Infralimbic GluN2A-Containing NMDA Receptors Modulate Reconsolidation of Cocaine Self-Administration Memory

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Addiction is characterized by high relapse susceptibility, and relapse can be triggered by drug-associated cues. Cue presentation induces retrieval of the drug-cue memory, which becomes labile and must be reconsolidated into long-term storage. Repeated unpaired cue presentation, however, promotes extinction. Cue-reactivity can be reduced by blocking reconsolidation or facilitating extinction, which are mediated by NMDA receptors (NMDArs). However, the role of NMDArs in either process following self-administration is unclear. Thus, to determine their role in extinction, rats learned to self-administer cocaine before receiving injections of the NMDAr antagonist CPP immediately after four 45-min extinction sessions. During a subsequent 90-min extinction retention test, CPP-treated rats lever pressed less than saline-treated rats indicating that NMDAr blockade facilitated extinction or disrupted drug-cue memory reconsolidation. In addition, infusing CPP into the infralimbic medial prefrontal cortex (IL-mPFC), a structure implicated in extinction, before four 45-min or immediately after four 30min extinction sessions, had similar results during the extinction retention tests. Next, the GluN2A-selective antagonist NVP or GluN2B-selective antagonist Ro25 was infused into IL-mPFC or nucleus accumbens (NAc) shell, another structure implicated in extinction, after four 45-min extinction sessions. Blocking GluN2A-, but not GluN2B-, containing NMDArs, in IL-mPFC or NAc shell reduced lever pressing during the extinction retention tests. Finally, to dissociate reconsolidation from extinction, NVP was infused into IL-mPFC after four 10-min reactivation sessions, which resulted in reduced lever pressing during the retention test. These results indicate that IL-mPFC GluN2A-containing NMDArs modulate reconsolidation, and suggest a novel treatment strategy, as reducing cue reactivity could limit relapse susceptibility.

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INTRODUCTION

Drug addiction is characterized by compulsive drug seeking and taking, and chronic relapse (McLellan *et al*, 2000). Addiction is maintained by cues associated with drug administration, as these cues can trigger retrieval of the original drug-cue memory, evoke craving, and promote relapse (Childress *et al*, 1986). Following memory retrieval, the memory is labile and requires restabilization into long-term storage through a process called reconsolidation (Miller and Marshall, 2005; Misanin *et al*, 1968; Nader *et al*, 2000). However, when the cue is repeatedly presented without drug reinforcement, a new inhibitory extinction memory forms (Millan *et al*, 2011; Quirk and Mueller, 2008). Both reconsolidation and extinction share similar, but distinct, underlying mechanisms (Suzuki *et al*, 2004; Tronson and Taylor, 2007), with some research suggesting they compete at the molecular level (Nader, 2003). Thus, dissociating these processes is challenging. Behaviorally, they are distinguished

by the amount of time the animal undergoes a reactivation or extinction session. Brief sessions reactive the original memory, which subsequently undergoes reconsolidation, but fail to result in extinction (reduced responding to the stimulus) as longer sessions would (Nader, 2003; Quirk and Mueller, 2008; Suzuki *et al*, 2004; Tronson and Taylor, 2007).

One mechanism underlying both extinction consolidation and memory reconsolidation is NMDA receptor (NMDAr) activity (Nader, 2003). NMDArs are necessary for learning and memory (eg, McLamb *et al*, 1990; Mondadori *et al*, 1989; Thompson *et al*, 1987), including extinction of conditioned fear (Liu *et al*, 2009; Santini *et al*, 2001; Suzuki *et al*, 2004) and drug seeking (Hafenbreidel *et al*, 2014; Hsu and Packard, 2008). Moreover, NMDArs are necessary for reconsolidation, as blocking NMDArs following a short reactivation session disrupts the reconsolidation of conditioned fear (Lee *et al*, 2006), contextual (Pedreira *et al*, 2002), appetitive (Lee and Everitt, 2008), odor discrimination (Torras-Garcia *et al*, 2005), and drug-induced conditioned place preference memories (Alaghband and Marshall, 2013; Kelley *et al*, 2007; Sadler *et al*, 2007). Whether reconsolidation of more complex memories, such as those formed during cocaine self-administration, can also be disrupted with NMDAr blockade remains unclear. For example, some research suggests that habitual learning, including operant

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conditioning, does not undergo reconsolidation (Brown et al, 2008; Hernandez and Kelley, 2004; Mierzejewski et al, 2009), whereas other research with different parameters suggests that it does (Exton-McGuinness and Lee, 2015; Exton-McGuinness et al, 2014; Milton et al, 2008; Wells et al, 2016; Wouda et al, 2010).

We previously found that blocking NMDArs before four 45-min extinction sessions disrupted extinction following cocaine self-administration (Hafenbreidel et al, 2014). Here, we aimed to determine if NMDArs were necessary for extinction consolidation, and in which brain region. We examined the effects of NMDAr blockade in the infralimbic region of the medial prefrontal cortex (IL-mPFC). This region is implicated in extinction consolidation of conditioned fear (Quirk and Mueller, 2008; Quirk et al, 2000; Santini et al, 2001), conditioned place preference (Otis et al, 2014), and self-administration (LaLumiere et al, 2010; Peters et al, 2008). We also targeted NMDArs in the nucleus accumbens (NAc) shell (NAc shell) as this structure receives projections from IL-mPFC (Groenewegen et al, 1999), and has been implicated in both extinction (Peters et al, 2008) and reconsolidation (Miller and Marshall, 2005). However, we found that blocking NMDArs after four 45-min extinction sessions resulted in a reduction in lever pressing, which suggested facilitated extinction or disrupted reconsolidation of the original drug-cue memory. Therefore, to determine which of these processes were being modulated by NMDAr blockade, we investigated the role of NMDArs in IL-mPFC during reconsolidation of cocaine self-administration memory.

MATERIALS AND METHODS

Subjects

Male Long-Evans rats (Harlan) weighing 250–300 g were housed and handled as previously described (Hafenbreidel et al, 2014). Rats had unlimited water access, but were food restricted (13–28 g rat chow/day) throughout experiments except during surgery and recovery. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee in accordance with National Institutes of Health guidelines.

Drugs

Cocaine HCl (NIDA) was dissolved in sterile 0.9% saline and administered intravenously (i.v.) at 0.25 mg per infusion. ($\pm$) 3-(2-carboxypiperazin-4yl)propyl-1-phosphonic acid (CPP; Tocris) was dissolved in sterile 0.9% saline and administered at a dose of 10 mg/kg, i.p., or was infused into IL-mPFC (36 µg/0.3 µl/side at a rate of 0.2 µl/min) 1 h before or immediately after extinction training. Ro25-6981 (Ro25; 2 µg/µl, Sigma-Aldrich), NVP-AAM077 (NVP; 1 µg/µl, Sigma-Aldrich), or vehicle (10% DMSO in dH$_2$O) was infused into IL-mPFC or NAc shell (0.3 µl per side at a rate of 0.5 µl/min) immediately following extinction or reactivation sessions. These doses were determined based on previous research (Burgos-Robles et al, 2007; Gilmartin et al, 2013; Hafenbreidel et al, 2014; Santini et al, 2001).

