drug-associated cue that can signal either drug availability (DS+) or unavailability (DS−) to guide drug seeking and taking [7]. They play important roles in relapse because they precede and guide drug seeking *prior* to drug taking and are difficult to extinguish [7, 10–21].

In most studies of DS-controlled drug seeking, the DS+ and DS− were presented in separate sessions and investigators used procedures that did not clearly isolate their effects from other drug-associated stimuli, such as context or discrete conditioned stimuli (CSs) [7, 10–13, 16–19, 22]. In contrast, trial-based procedures for studying DS-control allow isolation of DSs from other stimuli [14, 20, 23, 24] to identify their unique contribution to drug-seeking behavior. Incorporation of many, intermixed, repeated trials also enables investigation of DS-specific neuronal ensemble activity using *in vivo* electrophysiology or calcium imaging [25–31]. Based on these considerations, we developed a trial-based procedure in rats that used DS+ and DS− with a common lever manipulandum and no drug infusion-paired cues [21, 32], to isolate the unique effects of the DSs beyond that of previous studies [14, 23, 24]. Following trial-based discrimination, we assessed DS-control of cocaine seeking during abstinence and

showed persistent non-reinforced drug seeking during DS+ (but not DS−) presentations, up to 300 days after the last DS-drug pairing [21].

The neural mechanisms of trial-based DS control of drug [14, 20, 23, 24] or non-drug [25–31] reward seeking have not often been studied. The medial prefrontal cortex (mPFC) has been implicated in drug and non-drug reward seeking [33–38]. Early studies using the extinction-reinstatement model with cocaine suggested a functional dichotomy wherein prelimbic cortex (PL) activity promotes cocaine seeking while infralimbic cortex (IL) activity suppresses cocaine seeking [39–41]. However, recent studies have shown opposite and sometimes overlapping roles for these subregions in reward seeking, depending on the reinforcer type [42–47], abstinence-induced manipulation (forced abstinence versus extinction) [46, 48–50], the stimulus used to induce reward seeking [13, 18, 51–55], or the neural manipulation (global inactivation versus Daun02 selective inactivation of Fos-expressing ensemble neurons) [56, 57]. While some studies showed that prior extinction training is necessary for IL inhibitory control [58, 59] other studies suggest extinction-independent roles for PL and IL in drug seeking during abstinence [48, 60, 61]. However, these studies mainly focused on how mPFC activity guides drug seeking in response to contextual or drug-paired stimuli, or drug priming; [9, 62] only a few examined the role of PL and IL in DS-controlled drug and non-drug reward seeking and taking [13, 18, 20, 27, 28, 52, 53].

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We first investigated individual contributions of DS+ and DS− to persistent cocaine seeking after 21 abstinence days by measuring non-reinforced lever-presses during 4 trial types: *no-DSs*, *DS+*, *DS−*, and *both-DSs*. Next, we used microinjections of GABA<sub>A</sub> + GABA<sub>B</sub> receptor agonists (muscimol + baclofen, M + B) to examine the role of PL and IL in DS-controlled relapse to cocaine seeking after 21 abstinence days and in ongoing DS-controlled cocaine self-administration. Finally, we used whole-cell voltage clamp recordings in an ex vivo brain slice preparation to identify candidate synaptic mechanisms underlying M + B effects that might contribute to DS-control of relapse to cocaine seeking.

## MATERIALS AND METHODS

A detailed description of experimental subjects, apparatus, and procedures is included as Supplementary Online Methods. All procedures were approved by the NIDA IRP Animal Care and Use Committee and followed guidelines outlined in the Guide for the Care and Use of Laboratory Animals [63]. Below we provide an overview of the experiments.

### Individual contributions of DS+ and DS− to DS-controlled cocaine seeking during abstinence (Experiment 1)

The goal of this experiment was to determine how the two DSs used in our procedure [21] exert stimulus control over cocaine seeking during abstinence. We first trained male rats to either lever press for cocaine (0.75 mg/kg/infusion) when a light cue (DS+) signaled cocaine availability (DS+ trials) or suppress lever-pressing when a different light cue (DS−) signaled cocaine unavailability (DS− trials) during the same session; DS +/- trials were presented in pseudorandom order. After training, we tested the rats for DS-controlled cocaine seeking on day 1 and placed them in their homecage for 20 days of forced abstinence. We then investigated the individual contributions of DS+ and DS− to discriminated cocaine seeking on abstinence day 21. We measured non-reinforced responses during each of 4 possible combinations of DS+ (on, off) and DS− (on, off) trial types: *no-DSs*, *DS+*, *DS−*, or *both-DSs* (15 presentations per type; pseudorandom order).

### Role of IL (Experiment 2) and PL (Experiment 3) neural activity in DS-controlled cocaine seeking

The goal of these experiments was to examine the role of IL and PL activity in discriminated cocaine seeking. Following training and a period of forced abstinence, we tested whether M + B microinjections (0.03 nM muscimol + 0.3 nM baclofen per side; 0.5 μL injection) into IL or PL would affect DS-controlled cocaine seeking on abstinence day 21.

### Role of IL and PL neural activity in DS-controlled cocaine self-administration (Experiment 4)

The goal of this experiment was to examine the role of IL or PL activity in ongoing DS-controlled cocaine self-administration. Following training, we tested whether M + B microinjections into IL or PL would affect ongoing discriminated cocaine self-administration. We retrained rats between tests and tested those that showed stable discriminated drug-taking behavior.

### Effect of pharmacological manipulation of GABA receptors in IL neurons using ex vivo brain slice electrophysiology (Experiment 5)

The goal of this experiment was to determine the effect of M + B on synaptic activity using whole cell voltage-clamp recordings ( $V_{\text{hold}} = -70$  mV) in visually identified layer 5/6 pyramidal neurons within IL. We recorded spontaneous synaptic responses and then used electrical stimulation to evoke synaptic responses. After obtaining a stable baseline recording of both evoked and spontaneous excitatory postsynaptic currents (EPSCs), we bath-applied M + B for 10–15 min and determined the percent change from baseline after drug application.

## RESULTS

### Discrimination training

Discrimination training was performed identically for experiments 1–4 (see Figs. 1–4, and Fig. S1). Rats learned to lever press for

cocaine infusions (left graph panel B), continued responding during trial training (center graph panel B) and then learned to discriminate DS+ from DS− during discrimination training (right graph panel B). There were no group differences in acquisition of discrimination training or during DS-controlled cocaine seeking on day 1 for rats subsequently tested under the different experimental conditions. See supplementary tables S1–6 for a detailed listing of experimental subjects and statistical analyses.

