ARTICLE

Prelimbic cortical projections to rostromedial tegmental nucleus play a suppressive role in cue-induced reinstatement of cocaine seeking

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The prelimbic (PL) region of prefrontal cortex has been implicated in both driving and suppressing cocaine seeking in animal models of addiction. We hypothesized that these opposing roles for PL may be supported by distinct efferent projections. While PL projections to nucleus accumbens core have been shown to be involved in driving reinstatement of cocaine seeking, PL projections to the rostromedial tegmental nucleus (RMTg) may instead suppress reinstatement of cocaine seeking, due to the role of RMTg in behavioral inhibition. Here, we used a functional disconnection approach to temporarily disrupt the PL-RMTg pathway during cue- or cocaine-induced reinstatement. Male Sprague Dawley rats self-administered cocaine during daily 2-h sessions for ≥10 days and then underwent extinction training. Reinstatement of extinguished cocaine seeking was elicited by cocaine-associated cues or cocaine prime. Prior to reinstatement, rats received microinjections of the GABA agonists baclofen/muscimol (1/0.1 mM) into unilateral PL and the AMPA receptor antagonist NBQX (1 mM) into contralateral or ipsilateral RMTg. Functional disconnection of PL-RMTg via contralateral inactivation markedly increased cue-induced reinstatement, but did not increase cocaine-induced reinstatement or drive reinstatement of extinguished cocaine seeking in the absence of cues or cocaine. Enhanced cue-induced reinstatement was also observed with ipsilateral inactivation of PL and RMTg, but not with unilateral inactivation of PL or RMTg alone, indicating that both ipsilateral and contralateral projections from PL to RMTg have an inhibitory influence on behavior. These data further support a suppressive role for PL in cocaine seeking by implicating PL efferent projections to RMTg in inhibiting cue-induced reinstatement.

INTRODUCTION

The prelimbic (PL) region of prefrontal cortex (PFC) has been described as a driver of cocaine seeking. Inactivation studies have shown that PL is necessary for cue-, cocaine-, and stress-induced reinstatement of extinguished cocaine seeking in rats [1–4]. However, PL also has been associated with the inhibition of cocaine seeking under some circumstances, indicating a more complex role in reward processing [as reviewed by 5]. For example, PL plays a critical role in suppressing cocaine seeking in the presence of footshock punishment or discriminative stimuli that signal cocaine unavailability [6, 7]. These opposing roles may be related to distinct PL efferents that either drive or suppress cocaine seeking. Whereas PL projections to the nucleus accumbens (NAc) core are involved in driving cocaine- and cue-induced reinstatement of cocaine seeking [8–11], we hypothesized that PL projections to the rostromedial tegmental nucleus (RMTg) are involved in the suppression of cocaine seeking.

The RMTg, or tail of the ventral tegmental area (VTA), is a GABAergic nucleus that receives input from PL, projects to dopamine (DA) neurons in the VTA and substantia nigra, and is implicated in aversive valence encoding and behavioral inhibition [12–18]. In accordance with a role in behavioral inhibition, RMTg has been shown to inhibit drug seeking in rats. Lesions or temporary inactivation of RMTg led to increased alcohol seeking and consumption, as well as enhanced cue-induced reinstatement of cocaine seeking [19, 20]. In addition, allosteric potentiation of AMPA receptors in RMTg either before or after a short extinction session led to enhanced extinction learning and reduced cocaine seeking [20]. Finally, PL inputs to RMTg have been implicated in cue-induced behavioral inhibition. A recent study demonstrated that PL modulates RMTg responses to conditioned cues predictive of food reward or footshock, and that PL projections to RMTg are involved in reductions in reward seeking in the face of punishment [21].

We hypothesized that PL contributes to a suppression of cocaine seeking via projections to RMTg, and that inactivation of the PL-RMTg pathway would enhance cue-induced reinstatement of extinguished cocaine seeking. We used a functional disconnection approach based on previous anatomic work [12] and results presented here indicating that PL projections to RMTg are predominantly ipsilateral. We temporarily inactivated PL projections to RMTg via unilateral microinjection of an AMPA receptor antagonist into RMTg (to target glutamatergic inputs, including PL) and contralateral microinjection of GABA agonists into PL (to cause a more general inactivation of these neurons). This approach should impair any behavior that requires interactions

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of both structures, while leaving intact functions that only require these structures separately. We investigated the influence of the PL-RMTg pathway on cocaine seeking by disrupting this pathway prior to cue- or cocaine-induced reinstatement, or expression of extinguished cocaine seeking. We also tested control groups of rats that instead received ipsilateral PL-RMTg inactivation or unilateral inactivation of PL or RMTg alone.

MATERIALS AND METHODS

Animals
Male Sprague Dawley rats (initial weight 250–300 g; Charles River, Raleigh, NC) were single housed under a 12-h reverse light/dark cycle, and had access to food and water ad libitum. Animals were housed in a temperature- and humidity-controlled animal facility at Texas A&M University (AAALAC accredited). All experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University, and conducted according to specifications of the National Institutes of Health, as outlined in the Guide for the Care and Use of Laboratory Animals.

