Behavioral/Cognitive

Removal of Perineuronal Nets in the Medial Prefrontal Cortex Impairs the Acquisition and Reconsolidation of a Cocaine-Induced Conditioned Place Preference Memory

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Pyramidal neurons in the medial prefrontal cortex (mPFC) critically contribute to cocaine-seeking behavior in humans and rodents. Activity of these neurons is significantly modulated by GABAergic, parvalbumin-containing, fast-spiking interneurons, the majority of which are enveloped by specialized structures of extracellular matrix called perineuronal nets (PNNs), which are integral to the maintenance of many types of plasticity. Using a conditioned place preference (CPP) procedure, we found that removal of PNNs primarily from the prelimbic region of the mPFC of adult, male, Sprague Dawley rats impaired the acquisition and reconsolidation of a cocaine-induced CPP memory. This impairment was accompanied by a decrease in the number of c-Fos-positive cells surrounded by PNNs. Following removal of PNNs, the frequency of inhibitory currents in mPFC pyramidal neurons was decreased; but following cocaine-induced CPP, both frequency and amplitude of inhibitory currents were decreased. Our findings suggest that cocaine-induced plasticity is impaired by removal of prelimbic mPFC PNNs and that PNNs may be a therapeutic target for disruption of cocaine CPP memories.

Key words: **cocaine; conditioned place preference; memory; perineuronal net**

Introduction

The medial prefrontal cortex (mPFC) plays a significant role in cognitive function (Dalley et al., 2004) and contributes to cocaine-seeking behavior in both humans and rodents (Maas et al., 1998; Jentsch and Taylor, 1999; Ciccocioppo et al., 2001; McFarland and Kalivas, 2001; Kalivas and Volkow, 2005; Stefanik et al., 2013). Following exposure to cocaine or cocaine-associated cues, the activity of the mPFC increases (Grant et al., 1996; Maas et al., 1998; Childress et al., 1999; Kilts et al., 2001; Sun and Rebec, 2006; Goldstein and Volkow, 2011). Memories are dependent on the PFC (Rudy et al., 2005; Robbins et al., 2008), but few studies have focused on the neural plasticity underlying cocaine-associated memories in this brain region.

The deep layers (V and VI) of the prelimbic (PL) region of the mPFC provide a major excitatory driving force via glutamatergic pyramidal neurons projecting to the nucleus accumbens contributing to cocaine-seeking behavior (Grant et al., 1996). Interneurons within these layers contain GABA, regulate the firing of glutamatergic pyramidal neurons (Markram et al., 2004; Hearing et al., 2013), and modulate cocaine-seeking behavior. Repeated cocaine exposure increases GABA levels within the mPFC (Jayaram and Steketee, 2005) and cocaine-induced conditioned place preference (CPP) increases c-Fos levels in GABAergic interneurons within the PL mPFC (Miller and Marshall, 2004). Additionally, exposure to drug cues increases the frequency of IPSCs onto mPFC pyramidal neurons (Van den Oever et al., 2010). Collectively, these studies suggest that exposure to cocaine alters GABA circuits within the mPFC, and these GABAergic interneurons may regulate cocaine-associated memories.

Surrounding the proximal dendrites and soma of parvalbumin (PV)-containing, fast-spiking, GABAergic interneurons are aggregations of extracellular matrix (ECM) termed perineuronal nets (PNNs) (Härtig et al., 1992; Schüppel et al., 2002; Dityatev et al., 2007). Removal of PNNs in adult animals restores ocular dominance plasticity within the visual cortex (Pizzorusso et al., 2002), enhances reversal learning in the auditory cortex (Happel

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et al., 2014), recovers motor learning after spinal cord injury (Zhao and Fawcett, 2013) and cortical ischemia (Gherardini et al., 2015), and reinstates juvenile-like extinction of a fear conditioned memory when removed from the BLA (Gogolla et al., 2009). Based on these studies, PNNs are thought to be important for maintaining or restricting many types of plasticity.

In contrast, PNN removal has been shown to prevent plasticity induced by fear conditioning: PNN removal within the hippocampus or mPFC impairs, respectively, context-induced or cue-induced reinstatement of fear conditioning (Hylin et al., 2013). The mPFC is involved in learning and memory processes of both fear conditioning and addiction (Peters et al., 2009); therefore, the role of PNNs may be similar in attenuating plasticity associated with both fear-inducing stimuli and drugs of abuse. We hypothesized that PNN removal in the PL mPFC of adult rats would impair cocaine reward-associated memories. We tested this hypothesis by removing PNNs and determining the extent to which the acquisition, extinction, and reconsolidation of a cocaine-associated memory were suppressed in a cocaine-induced CPP task.

Materials and Methods

Subjects. A total of 229 male Sprague Dawley rats were obtained from Simonsen Laboratories. Animals weighed 280–300 g at the start of the experiment and were singly housed in a temperature- and humidity-controlled room with a 12 h light/dark cycle. They were given food and water *ad libitum* throughout the course of the experiment, with exception of the time they were placed in the CPP apparatus. All experiments were approved by the Institutional Animal Care and Use Committee and according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All efforts were made to reduce the number of animals used in the experiments and to minimize pain and suffering.

Surgery and microinjection. Rats were anesthetized with brief exposure to isoflurane followed by an intramuscular injection of zyket (ketamine 87 mg/kg + xylazine 13 mg/kg) and were placed into a stereotaxic apparatus. Bilateral surgical-grade steel cannulae (26 gauge) were implanted into the PL mPFC (3.0 mm from bregma, 0.8 mm from midline, and −2.5 mm from skull) (Paxinos and Watson, 2007) or infralimbic (IL) mPFC (IL; 7° angle from midline, 3.0 mm from bregma, 1.4 mm from midline, and −4.0 mm from skull) (Paxinos and Watson, 2007) and fixed with dental acrylic cement. Obturators (33 gauge) measuring the same length as the cannulae were inserted into the cannulae following surgery and remained in place until the time of microinjection. Following surgery, all animals received an intramuscular injection of ketoprofen (10 mg/kg). Rats recovered for 5–7 d before the start of the experiment.

A surgical-grade steel needle (1 mm longer than cannulae, 33 gauge) was connected to tubing attached to a 1.0 μl Hamilton syringe and inserted into the cannulae. Using an infusion pump, a volume of 0.6 μl was injected bilaterally over a period of 108 s. Following injection, the needles remained in place for an additional 30 s. All cannulae placements were verified at the end of the experiment. All animals with treatment reaching part of the PL or IL mPFC were included in analysis.

Drugs. Cocaine hydrochloride was a gift from the National Institute on Drug Abuse. The cocaine salt was dissolved in saline as weight of the salt. For CPP experiments, the training dose of cocaine was 12 mg/kg and the reinstatement dose was 10 mg/kg; these doses were based on our previous work (Brown et al., 2007). Protease-free chondroitinase-ABC (Ch-ABC) was obtained from Sigma-Aldrich and dissolved in sterile water to a final concentration of 0.09 U/μl. This dose was chosen based on the range of doses used in several other studies (Pizzorusso et al., 2002; Hylin et al., 2013; Xue et al., 2014).

CPP. All CPP experiments were conducted during the light phase. The CPP apparatus comprises three Plexiglas compartments (Med Associates), including two main outer compartments (28 × 21 × 21 cm), one of which has black walls with a wire mesh floor and the other of which has white walls with a metal rod floor. The central compartment (12 × 21 × 21 cm) has gray walls with solid gray flooring. Locomotor activity and side preference were automatically recorded with infrared photocell beams within the apparatus. A manual guillotine door separates each compartment, allowing for confinement of the rat to one side of the apparatus.

Rats were handled briefly for 2 d before the start of each experiment, unless specified elsewhere. Animals received two initial preference days: the first serving as a habituation day and the second serving as a test for initial preference. At the beginning of the initial preference days, animals were placed in the central compartment of the apparatus and allowed free access to all compartments for 15 min. At the end of each session, the compartments were cleaned and dried, and time spent within each compartment was recorded.