Self-administration

Self-administration procedures were conducted using 12 sound-attenuated operant conditioning chambers (MED Associates) as previously described (Hafenbreidel et al, 2014). Briefly, 1 week after arrival, rats were food restricted for 3 days before sucrose training sessions on a fixed ratio-1 (FR1) schedule of reinforcement. Next, rats were anesthetized with a mixture of ketamine/xylazine (87/13 mg/kg, i.p.) and implanted with chronic intravenous catheters (assembled by Access Technologies) as described previously (Hafenbreidel et al, 2014). Rats in infusion experiments were implanted with a 26-gauge double-barrel guide cannula aimed bilaterally at IL-mPFC (AP, +2.8, ML, ±0.6, DV, − 4.4 mm relative to bregma) or NAc shell (AP, +1.5, ML, ± 0.6, DV, − 6 mm relative to bregma; see Otis et al, 2013 for cannula surgery details). Following surgery, rats were administered penicillin g procaine (75,000 units/0.35 ml, s.c.) and carprofen (5 mg/0.1 ml, s.c.). Following recovery, catheter patency was verified with 0.3 ml of 1% Propofol (i.v.), a short acting anesthetic that causes immediate loss of muscle tone. Rats were given a minimum of 7 days to recover before behavioral testing. Catheter patency was maintained daily with heparinized saline (~0.2 cm^3 of 60 i.u./ml).

Rats were trained to self-administer cocaine daily during 90-min sessions on a FR1 schedule or until the progressive daily cap was achieved (20–35 infusions/session) for 15–25 sessions, as previously described (Hafenbreidel et al, 2014). Briefly, each session began with the house light and right stimulus light turning on, and the right and left lever extending. Responding on the active (right) lever resulted in initiation of an infusion of cocaine, onset of a tone, and offset of the stimulus light for a 20 s timeout period during which no lever presses were reinforced. All lever presses, reinforced or not, are reported as active lever presses during acquisition, extinction, and reinstatement. Responding on the inactive lever was recorded, but had no programmed consequences. Groups were matched on average number of infusions received during self-administration, the average number of infusions and total active and inactive lever presses made over the past 3 days of self-administration, and if rats received priming infusions or extra sessions to achieve stable drug seeking ($n = 58$).

Experimental Manipulations

Experiment one. To determine the role of NMDArs during extinction consolidation, NMDArs were blocked systemically following four 45-min extinction sessions and retention was tested on the following drug-free extinction session. Following cocaine self-administration, rats were matched and assigned to two groups receiving saline or CPP injections. Forty-five-min extinction sessions were used as previously described (Hafenbreidel et al, 2014; Hafenbreidel et al, 2015; LaLumiere et al, 2010) to minimize extinction during the first four extinction sessions, in order to better manipulate initial learning by limiting the amount of time and number of cue presentations available. Retention of the initial extinction learning was then tested during a full 90-min extinction session, after a 2-day drug wash-out period, followed by subsequent 90-min sessions to measure extinction across days. During the first 90-min extinction session

(extinction retention test), poor extinction memory retention from the first four 45-min sessions would be demonstrated by a significant increase in lever presses compared with controls, normal extinction retention would be demonstrated by no change in lever presses compared with controls, and good or facilitated extinction retention would be demonstrated by a significant decrease in lever presses compared with controls. During both 45- and 90-min extinction sessions, lever presses resulted in the same programmed consequences as during self-administration, but no cocaine was administered.

Experiment two. To determine the role of NMDArs in IL-mPFC during extinction of drug seeking, NMDArs were blocked before four 45-min extinction sessions and extinction retention was tested on the following 90-min drug-free extinction session. In a separate group, NMDArs were blocked immediately after four 30-min extinction sessions and extinction retention was tested on the following 90-min drug-free extinction session to determine the role of NMDArs in IL-mPFC during the consolidation of extinction of drug seeking. Thirty-min sessions were selected for post-session infusions to further reduce extinction learning, in order to better manipulate initial extinction learning, as less time is available to acquire the new extinction memory before NMDArs are blocked during consolidation. Following cocaine self-administration, rats were matched and assigned to two groups receiving saline- or CPP-infusions into IL-mPFC. Next, rats were adapted to microinfusion procedures. On the first day, injectors extending 1.0 mm past the guide cannula were lowered to the infusion site for two min. The next day, saline was infused at the same rate and volume as during drug manipulation to allow the rats to adapt to changes in cranial pressure and mechanical stimulation. On the following day, saline or CPP was infused into IL-mPFC before or after the first four extinction sessions, followed by a 2-day drug wash-out break before extinction retention was tested during 90-min extinction sessions.

Experiment three. To determine the role of GluN2A- or GluN2B-containing NMDArs (GluN2A-NMDAr; GluN2B-NMDAr) in IL-mPFC or NAc shell during extinction of drug seeking, GluN2A-NMDAr or GluN2B-NMDArs were blocked following four 45-min extinction sessions and retention was tested on the following drug-free extinction session. Following cocaine self-administration, rats were matched and assigned to six groups receiving vehicle, NVP, or Ro25 into IL-mPFC or NAc shell. Rats were adapted to microinfusion procedures and then infused with their respective treatment immediately following four 45-min extinction sessions. This was followed by a 2-day drug wash-out break before extinction retention was tested during 90-min extinction sessions.

Experiment four. To determine the role of GluN2A-NMDARs in IL-mPFC during reconsolidation of the original drug-cue memory, GluN2A-NMDArs were blocked following four 10-min reactivation sessions (under extinction conditions) and memory retention was tested on the following drug-free extinction session. Following cocaine self-administration, rats were matched and assigned to four groups receiving vehicle or NVP into IL-mPFC immediately following four reactivation sessions, or in the absence of behavioral testing (immediately returned to their home cages with no exposure to operant chambers) for 4 days. Similarly to experiment two, rats were adapted to microinfusion procedures, infused with their respective treatments, and tested in subsequent daily 90-min extinction sessions.

Reinstatement procedures. Following extinction, rats received a priming injection of cocaine (10 mg/kg; i.p.) before undergoing normal extinction procedures to test for cocaine-induced reinstatement.

Data Analysis

Lever presses and infusions during acquisition were analyzed by comparing the average of the last 3 days of cocaine self-administration between groups using *t*-tests. For extinction, lever presses were analyzed across days and between groups using a two-way repeated-measures analysis of variance (ANOVA). The last two extinction sessions for each rat were averaged and shown as the last extinction day to account for variability in the number of extinction sessions to criterion, which was defined as a significant reduction in the average active lever presses made during the last two extinction sessions compared with the first 90-min extinction session with a paired *t*-test. Reinstatement was measured by comparing the average active lever presses made during the last two extinction sessions to those made after a priming injection of cocaine, and was analyzed separately with two-way repeated measures ANOVAs. All *post hoc* tests were conducted, when appropriate, using Fisher's least significant difference (LSD) test. Some rats were removed from the analysis due to blocked or non-patent catheters ($n = 11$), not acquiring self-administration ($n = 5$), being two or more standard deviations away from the group mean throughout extinction ($n = 1$), or incorrect injector tip placement ($n = 3$). Final group sizes are reported in Table 1 and Figures. After behavioral procedures, verification of injector tip location was performed on cresyl violet-stained coronal sections.