Surgery for catheter/cannula implantation
Rats were anesthetized with vaporized isoflurane (induced at 5%, maintained at 1–2%), given the analgesic ketoprofen (2 mg/kg, s.c.), and implanted with chronic indwelling intravenous jugular catheters, as previously described (Smith et al., 2009). Immediately following catheter implantation, rats were placed in a stereotaxic frame (Kopf, Tujunga, CA) to implant intracranial guide cannulae (26 gauge, 10 mm length for PL, and 15 mm length for RMTg, Plastics One, Roanoke, VA) aimed at either contralateral PL and RMTg (for functional disconnection), or ipsilateral PL and RMTg (for ipsilateral inactivation). Left and right hemisphere were counterbalanced for receiving PL and RMTg cannulae, although all contralateral and ipsilateral placements are shown in the left and right hemisphere, respectively, in Fig. 2B for clarity. For stereotaxic surgery, a skin incision was made over the skull, small screws were secured to each skull plate, and holes were drilled in the skull over the target sites. Cannulae were inserted 2 mm above PL (surgical coordinates: AP +3.1, ML +1.5 to +1.7, DV −1.5 to −1.7 from skull, 12° angle) and RMTg (AP −7.3 to −7.8, ML +2.2 to +2.6, DV −5.7 from dura, 10° angle), and secured in place with orthodontic acrylic. Stainless steel dummy wires were inserted into the guide cannulae to prevent clogging. Beginning 3 days after surgery, intravenous catheters were flushed once daily with 0.1 ml of cefazolin (100 mg/ml) and 0.05 ml of heparin (10 units/ml). Self-administration sessions began at least 5 days after surgery. Catheters were flushed with 0.1 ml saline before each self-administration session to test for patency, and then flushed with cefazolin and heparin after the session.

Cocaine self-administration and extinction
Following recovery from surgery, rats were trained to self-administer cocaine in operant conditioning chambers housed in sound-attenuating cubicles and controlled by a MED-PC IV program (Med-Associates, St. Albans, VT). Rats learned to self-administer cocaine in daily 2-h sessions, in which a press on the active lever led to an infusion of intravenous cocaine (fixed ratio 1; 0.2 mg/50 µl infusion; cocaine HCl powder generously provided by the NIDA Drug Supply Program). Each infusion was paired with a compound light/tone cue (white LED light above the active lever; 78 dB, 2900 Hz tone) and followed by a 20-s timeout. The inactive lever in the chamber had no programmed consequences. After ≥10 days of self-administration with ≥20 infusions per session, rats underwent extinction (no cocaine or cues) for ≥7 days and until they had two consecutive sessions with <25 active lever presses. Rats were excluded from reinstatement testing if they failed to meet the criterion for extinction within 20 days ($n = 3$).

Reinstatement and microinjections
After reaching the extinction criterion, rats underwent reinstatement testing. For cue-induced reinstatement, active lever presses led to the presentation of the tone/light cue previously paired with cocaine, but no cocaine was delivered. Previous work has demonstrated that similar stimulus lights and/or tones have little to no reinforcement value alone, and do not drive reinstatement of responding unless they are previously associated with reward or are novel [22–24], indicating that cue-induced reinstatement is driven by the conditioned association between the cues and cocaine. For cocaine-induced reinstatement, rats were given 10 mg/kg cocaine (i.p.) prior to being placed in the operant conditioning chamber under extinction conditions. All rats received at least two tests for cue-induced reinstatement (vehicle and drug), with a subset of rats in the contralateral and ipsilateral groups also receiving unilateral inactivation (total of three tests, counterbalanced order). Some rats also received two subsequent tests for cocaine-induced reinstatement. Prior to each reinstatement session, rats were given microinjections of drug or vehicle in a counterbalanced order for within-subject comparisons. A subset of rats received microinjections of drug or vehicle (counterbalanced order) prior to extinction sessions that occurred before reinstatement testing. After each test, rats were given additional extinction sessions and required to meet the criterion of ≥2 extinction sessions with <25 presses prior to the next test.

One day prior to the first microinjection, sham microinjectors were inserted 2 mm beyond guide cannulae, while rats were acclimated to the gentle towel restraint used for the microinjection procedure. For microinjections prior to testing, injector cannulae were inserted into PL and RMTg (33 gauge; extending 2 mm beyond guide cannulae). PL was injected with a cocktail of GABA agonists comprised of R-baclofen and muscimol (1 and 0.1 mM, 0.3 µl total volume; Tocris, Minneapolis, MN), and RMTg was injected with the AMPA receptor antagonist NBQX disodium salt (1 mM, 0.3 µl; Tocris). These concentrations are similar to or lower than previous behavioral studies using baclofen/muscimol [3, 25] or NBQX [26–28]. The vehicle used for all drugs was filtered 1× phosphate-buffered saline (PBS). Microinjections occurred over 60 s and then microinjectors were left in place for an additional 60 s to allow for diffusion. Rats were placed into the operant conditioning chamber 5 min later for testing.