### Experiment 1: Individual contributions of DS+ and DS− to persistent DS-controlled cocaine seeking

Following discrimination training, we first tested rats for discriminated cocaine seeking on abstinence day 1. The number of trials with at least one lever press (denoted as *trials*) and total number of lever presses (denoted as *lever presses*) were recorded separately for each DS trial type during each session and analyzed using the within-subject factor of DS (DS+, DS−). Rats responded on more DS+ trials than DS− trials ( $t_{26} = 6.7$ ,  $p < 0.0001$ ); they also made more lever presses during DS+ trials ( $t_{26} = 6.5$ ,  $p < 0.0001$ ), indicating that cocaine seeking was under DS control (Fig. 1C, left panel). We then placed rats in their homecage for 20 days and tested for DS-controlled cocaine seeking on abstinence day 21 using a modified version of the day 1 seeking test with 4 trial conditions: *no-DSs*, *DS+*, *DS−*, or *both-DSs*. We analyzed both *trials* and *lever presses* measures using two-way ANOVAs with within-subject factors of DS+ (on, off) and DS− (on, off) and observed significant interaction between the two factors (*trials*:  $F_{1,26} = 10.4$ ,  $p = 0.0033$ ; *lever presses*:  $F_{1,26} = 12.7$ ,  $p = 0.0014$ ). For both measures, responding was low during 'no-DSs' and 'DS−' trials, intermediate during 'both-DSs' trials, and maximal during 'DS+' trials (see Table S2 for full statistical results). These results indicate that DS+ and DS− independently control the expression (i.e., increased responding during 'DS+' relative to 'no-DSs' trials) and suppression (i.e., decreased responding during 'both-DSs' relative to 'DS+' trials) of cocaine seeking during abstinence.

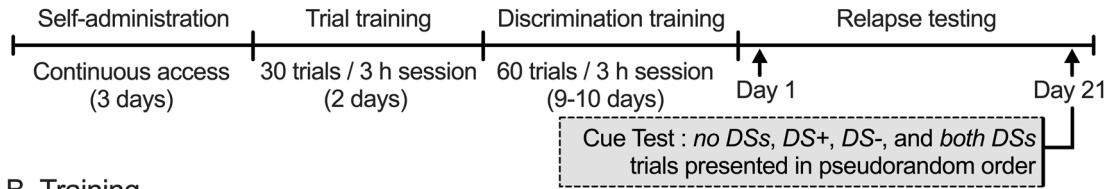
### Experiment 2: Microinjections of M + B into IL prior to DS-controlled cocaine seeking

Following discrimination training, we first tested rats for cocaine seeking on abstinence day 1 (Fig. 2C, left panel). We then placed the rats in their homecage for 20 days and tested whether M + B microinjections into IL would affect DS-controlled cocaine seeking on abstinence day 21. We analyzed the number of non-reinforced lever presses during the test session using mixed ANOVA with within-subject factor of DS (DS+, DS−) and between-subjects factor of M + B dose (Vehicle, M + B). The analysis showed significant effects of DS ( $F_{1,33} = 43.0$ ,  $p < 0.0001$ ), M + B dose ( $F_{1,33} = 4.4$ ,  $p = 0.0029$ ), and DS x M + B dose ( $F_{1,33} = 4.3$ ,  $p = 0.045$ ), indicating that IL microinjections of M + B suppressed DS-controlled cocaine seeking (Fig. 2C, right panel). Bonferroni posthoc analysis showed that M + B decreased cocaine seeking during both DS+ trials ( $t_{33} = 2.8$ ,  $p = 0.018$ ) and DS− trials ( $t_{33} = 2.9$ ,  $p = 0.013$ ). Further, M + B did not affect discriminated cocaine seeking (Fig. S2) during the test ( $t_{33} = 0.03859$ ,  $p = 0.96945$ ).

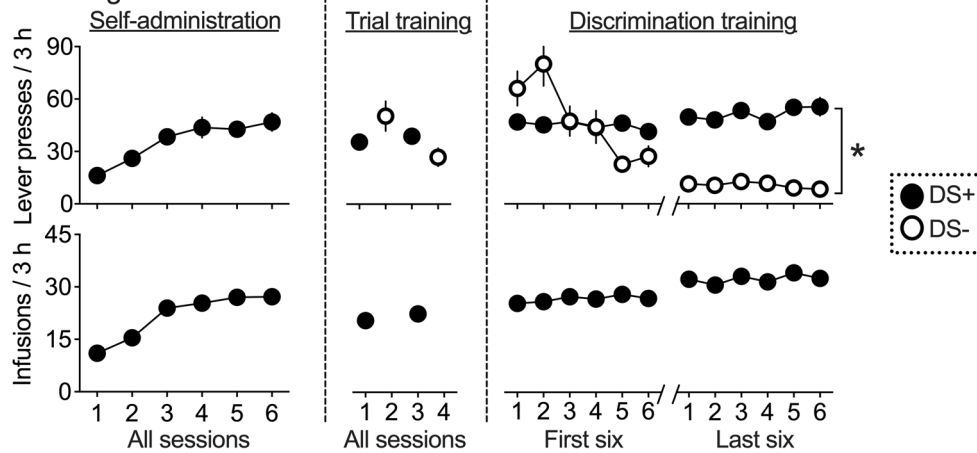
### Experiment 3: Microinjections of M + B into PL prior to DS-controlled cocaine seeking

Following discrimination training, we first tested rats for cocaine seeking on abstinence day 1 (Fig. 3C, left panel). We then placed the rats in their homecage for 20 days and tested whether M + B microinjections into PL would affect DS-controlled cocaine seeking on abstinence day 21. Mixed ANOVA analysis with within-subject factor of DS and between-subjects factor of M + B dose showed a significant main effect of DS ( $F_{1,28} = 100.4$ ,  $p < 0.0001$ ) but not M + B dose or interaction ( $p$  values > 0.05), indicating that PL microinjections of M + B did not affect DS-controlled cocaine seeking (Fig. 3C, right panel).

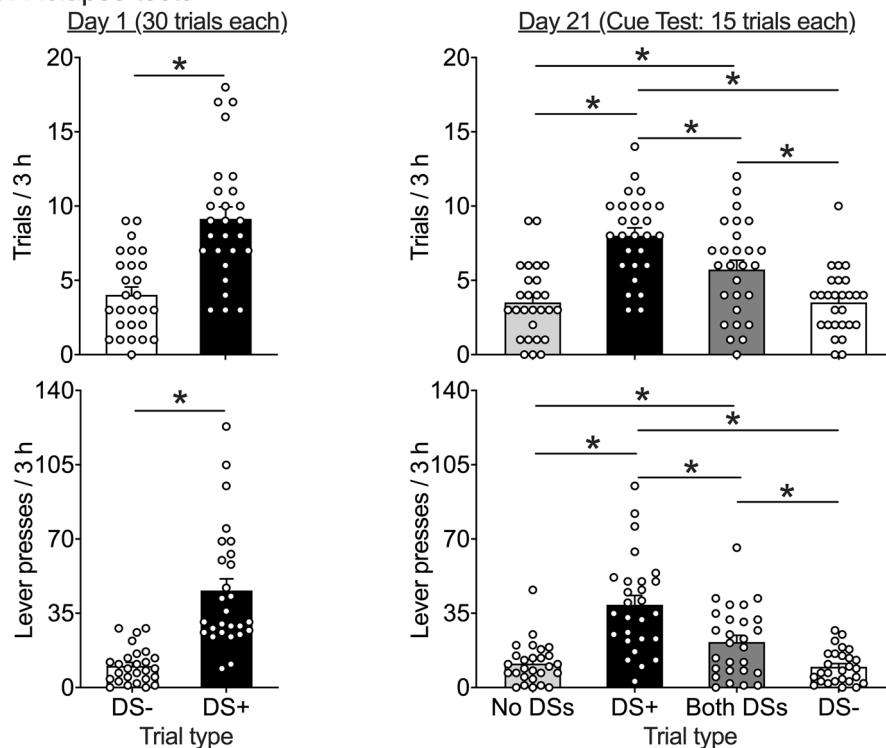
## A. Experimental timeline



## B. Training



## C. Relapse tests

**Experiment 4: Microinjections of M + B into IL or PL prior to DS-controlled cocaine self-administration**

Following discrimination training, we used a within-subjects design and tested for DS-controlled cocaine self-administration after counterbalanced vehicle and M + B microinjections into IL or PL (Fig. 4C). For each subregion, we analyzed the number of lever presses made during the test session using repeated measures ANOVA: within-subjects factors of DS and M + B dose. For both subregions, we observed a significant main effect of DS (PL:  $F_{1,10} = 82.8, p < 0.0001$ ; IL:  $F_{1,11} = 57.6, p < 0.0001$ ) but no effect of M +