The cocaine-paired compartment was determined by counterbalancing the preferred and nonpreferred sides as well as the black and white compartments. Cocaine and saline were administered on alternate days for six training days. Every animal received three saline (1 ml/kg, i.p.) and three cocaine (12 mg/kg, i.p.) pairings. Immediately following administration of saline or cocaine, each animal, except those in the pseudo-conditioned group (see below), was confined to the assigned compartment for 25 min (modified from Brown et al., 2007). A pseudo-conditioned group was run as a control to test for the effect of CPP training on c-Fos levels. These rats received cocaine or saline in an unpaired manner with the CPP apparatus. Each rat received three saline (1 ml/kg, i.p.) and three cocaine (12 mg/kg, i.p.) pairings in their home cage and, 6 h later, were confined to either the black or white compartment for 25 min (Chefer et al., 2011).

A place preference test was conducted following training for all animals. Rats were tested in a drug-free state. They were placed into the central compartment and allowed free access to all compartments for 15 min. Animals spending more time in the cocaine-paired compartment than on the initial preference day were considered to have acquired a place preference.

To test the effect of CPP training on c-Fos levels, the day following the CPP test, animals were confined to one compartment for 30 min. Animals trained in a paired manner were confined to either the saline- or cocaine-paired compartment in a counterbalanced manner. Animals pseudo-conditioned were also confined to either the saline or cocaine pseudo-conditioned compartment in a counterbalanced manner.

For all other experiments, following the test day, animals underwent extinction training for a minimum of 7 d. During extinction training, animals were placed into the central compartment and allowed free access to all compartments for 15 min. No drugs were administered during extinction training. This procedure continued until the group of rats reached a criterion of no significant difference compared with the time spent in the cocaine-paired compartment on the initial preference day. Between reactivation and reinstatement, animals underwent additional extinction training to allow for baseline levels of preference to be restored before testing for cocaine-induced reinstatement. A second group of rats underwent the same training and initial extinction period, but after the reactivation or no reactivation day, were subsequently given a period of forced abstinence to control for the possibility that the second extinction period would alter subsequent cocaine-primed reinstatement. During forced abstinence, animals were handled briefly once daily within the housing facility.

Reactivation and reinstatement for place preference behavior were tested by administering a dose of cocaine (10 mg/kg, i.p.). This lower dose was given for reinstatement to create a discrepancy between the dose used for training and the dose used for reinstatement, increasing reward prediction error (Itzhak and Anderson, 2012). This prediction error should increase the opportunity for memory updating that is believed to be important for memory activation and subsequent reconsolidation (Lee, 2010). After injection, rats were immediately placed into the central compartment and allowed free access to all compartments for 15 min.

One hour following the CPP test or reinstatement test, animals were perfused intracardially with 4% PFA in PBS. The brains were removed and stored overnight in 4% PFA at 4°C. The next day, brains were moved to a 20% sucrose solution; and 24–48 h later, brains were frozen at −80°C and stored until analysis.

Time course of Ch-ABC in naive rats. Rats were handled briefly once daily for 5 d. The next day, Ch-ABC or vehicle was administered into the mPFC as described previously. On days 3, 9, 13, or 30 following injection,

Table 1. Categorization of PV- and WFA-positive neurons within the PL and IL mPFC

		Total number of cells ($n = 6$)			Mean number of cells/20× field ± SEM				
	Layer	PV	WFA	PV/WFA	PV	WFA	PV/WFA	% PV/WFA of WFA	% PV/WFA of PV
PL	V	351	309	248	7.31 ± 1.15	6.43 ± 0.76	5.17 ± 0.80	80.62 ± 8.58	73.59 ± 7.01
	VI	320	319	227	6.67 ± 1.40	6.65 ± 0.97	4.73 ± 1.14	70.19 ± 9.16	72.20 ± 8.55
IL	V	287	264	198	5.98 ± 0.86	5.50 ± 0.79	4.13 ± 0.60	72.72 ± 10.50	70.67 ± 10.20
	VI	247	322	189	5.15 ± 0.74	6.71 ± 0.97	3.94 ± 0.57	60.44 ± 8.72	76.36 ± 11.02

animals were perfused as described previously. These time points were selected to examine the number and intensity of PNNs following Ch-ABC treatment at behaviorally relevant points used during the CPP experiments.

Histology and immunohistochemistry. Coronal brain sections through the mPFC were taken at 30 μm using a freezing microtome. Cannula placement was verified following all experiments; placements not including the PL or IL mPFC were omitted from analysis. Immunohistochemistry was performed by washing free-floating sections three times for 5 min in PBS. Tissue was then placed in 50% ethanol for 30 min. After a set of three 5 min washes in PBS, the tissue was placed in 3% goat blocking serum (Vector Laboratories) for 1 h and then coincubated overnight at 4°C on a shaker table with c-Fos (1:1000, EMD Millipore), NeuN (1:100, EMD Millipore), or PV (1:1000, Sigma) antibodies and *Wisteria floribunda* agglutinin (WFA, 1:500, Vector Laboratories) (Härtig et al., 1992) in PBS containing 2% goat serum. After three 10 min washes in PBS, the tissue was incubated for 2 h with the secondary antibody (AlexaFluor-594 goat anti-rabbit for the c-Fos antibody or AlexaFluor-594 goat anti-mouse for the NeuN or PV antibody) in PBS with 2% normal goat serum. The tissue was washed three times for 10 min each in PBS and mounted onto Frost plus slides in diluted PBS (30:200) with 0.0015% Triton. After drying, the tissue was coverslipped with ProLong (Vector Laboratories). Images of the PFC were photographed using a Zeiss Axioplan fluorescent microscope with an Infinity2 digital camera. c-Fos, NeuN, or PV labeling was quantified by counting the number of c-Fos-, NeuN-, or PV-positive cells within a 20× field. For the c-Fos-, NeuN-, or PV- labeled cells that were double-labeled with WFA, the images were photographed in the green and red channels and the microscope switched between the two fields to evaluate double labeling.

To measure the intensity of WFA, tissue was stained with WFA and coverslipped as described above. Images of the mPFC were taken using an Olympus IX81 confocal microscope equipped with Slidebook software. Confocal image stacks consisted of 4 images with a 2 μm interimage distance. WFA staining was quantified bilaterally in an area below the cannula within a fixed area frame (360 μm × 265 μm). Background threshold levels were set and applied to all images for comparison. Pixel intensities above this threshold were used for quantification measures (area occupied by pixels). The number of WFA-positive cells within the frame was also assessed by counting all cells surrounded by WFA immunolabeling. An experimenter blinded to the treatment conditions performed all immunohistochemical analyses.

Electrophysiology. Rats were anesthetized with isoflurane followed by intracardial perfusion with a recovery solution oxygenated with 95% O_2–5% CO_2 at ice-cold temperatures. The composition of the recovery solution was (in mM) as follows: 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 4 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4(H_2O)_7$, and 0.5 $CaCl_2(H_2O)_2$. Following perfusion, rats were decapitated, and coronal slices (300 μm) containing the PL region of the mPFC (3.0 mm from bregma) (Paxinos and Watson, 2007) were prepared using a vibrating microtome (Leica VT1200S; Leica). Brain slices were cut in an ice-cold recovery solution (described above) oxygenated with 95% O_2–5% CO_2 and then placed in a holding chamber containing recovery solution oxygenated with 95% O_2–5% CO_2 at 37°C for 10 min. The slices were then placed in a storage chamber containing holding solution oxygenated with 95% O_2–5% CO_2 and kept at room temperature for at least 1 h before recording. The composition of the holding solution was (in mM) as follows: 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 2 $MgSO_4(H_2O)_7$, and 2 $CaCl_2(H_2O)_2$. For whole-cell patch-clamp recording, one slice was transferred to a recording chamber and fixed to the bottom of the chamber with a nylon grid on a platinum frame. The chamber was perfused constantly at 31.0 ± 0.5°C at a rate of 5–6 ml/min of aCSF. The composition of the aCSF was (in mM) as follows: 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 11.0 dextrose, 1.30 $MgSO_4(H_2O)_7$, and 2.5 $CaCl_2(H_2O)_2$. Whole-cell recordings were made with a patch-clamp amplifier (Axon MultiClamp 700B, Molecular Devices) using an infrared differential contrast microscope (Olympus). Electrical signals were low-pass filtered at 2 kHz, digitized at 10 kHz (Digidata 1440A, Molecular Devices). Online analysis was conducted with pCLAMP software (version 10; Molecular Devices) and analyzed with MiniAnalysis (Synaptosoft).