Histology
Rats were deeply anesthetized with isoflurane and then transcardially perfused with 0.9% NaCl followed by 10% neutral-buffered formalin via a peristaltic pump (Cole Parmer, Vernon Hills, IL). Brains were collected, postfixed in 10% formalin overnight, placed in 20% sucrose with 0.02% sodium azide for ≥2 days, frozen in dry ice, and then sectioned at 40 µm using a cryostat. To assess cannula placements, sections were mounted onto glass slides, Nissl stained (0.045% thionin), and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). PL borders were defined according to Paxinos and Watson [29], and RMTg borders were defined according to Smith et al. [30].

Retrograde tracing
A separate group of rats underwent stereotaxic surgery to receive unilateral injections of the retrograde tracer cholera toxin B (CTB, 0.2% dissolved in 0.9% NaCl, List Biological Laboratories) in RMTg (100 nl, left hemisphere, surgical coordinates: AP −7.2, ML +1.9, DV −7.6 from skull, 10° angle). Intracranial injections were made using a pulled glass micropipette and Nanoject 2010 injector (World Precision Instruments). After ≥1 week of recovery, rats were sacrificed and brain tissue was processed for CTB immunohistochemistry. Free-floating coronal brain sections were incubated overnight at room temperature in goat anti-CTB (1:50 K, List Biological Labs cat# 703, RRID:AB_10013220) in PBS with 0.25% Triton X-100 (Sigma-Aldrich), and then incubated for 1 h in Alexa

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Fluor 594-conjugated donkey anti-goat (1:500, Jackson Immunoresearch Labs cat# 705-585-147, RRID:AB_2340433). Following each incubation step, tissue was rinsed three times in PBS for 1 min each. Sections were mounted onto glass slides, coverslipped with ProLong Diamond Antifade (Thermofisher Scientific), sealed with clear nail polish, and stored at 4 °C. Fluorescent images were acquired using an Olympus BX51 microscope. For each rat, CTB-labeled neurons were manually counted within a 0.60×0.45 mm counting window on 20× images of ipsilateral or contralateral PL from six brain sections.

Statistical analyses
A two-way ANOVA was used to analyze the percentage of CTB-labeled neurons in contralateral vs. ipsilateral PL neurons projecting to RMTg, with AP level as the second factor in the analysis. A three-way ANOVA was used to analyze cue-induced reinstatement tests, comparing lever (active, inactive), drug (vehicle, drug), and group (contralateral, ipsilateral, unilateral PL, unilateral RMTg), with repeated measures for the first two factors. Two-way ANOVAs were used to analyze drug × group effects separately for the active and inactive levers, and to compare rats prior to combining for the unilateral groups. Normality of distribution was tested via Shapiro–Wilk (significance set to $p < 0.05$) and outliers were identified via ROUT (Q set to 10% to detect all likely outliers) for all eight data groups (vehicle and drug conditions for the four experimental groups). Wilcoxon signed-rank tests were used for nonparametric comparisons of within-subject cue-induced reinstatement effects. Time course effects for cue-induced reinstatement were analyzed via two- and three-way ANOVAs comparing drug (repeated measure), time bin (repeated measure), and group (for three-way analysis). Sidak's multiple comparisons tests were used for all post hoc analyses of ANOVA effects. Two-way ANOVAs were used to analyze drug × lever effects for cocaine-induced reinstatement and extinction tests. Statistical analyses were performed using GraphPad Prism (V8.3). Figures show data expressed as mean ± SEM. Group numbers are shown on bar graphs. Baseline extinction data are the mean of the last 2 days of extinction.

RESULTS
Retrograde tracing in PL-RMTg pathway
Injections of CTB into unilateral RMTg (Fig. 1A, $n = 5$ rats) revealed afferent neurons in PL (Fig. 1B). We found that the majority of PL projections to RMTg are ipsilateral (75.7 ± 1.7%; average number of CTB neurons counted per rat = 366.6 ± 77.3). The percentage of ipsilateral vs. contralateral inputs to RMTg was consistent across the rostral-caudal axis of PL (Fig. 1C; main effect of ipsilateral vs. contralateral: $F_{(1,4)} = 233.8$, $p = 0.0001$; no interaction with AP level: $F_{(5,20)} = 1.14$, $p = 0.37$).

Cue-induced reinstatement
Representative cannulae placements are shown in Fig. 2A. Rats were included in the contralateral group or ipsilateral group only if both microinjectors were within the boundaries of PL and RMTg (Fig. 2B). Rats were included in the PL unilateral group or RMTg unilateral group (Fig. 2C) if they received either: (1) a drug microinjection in the target area and a vehicle microinjection in the second area, or (2) a drug microinjection in the target area and a drug microinjection that missed (i.e., was outside the boundaries of) the second area. These rats were combined for the unilateral groups only after statistical analyses found no significant difference between them (two PL conditions: $F_{(1,24)} = 1.18$, $p = 0.68$; two RMTg conditions: $F_{(1,12)} = 0.36$, $p = 0.56$). Rats were excluded from all analyses if microinjectors missed both PL and RMTg ($n = 5$).