B dose or interaction ( $p$  values  $> 0.05$ ), indicating that inactivation of IL or PL did not affect DS-controlled cocaine self-administration.

**Experiment 5: Effect of M + B on IL synaptic activity using ex vivo brain slice electrophysiology**

We used whole cell voltage clamp recordings in an ex vivo brain slice preparation to determine the effect of GABAergic receptor agonism on IL synaptic activity. We used either paired pulses or a pulse train of electrical stimulation to elicit postsynaptic responses in visually identified Layer 5/6 pyramidal neurons (Fig. 5A). After

**Fig. 1 Individual contributions of DS+ and DS- to persistent DS-controlled cocaine seeking during abstinence.** **A** Experimental timeline. **B** Training data. *Self-administration*: Rats learned to self-administer cocaine over 6 sessions. Mean ( $\pm$ SEM) number of cocaine infusions and lever presses during each 3-h session. *Trial training*: Mean ( $\pm$ SEM) number of lever presses and infusions received during the 3-h sessions (30 DS+ trials in the AM session, 30 DS- trials in the PM session). *Discrimination training*: All rats learned to discriminate DS+ from DS- trials within the same session (30 trials each of DS+ and DS- trials presented in a pseudorandomized manner). Mean ( $\pm$ SEM) number of lever presses during each trial type (top), and infusions received (bottom) during a 3-h discrimination training session. \* indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types ( $n = 27$ ). **C**: Relapse tests. *Day 1*: Rats showed reliable DS-controlled cocaine seeking on abstinence day 1 when presented with 30 trials each of DS+ and DS- (pseudorandomized order, extinction conditions). Columns indicate Mean ( $\pm$ SEM) number of trials and lever presses during the 3-h day 1 relapse test session separated by 2 trial types, while dots indicate values for individual rats. \* denotes significant difference ( $p < 0.05$ ) in responding between DS+ and DS- trials. *Day 21 (Cue test)*: Rats maintained DS-controlled cocaine seeking on abstinence day 21 when presented with 15 each of *no-DSs*, DS+, DS-, and *both-DSs* trials (pseudorandomized order, extinction conditions). Non-reinforced responding was low during *no-DSs* and DS- trials, intermediate during *both-DSs* trials, and maximal during DS+ trials indicating that following discrimination training in this task, the DS+ and DS- independently control the expression and suppression of DS-controlled drug seeking during abstinence. Columns indicate Mean ( $\pm$ SEM) number of trials and lever presses during the 3-h day 21 relapse test session separated by 4 trial types, while dots indicate values for individual rats. \* denotes significant difference ( $p < 0.05$ ) in responding between trial types indicated by the solid line ( $n = 27$ ).

establishing a stable baseline level of synaptic responding, we bath-applied M + B (0.03 nM muscimol + 0.3 nM baclofen) onto the slice. M + B application caused  $76.1 \pm 5.2$  percent change in the amplitude of the synaptic response for EPSC<sub>1</sub>,  $66.0 \pm 7.2$  percent change in EPSC<sub>2</sub> and significantly increased paired-pulse response ( $t_{10} = 6.01$ ,  $p = 0.0001$ ). M + B application caused  $68.5 \pm 5.2$  percent change in P1,  $60.0 \pm 6.6$  percent change in P2,  $55.4 \pm 7.4$  percent change in P3,  $51.0 \pm 8.0$  percent change in P4,  $48.3 \pm 7.5$  percent change in P5,  $45.7 \pm 8.7$  percent change in P6, and  $46.7 \pm 8.7$  percent change in P7. We also recorded spontaneous excitatory postsynaptic responses (sEPSCs) before and after drug application by recording continuously for 5 min during the baseline period and once again after drug application for 5–10 min (Fig. 5B). M + B application significantly reduced the frequency of sEPSCs in recorded neurons ( $t_{10} = 3.0$ ,  $p = 0.013$ ) and had no effect on sEPSC amplitude ( $t_{10} = 0.76$ ,  $p = 0.466$ ) (Fig. 5B, middle and right panel).

## DISCUSSION

We examined behavioral and neurobiological mechanisms underlying trial-based DS-controlled cocaine seeking and taking. We report four main findings. First, after 21 abstinence days, DS+ alone increased cocaine seeking relative to *no DS* trials while DS- decreased (DS+)-induced cocaine seeking when presented together with the DS+ in *both-DSs* trials. Second, IL (but not PL) M + B microinjections reduced cocaine seeking during both DS+ and DS- trials but did not affect discriminated responding. Third, IL or PL M + B microinjections did not affect ongoing DS-controlled cocaine self-administration. Finally, in mPFC slices M + B application suppressed the magnitude of electrically evoked postsynaptic responses and decreased spontaneous EPSC frequency. Overall, our data indicate that during abstinence, DS+ and DS- independently control the expression and suppression of DS-controlled drug seeking, and that DS-controlled cocaine seeking (but not discriminated responding) is mediated by IL activity, likely via a presynaptic mechanism.

### Behavioral mechanisms underlying trial-based DS-controlled relapse to cocaine seeking

During discrimination training in our trial-based procedure [21], the two DSs are presented within the same session and set the occasion for responding (or not responding) on a common retractable lever. Thus, the only aspect that discriminates between trial types is the DS itself. In our previous study [21], we assessed cocaine seeking only during DS+ or DS- trials. Thus, it was unknown whether it is the *presence* of DS+ or *absence* of DS- that induces cocaine seeking. To address this question, we assessed the individual contributions of the DS+ and DS- to cocaine seeking, relative to presentation of the lever alone (*no-DSs* trials) or when both DSs were presented together (*both-DSs* trials). While

the DS+ increased responding relative to *no-DSs* trials, the DS- did not alter responding relative to *no-DSs* trials, possibly due to a floor effect (responding was low in *no-DSs* trials). Critically, when both DSs were presented together (*both-DSs* trials), the DS- decreased cocaine seeking induced by the DS+ and served as a conditioned inhibitor of the DS+ [64]. We did not observe higher responding in *no-DSs* trials vs. DS- trials, indicating that increased responding in DS+ trials was not due to disinhibition of responding due to DS- removal. Overall, our data indicate that DS+ and DS- contribute independently to DS control of drug seeking during abstinence.