For recording miniature IPSCs (mIPSCs), neurons were voltage-clamped at −70 mV in the presence of DNQX (10 μM), D-AP5 (50 μM), and TTX (1 μM). Patch pipettes were pulled from borosilicate capillary tubing, and the electrode resistance was typically 3–4 MΩ. The composition of the intracellular solution was (in mM) as follows: 117 CsCl, 2.8 NaCl, 5 $MgCl_2$, 20 HEPES, 2 Mg^{2+}ATP, 0.3 Na^{2+}GTP, 0.6 EGTA, and sucrose to bring osmolarity to 275–280 mOsm and pH to ~7.25. For recordings assessing action potentials, CsCl was replaced with KCl (125.0 mM) in the internal solution. To assess the efficacy of Ch-ABC in depleting PNNs, fluorescein-labeled WFA (1:100) was added to the holding solution. The cells' input resistance and series resistance were monitored throughout each experiment; cells were discarded if these values changed by >10% during the experiment. For animals undergoing cocaine-induced CPP (see CPP testing), slices were prepared immediately after the test session (15 min long) for CPP. The experimenter for all electrophysiology experiments was not blinded to the treatment conditions.

Statistics. All statistical tests were conducted using Prism 6 (GraphPad Software). All CPP experiments were analyzed using a two-way, repeated-measures ANOVA (vehicle vs Ch-ABC treatment as the between-subjects measure; CPP day as the within-subjects measure). When applicable, a Bonferroni *post hoc* analysis was used. Immunohistochemical data were analyzed using an unpaired, Student's two-tailed *t* test or one-way ANOVA. Statistical comparisons of the synaptic amplitudes (mIPSCs) were made using the Kolmogorov–Smirnov test; statistical comparisons of mIPSC frequencies were analyzed using one-way ANOVA followed by a Dunnet's multiple-comparisons test. Action potential experiments were analyzed using a two-way, repeated-measures ANOVA (control vs Ch-ABC treatment as the between-subjects measure; injection current as the within-subjects measure). Sidak's multiple-comparisons test was used to analyze differences. Differences were considered significant when $p < 0.05$.

Results

c-Fos levels are increased in PNN-surrounded neurons within the PL mPFC following exposure to a cocaine-associated environment

We first determined the extent to which PNNs colocalized with PV neurons within the PL and IL mPFC, which had not been previously measured. We labeled PNNs using WFA because it is a standard method of PNN visualization (Härtig et al., 1992; Pizzorusso et al., 2002; Gogolla et al., 2009; Hylin et al., 2013). Within layers V and VI of the PL mPFC, ~70%–80% of WFA cells were double-labeled for PV, and a similar percentage of PV-labeled cells were double-labeled for WFA (Table 1). Within layers V and VI of the IL mPFC, ~60%–73% of WFA cells were