RESULTS

Blocking NMDArs Systemically or in IL-mPFC Does not Disrupt Extinction

We previously found that blocking NMDArs with the NMDAr antagonist CPP before four 45-min extinction sessions disrupted extinction (Hafenbreidel *et al*, 2014). To determine the necessity of NMDArs during extinction consolidation following cocaine self-administration, rats were injected with saline or CPP immediately following four 45-min extinction sessions. Extinction retention was tested on days 5–23 with 90-min drug-free extinction sessions. Groups were matched by the average number of infusions, and active or inactive lever presses made during the last 3 days of cocaine self-administration (Table 1), and thus rats were anticipated to lever press similarly during extinction day 1. Both groups reduced lever pressing across the first 4 days of extinction (Figure 1a, left), but CPP-treated rats had reduced lever presses compared with saline-treated rats during the 45- and 90-min drug-free extinction sessions

Table 1 Average Number of Active and Inactive Lever Presses Made or Infusions Received during the Last 3 Days of Cocaine Self-administration

Experiment	Treatment	Ext days 1–4 time	Active lever	Inactive lever	Infusions
1	Post Sal, i.p. (6)	45	47.39 ± 12.74	0.78 ± 0.46	30.00 ± 1.72
	Post CPP, i.p. (7)	45	41.95 ± 7.51	1.95 ± 1.73	28.67 ± 2.22
2	Pre Sal, IL (10)	45	34.70 ± 3.42	1.97 ± 1.24	28.20 ± 1.55
	Pre CPP, IL (11)	45	35.88 ± 4.09	1.15 ± 0.52	27.55 ± 1.77
	Post Sal, IL (11)	30	32.76 ± 2.13	39.58 ± 35.25	27.45 ± 1.19
3	Post CPP, IL (11)	30	34.39 ± 2.37	5.45 ± 2.73	27.52 ± 1.28
	Post Veh, IL (9)	45	41.44 ± 7.27	2.67 ± 1.16	25.89 ± 1.28
	Post Ro25, IL (10)	45	35.40 ± 5.47	6.37 ± 4.97	25.43 ± 1.79
	Post NVP, IL (10)	45	37.80 ± 10.25	45.57 ± 42.24	24.97 ± 1.83
	Post Veh, NAc (10)	45	31.23 ± 4.01	2.00 ± 1.24	26.03 ± 1.75
	Post Ro25, NAc (10)	45	39.20 ± 8.24	1.53 ± 0.50	26.90 ± 1.48
	Post NVP, NAc (10)	45	41.40 ± 7.80	1.67 ± 0.57	26.50 ± 1.35
4	Post Sal, IL (10)	10	38.00 ± 4.81	35.80 ± 32.49	26.77 ± 1.64
	Post NVP, IL (10)	10	38.17 ± 3.97	2.73 ± 2.15	26.60 ± 1.40
	Sal, IL (10)	—	37.33 ± 6.28	5.60 ± 2.16	27.12 ± 1.73
	NVP, IL (11)	—	48.09 ± 10.31	2.18 ± 1.73	27.12 ± 1.22

Numbers in the parentheses are group sizes.

(Figure 1a, right). Across the 45-min extinction sessions (1–4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,44} = 3.726$, $p = 0.018$) and treatment ($F_{1,44} = 6.241$, $p = 0.016$), but no day by treatment interaction. For the 90-min extinction sessions (5–23; days 11–22 not shown; active lever presses), ANOVA revealed a significant effect of treatment ($F_{1,209} = 56.044$, $p < 0.0001$) but no effect of day or day by treatment interaction. A summary of the statistics for inactive lever presses for all experiments are shown in Table 2, and are described in Supplementary Results (data not shown). Moreover, further analysis of extinction day 5 (day 1 for the final experiment) within session lever pressing is described in Supplementary Results for each experiment and shown in Supplementary Figures 1 and 2. Following extinction, all rats were tested for cocaine-induced reinstatement of drug seeking. Rats were given a non-contingent priming injection of cocaine (Figure 1b) before a 90-min extinction session. ANOVA revealed a significant increase in active lever pressing during the reinstatement test compared with the last extinction session ($F_{1,22} = 22.942$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Overall, these results are the opposite of our previous findings, and indicate that CPP treatment reduced active lever presses during 45- and 90-min extinction sessions, suggesting facilitated extinction or disrupted reconsolidation of the original drug-cue memory. However, both groups reinstated following a priming injection of cocaine, which suggests that the effects observed were either transient or partial.