### Role of mPFC activity in (DS+)-induced expression of cocaine seeking

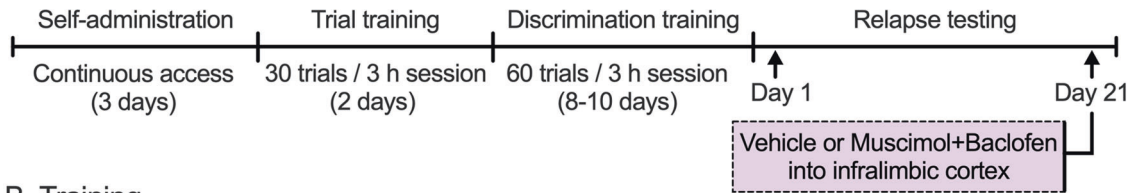
IL (but not PL) microinjections of M + B decreased cocaine seeking during DS+ trials, indicating that (DS+)-induced cocaine seeking during abstinence is mediated by IL activity. Previous studies of (DS+)-induced cocaine seeking primarily used session-based procedures, where rats' lever pressing was assessed in separate sessions while they were exposed continuously to either a DS+ or DS- [65, 66]. Weiss and colleagues found that Fos expression was higher in PL following DS+ (versus DS-) induced reinstatement after extinction, an effect reversed by systemic injections of SCH39166 (a dopamine D<sub>1</sub>/D<sub>5</sub> receptor antagonist); IL activity was not assessed [65, 66]. However, the authors did not inactivate either region to assess their causal role in (DS+)-induced reinstatement.

Our results agree with those of Suto and colleagues who used the Daun02 inactivation procedure [67] and found a role for IL in (DS+)-induced reinstatement of food seeking following session-based self-administration and extinction training [68]. While these results suggest a general role for IL in (DS+)-induced relapse to reward seeking, additional studies using the same procedure and other reinforcers (e.g. food, heroin) are necessary to test this possibility.

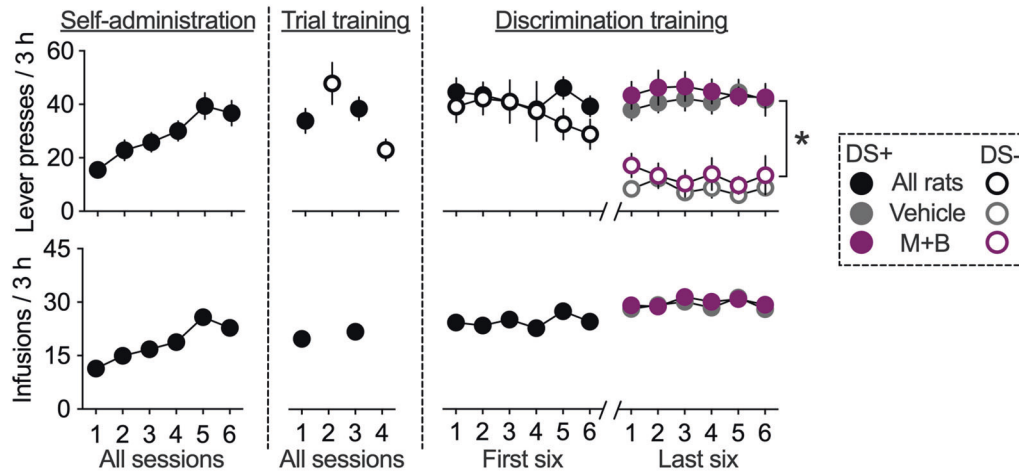
### Role of mPFC neural activity in (DS-)-based inhibitory control of cocaine seeking

Cocaine seeking during DS- trials did not increase following PL or IL M + B microinjections. However, these negative results should be interpreted with caution, because under our experimental conditions, the DS- did not inhibit cocaine seeking relative to the *no-DSs* trials. In a previous study where a signaled DS- decreased cocaine seeking, this effect was associated with increased Fos expression in PL [69]. Further, PL (but not IL) inactivation with muscimol reversed inhibitory DS- control [13]. In a different study, Daun02 inactivation of (DS-)-responsive IL Fos-labeled neurons (using a different procedure also containing only a signaled DS-), reversed the inhibitory effect of the DS- on cocaine and alcohol seeking [18]. In contrast, we observed decreased cocaine seeking during DS- trials following IL M + B microinjections. A possible explanation for this inhibitory effect is