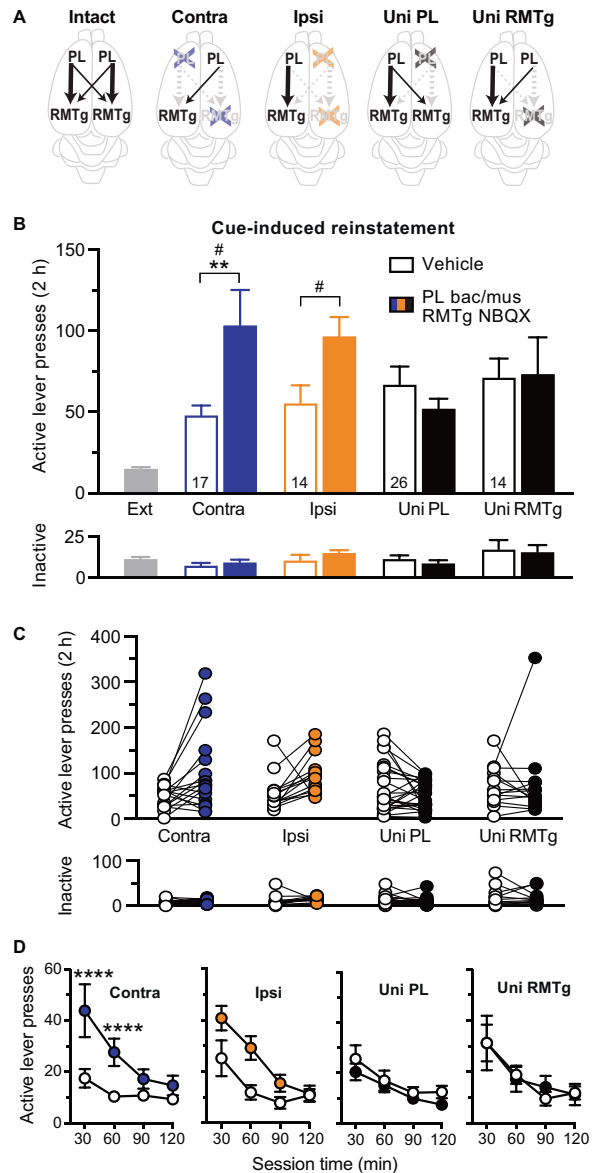Functional disconnection of the PL-RMTg pathway was performed by microinjecting baclofen/muscimol into unilateral PL
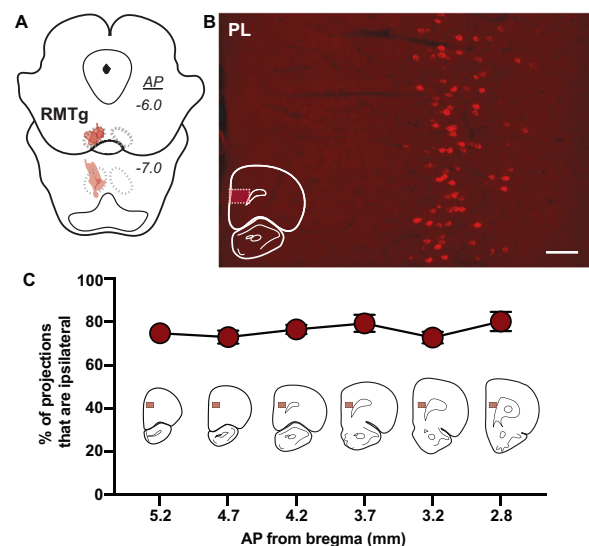
Fig. 1 Retrogradely-labeled PL neurons projecting to RMTg. A Drawings of brain sections showing location and spread of CTB injections targeting RMTg ($n = 5$). **B** Representative photograph of CTB-labeled neurons in PL. Scale bar = 100 μm. Drawing of brain section represents AP level at which this image was taken, and the rectangle within this drawing indicates the size and location of the image. **C** The percentage of PL projections to RMTg that are ipsilateral is consistent across the rostral-caudal axis of PL (mean ± SEM). Drawings of brain sections represent each AP level used for counting of CTB-labeled cells, and a rectangle within PL represents the size and location of the counting area.