Next, to determine if our conflicting results were due to brain penetration issues, CPP was infused directly into the brain. NMDArs in IL-mPFC have been implicated in extinction in other paradigms (eg, Otis et al, 2014; Sotres-Bayon et al, 2009), prompting us to examine their role in extinction of cocaine seeking following self-administration. Rats were infused with saline or CPP before four 45-min extinction sessions (infusion sites depicted in Figure 1c), and retention was tested on extinction days 5–21 (90 min). Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Overall, little extinction was observed in the saline-infused rats, but increased seeking was observed in the CPP-infused rats, across the first four 45-min extinction sessions (Figure 1d, left). However, CPP-infused rats had reduced lever presses compared with saline-infused rats during 90-min drug-free extinction sessions (Figure 1d, right). Across 45-min extinction sessions (1–4; active lever presses), ANOVA revealed a significant effect of treatment ($F_{1,76} = 13.611$, $p < 0.0001$) and a day by treatment interaction ($F_{3,76} = 3.876$, $p = 0.012$), but no effect of day. Post hoc analysis revealed that CPP-infused rats lever pressed significantly less than saline-infused rats on extinction day 1 ($p = 0.047$), but pressed more than saline-infused rats on days 2 ($p = 0.022$), 3 ($p = 0.026$), and 4 ($p = 0.014$). The increase in lever pressing in CPP-infused rats during extinction days 2–4 was likely a state-dependent effect (see Supplementary Results for locomotion results; data not shown). For the 90-min extinction sessions (5–21; 11–20 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{16,323} = 7.792$, $p < 0.0001$) and day by treatment interaction ($F_{16,323} = 3.421$, $p < 0.0001$), but no effect of treatment. Post hoc analysis revealed that CPP-infused rats lever pressed significantly less than saline-infused rats on extinction day 5 ($p = 0.001$) and 6 ($p = 0.023$). Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 1e). ANOVA revealed a significant increase in active lever pressing during the reinstatement test compared with the last extinction session ($F_{1,38} = 54.410$, $p < 0.0001$), as well as a significant effect of treatment ($F_{1,38} = 5.389$, $p = 0.026$) and day by treatment interaction ($F_{1,38} = 4.953$, $p = 0.032$). Post hoc analysis revealed that previously CPP-treated rats lever pressed more than
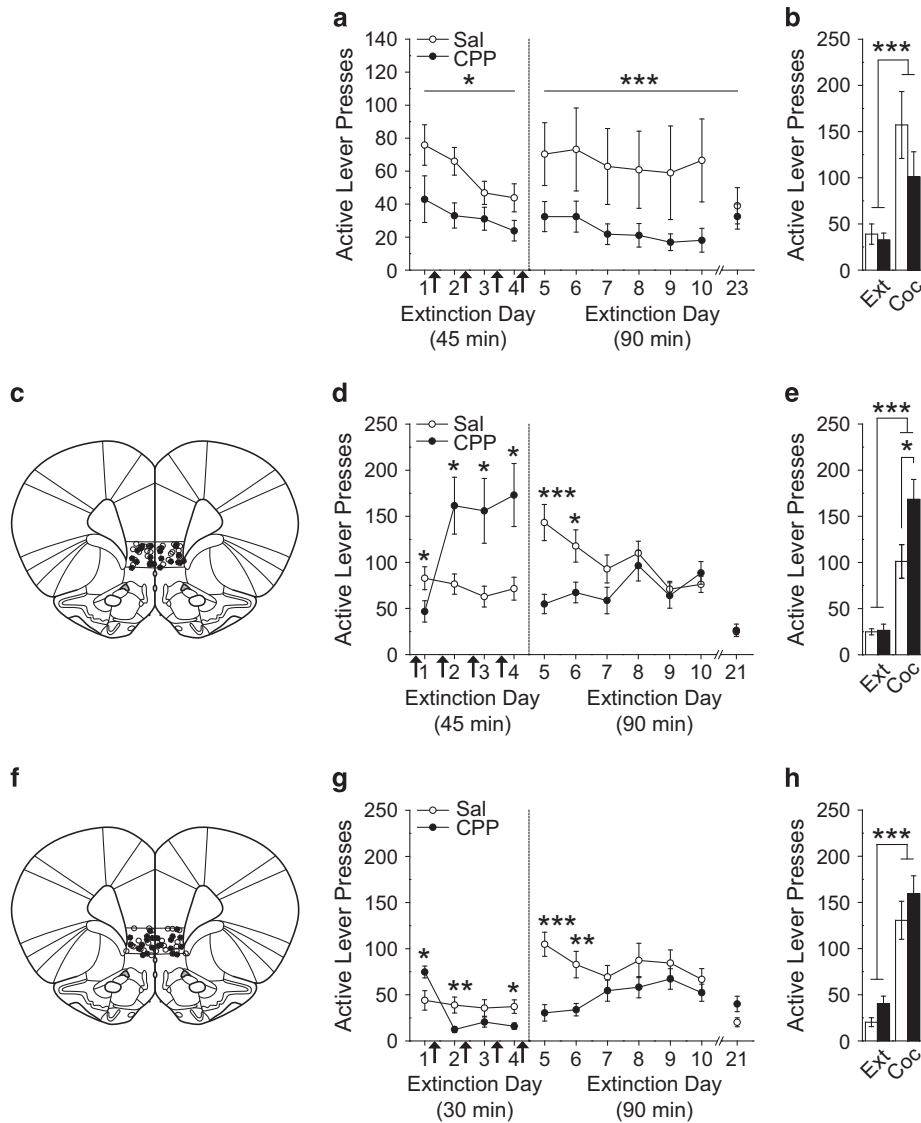
Figure 1 Blocking NMDArs systemically or in IL-mPFC does not disrupt extinction consolidation, but rather, reduces subsequent lever pressing. (a) Post-extinction injections of CPP (arrows) decreased lever pressing during extinction, specifically during the 90-min extinction session when retention was tested. Sal, $n = 6$; CPP, $n = 7$. (b) All rats reinstated following a priming injection of cocaine. (c) Injector tip locations for saline (open circles; $n = 10$) or CPP (closed octagons; $n = 11$) in IL-mPFC with pre-extinction infusions. (d) Pre-extinction infusions of CPP (arrows) into IL-mPFC increased lever pressing during extinction days 2–4, but resulted in reduced lever pressing during the 90-min extinction sessions when retention was tested. (e) All rats reinstated following a priming injection of cocaine, but previously CPP-treated rats were potentiated. (f) Injector tip locations for saline (open circles; $n = 11$) or CPP (closed octagons; $n = 11$) in IL-mPFC with post-extinction infusions. (g) Post-extinction infusions of CPP (arrows) in IL-mPFC reduced lever pressing on extinction days 2 and 4, and during the 90-min extinction sessions when retention was tested. (h) All rats reinstated following a priming injection of cocaine. Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

previously saline-treated rats following a priming injection of cocaine ($p = 0.029$). Overall, blocking infralimbic NMDArs one h before four 45-min extinction sessions resulted in similar effects as post-session systemic CPP administration during extinction day 5, as rats lever pressed less than saline-treated rats. Moreover, both groups reinstated following a priming injection of cocaine, but previously CPP-treated rats had potentiated seeking, which may be due to nonspecific drug effects of CPP (Del Arco et al, 2011; Del Arco et al, 2008) being active during memory retrieval.

To dissociate acquisition from consolidation of extinction, we examined the role of NMDArs in IL-mPFC following extinction of cocaine seeking. Rats were infused with saline

or CPP immediately following four 30-min extinction sessions (infusion sites depicted in Figure 1f), and retention was tested on extinction days 5 – 21 (90 min). Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Both treatment groups reduced lever pressing across the first 4 days of extinction (Figure 1g, left), but CPP-infused rats had reduced lever presses compared with saline-infused rats during the 90-min drug-free extinction sessions (Figure 1g, right). Across the 30-min extinction sessions (1 – 4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,80} = 10.338$, $p < 0.0001$) and day by treatment interaction ($F_{3,80} = 6.706$, $p < 0.0001$),

Table 2 Statistical Summary of Inactive Lever Presses Made During Extinction and Cocaine-Induced Reinstatement

Experiment	Treatment	Ext days 1–4 time	Ext 1–4 days	Ext 5+ (90 min) days	Coc reinstatement
1	Post Sal, i.p.	45	Day[NS]	Day**	Day**
	Post CPP, i.p.	45	Treat***	Treat***	Treat*
			DayXTreat[NS]	DayXTreat*	DayXTreat[NS]
2	Pre Sal, IL	45	NS	NS	NS
	Pre CPP, IL	45			
	Post Sal, IL	30	Day*	NS	NS
	Post CPP, IL	30	Treat[NS]		
			DayXTreat[NS]		
3	Post Veh	45	Day[NS]	NS	NS
	Post NVP, IL	45	Treat*		
			DayXTreat[NS]		
	Post Veh	45	NS	Day**	Day*
	Post Ro25, IL	45		Treat[NS]	Treat[NS]
				DayXTreat[NS]	DayXTreat[NS]
	Post Veh	45	Day[NS]	Day[NS]	NS
	Post NVP, NAc	45	Treat*	Treat***	
			DayXTreat[NS]	DayXTreat[NS]	
	Post Veh	45	Day*	NS	NS
	Post Ro25, NAc	45	Treat[NS]		
			DayXTreat[NS]		
4	Post Veh, IL	10	NS	Day***	Day*
	Post NVP, IL	10		Treat[NS]	Treat[NS]
				DayXTreat[NS]	DayXTreat[NS]
	Veh, IL	—	—	Day***	Day**
	NVP, IL	—		Treat**	Treat[NS]
				DayXTreat[NS]	DayXTreat[NS]