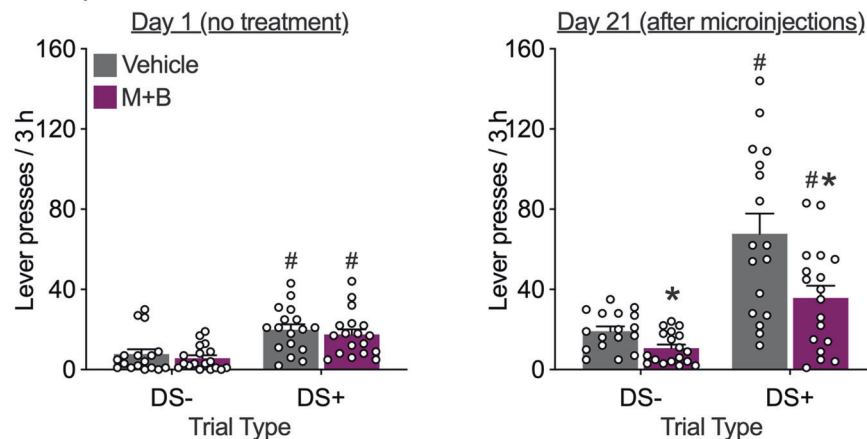
## A. Experimental timeline



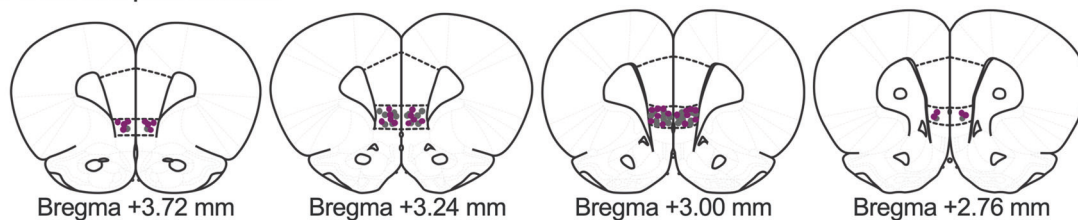
## B. Training



## C. Relapse tests



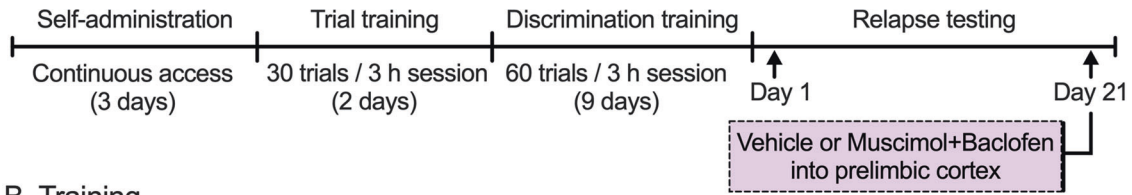
## D. Cannula placements



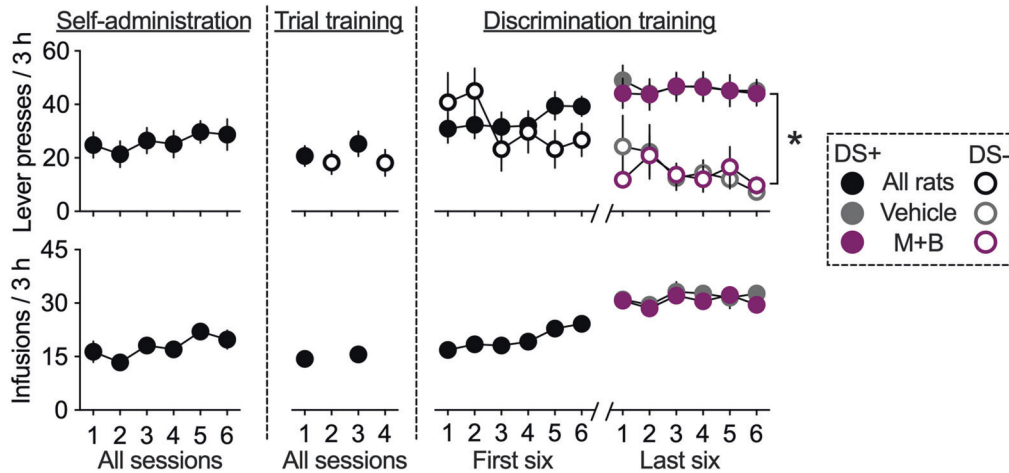
**Fig. 2** Microinjections of muscimol + baclofen into infralimbic cortex prior to DS-controlled cocaine relapse following abstinence.

**A** Experimental timeline. **B** Training data. *Self-administration*: Rats learned to self-administer cocaine over 6 sessions. Mean ( $\pm$ SEM) number of cocaine infusions and lever presses during each 3-h session. *Trial training*: Mean ( $\pm$ SEM) number of lever presses and infusions received during the 3-h sessions (30 DS+ trials in the AM session, 30 DS- trials in the PM session). *Discrimination training*: All rats learned to discriminate DS+ from DS- trials within the same session (30 trials each of DS+ and DS- trials presented in a pseudorandomized manner). Mean ( $\pm$ SEM) number of lever presses during each trial type (top), and infusions received (bottom) during a 3-h discrimination training session. \* indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types ( $n = 35$ ). **C** Relapse tests. There were no group differences in DS-controlled cocaine seeking after 1 day of abstinence (left panel, no microinjections). Microinjections of M+B into IL significantly reduced cocaine seeking during both DS+ and DS- trials following 21 days of abstinence as compared to vehicle-injected controls. Mean ( $\pm$ SEM) number of lever presses during the entire 3-h relapse test session (day 1—left panel; day 21—right panel). \* indicates significant difference ( $p < 0.05$ ) in responding during DS trials between vehicle and M+B groups. # indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types within a treatment group ( $n = 17$  for vehicle;  $n = 18$  for M+B). **D** Cannula placements. Placement of injector tips was determined using cresyl violet counterstaining of formalin-fixed tissue.