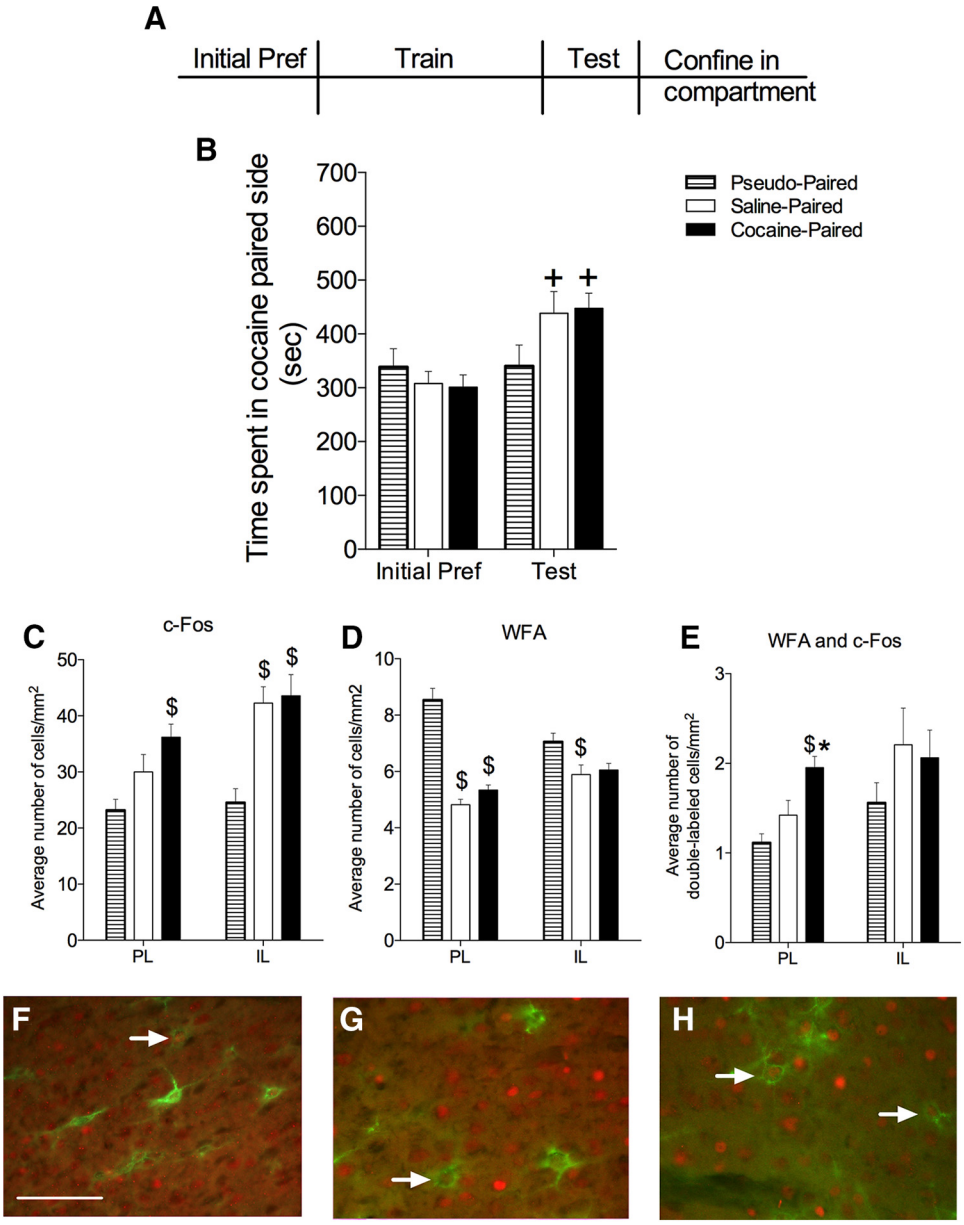

Figure 1. Cocaine-associated reward increases WFA/c-Fos double-labeled neurons in the PL mPFC. ***A***, Timeline of experimental procedure. ***B***, Time spent in cocaine-paired chamber on initial preference and test day for CPP. Animals conditioned in a paired manner ($n = 8$/group) show place preference, but animals conditioned in an unpaired manner ($n = 8$) do not show preference. ***C***, c-Fos-labeled cells within layers V and VI of the PL mPFC are increased 60 min after confinement to the cocaine-paired, but not saline-paired, chamber in animals trained in a paired manner. c-Fos-labeled cells within layers V and VI of the IL mPFC are increased 60 min after confinement to either the cocaine-paired or saline-paired chamber in animals trained in a paired manner. ***D***, WFA-labeled cells within layers V and VI of the PL mPFC are decreased 60 min after confinement to the cocaine- or saline-paired chamber in animals trained in a paired manner. WFA-labeled cells within layers V and VI of the IL mPFC are decreased only in animals trained in a paired manner and confined to the saline-paired chamber. ***E***, WFA/c-Fos double-labeled neurons within layers V and VI of the PL mPFC, but not the IL mPFC, were increased in animals confined to the cocaine-paired chamber compared with those confined to the saline-paired chamber or to those confined to a pseudo-conditioned chamber. ***F–H***, Representative images from the PL mPFC of pseudo-conditioned (***F***), saline-paired (***G***), and cocaine-paired (***H***) animals. Arrows indicate double-labeled cells for WFA (green) and c-Fos (red). Scale bar, 100 μm. Data are mean ± SEM. *$p < 0.05$, compared with vehicle-treated animals. $^{\$}p < 0.05$, compared with pseudo-conditioned animals. $^{+}p < 0.05$, compared with initial preference day.

PV labeled and 70%–76% of PV cells were double-labeled for WFA (Table 1).

To determine whether PNN-surrounded neurons, the majority of which are GABAergic interneurons, were involved in cocaine reward, we used WFA as a marker for PNNs and measured double labeling for c-Fos following a CPP task. Animals ($n = 8$/group) were trained and tested for CPP behavior and confined to either the cocaine-paired compartment or the saline-paired compartment (Fig. 1*A*). An additional group of animals ($n = 8$) was exposed to the same training procedure but in an unpaired manner and were confined to either the saline or cocaine pseudo-conditioned compartment. No difference in behavior or immunohistochemistry was observed within the pseudo-conditioned group whether they were confined to the saline or cocaine pseudo-conditioned compartment; thus, all pseudo-conditioned animals were pooled. A two-way ANOVA revealed a significant CPP day effect ($F_{(1,21)} = 14.12$, $p < 0.0012$). No difference was observed in CPP behavior before confinement in animals trained in a paired manner, whereas animals trained in an unpaired manner did not show place preference (Fig. 1*B*).

An increased number of WFA/c-Fos double-labeled neurons were present in layers V and VI of the PL mPFC following confinement in the cocaine-paired compartment compared with confinement in the saline-paired compartment or after pseudoconditioning (Fig. 1*E–H*; one-way ANOVA, $F_{(2,21)} = 10.29$, $p < 0.0008$). The average number of WFA-positive cells was different between the paired and unpaired training groups (Fig. 1*D*,*F–H*; one-way ANOVA, $F_{(2,21)} = 52.00$, $p < 0.0001$), suggesting that learning associated with CPP can decrease the number of PNNs. Additionally, the average number of c-Fos-positive cells was also different between groups (Fig. 1*C*,*F–H*; one-way ANOVA, $F_{(2,21)} = 6.84$, $p < 0.005$), suggesting that learning associated with CPP can increase c-Fos activation. In contrast, no difference in the number of WFA/c-Fos double-labeled neurons was observed in layers V and VI of the IL mPFC between confinement conditions (Fig. 1*E*; one-way ANOVA, $F_{(2,21)} = 1.11$, $p = 0.34$). However, the average number of WFA-positive cells within the IL mPFC was altered similarly to that of the PL following confinement (Fig. 1*D*; one-way ANOVA, $F_{(2,21)} = 4.66$, $p < 0.05$), as well as the average number of c-Fos-positive cells (Fig. 1*C*; one-way ANOVA, $F_{(2,21)} = 11.74$, $p < 0.0005$). These results suggest that PNN-surrounded neurons within the PL mPFC are preferentially activated by a cocaine reward memory because the increase in double-labeled cells is observed only within the PL mPFC following confinement in the cocaine-paired compartment.

Removal of intra-PL, but not intra-IL, mPFC PNNs attenuates acquisition of cocaine-induced CPP

To test whether PNN-surrounded neurons were involved in the formation of a cocaine-induced CPP memory, we removed PNNs primarily from the PL mPFC using Ch-ABC ($n = 11$ vehicle, 13 Ch-ABC) before acquisition training for CPP (targeted sites are shown in Fig. 2). The time line of the experiment is shown in Figure 3*A*. No difference was observed between treatment groups during initial preference testing (Fig. 3*B*). A two-way ANOVA revealed a significant CPP day effect ($F_{(1,22)} = 43.79$, $p < 0.0001$), showing that both treatment groups displayed preference for the cocaine-paired compartment after training. Locomotor activity between the groups was not different during the CPP test (infrared photocell beam breaks: vehicle, 2619 ± 460; Ch-ABC, 2121 ± 377; Student's two-tailed *t* test, $p = 0.41$). However, Ch-ABC-treated animals spent less time in the cocaine-paired chamber than did vehicle-treated animals, as revealed by a significant treatment effect (Fig. 3*B*; $F_{(1,22)} = 5.291$, $p < 0.05$), suggesting that Ch-ABC-treated rats have a weaker memory for the cocaine-paired compartment than vehicle-treated rats.

One hour following the CPP test, animals were killed and immunohistochemistry was performed. Compared with vehicle-treated animals ($n = 5$), Ch-ABC-treated animals ($n = 5$) had fewer WFA/c-Fos double-labeled neurons within the mPFC (Fig. 3*D–F*; Student's two-tailed *t* test, $p < 0.05$). No difference was observed in the number of cells positive for WFA (Table 2; Student's two-tailed *t* test, $p = 0.68$) or c-Fos (Table 2; Student's two-tailed *t* test, $p = 0.21$).

Together, these results indicate that PNN-surrounded neurons within the PL mPFC may contribute to the acquisition of cocaine-induced CPP because the impaired CPP behavior was mirrored by a reduction in c-Fos-expressing neurons that are surrounded by PNNs.

Because removal of PNNs from the PL mPFC decreased acquisition of cocaine-induced CPP, we tested whether removal of PNNs within the IL mPFC would have a similar effect. Before training on the CPP task, Ch-ABC ($n = 6$) or vehicle ($n = 5$) was administered into the IL mPFC. No difference was observed in behavior on the initial preference day or on the test day (Fig. 3*C*). A two-way ANOVA revealed a significant CPP day effect ($F_{(1,9)} = 5.44$, $p < 0.05$), but no treatment effect. No difference was observed in locomotor activity on the test day between the treatment groups (infrared photocell beam breaks: vehicle, 1723 ± 131; Ch-ABC, 1688 ± 380; Student's two-tailed *t* test, $p = 0.93$). This suggests that PNNs within the IL mPFC do not contribute to the acquisition of cocaine-induced CPP.