Abbreviation: NS, not significant.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

but no effect of treatment. *Post hoc* analysis revealed that CPP-infused rats lever pressed significantly more than saline-infused rats on extinction day 1 ($p = 0.021$), but significantly less on extinction days 2 ($p = 0.009$), and 4 ($p = 0.016$). The significant increase in lever pressing by CPP-infused rats on extinction day 1 was not expected as rats did not significantly differ on average number of infusions, active or inactive lever presses made during the last 3 days of acquisition (Table 1), and CPP was infused following the extinction session. For 90-min extinction days (5 – 21; 11–20 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{16,340} = 3.361$, $p < 0.0001$) and day by treatment interaction ($F_{16,340} = 3.795$, $p < 0.0001$), but no effect of treatment. *Post hoc* analysis revealed that CPP-infused rats lever pressed significantly less than saline-infused rats on extinction days 5 ($p < 0.0001$), 6 ($p = 0.006$), but significantly more on day 20 ($p = 0.049$; data not shown). The significant increase in responding on extinction day 20 could be due to spontaneous recovery in the CPP-infused rats, which would suggest that the effect observed on days 5 and 6 was transient or partial. Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 1h). ANOVA revealed a significant increase in active lever pressing during the reinstatement test compared with the last extinction session ($F_{1,38} = 58.104$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Overall, blocking infralimbic NMDArs immediately following four 30-min extinction sessions did not impair extinction, but instead resulted in effects similar to post-session systemic CPP administration. Moreover, similar to post-session systemic injections of CPP, both groups reinstated following a priming injection of cocaine similarly to saline-treated rats, which suggests that post-session administration of CPP (systemic or into IL-mPFC) induces a transient or partial reduction in lever pressing during the 90-min extinction sessions.

In IL-mPFC and NAc Shell, Blocking GluN2A-NMDArs Does not Disrupt Extinction

CPP has a higher affinity for GluN2A-NMDArs than to GluN2B-NMDARs (Lehmann *et al*, 1986; Lozovaya *et al*, 2004) and has some nonspecific effects (Del Arco *et al*, 2011; Del Arco *et al*, 2008), which could underlie our conflicting results. Therefore, to control for drug-specific effects and to dissociate the role of NMDAr subtypes in IL-mPFC and its downstream target the NAc shell during extinction, we first isolated the effects of GluN2A-NMDAr inhibition following extinction. Rats were infused with vehicle or NVP
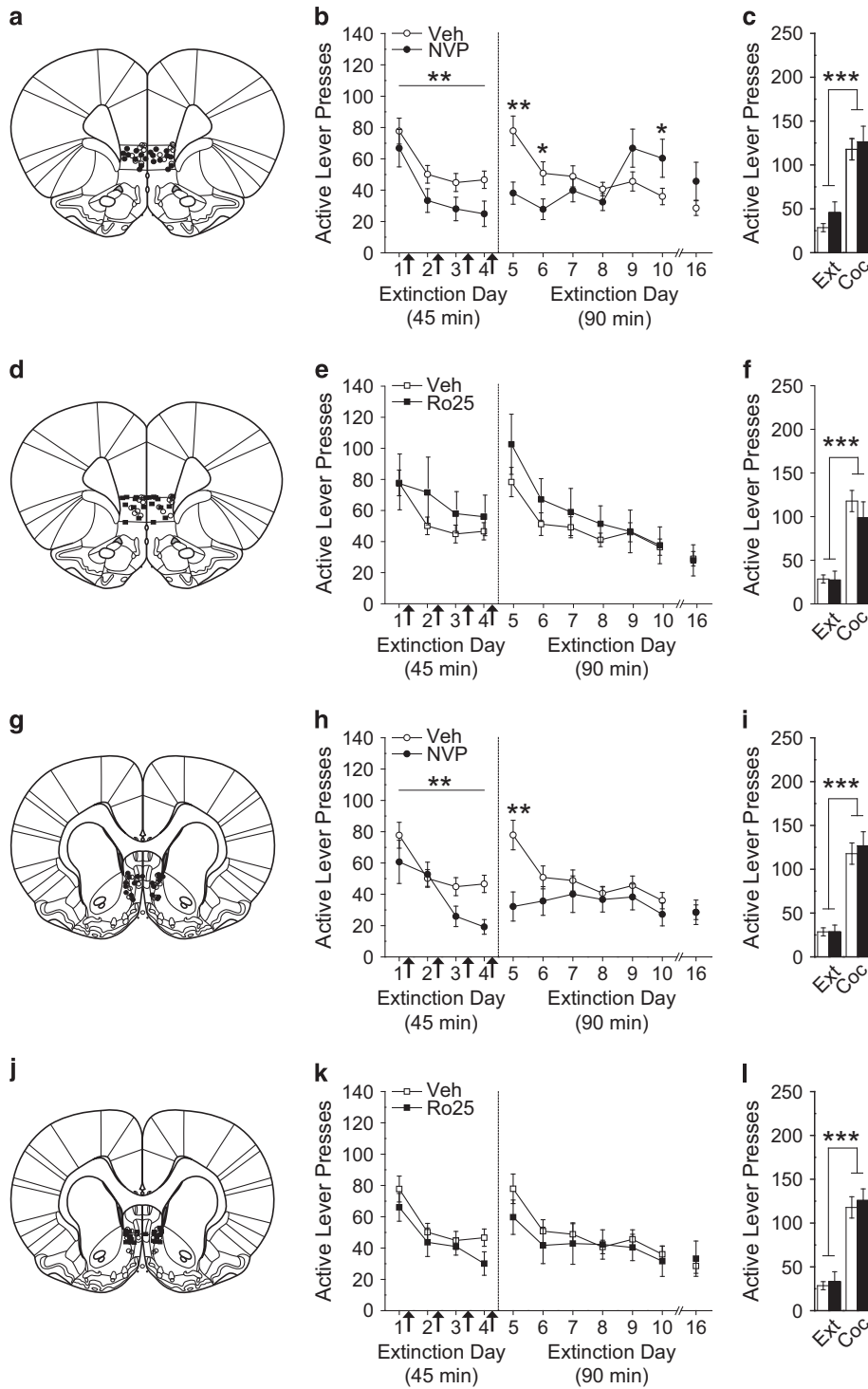
Figure 2 In IL-mPFC or NAc shell, blocking GluN2A-containing NMDArs does not disrupt extinction, but rather, reduces subsequent lever pressing. (a) Injector tip locations for vehicle (open circles; $n = 9$) or NVP (closed circles; $n = 10$) in IL-mPFC. (b) Post-extinction infusions of GluN2A-specific NVP (arrows) into IL-mPFC resulted in reduced lever pressing during extinction, specifically during the 90-min extinction sessions when retention was tested. (c) All rats reinstated following a priming injection of cocaine. (d) Injector tip locations for vehicle (open circles; $n = 9$) or Ro25 (closed squares; $n = 10$) in IL-mPFC. (e) Post-extinction infusions of GluN2B-specific Ro25 (arrows) into IL-mPFC had no effect on extinction. (f) All rats reinstated following a priming injection of cocaine. (g) Injector tip locations for vehicle (open circles; $n = 10$) or NVP (closed circles; $n = 10$) in NAc shell. (h) Post-extinction infusions of GluN2A-specific NVP (arrows) into NAc shell resulted in reduced lever pressing during extinction, specifically during the 90-min extinction sessions when retention was tested. (i) All rats reinstated following a priming injection of cocaine. (j) Injector tip locations for vehicle (open circles; $n = 10$) or Ro25 (closed squares; $n = 10$) in NAc shell. (k) Post-extinction infusions of GluN2B-specific Ro25 (arrows) into NAc shell had no effect on extinction. (l) All rats reinstated following a priming injection of cocaine. Error bars are ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