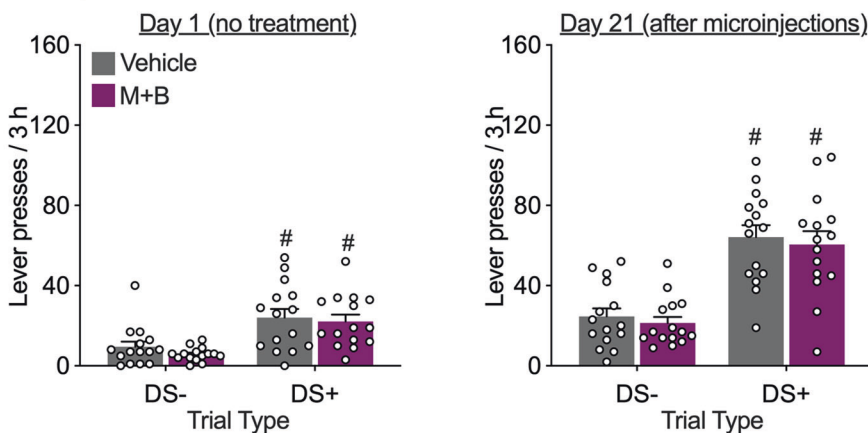
## A. Experimental timeline



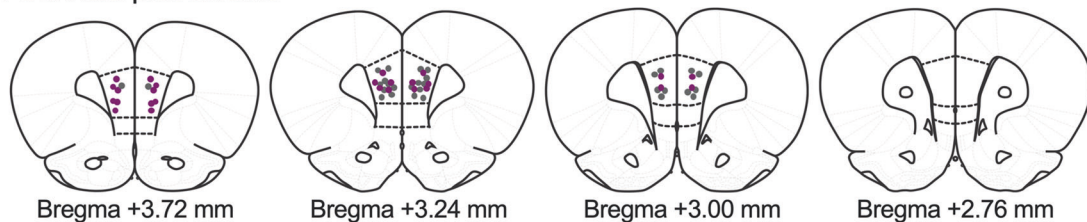
## B. Training



## C. Relapse tests

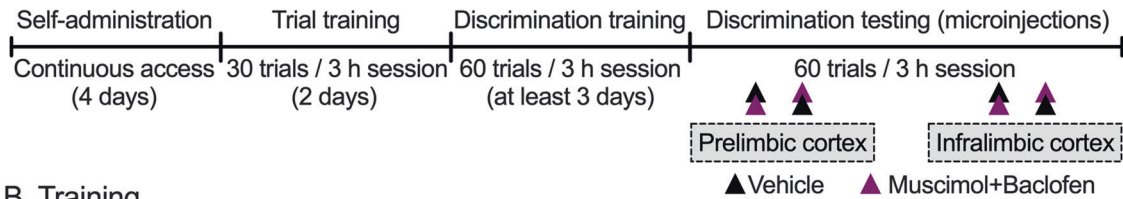


## D. Cannula placements

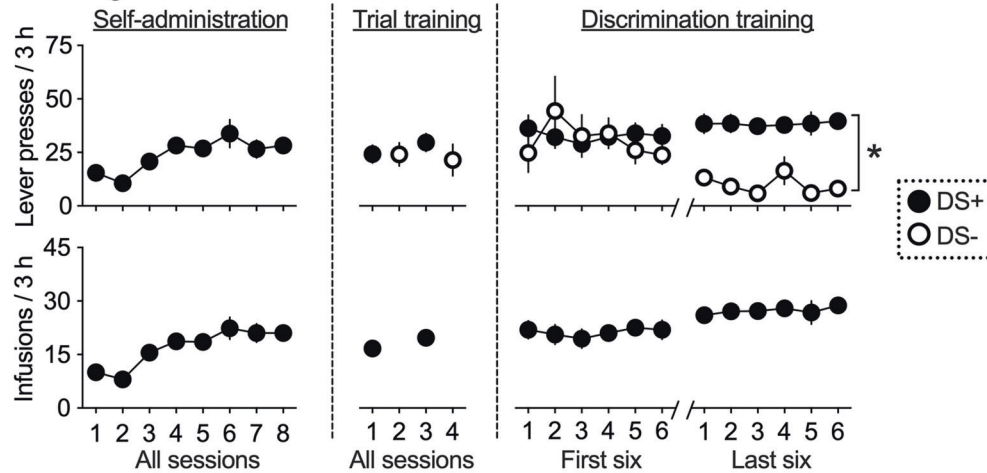


**Fig. 3 Microinjections of muscimol + baclofen into prelimbic cortex prior to DS-controlled cocaine relapse following abstinence.** **A** Experimental timeline. **B** Training data. *Self-administration*: Rats learned to self-administer cocaine over 6 sessions. Mean ( $\pm$ SEM) number of cocaine infusions and lever presses during each 3-h session. *Trial training*: Mean ( $\pm$ SEM) number of lever presses and infusions received during the 3-h sessions (30 DS+ trials in the AM session, 30 DS- trials in the PM session). *Discrimination training*: All rats learned to discriminate DS+ from DS- trials within the same session (30 trials each of DS+ and DS- trials presented in a pseudorandomized manner). Mean ( $\pm$ SEM) number of lever presses during each trial type (top), and infusions received (bottom) during a 3-h discrimination training session. \* indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types ( $n = 30$ ). **C** Relapse tests. There were no group differences in DS-controlled cocaine seeking after 1 day of abstinence (left panel, no microinjections). Microinjections of M+B into PL did not disrupt DS-controlled cocaine seeking following 21 days of abstinence as compared to vehicle-injected controls. Mean ( $\pm$ SEM) number of lever presses during the entire 3-h relapse test session (day 1—left panel; day 21—right panel). # indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types within a treatment group ( $n = 15$  for vehicle;  $n = 15$  for M+B). **D** Cannula placements. Placement of injector tips was determined using cresyl violet counterstaining of formalin-fixed tissue.

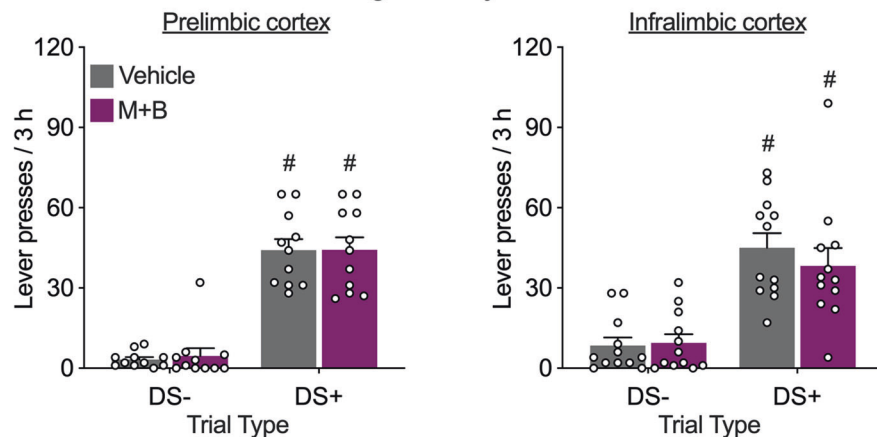
## A. Experimental timeline



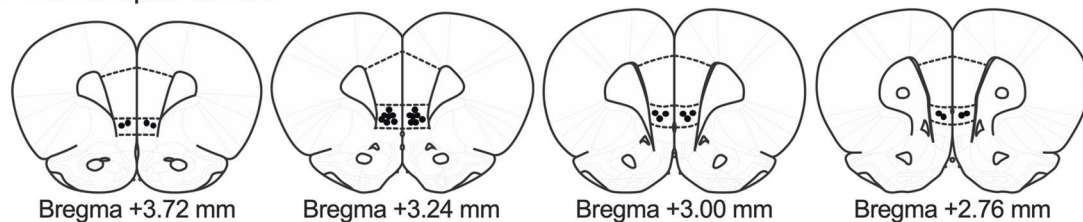
## B. Training



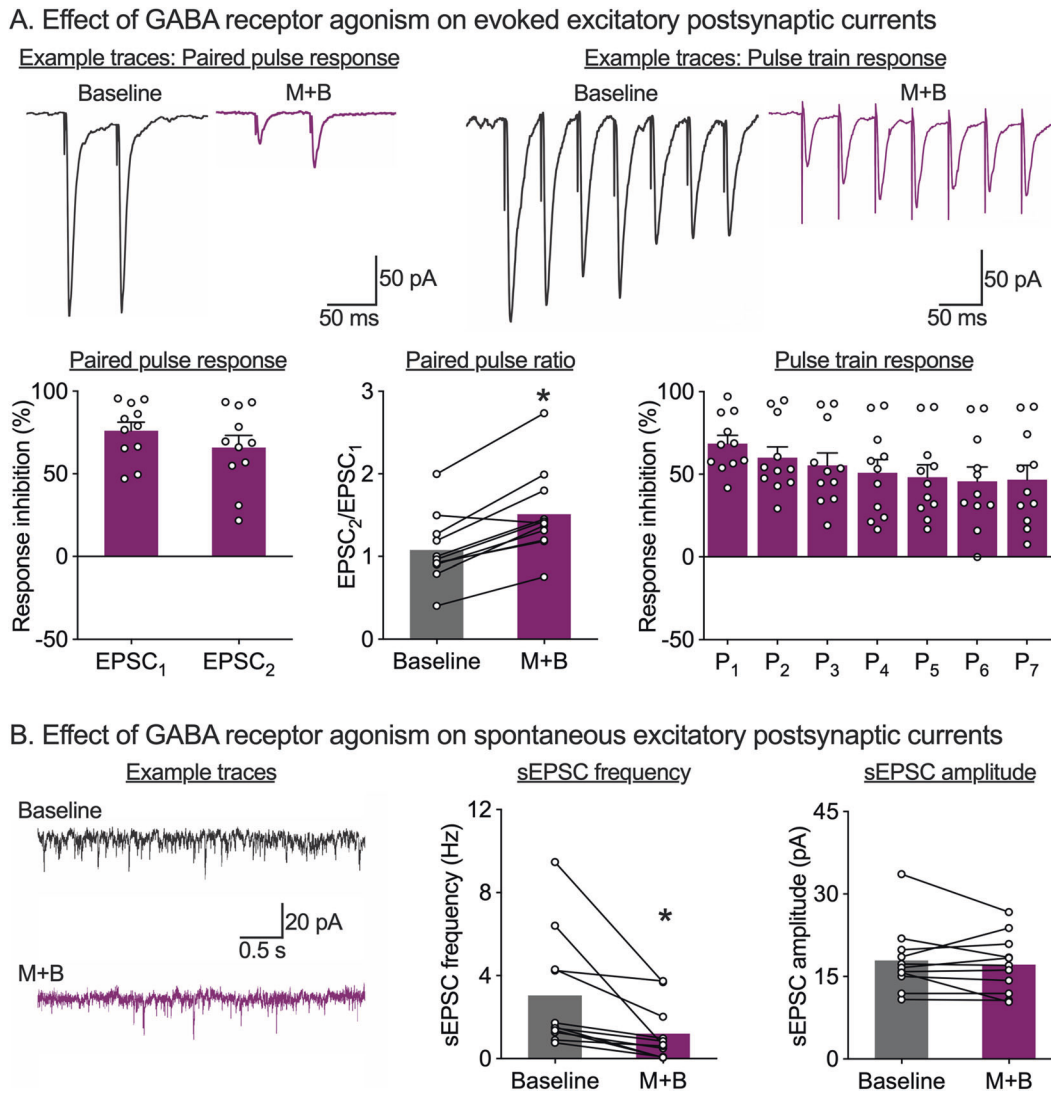
## C. Discrimination tests following microinjections