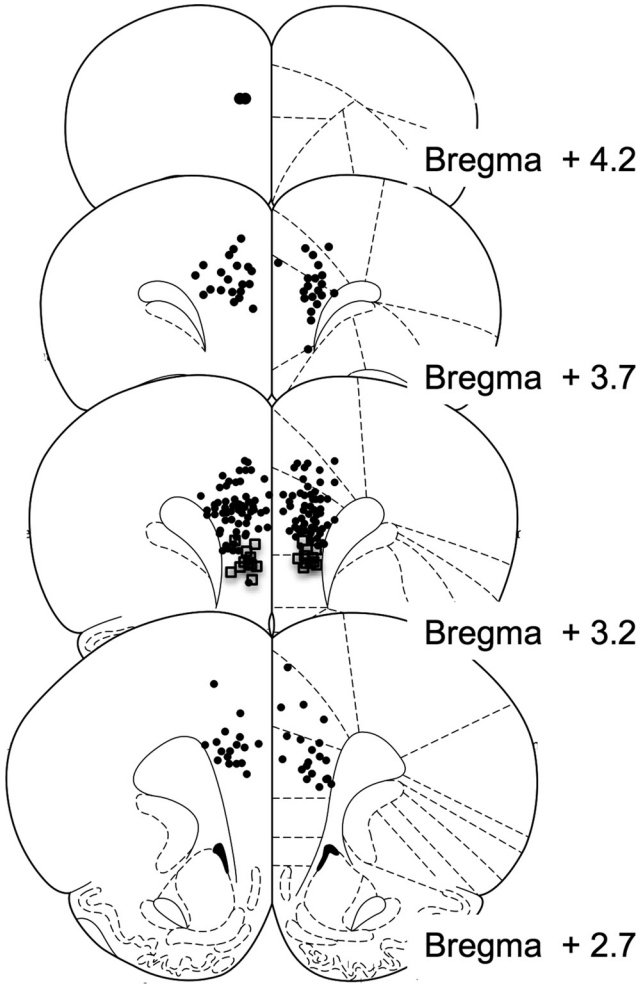

Figure 2. Intra-mPFC cannulae placements. Circles represent the most ventral point of the cannula tract for each animal receiving treatment primarily within the PL mPFC. Squares represent the most ventral location of cannulae tracts for each animal receiving intra-IL treatment. Numbers indicate the distance from bregma in millimeters.

Removal of intra-PL mPFC PNNs does not alter extinction rate

Based on previous work demonstrating an effect of intra-BLA Ch-ABC treatment on extinction of both fear memory and drug memory (Gogolla et al., 2009; Xue et al., 2014), we determined whether removal of PNNs within the PL mPFC altered extinction learning. We administered Ch-ABC ($n = 6$ vehicle, 5 Ch-ABC) into the mPFC after the CPP memory had been established, but before extinction training (Fig. 4*A*). Ch-ABC administration did not modify the rate of extinction learning (Fig. 4*B*) or subsequent cocaine-induced reinstatement (Fig. 4*C*). A two-way ANOVA revealed a significant CPP day effect ($F_{(1,9)} = 26.67$, $p < 0.001$),

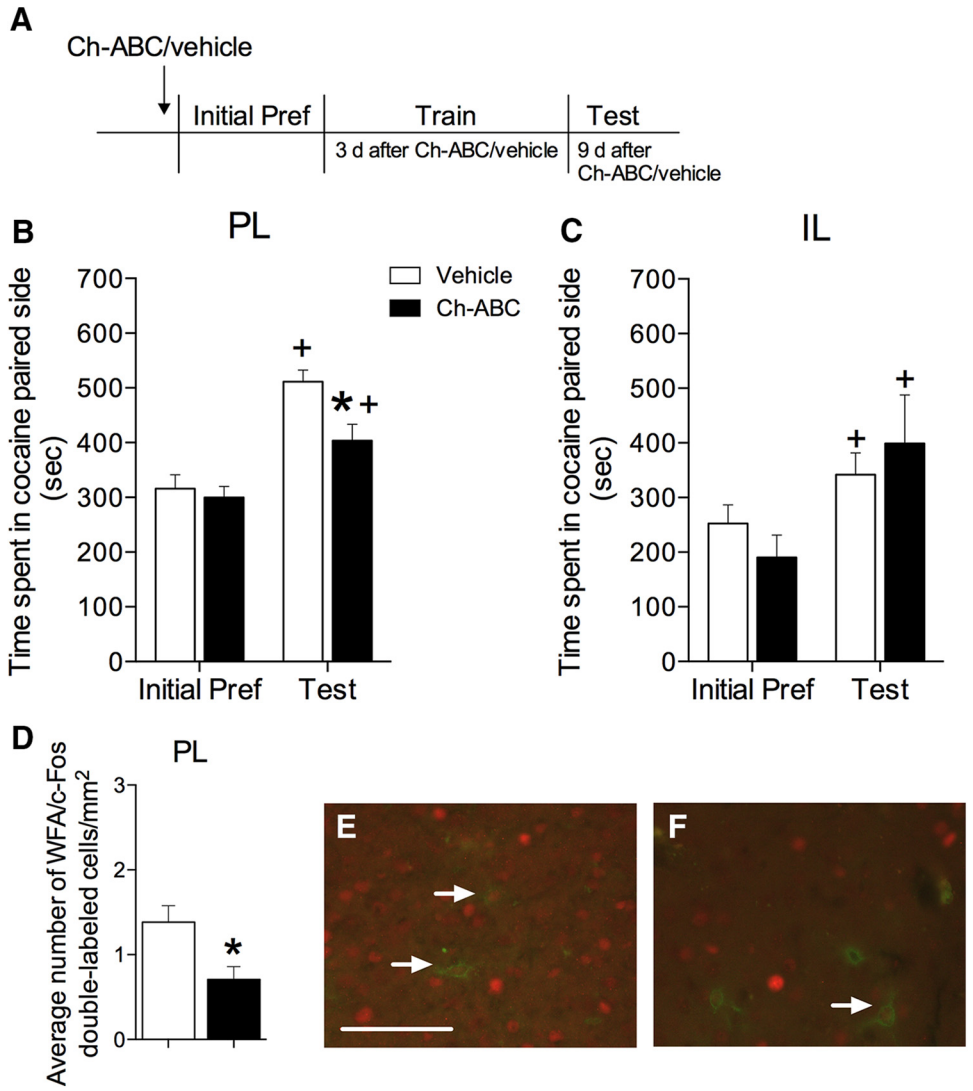

Figure 3. Intra-PL administration of Ch-ABC attenuates acquisition of cocaine-induced CPP. ***A***, Timeline of experimental procedure. ***B***, Time spent in cocaine-paired chamber on initial preference and test day for CPP is shown after pretreatment with intra-PL administration of vehicle ($n = 11$) or Ch-ABC ($n = 13$). ***C***, Time spent in cocaine-paired chamber on initial preference and test day for CPP is shown after pretreatment with intra-IL administration of vehicle ($n = 5$) or Ch-ABC ($n = 6$). ***D***, Average number of WFA/c-Fos double-labeled neurons in the PL mPFC treated with vehicle ($n = 5$) and Ch-ABC ($n = 5$). Rats treated with Ch-ABC had fewer WFA/c-Fos double-labeled neurons than rats treated with vehicle. ***E***, ***F***, Representative images from rats treated with vehicle (***E***) and Ch-ABC (***F***) of WFA (green) and c-Fos (red). Arrow indicates double-labeled cells. Scale bar, 100 μm. Data are mean ± SEM. *$p < 0.05$, compared with vehicle-treated animals. $^{+}p < 0.05$, compared with initial preference day.

Table 2. WFA- and c-Fos-positive cells following behavioral procedures with intra-PL vehicle or Ch-ABC administration[a]

Ch-ABC effect on: (corresponding figure)	WFA		c-Fos	
	Vehicle	Ch-ABC	Vehicle	Ch-ABC
Acquisition (Fig. 3)	3.35 ± 0.37	3.54 ± 0.29	32.11 ± 2.94	25.62 ± 3.62
Extinction (Fig. 4)	5.42 ± 0.21	4.30 ± 0.35*	78.85 ± 3.18	68.83 ± 3.38
Reconsolidation (Fig. 5)				
No reaction	4.97 ± 0.36	4.19 ± 0.25	43.79 ± 8.34	40.17 ± 6.09
Reaction	4.51 ± 0.20	3.90 ± 0.33	40.10 ± 4.79	33.53 ± 4.08

[a]Data are mean number of cells/mm^2 ± SEM.