and NBQX into contralateral RMTg (Fig. 3A). Additional experimental groups included ipsilateral injections into PL and RMTg, or unilateral injections into PL or RMTg alone (Fig. 3A). We found that cue-induced reinstatement was enhanced following contralateral or ipsilateral inactivation of the PL-RMTg pathway, but not after unilateral inactivation of PL or RMTg alone (Fig. 3B). A three-way comparison of the four experimental groups (contralateral, ipsilateral, unilateral PL, and unilateral RMTg) across both testing conditions (vehicle, drug) and both levers (active, inactive) revealed main effects for drug ($F_{(1,67)} = 139.6$, $p < 0.0001$) and lever ($F_{(1,67)} = 5.63$, $p = 0.02$) and several interactions (lever × group: $F_{(3,67)} = 4.44$, $p = 0.007$; lever × drug: $F_{(1,67)} = 7.40$, $p = 0.008$; group × lever × drug: $F_{(3,67)} = 4.76$, $p = 0.005$). Based on a main effect of lever and a significant three-way interaction, we further explored these effects with separate analyses of the active and inactive lever via two-way ANOVAs. While we found no significant effect of drug for the inactive lever ($F_{(1,67)} = 0.16$, $p = 0.69$), we found a significant effect of drug for the active lever ($F_{(1,67)} = 6.59$, $p = 0.01$) that varied across groups (group × drug interaction: $F_{(3,67)} = 4.72$, $p = 0.005$). Post hoc analysis of group differences for the active lever revealed a significant difference between the contralateral group and the unilateral PL group ($p = 0.018$), but no other differences. As might be expected based on the graphs, post hoc analysis revealed a significant difference for vehicle vs. drug within the contralateral group ($p = 0.004$) and no significant differences within the unilateral groups (PL $p = 0.90$; RMTg $p > 1.0$). Surprisingly, post analysis revealed no significant difference within the ipsilateral group ($p = 0.09$), despite the fact that the vast majority of rats showed increased cue-induced reinstatement following ipsilateral inactivation (Fig. 3C). We speculated that this lack of significance might be attributed to a non-normal distribution of the data (observed in 5/8 data groups) and the presence of statistical outliers (1–3 outliers observed in 3/8 data groups). Thus, we used nonparametric statistical tests to compare drug effects within each group and found that cue-induced reinstatement was significantly enhanced following

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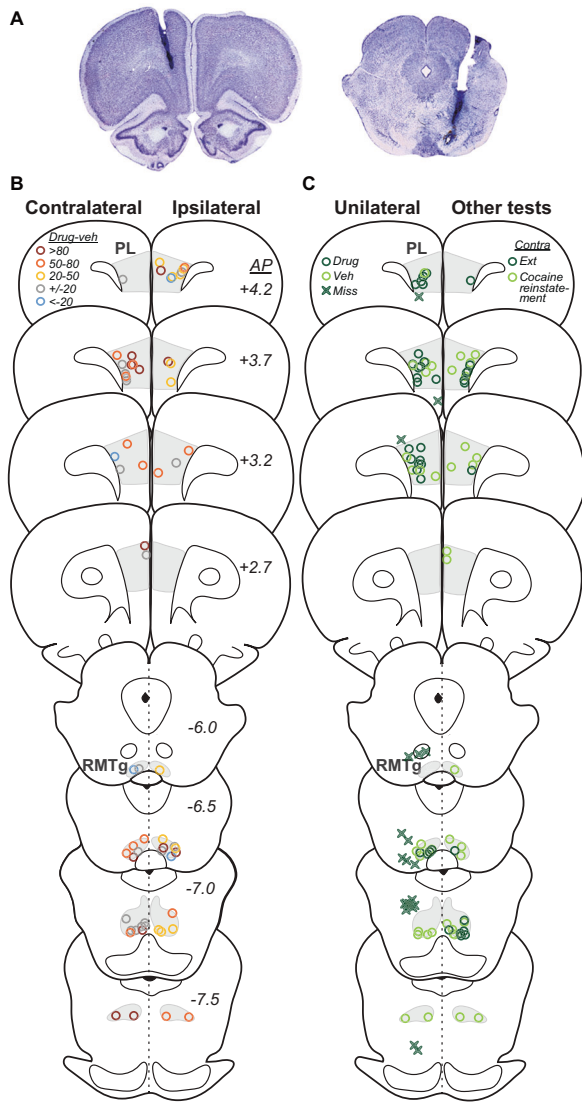
Fig. 2 Microinjection cannulae placements for PL and RMTg. A Representative photographs of microinjection cannulae targeting PL and RMTg. **B** Drawings of brain sections showing microinjector placements for rats receiving contralateral injections into PL and RMTg (shown on left side), and ipsilateral injections into PL and RMTg (shown on right side) during cue-induced reinstatement. The legend indicates that symbols are color coded according to the magnitude of the change in cue-induced reinstatement behavior (difference score = drug session minus vehicle session) to show rats with enhanced reinstatement (yellow circles, 20–50 difference score; orange circles, 50–80 difference score; red circles, >80 difference score), no change in reinstatement (gray circles, ±20 difference score), or reduced reinstatement (blue circles, ≤20 difference score). **C** Drawings of brain sections showing microinjector placements for rats receiving unilateral injections into PL or RMTg (shown on left side), or contralateral injections into PL and RMTg during extinction or cocaine-induced reinstatement (shown on right side). For unilateral injections, symbols are color coded to represent drug injections into target area (dark green circles), vehicle injections into the second area (light green circles), or drug injections outside of the second area (i.e., misses, dark green Xs). For the other tests, symbols are color coded to represent extinction (dark green circles) or cocaine-induced reinstatement (light green circles). Anterior–posterior (AP) levels represent distance from bregma (in mm).