## D. Cannula placements



**Fig. 4** Microinjections into IL or PL prior to DS-controlled cocaine self-administration. **A** Experimental timeline. **B** Training data. *Self-administration*: Rats learned to self-administer cocaine over 8 sessions. Mean ( $\pm$ SEM) number of cocaine infusions and lever presses during each 3-h session. *Trial training*: Mean ( $\pm$ SEM) number of lever presses and infusions received during the 3-h sessions (30 DS+ trials in the AM session, 30 DS- trials in the PM session). *Discrimination training*: All rats learned to discriminate DS+ from DS- trials within the same session (30 trials each of DS+ and DS- trials presented in a pseudorandomized manner). Mean ( $\pm$ SEM) number of lever presses during each trial type (top), and infusions received (bottom) during a 3-h discrimination training session. \* indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types ( $n = 14$ , all tested rats). **C** Discrimination tests following microinjections. M + B (counterbalanced with corresponding vehicle using a within-subjects design, 2 tests/region) was microinjected into either IL or PL prior to a discriminated cocaine self-administration session. Rats were retrained between tests and testing was restricted to rats that maintained discriminated drug-taking behavior. Microinjections of M + B into either PL (bottom left,  $n = 11$ ) or IL (bottom right,  $n = 12$ ) did not disrupt DS-controlled cocaine taking. Mean ( $\pm$ SEM) number of lever presses during each trial type during 3-h DS-controlled cocaine self-administration sessions following microinjections into IL or PL. # indicates significant difference ( $p < 0.05$ ) between responding during DS+ and DS- trial types within a treatment group. **D** Cannula placements. Placement of injector tips was determined using cresyl violet counterstaining after the last test ( $n = 14$ , all tested rats). PL injection locations were 2.2 mm dorsal to those shown in the figure.



**Fig. 5 Pharmacological manipulation of GABA receptors in infralimbic neurons using ex vivo brain slice electrophysiology.** **A** Example traces showing evoked paired-pulse synaptic responses before and after drug application (upper left), and example traces showing synaptic responses to a train of seven pulses (upper right). Summary graph showing the percent amplitude change in the paired-pulse response after application of M + B (bottom left). Paired-pulse ratios before and after drug application (bottom middle). Summary graph showing the percent amplitude change in the pulse train response after M + B application ( $n = 11$  cells, 9 rats). \* indicates significant difference ( $p < 0.05$ ) between vehicle and M + B groups. **B** Example traces showing spontaneous synaptic response before and after drug application (left). Summary graph showing the effect of M + B on sEPSC frequency (middle) and sEPSC amplitude (right) ( $n = 11$  cells, 9 rats). \* indicates significant difference ( $p < 0.05$ ) between vehicle and M + B groups.

that IL M + B microinjections inhibited residual excitatory drive due to exposure to the common active lever during DS– presentation after 21 abstinence days. It is also possible that IL M + B microinjections decreased the time-dependent potentiation of cocaine seeking during abstinence (‘incubation’) [70, 71], because responding in the M + B group during both DS+ and DS– trials on day 21 was similar to day 1 responding without intracranial injections; however, M + B microinjections prior to day 1 relapse test are necessary to verify this hypothesis. It is unlikely that this decrease is due to non-specific suppression of operant responding during the relapse test as IL M + B microinjections had no effect on (DS+)–controlled cocaine self-administration in our study. Additionally, previous studies have shown that IL M + B inactivation is ineffective at decreasing stress- or cocaine priming-induced reinstatement of cocaine seeking [72, 73], and after extinction, this manipulation potentiates spontaneous recovery [74] and reinstates cocaine seeking [40].

It is possible that previous studies detected mPFC contributions to inhibitory effects of DS– because training and testing were conducted under conditions where baseline responding (in the absence of DS–) was higher than in our task, likely due to the continued presence of excitatory stimuli such as cocaine availability prior to DS– presentation [13], response-contingent cocaine-paired CSs [18], or DS– presentation together with cocaine-predictive cues [13, 18]. This higher baseline would allow for an observable suppression of responding in the presence of the DS– that could then be manipulated pharmacologically. Thus, in these studies the no-DS– condition was likely more similar to our excitatory DS+ condition (vs. our no-DS condition) and it is possible that we would also have observed disinhibition of DS– control following our pharmacological manipulations if we had compared rats’ responding during trials with or without the DS–, in the presence of the excitatory DS+ in both conditions (i.e. *both-DSs* versus *DS+* trials in Experiment 1).



Altogether, in studies where the DS<sup>-</sup> decreased cocaine seeking or taking, the IL and PL both appear to play a role in (DS<sup>-</sup>)-based inhibitory control. Future studies are necessary to examine IL and PL role in the inhibitory effect of DS<sup>-</sup> on cocaine seeking induced by DS<sup>+</sup> in our trial-based procedure.

#### Role of mPFC neural activity in DS-controlled cocaine self-administration

IL or PL M + B microinjections had no effect on ongoing DS-controlled cocaine self-administration in our task. In contrast, Gutman et al. [20] found that IL and PL M + B microinjections prior to discriminated cocaine self-administration decreased responding during DS<sup>+</sup> trials and increased responding during DS<sup>-</sup> trials. This study did not assess relapse to cocaine seeking during abstinence.

Both our task and the Gutman task employed discrete trials and counterbalanced presentations of DS<sup>+/-</sup>. However, in the Gutman study, lever-presses during DS<sup>+</sup> trials (but not DS<sup>-</sup> trials) caused retraction of both levers and led to DS<sup>+</sup> turning off. In contrast, in our task lever-presses were reinforced on an FR1 reinforcement schedule during DS<sup>+</sup> (but not DS<sup>-</sup>) trials, the lever and DS<sup>+</sup> stayed on for 60-s, and multiple infusions could be earned during each trial. While no explicit tone/light CS<sup>+</sup> was paired with drug deliveries in the Gutman study, immediate lever retraction after a lever press only during DS<sup>+</sup> trials likely served as a CS<sup>+</sup> in their procedure. Additionally, the rats in the Gutman study were given initial food self-administration training, were limited to 2-h daily cocaine self-administration, and were allowed to respond only once during each 10-s DS<sup>+</sup> trial to receive a single cocaine infusion. These differences in task structure and training methodology likely caused differential engagement of mPFC and also affected how DS<sup>+/-</sup> in the two studies exerted behavioral control during cocaine self-administration. Our results do agree, however, with those from Moorman and Aston-Jones who found a role for IL, but not PL, in DS<sup>+</sup> control of discriminated sucrose self-administration [53].