immediately following four 45-min extinction sessions (infusion sites depicted in Figure 2a), and retention was tested on extinction days 5–16 (90 min). Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Vehicle-infused rats in IL-mPFC or NAc shell were not significantly different during acquisition (Table 1; active or inactive, all $ts < 1.264$, $ps > 0.05$), extinction (active or inactive, all $Fs < 0.600$, $ps > 0.05$), or reinstatement (active or inactive, all $Fs < 0.469$, $ps > 0.05$), and therefore were collapsed into a single vehicle group. Both vehicle and NVP-infused groups reduced lever pressing across the first 4 days of extinction (Figure 2b, left), but NVP-infused rats had reduced lever presses compared with vehicle-infused rats during the 90-min drug-free extinction sessions (Figure 2b, right). Across the 45-min extinction sessions (1–4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,108} = 10.037$, $p < 0.0001$) and treatment ($F_{1,108} = 9.155$, $p = 0.003$), but no day by treatment interaction. For the 90-min extinction sessions (5–16; 11–15 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{11,324} = 2.362$, $p = 0.008$) and day by treatment interaction ($F_{11,324} = 3.006$, $p = 0.001$), but no effect of treatment. *Post hoc* analysis revealed that NVP-infused rats lever pressed significantly less than saline-infused rats on extinction days 5 ($p = 0.009$) and 6 ($p = 0.049$), but pressed significantly more

than saline-infused rats on extinction day 10 ($p = 0.040$). The significant increase in responding on extinction day 10 could be due to spontaneous recovery in the NVP-infused rats, which would suggest that the effect observed on days 5 and 6 was transient or partial. Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 3c). ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,54} = 51.211$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Overall, blocking infralimbic GluN2A-NMDArs immediately following four 45-min extinction sessions produced results similar to infusions of the nonspecific NMDAr antagonist CPP into IL-mPFC during the 90-min extinction sessions. These results suggest that the reduction in lever pressing observed during the extinction retention tests (eg, extinction day 5) were due to GluN2A-NMDArs blockade selectively and not likely due to a drug-specific effect. In addition, both groups reinstated following a priming injection of cocaine, which suggests that post-session blockade of GluN2A-NMDARs induces transient or partial reduction in lever pressing during the 90-min extinction sessions.

Next, we examined the role of GluN2B-NMDArs in IL-mPFC during extinction by infusing rats with vehicle or Ro25 immediately following four 45-min extinction sessions (infusion sites depicted in Figure 2d), and testing retention
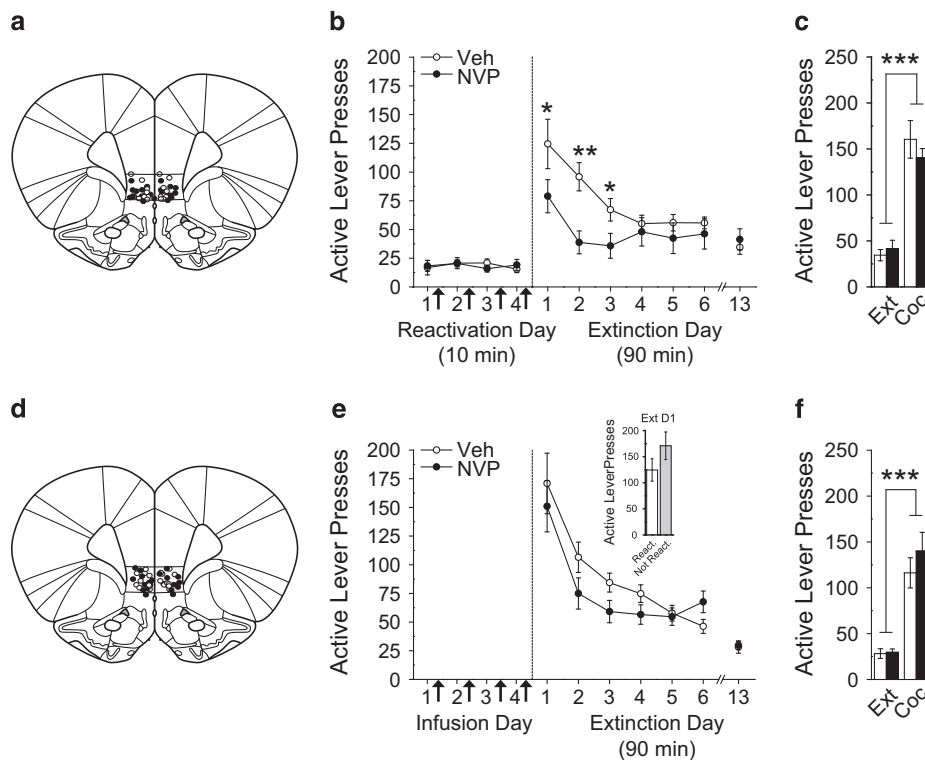

Figure 3 Blocking GluN2A-containing NMDArs in IL-mPFC following four 10 min reactivation sessions reduced subsequent lever pressing. (a) Injector tip locations for vehicle (open circles; $n = 10$) or NVP (closed circles; $n = 10$) in IL-mPFC with memory reactivation. (b) Post 10-min reactivation session infusions of GluN2A-specific NVP (arrows) in IL-mPFC resulted in reduced lever pressing during the 90-min extinction sessions when retention was tested. (c) All rats reinstated following a priming injection of cocaine. (d) Injector tip locations for vehicle (open circles; $n = 10$) or NVP (closed circles; $n = 11$) in IL-mPFC without memory reactivation. (e) Infusions of NVP into IL-mPFC without memory reactivation had no effect on lever pressing during the 90-min extinction sessions when retention was tested. (e, inset) Vehicle-treated rats that did undergo the 10-min reactivation sessions lever pressed similarly to vehicle-treated rats that did not undergo the 10-min reactivation sessions during extinction day 1. (f) All rats reinstated following a priming injection of cocaine. Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

during extinction days 5–16 (90 min). Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Both treatment groups lever pressed similarly throughout extinction (Figure 2e). Across the 45-min extinction sessions (1 – 4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,108} = 2.741$, $p = 0.047$), but no effect of treatment or day by treatment interaction. For the 90-min extinction sessions (5 – 16; 11 – 15 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{11,324} = 9.101$, $p < 0.0001$), but no effect of treatment or a day by treatment interaction. Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 2f). ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,54} = 47.581$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Thus, blocking infralimbic GluN2B-NMDArs following four 45-min extinction sessions had no effect on extinction or cocaine-induced reinstatement.