Fig. 3 Effects of PL-RMTg functional disconnection on cue-induced reinstatement. A Experimental approach showing PL-RMTg projections in the intact condition and disrupted conditions, including contralateral inactivation (functional disconnection), ipsilateral inactivation, unilateral PL inactivation, or unilateral RMTg inactivation. For each experimental condition, intact PL-RMTg projections (ipsilateral and contralateral) are represented by solid black lines, whereas disrupted PL-RMTg projections are represented by dashed gray lines. **B** Cue-induced reinstatement was enhanced following contralateral inactivation of PL-RMTg and ipsilateral inactivation of PL-RMTg, as compared to within-subject vehicle injections, but not following unilateral inactivation of PL or RMTg alone (**$p = 0.004$ for Sidak post hoc, #$p = 0.01$ for nonparametric Wilcoxon test). Lever pressing was different for the active lever, but not the inactive lever. Baseline extinction is the mean lever presses for the last 2 days of extinction for all groups combined. **C** Individual data is shown for each group for cue-induced reinstatement. **D** The time course for cue-induced reinstatement revealed significantly enhanced lever pressing during the first and second 30-min time bins (****$p < 0.0001$) following contralateral inactivation, as compared to vehicle injections (mean ± SEM).

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contralateral inactivation ($p = 0.01$) and ipsilateral inactivation ($p = 0.01$), but not following unilateral inactivation of PL ($p = 0.23$) or RMTg ($p = 0.77$; Fig. 3B).

Rats within each group received vehicle vs. drug in a counterbalanced order. To ensure that the order of injections did not influence reinstatement effects, we analyzed order effects for cue-induced reinstatement (comparing first vs. second test order for vehicle vs. drug). We found main effects for drug in ipsilateral ($F_{(1,24)} = 5.67$, $p = 0.03$) and contralateral groups ($F_{(1,30)} = 7.30$, $p = 0.01$), but not unilateral groups (p's > 0.6). The contralateral group alone also showed a main effect of order ($F_{(1,30)} = 5.61$, $p = 0.02$) and an interaction of drug × order ($F_{(1,30)} = 6.87$, $p = 0.01$), with the first vs. second tests different for drug ($p = 0.003$) but not vehicle ($p = 0.98$).

An examination of the time course of lever pressing during cue-induced reinstatement (Fig. 3D) indicated that both contralateral and ipsilateral inactivation enhanced lever pressing evoked by cocaine-associated cues, with no effect of unilateral PL or RMTg inactivation. All four groups showed a significant main effect of time, likely due to within-session extinction (contralateral: $F_{(3,48)} = 9.86$, $p < 0.0001$; ipsilateral: $F_{(3,39)} = 20.64$, $p < 0.0001$; unilateral PL: $F_{(3,75)} = 10.17$, $p < 0.0001$; unilateral RMTg: $F_{(3,39)} = 10.64$, $p < 0.0001$). However, a main effect of drug was only observed in the contralateral ($F_{(1,16)} = 7.94$, $p = 0.01$) and ipsilateral groups ($F_{(1,13)} = 6.88$, $p = 0.02$). Although there was no significant drug × time interaction for the ipsilateral group ($F_{(3,39)} = 2.35$, $p = 0.088$), there was a significant interaction for the contralateral group ($F_{(3,48)} = 8.17$, $p = 0.0002$), due to significantly enhanced reinstatement during the first ($p < 0.0001$) and second 30-min bins ($p < 0.0001$) of the 2-h session. A three-way comparison of the contralateral and ipsilateral groups across time and testing conditions (vehicle, drug) revealed an effect of drug ($F_{(1,29)} = 13.85$, $p = 0.0008$) and time ($F_{(3,87)} = 26.90$, $p < 0.0001$), but no interaction with group (time × group × drug interaction: $F_{(3,87)} = 0.83$, $p = 0.48$).

Cocaine-induced reinstatement and extinction

To determine whether inactivation of the PL-RMTg pathway enhanced cocaine seeking in general, we also tested the effects of functional disconnection on cocaine-induced reinstatement and extinction responding. Rats were included in the contralateral group only if both microinjectors were within the boundaries of PL and RMTg (Fig. 2C). We found that cocaine-induced reinstatement was not significantly altered by contralateral injections of baclofen/muscimol in PL and NBQX in RMTg, as compared to vehicle injections (Fig. 4A, B). Analysis revealed a main effect of lever ($F_{(1,10)} = 38.37$, $p = 0.0001$), but no lever × drug interaction ($F_{(1,10)} = 0.63$, $p = 0.45$). Furthermore, contralateral injections did not drive reinstatement when administered prior to an extinction session (Fig. 4C, D; lever × drug interaction: $F_{(1,7)} = 2.25$, $p = 0.18$).