#### Effects of GABA receptor agonists on mPFC neuronal activity

We performed whole-cell recordings of IL pyramidal neurons in layer 5/6 to determine synaptic mechanisms for the inhibitory effect of M + B on neuronal activity. We chose layer 5/6 instead of superficial layers 2/3 since this is the primary mPFC output layer and is most likely to affect downstream circuits and behavior [75]. We performed recordings with network activity intact (i.e., no synaptic blockers present) to keep recording conditions similar to the *in vivo* conditions and chose M + B concentrations similar to those used for mPFC microinjections. Although GABA receptor agonists are frequently used to inactivate brain regions of interest [28, 39, 40, 43, 48], their effect on mPFC pyramidal neuron synaptic activity has not been characterized. M + B application reduced amplitude of electrically evoked postsynaptic currents by ~75%. The apparent efficacy of M + B lessened over the course of a seven-pulse train at 25 Hz due primarily to a lessening of overall magnitude of postsynaptic responses without M + B and little change of postsynaptic responses with M + B. We observed increased paired-pulse ratio following M + B application. One putative mechanism is inhibition of presynaptic calcium release via activation of presynaptic GABA<sub>B</sub> receptors [76, 77]. The contribution of presynaptic GABA<sub>A</sub> receptors is more difficult to discern given that the ultimate effect of their activation depends on several factors, including modulation by surrounding GABA<sub>B</sub> receptors and overall GABA<sub>A</sub> receptor activation level [78]. M + B reduced frequency of spontaneous postsynaptic currents in most recorded neurons, but had no effect on amplitude of these currents, similar to a previous study [51].

Together, our data suggest that the predominating effect of M + B on synaptic transmission occurs via a presynaptic mechanism. Increased paired-pulse ratio coupled with decreased spontaneous

event frequency support this conclusion. Since M + B effectively silenced synaptic activity onto IL neurons, it appears that excitatory synaptic activity onto IL neurons is necessary for DS-controlled cocaine seeking. Of note, our recordings were performed in brain slices of drug-naïve rats. Thus, while unlikely, we cannot rule out that modulation of synaptic activity by M + B is altered by cocaine experience [79, 80].

#### General role of PL versus IL neural activity in drug seeking

Early studies using the extinction-reinstatement model [81] where rats were trained to self-administer cocaine and exposed to discrete cues or cocaine priming to induce reinstatement of cocaine seeking led to the hypothesis that PL promotes drug seeking while IL suppresses drug seeking [34, 38–40, 82, 83]. At least for cocaine, a number of studies support this hypothesis, although recent evidence suggests that prior extinction training might be required to engage IL inhibitory control during cue- but not cocaine-primed reinstatement of cocaine seeking [58, 59, 84] (but see [60]). However, results from studies using the extinction-reinstatement model with heroin, alcohol, methamphetamine, and sucrose where reinstatement was induced by drug priming, discrete cues, and contextual cues did not support this hypothesis [42, 43, 51, 85–87]. Further, evidence that the PL-go/IL-stop hypothesis generalizes to other relapse-related models (that do not rely on extinction to suppress drug seeking) is mixed. Koya et al. [48] showed that IL but not PL inactivation using M + B decreases cocaine seeking after 30 abstinence days. In contrast, Cameron et al. [60] showed that optogenetic activation of the IL to nucleus accumbens pathway suppresses cocaine seeking regardless of the period of abstinence. Additionally, PL neurons have been shown to encode incubated cocaine seeking [49] and projections from PL and IL to nucleus accumbens promote and inhibit incubation of cocaine seeking, respectively [61]. Finally, our studies using the Daun02 inactivation procedure suggest a role for IL ensembles in both promotion and inhibition of non-reinforced cocaine (and food) seeking [56, 57]. Together, these results do not support the hypothesis that PL and IL always play opposing roles in drug seeking.

Specifically regarding DS control of cocaine seeking, our finding that inhibition of IL but not PL activity decreases DS-controlled relapse after prolonged abstinence also does not support the PL-go/IL-stop hypothesis. Previous studies using different DS-based procedures also suggest a more complicated role, with evidence for IL involvement in promoting DS<sup>+</sup> controlled food and cocaine seeking [20, 52, 53] and for both PL and IL in inhibition of reward seeking by DS<sup>-</sup> [13, 18, 20, 53].

One possibility for the discrepant results described above is that mPFC neural activity represents a higher order associative structure that underlies cue- and context-guided expression of learned behaviors rather than simply promoting or inhibiting these behaviors. Indeed, mPFC neurons respond not only during reward-related actions, but also to reward-associated contexts and cues, and to non-contingent reward delivery [37, 49, 53, 88–91]. In support of this hypothesis, and specifically for DS-control, Moorman and Aston-Jones [53] conducted electrophysiological recordings in PL and IL during a sucrose DS-task (before and during extinction) and found that neuronal activity in neither region is specifically locked to simply the DS or the action (i.e. going or stopping), but instead represents the appropriate behavioral action based on 'context' (e.g., responding during DS<sup>+</sup> vs. inhibiting responses following extinction training). In most previous studies, only one 'context' (training or extinction; no DS or DS<sup>+</sup>) and associated action pair (press or withhold) was tested at a time, making it difficult to ascertain whether mPFC activity represented (1) the behavioral action measured, (2) the cue-response association needed to perform the correct action, or (3) some higher order information requiring input from other upstream brain regions.

In contrast, the rats in our study had to recognize and make appropriate responses during two orthogonal DS-action pairs. Under these conditions, we found that suppression of IL activity reduced DS-controlled cocaine seeking but did not lead to a breakdown of DS-guided discriminated responding. This suggests that the DS-cocaine association is likely processed in other regions upstream of the IL and that IL activity in our procedure integrates this information to support DS-controlled cocaine seeking during abstinence.

In line with the idea of mPFC processing higher order associative structures [91], it is likely that the complex computations needed for appropriate task performance in drug seeking are mediated by separate but intermingled neuronal ensembles within the mPFC that allow more flexible high-resolution responses to different conditions (e.g. cues, context, DS) than would be allowed by uniform action (promoting or suppressing) of all neurons in a brain area [92]. In support of this hypothesis, targeted ablation of only cue- and context-induced drug-seeking specific neurons is sufficient to disrupt ongoing promotion or suppression of reward seeking [18, 42, 56, 57, 68, 92–102]. Additionally, we and others have shown that separate ensembles within the same brain region can control opposing effects on drug and non-drug reward seeking [18, 56, 57, 68, 92, 102]. Based on our current findings and those of others, we hypothesize that future ensemble-level manipulations are likely to identify DS-drug-specific ensembles in regions upstream of mPFC, and cue-action-specific ensembles within the mPFC, that act together to induce the appropriate DS-controlled behavioral response.

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## AUTHOR CONTRIBUTIONS

R.M., L.A.R., B.J.T., J.M.B., Y.S., and B.T.H. designed the experiments; R.M., L.A.R., B.J.T., J.M.B., S.J.W., M.B.B., V.A.L., O.R.D., and L.E.K. ran the experiments and collected the data; R.M., L.A.R., B.J.T., S.J.W., M.B.B., V.A.L., O.R.D., and L.E.K. analyzed the data; R.M., L.A.R., B.J.T., Y.S., and B.T.H. wrote the paper. All authors reviewed and approved the final version prior to submission.

## ADDITIONAL INFORMATION

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