*$p < 0.05$ (Student's, two-tailed, *t* test).

but no treatment effect. No differences were observed in locomotor activity between treatment groups during the reinstatement test (infrared photocell beam breaks: vehicle, 4559 ± 380; Ch-ABC, 3503 ± 432; Student's two-tailed *t* test, $p = 0.09$). Additionally, no significant difference was observed between treatment conditions for the number of WFA/c-Fos double-labeled neurons (Fig. 4*D*; Student's two-tailed *t* test, $p = 0.15$). Interestingly, we did observe a reduction in the number of cells positive for WFA (Table 2; Student's two-tailed *t* test, $p = 0.02$) and a trend toward a reduction in the number of cells positive for c-Fos (Table 2; Student's two-tailed *t* test, $p = 0.06$). We are unable to explain this 20% reduction in WFA staining in the Ch-ABC group. The trend toward decreased WFA/c-Fos double-labeled neurons may indicate that the first extinction session(s) serve as a weak memory reactivation, but this is not manifest as a decrease in behavioral responding on the test for reinstatement. Our results suggest that PNN-surrounded neurons in the PL mPFC do not contribute to the extinction of cocaine-induced CPP.

Removal of intra-PL mPFC PNNs disrupts reconsolidation of cocaine-induced CPP

Because Ch-ABC pretreatment disrupted the acquisition and/or consolidation of cocaine-induced CPP, we determined whether this treatment would also disrupt the reconsolidation of cocaine-induced CPP. That is, we assessed whether PNN removal by

Ch-ABC would alter cocaine-induced reinstatement of CPP in a memory reactivation-dependent manner. To test this, we treated rats with Ch-ABC before reactivating the CPP memory (Fig. 5*A*). The reactivated group ($n = 18$ vehicle, 15 Ch-ABC) received a 15 min, cocaine-induced memory reactivation session 3 d following treatment, whereas the nonreactivated group ($n = 14$ vehicle, 16 Ch-ABC) did not receive a memory reactivation session. The 3 d interval was chosen to match the timing used in the acquisition experiment between treatment and the first training day. No differences were observed in behavior during the cocaine-induced memory reactivation session (seconds in cocaine-paired compartment: vehicle, 485.4 ± 43.6; Ch-ABC, 467.3 ± 49.3; Student's two-tailed *t* test, $p = 0.78$). Five days following treatment, all animals underwent either extinction training or a period of forced abstinence. All animals received a cocaine-induced reinstatement test 11–14 d following treatment. This interval was selected to provide sufficient time for animals within the extinction group to reach the extinction criterion. No difference was observed in the time spent in the cocaine-paired compartment during the reinstatement test between animals in the extinction or forced abstinence groups for each reactivation condition; hence, the results from the extinction and forced abstinence groups were pooled. Ch-ABC treatment in the PL mPFC reduced preference for the cocaine-paired chamber compared with vehicle treatment only in rats that were given a memory reactivation session (Fig. 5*B*). A two-way ANOVA revealed a significant CPP day effect ($F_{(1,31)} = 31.49$, $p < 0.0001$) and a significant treatment effect ($F_{(1,31)} = 8.282$, $p < 0.01$). In contrast, Ch-ABC treatment had no effect in rats that did not receive a memory reactivation session, displaying a level of reinstatement similar to that of vehicle controls (Fig. 5*F*; CPP day effect, $F_{(1,28)} = 54.08$, $p < 0.0001$). Thus, the impact of PNN removal in the mPFC was dependent on memory reactivation, indicating that intact PNNs contribute to memory maintenance of a cocaine-associated memory and that the resultant effects of removing PNNs impair memory reconsolidation. No differences were observed in locomotor activity during the reinstatement test (infrared photocell beam breaks: reactivated: vehicle, 2851 ± 395; Ch-ABC, 2770 ± 470; Student's two-tailed *t* test, $p = 0.89$; nonreactivated: vehicle, 1796 ± 92; Ch-ABC, 1946 ± 187; Student's two-tailed *t* test, $p = 0.50$).

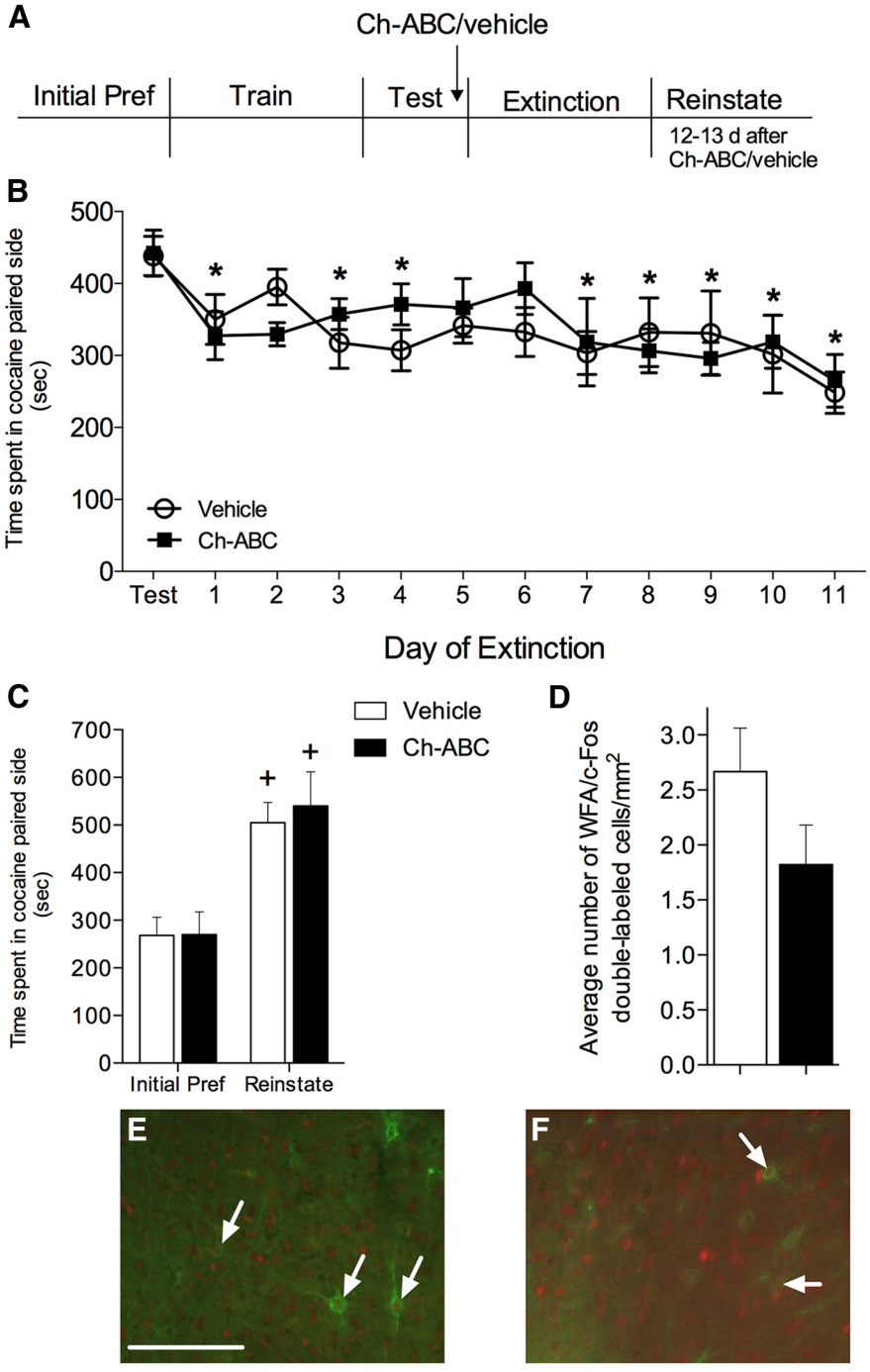

Figure 4. Intra-PL administration of Ch-ABC before extinction does not alter the rate of extinction training or suppress subsequent reinstatement of cocaine-induced CPP. ***A***, Timeline of experimental procedure. ***B***, Time course of extinction rate for vehicle-treated ($n = 6$) and Ch-ABC-treated ($n = 5$) rats. Treatment did not alter the rate of extinction. ***C***, Time spent in cocaine-paired chamber on initial preference and reinstatement day (Reinstate) is shown. ***D***, Average number of WFA/c-Fos double-labeled neurons following the reinstatement test was not different between treatment groups. ***E***, ***F***, Representative images from rats treated with vehicle (***E***) and Ch-ABC (***F***) of WFA (green) and c-Fos (red). Arrows indicate double-labeled cells. Scale bar, 100 μm. Data are mean ± SEM. $^{+}p < 0.05$, compared with initial preference day. $^{*}p < 0.05$, compared with test day.