DISCUSSION

These studies establish a suppressive influence of PL projections to RMTg on cue-induced reinstatement of cocaine-seeking. We found that functional disconnection of the PL-RMTg pathway via contralateral inactivation enhanced cue-induced reinstatement of cocaine seeking (Fig. 3B), without enhancing cocaine-induced reinstatement or extinction responding (Fig. 4), indicating that inactivation of the PL-RMTg pathway does not have a general effect on all cocaine seeking. We found that ipsilateral inactivation of PL and RMTg also enhanced cue-induced reinstatement (Fig. 3B), but that unilateral inactivation of either PL or RMTg alone did not change cue-induced reinstatement. Together, these data indicate that contralateral PL-RMTg projections (which account for ~24% of projections, Fig. 1C) also play a suppressive role in cue-induced reinstatement. Importantly, these data demonstrate that PL can play a suppressive role in cue-induced
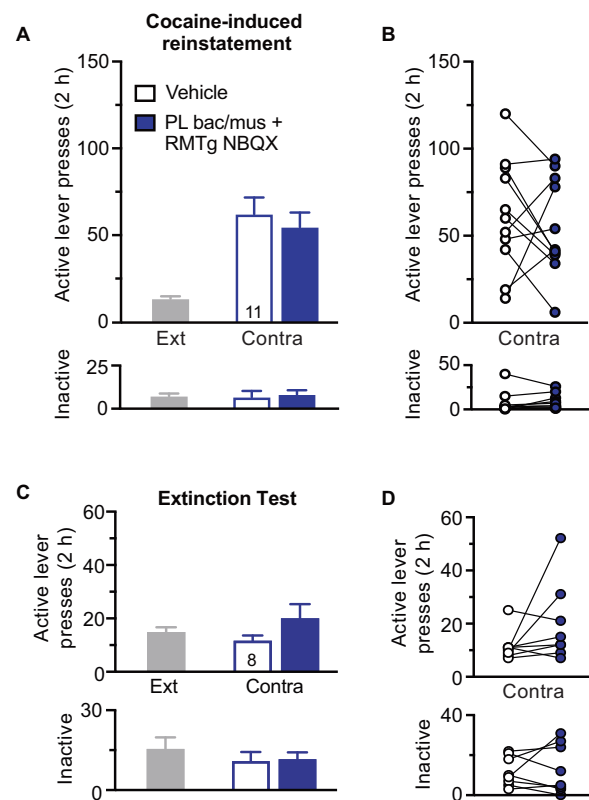
Fig. 4 Effects of PL-RMTg functional disconnection on cocaine-induced reinstatement and extinction responding. A Cocaine-induced reinstatement was not significantly different following contralateral inactivation of the PL-RMTg pathway, as compared to within-subject vehicle injections. **B** Individual data is shown for cocaine-induced reinstatement. **C** Extinction responding was not significantly different following contralateral inactivation of the PL-RMTg pathway. **D** Individual data is shown for extinction. No differences were observed for active or inactive lever. Baseline extinction is the mean lever presses for the last 2 days of extinction (mean ± SEM).

reinstatement of cocaine seeking and that PL efferent projections to RMTg support this inhibitory role in reinstatement.

These data contrast with a role for PL as a driver of cocaine seeking, particularly in terms of reinstatement [1, 11]. However, opposing roles for PL in driving and suppressing cocaine seeking may be related to distinct PL efferents. Previous work has established that PL projections to NAc core are necessary for cocaine- and cue-induced reinstatement [8–10, 31]. Here, we show that PL projections to RMTg play a suppressive role in cue-induced reinstatement, indicating that PL can support opposing roles in cocaine seeking through distinct projections. While previous work has demonstrated that different subregions within medial PFC (e.g., PL and infralimbic) can have opposing influences during behavior [5, 32, 33], the current studies suggest that a single PFC subregion can also exert opposing influences on behavior via different efferent projections. This result parallels previous findings that medial PFC projections to the dorsal raphe nucleus drove escape behavior in a forced swim test, while projections to the lateral habenula suppressed escape behavior [34]. Given that PL projections to RMTg have an opposing influence on cue-induced cocaine seeking, as compared to PL projections to NAc core, it is possible that PL neurons projecting to RMTg vs. NAc core are differentially recruited to regulate cue-induced cocaine seeking. One possibility is that two separate subpopulations of PL neurons project to NAc core vs. RMTg, and that these two subpopulations are recruited via distinct afferents to facilitate the

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regulation of cocaine seeking. Alternatively, both subpopulations of PL neurons may be equally recruited during cue-induced reinstatement, resulting in opposing influences on cocaine seeking.