Furthermore, to determine if our results were specific to infralimbic GluN2A-NMDArs, rats were infused with vehicle or NVP into NAc shell immediately following four 45-min extinction sessions (infusion sites depicted in Figure 2g), and retention was tested during extinction days 5 – 16 (90 min). Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Both treatment groups reduce lever pressing across the first 4 days of extinction (Figure 2e, left), but NVP-infused rats had reduced lever presses compared with vehicle-infused rats during the 90-min drug-free extinction sessions (Figure 2e, right). Across the 45-min extinction sessions (1–4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,108} = 9.485$, $p < 0.0001$) and treatment ($F_{1,108} = 7.761$, $p = 0.006$), but no day by treatment interaction. For the 90-min extinction days (5 – 16; 11 – 15 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{11,324} = 3.191$, $p < 0.0001$), treatment ($F_{1,324} = 6.426$, $p = 0.012$), and day by treatment interaction ($F_{11,324} = 1.894$, $p = 0.039$). Post hoc analysis revealed that NVP-infused rats lever pressed significantly less than saline-infused rats on extinction day 5 ($p = 0.004$). Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 2i). ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,54} = 71.671$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Overall, blocking GluN2A-NMDArs in NAc shell immediately following four 45-min extinction sessions resulted in reduced lever pressing during the initial 90-min extinction sessions, but had no effect on cocaine-induced reinstatement, which are similar to the results observed following infusions into IL-mPFC. These results suggest that blocking GluN2A-containing NMDArs in the NAc shell resulted in a transient or partial reconsolidation disruption or facilitated extinction.

In addition, to determine the role of GluN2B-NMDArs in NAc shell during extinction, rats were infused with vehicle or Ro25 immediately following four 45-min extinction sessions (infusion sites depicted in Figure 2j), and retention was tested during extinction days 5–16 (90 min). Lever presses

and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Both treatment groups lever pressed similarly throughout extinction (Figure 2k). Across the 45-min extinction sessions (1 – 4; active lever presses), ANOVA revealed a significant effect of day ($F_{3,108} = 8.107$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. For the 90-min extinction sessions (5 – 16; 11 – 15 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{11,324} = 5.109$, $p < 0.0001$), but no effect of treatment or a day by treatment interaction. Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 2l). ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,54} = 68.773$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Thus, blocking GluN2B-NMDArs in NAc shell immediately following four 45-min extinction sessions had no effect on extinction or cocaine-induced reinstatement.

In IL-mPFC, GluN2A-NMDArs Modulate Reconsolidation of the Original Drug-Cue Memory

We found that post 30- or 45-min extinction session systemic injections or infusions into IL-mPFC of CPP, or infusions into IL-mPFC or NAc of NVP (but not Ro25) resulted in a reduction of lever pressing when tested during a subsequent 90-min extinction retention test. These results suggest that NMDAr blockade results in either facilitated extinction or disrupted reconsolidation of the original drug-cue memory. To distinguish which of these processes resulted in the significant reduction in lever pressing observed during the extinction retention tests, we directly tested whether infralimbic GluN2A-NMDArs are necessary for reconsolidation of the original drug memory by utilizing 10-min reactivation sessions that are insufficient to induce extinction (see below for statistical anaylsis and Figure 3e inset; see also Fuchs et al, 2009; Nader et al, 2000; Tronson and Taylor, 2007). Following cocaine self-administration, rats were infused with vehicle or NVP immediately following four 10-min reactivation sessions (infusion sites depicted in Figure 3a). Memory retention was then tested during subsequent 90-min extinction sessions. Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Both treatment groups lever pressed similarly during the four 10-min reactivation sessions (Figure 3b, left), but NVP-infused rats had reduced lever presses compared with vehicle-infused rats during the 90-min drug-free extinction sessions (Figure 3b, right). Across the 10-min reactivation sessions (1–4; active lever presses), ANOVA did not reveal a significant effect of day, treatment, or a day by treatment interaction. For the 90-min extinction days (1–13; 7–12 not shown; active lever presses), ANOVA revealed a significant effect of day ($F_{12,234} = 10.148$, $p < 0.0001$), treatment ($F_{1,234} = 8.510$, $p = 0.004$), and day by treatment interaction ($F_{12,234} = 3.122$, $p < 0.0001$). Post hoc analysis revealed that NVP-infused rats lever pressed significantly less than saline-infused rats on extinction day 1 ($p = 0.019$), 2 ($p = 0.002$), and 3 ($p = 0.045$). Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 3c).

ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,36} = 78.525$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Thus, blocking infralimbic GluN2A-NMDArs immediately following four reactivation sessions significantly reduced lever pressing suggesting that reconsolidation of the original drug-cue memory was disrupted. However, both treatment groups reinstate following a priming injection of cocaine, which suggests a partial or transient disruption.

To determine if reactivation was required for reconsolidation disruption, we blocked infralimbic GluN2A-NMDArs in the absence of reactivation sessions. Rats were infused with vehicle or NVP for 4 days and immediately returned to their home cages without behavioral testing (infusion sites depicted in Figure 3d). Memory retention was then tested during subsequent 90-min extinction sessions. Lever presses and number of infusions were equivalent between groups across the average of the last 3 days of cocaine self-administration (Table 1). Rats were infused with vehicle or NVP into IL-mPFC for 4 days and immediately returned to their home cages without behavioral testing (Figure 3e, left). Treatment groups did not differ during the 90-min drug-free extinction sessions (Figure 3e, right; days 1–13; 7–12 not shown; active lever presses), as ANOVA revealed a significant effect of day ($F_{12,247} = 21.535$, $p < 0.0001$), but no effect of treatment or a day by treatment interaction. In addition, active lever presses made during extinction day 1 by vehicle-infused rats that did undergo four 10-min reactivation sessions were not different than vehicle-infused rats that did not undergo four 10-min reactivation sessions ($t(18) = -1.121$, $p = 0.277$; Figure 3e inset). Thus, four 10-min reactivation sessions were not sufficient to induce extinction, as evident by a lack of a significant reduction in lever pressing, as shown by others (Fuchs et al, 2009; Nader et al, 2000; Tronson and Taylor, 2007). Following extinction, all rats were tested for cocaine-induced reinstatement (Figure 3f). ANOVA revealed a significant increase in active lever pressing during the cocaine-induced reinstatement test compared with the last extinction session ($F_{1,38} = 52.436$, $p < 0.0001$), but no effect of treatment or day by treatment interaction. Overall, blocking GluN2A-NMDArs in IL-mPFC following memory reactivation transiently or partially disrupted reconsolidation of the original drug-cue memory, but blocking GluN2A-NMDArs without memory reactivation had no effect on subsequent behavior. However, blocking GluN2A-NMDArs in IL-mPFC with or without memory reactivation did not alter cocaine-induced reinstatement.