The pattern of WFA/c-Fos double-labeled neurons following cocaine-induced reinstatement followed the pattern of behavior changes described above. The number of WFA/c-Fos doubled-labeled neurons was decreased in rats treated with Ch-ABC that received a memory reactivation session (Fig. 5*C–E*; Student's two-tailed *t* test, $p < 0.01$) but was not altered between treatment groups in rats that did not receive a memory reactivation session (Fig. 5*G–I*; Student's two-tailed *t* test, $p = 0.85$). No difference was observed in animals receiving a memory reactivation for total number of cells positive for WFA (Table 2; Student's two-tailed *t* test, $p = 0.12$) or c-Fos (Table 2; Student's two-tailed *t* test, $p =$

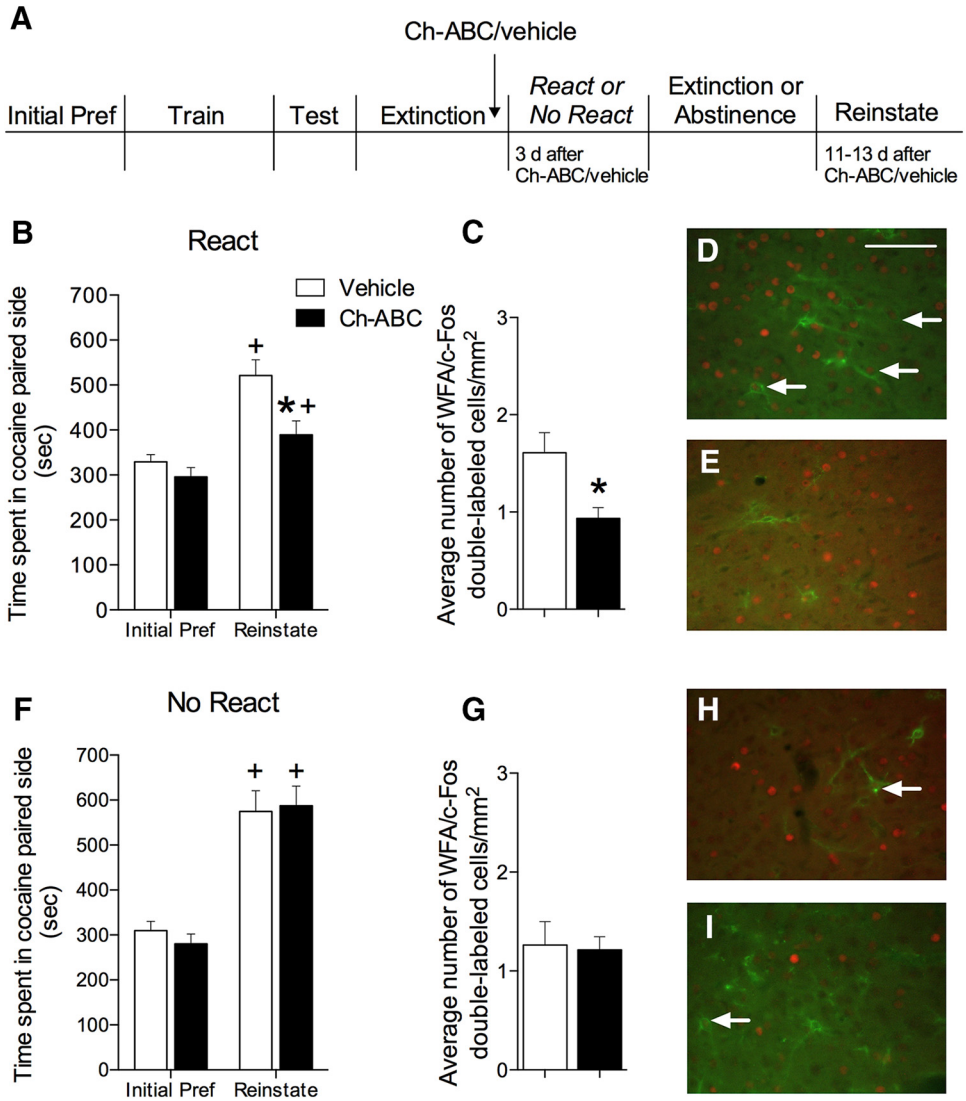

Figure 5. Intra-PL administration of Ch-ABC attenuates reconsolidation of cocaine-induced CPP. ***A***, Timeline of experimental procedure. ***B***, Time spent in cocaine-paired chamber on initial preference day and reinstatement day (Reinstate) is shown for animals (vehicle: $n = 18$; Ch-ABC: $n = 15$) receiving treatment 3 d before a reactivation session (React). ***C***, WFA/c-Fos double-labeled neurons within the PL mPFC decreased in animals treated with Ch-ABC compared with animals treated with vehicle. ***D***, ***E***, Representative images from rats treated with vehicle (***D***) and Ch-ABC (***E***) of WFA (green) and c-Fos (red). Arrows indicate double-labeled cells. ***F***, Time spent in cocaine-paired chamber on initial preference day and reinstatement day (Reinstate) is shown for animals (vehicle: $n = 14$; Ch-ABC: $n = 16$) not receiving a memory reactivation session (No React). ***G***, Average number of WFA/c-Fos double-labeled cells within the PL mPFC was not different between groups. ***H***, ***I***, Representative images from rats treated with vehicle (***H***) and Ch-ABC (***I***) of WFA (green) and c-Fos (red). Arrows indicate double-labeled cells. Scale bar, 100 μm. Data are mean ± SEM. $^{*}p < 0.05$, compared with vehicle treated animals. $^{+}p < 0.05$, compared with initial preference day.

0.32). Additionally, no difference was observed in animals not receiving a memory reactivation for total number of cells positive for WFA (Table 2; Student's two-tailed t test, $p = 0.08$) or c-Fos (Table 2; Student's two-tailed t test, $p = 0.72$). Together, these results indicate that c-Fos activation within PNN-surrounded neurons of the PL mPFC contribute to the reconsolidation of a cocaine-induced memory.

Intra-mPFC administration of Ch-ABC decreases intensity of WFA staining

Because we did not observe changes in the number of WFA-positive neurons following most of the behavioral procedures (Table 2), we determined the time course for PNNs to reassemble following removal with Ch-ABC. To test this, we administered Ch-ABC into the PL mPFC and killed animals at time points that matched the intervals used for the behavioral experiments presented above: 3, 9, and 13 d. The 3 d interval is the time between treatment and memory training (Fig. 3*A*) and the time between treatment and memory reactivation (Fig. 5*A*); 9 d is the interval between treatment and the CPP test (Fig. 3*A*); and 13 d is the duration of time animals underwent extinction training (Fig. 4*A*) and the interval between treatment and cocaine-induced reinstatement (Fig. 5*A*). At each time point, WFA intensity was decreased in animals treated with Ch-ABC compared with animals treated with vehicle (Fig. 6*C*,*G*,*K*; Student's two-tailed t test, 3 d: $p < 0.005$; 9 d: $p < 0.0001$; 13 d: $p < 0.05$). Additionally, we determined whether PNNs were reassembled after a more extended time period. WFA intensity was not different between treatment groups by 30 d following treatment (vehicle, 100 ± 30%; Ch-ABC, 60 ± 22%; $n = 3$ vehicle, 5 Ch-ABC; $p = 0.32$). In contrast to changes in intensity at the early time points, the number of WFA-labeled cells was significantly decreased only at the 3 d time point (Fig. 6*D*,*H*,*L*; Student's two-tailed t test, 3 d: $p < 0.005$; 9 d: $p = 0.08$; 13 d: $p = 0.81$). We used NeuN staining to