Interestingly, we found that ipsilateral inactivation of PL and RMTg was sufficient to drive enhanced cue-induced reinstatement, indicating that both ipsilateral and contralateral projections between PL and RMTg have a suppressive influence (Fig. 3A, B). An alternative explanation for these observations is that they are due to additive/synergistic effects of unilateral inactivation of PL and RMTg, and not necessarily due to disrupting projections between PL and RMTg. However, an additive effect seems unlikely given that we observed no effect of unilateral inactivation of PL or RMTg alone (Fig. 3B), and given that bilateral inactivation of PL vs. RMTg has opposite effects on reinstatement [1, 20]. Previous work has also demonstrated a role for contralateral PFC projections in reward seeking. Ipsilateral inactivation of PFC projections to NAc or basolateral amygdala led to significant effects on reinstatement of drug seeking [35–37]. Further, PL neurons projecting to contralateral NAc core showed Fos activation after cue-induced reinstatement of cocaine seeking [31]. Finally, Hart et al. [38] investigated pathways between PL and dorsomedial striatum, and found that one ipsilateral connection and one contralateral connection was sufficient for normal devaluation behavior (similar to the connections that are left intact with unilateral PL or RMTg inactivation, Fig. 3A), but one ipsilateral connection alone was not sufficient for maintaining normal behavior (similar to the connections left intact with PL-RMTg ipsilateral inactivation, Fig. 3A). Altogether, these findings indicate that both ipsilateral and contralateral projections from PFC have a crucial influence on behavior, but that a critical degree of disruption of these pathways is needed before their effects are attenuated.

The current results indicate that PL is normally providing a suppressive influence on cue-induced reinstatement via projections to RMTg. In principle, this suppressive influence may operate either by directly counteracting the cue-induced drive to seek cocaine, or instead by slowing the within-session extinction that is often observed across a 2-h reinstatement session. An examination of the time course of effects across the session (Fig. 3D) supports the former, even though previous studies have supported a role for RMTg in the expression of extinction learning after cocaine self-administration [20, 39]. We observed that disruption of the PL-RMTg pathway increased cue-induced cocaine seeking at the beginning of the session (the first and second 30-min time bins), but not later in the session. Therefore, functional disconnection did not block within-session extinction during cue-induced reinstatement, indicating that PL projections to RMTg are instead directly counteracting cue-induced cocaine seeking.

Further, we found that PL-RMTg functional disconnection did not drive reinstatement on its own (i.e., in the absence of cues or cocaine), indicating that this pathway is not necessary for the expression of extinguished cocaine seeking. In contrast, previous work found that bilateral inactivation of RMTg increased lever pressing during extinction, and this effect was observed for both the active and inactive lever, indicating a general disinhibitory effect [20]. However, that study did not distinguish RMTg from surrounding regions or examine the contributions of specific RMTg inputs, which may account for the different observations. In the current study, we found that disruption of the PL-RMTg pathway was not sufficient to drive increased cocaine seeking, and that increased drug seeking was only observed in the presence of cues. This corresponds with a recent study demonstrating that PL projections modulate RMTg responses to reward- and aversion-associated cues [21].

Interestingly, we found that the suppressive influence of the PL-RMTg pathway is specific to cue-induced cocaine seeking. Functional disconnection of the PL-RMTg pathway did not

enhance cocaine-induced reinstatement. This may be due to differences in the underlying mechanisms for cue- and cocaine-induced reinstatement. DA is necessary for both cue- and cocaine-induced reinstatement [4, 10, 31, 40]. However, while cocaine-associated cues increase DA release via enhanced firing of DA neurons in VTA, cocaine increases DA via direct actions at axon terminals [41–46]. Therefore, RMTg may have a larger inhibitory influence on cue-induced reinstatement due to its regulation of DA neuron firing [16, 47]. Inactivation of PL projections to RMTg might reduce inhibition of DA neurons, enhancing cue-induced DA release and drug seeking.

It is important to note that during the course of our studies, we did not observe changes to motor activity (data not shown) following functional disconnection of the PL-RMTg pathway, using GABA agonists in PL and an AMPA receptor antagonist in RMTg. Previous work has shown increased amphetamine-induced rotational behavior following unilateral RMTg lesion, and increased locomotor activity after bilateral RMTg lesions or microinjections of GABA agonists [17, 48, 49]. However, in contrast to GABA agonists or lesions that would reduce all RMTg activity, we specifically targeted AMPA receptors in RMTg with NBQX because PL inputs are glutamatergic, and recent findings showed that inactivation of PL or other glutamatergic inputs to RMTg does not change baseline RMTg firing [21]. Therefore, disruption of AMPA receptor signaling in unilateral RMTg does not seem sufficient to drive increased locomotor behavior. Finally, an important caveat to note is that these studies were performed only in male rats. Given that the neural circuitry underlying cocaine seeking is sexually dimorphic, more studies are required to determine the role of the PL-RMTg pathway in females [50–53].

CONCLUSIONS
The current results show that PL projections to RMTg play a suppressive role in cue-induced cocaine seeking. Together with previous work, these results demonstrate that PL plays a role not only in promoting reinstatement, but also in suppressing reinstatement. These opposing roles for PL may be supported by distinct efferent projections.

AUTHOR CONTRIBUTIONS
RJS and TCJ conceived the study. RJS and AMC designed and analyzed experiments, and wrote the manuscript, with TCJ contributing revisions. AMC, HFS, and THK conducted the experiments. All authors approved the final version of the manuscript.

ADDITIONAL INFORMATION
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