DISCUSSION

Here, we aimed to determine the role of NMDArs during extinction consolidation following self-administration. Instead, we found that inhibiting NMDArs, specifically the GluN2A-NMDAr, systemically or in IL-mPFC or NAc shell resulted in reduced lever pressing during the extinction retention test (extinction day 5). These results suggested that blocking NMDArs after four 45- or 30-min extinction sessions either enhanced extinction consolidation or disrupted the reconsolidation of the original drug-cue memory.

We directly tested these alternatives by blocking infralimbic GluN2A-NMDArs following four 10-min reactivation sessions, which resulted in a reduction in lever pressing during the retention test (extinction day 1) in NVP-infused rats compared with vehicle-infused rats. However, in each experiment, rats treated with the NMDAr antagonist reinstated lever pressing following a priming injection of cocaine. Therefore, we conclude that the effect of GluN2A-NMDAr blockade observed during the 90-min retention sessions was likely due to partial or transient reconsolidation disruption rather than facilitated extinction.

To date, the role of GluN2A-NMDArs in IL-mPFC during reconsolidation or extinction following cocaine self-administration had not been investigated. Our results were surprising, as previous research has demonstrated that blocking NMDArs bidirectionally mediates learning. For example, blocking NMDArs disrupts extinction (eg, Hafenbreidel et al, 2014; Hsu and Packard, 2008; Liu et al, 2009; Santini et al, 2001) and enhancing NMDAr function facilitates extinction in a number of paradigms (Botreau et al, 2006; Fiorenza et al, 2012; Hammond et al, 2012; Langton and Richardson, 2008; Ledgerwood et al, 2003; Matsuda et al, 2010; Walker et al, 2002), including with methods used here (Hafenbreidel et al, 2014). Therefore, it seemed improbable that blocking and enhancing NMDArs would both facilitate extinction. However, under different parameters, NMDAr blockade can disrupt reconsolidation (Alaghband and Marshall, 2013; Kelley et al, 2007; Lee and Everitt, 2008; Lee et al, 2006; Pedreira et al, 2002; Sadler et al, 2007; Torras-Garcia et al, 2005), and it is more probable that NMDAr blockade would disrupt new learning and memory reconsolidation, than disrupting and facilitating new learning. Nevertheless, it is possible that NMDAr blockade with these specific parameters was facilitating extinction. Therefore, we directly tested if infralimbic GluN2A-NMDArs were necessary for reconsolidation, and concluded that infralimbic GluN2A-NMDArs were partially or transiently modulating the reconsolidation of the original drug-cue memory.

Until now, IL-mPFC did not appear to have a role in reconsolidation, as it does not mediate contextual fear reconsolidation (Mamiya et al, 2009; Stern et al, 2014). However, others have reported findings similar to ours during extinction of conditioned fear (Kwapis et al, 2014; Leaderbrand et al, 2014; Song et al, 2015). One possibility is that the mechanisms underlying reconsolidation of instrumental or complex memories may differ from classical conditioning memories due to variables such as habituation (eg, Exton-McGuinness and Lee, 2015; Hernandez and Kelley, 2004; Wells et al, 2016; Wouda et al, 2010). However, NAc has been implicated in both extinction (Peters et al, 2008) and reconsolidation (Miller and Marshall, 2005), receives projections from mPFC (Groenewegen et al, 1999), and is thought to underlie expression of drug seeking (Millan et al, 2011). Thus, this interaction between IL-mPFC and NAc may be critical for reconsolidation of cocaine self-administration memory.

Our results could be explained by the different roles of NMDAr subunits in learning and memory. NMDArs are composed of four subunits, including two GluN1 subunits and typically two GluN2 subunits (GluN2A-D). The NMDAr antagonist CPP is more selective for the GluN2A-NMDAr (Lehmann et al, 1986; Lozovaya et al, 2004), which led us to

test whether our initial results (Figure 1) were due to GluN2A-NMDAr selective blockade, as GluN2A-NMDArs are necessary for memory acquisition (Bannerman et al, 2008; Brigman et al, 2008; Dalton et al, 2012; Gilmartin et al, 2013). Moreover, blocking GluN2A-NMDArs in BLA disrupts reconsolidation of conditioned fear memory, but does not affect retrieval or memory destabilization (Milton et al, 2013), and blocking GluN2A-NMDArs in the hippocampus can impair reconsolidation of context-specific drug-associated memories following self-administration (Wells et al, 2016). Conversely, infralimbic GluN2B-NMDArs are necessary for consolidation of extinction of conditioned fear (Dalton et al, 2012; Sotres-Bayon et al, 2009) and drug-associated conditioned place preference memories (Otis et al, 2014). Overall, specific NMDAr subunits likely mediate different aspects of learning and memory, specifically in the formation and consolidation of the initial memory and during extinction.

Overall, our data suggests that infralimbic GluN2A-NMDArs modulate reconsolidation of the original drug-cue memory. However, rats reinstated drug seeking following a priming injection of cocaine, which suggests that the observed effect was partial or transient. Conversely, the priming injection of cocaine might have been too salient of a cue, because the drug itself is the primary reinforcer, and may be resistant to reconsolidation disruption. For example, in the conditioned place preference paradigm, mice will reinstate cocaine seeking following a priming injection of cocaine even with previous reconsolidation disruption (Kelley et al, 2007). Moreover, rats were tested for cocaine reinstatement 2–5 weeks following the initial memory disruption. Little research has examined the longevity of reconsolidation disruption in a drug-associated memory following self-administration, which can potentiate over time (Pickens et al, 2011). Drug-associated memories are complex and include numerous salient and vague cues, and all associated cues may not have been retrieved and thus disrupted following GluN2A-NMDAr blockade, which could underlie the transient or partial disruption observed. Alternatively, even though our final experiment controlled for initial extinction learning, it is highly unlikely but still possible that NMDAr blockade affected extinction. Future research should examine the parameters of reconsolidation disruption by infralimbic GluN2A-NMDAr blockade. Specifically, less salient drug-associated cues should be examined during reinstatement and context-induced renewal, and the duration of the disruption including susceptibility to spontaneous recovery should be determined.

In conclusion, we demonstrated that blocking NMDArs, specifically GluN2A-NMDARs, in the IL-mPFC and NAc shell before four short extinction sessions resulted in reduced lever pressing during the extinction retention test (extinction day 5). Our findings indicate that NMDAr blockade was not facilitating extinction, but rather that the reconsolidation of the original drug-cue memory was being transiently or partially disrupted. These results suggest a potential therapeutic approach, as reducing cue-reactivity, whether through facilitated extinction or disrupted reconsolidation, could reduce relapse rates.

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