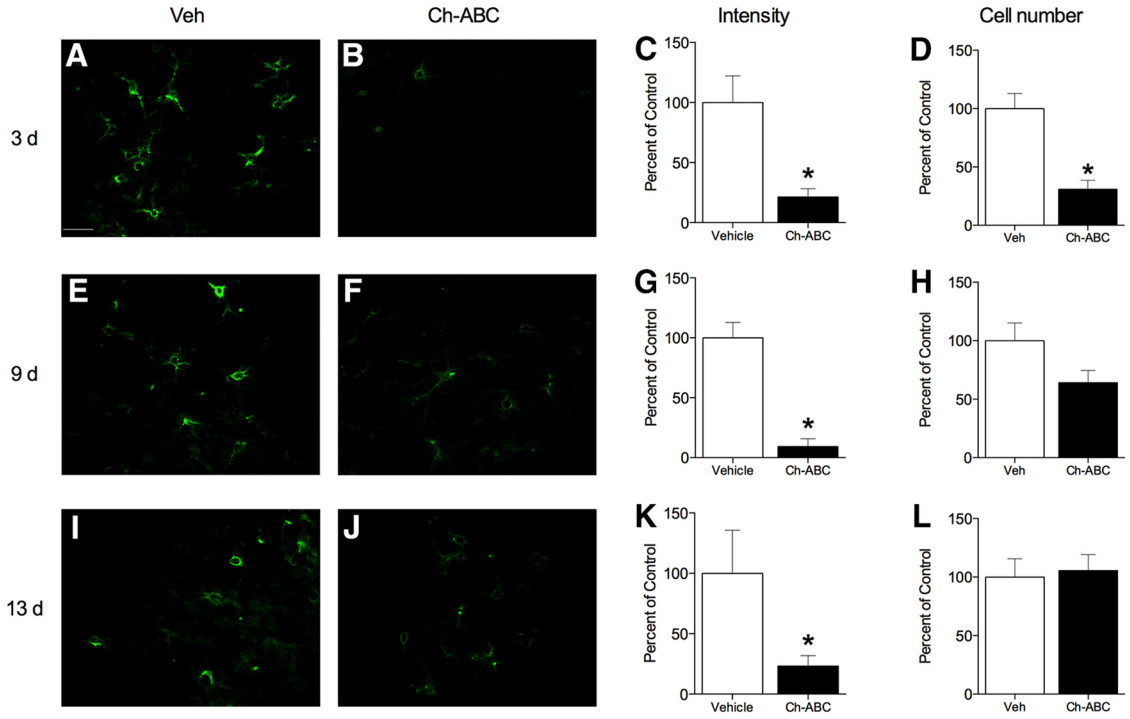

Figure 6. Time course of WFA intensity following Ch-ABC administration. Ch-ABC treatment condition was normalized to control levels for each time point and for both the intensity and cell number analysis. ***A–D***, Three days following treatment, WFA intensity (***C***) is decreased in Ch-ABC-treated animals compared with vehicle-treated animals ($n = 8$/group). Representative single-channel confocal micrographs of vehicle-treated (***A***) and Ch-ABC-treated (***B***) tissue. ***D***, The relative number of WFA-surrounded cells is decreased following Ch-ABC treatment compared with vehicle treatment (raw cell count: vehicle, 10.31 ± 1.34 cells; Ch-ABC, 3.19 ± 0.78 cells, $p < 0.005$). ***E–H***, Nine days following treatment, WFA intensity (***G***) is decreased in Ch-ABC-treated ($n = 6$) animals compared with vehicle-treated ($n = 4$) animals. Representative single-channel confocal micrographs of vehicle-treated (***E***) and Ch-ABC-treated (***F***) tissue. ***H***, The relative number of WFA-surrounded cells was not different between treatment groups (raw cell count: vehicle, 5.46 ± 0.83; Ch-ABC 3.5 ± 0.57, $p = 0.08$). ***I–L***, Thirteen days following treatment, WFA intensity (***K***) is decreased in Ch-ABC-treated ($n = 8$) compared with vehicle-treated ($n = 4$) animals. Representative single-channel confocal micrographs of vehicle-treated (***I***) and Ch-ABC-treated (***J***) tissue. ***L***, The relative number of WFA-surrounded cells was not different between treatment groups (raw cell count: vehicle, 7.88 ± 1.23; Ch-ABC, 8.31 ± 1.08, $p = 0.81$). Data are mean ± SEM. *$p < 0.05$, compared with vehicle-treated animals. Scale bar, 50 μm.

determine whether the decrease in WFA intensity was due to toxic effects of Ch-ABC; however, we observed no difference in the number of neurons positive for NeuN between vehicle- and Ch-ABC-treated rats (NeuN-positive nuclei: vehicle, 138 ± 6.50; Ch-ABC, 146 ± 3.22; $n = 7$ vehicle, 8 Ch-ABC; Student's two-tailed t test, $p = 0.27$). These results demonstrate that the intensity of PNNs was decreased at behaviorally relevant time points, but that the number of PNN-surrounded neurons did not differ.

GABA-mediated inhibition of mPFC pyramidal neurons is decreased, and pyramidal cell excitability is increased, following Ch-ABC administration

To begin to identify a potential mechanism for attenuated CPP acquisition in Ch-ABC-treated rats, we examined properties of mIPSCs on pyramidal neurons within the PL mPFC following cocaine-induced CPP and 9 d following Ch-ABC administration (at which time, we observed an impaired acquisition of cocaine-induced CPP). Slices for control experiments were taken from either naive rats or those given an intra-mPFC administration of vehicle. There were no differences between naive and vehicle-treated animals (data not shown); hence, the two groups were pooled together. Control experiments ($n = 11$ rats, 46 neurons) were interleaved with all other experimental groups. Following cocaine-induced CPP training ($n = 7$ rats, 16 neurons), the amplitude of mIPSCs on pyramidal neurons was attenuated compared with control levels (Fig. 7*A–C*). However, microinjection of Ch-ABC into the mPFC of naive rats ($n = 4$ rats, 18 neurons) had no effect on mIPSC amplitudes (Fig. 7*A–C*). Interestingly, both cocaine-induced CPP training and microinjection of Ch-ABC significantly reduced the frequency of mIPSCs (events/s) onto pyramidal cells in the mPFC compared with slices from control animals (Fig. 7*D*,*E*; control: 8.6 ± 0.62; cocaine-induced CPP: 3.7 ± 0.42; 9 d Ch-ABC: 5.6 ± 0.71). These results suggest that cocaine-induced CPP alters presynaptic and postsynaptic properties of mIPSCs, whereas Ch-ABC administration alters only presynaptic properties.

To assess whether depleting PNNs modulates action potential firing, we used a current ramp protocol and found that microinjection of Ch-ABC (9 d before recording) significantly increased the number of action potentials at various holding currents compared with slices from control animals (Fig. 7*F*,*G*; control, $n = 4$ rats, 10 neurons; Ch-ABC, $n = 3$ rats, 6 neurons). Both the mIPSC data and the action potential data suggest that depletion of PNNs causes hyperexcitability in the pyramidal cells.

Discussion

In the present study, we provide evidence that PNNs within the PL mPFC contribute to the maintenance of a cocaine-associated memory. First, PNN-surrounded neurons within the PL mPFC were preferentially activated following exposure to a cocaine-associated context. Second, removal of intra-PL PNNs with Ch-ABC blunted the acquisition of cocaine-induced CPP. Third, removal of intra-PL PNNs impaired the reconsolidation of a cocaine-associated memory because impaired CPP behavior was dependent on memory reactivation. Fourth, these behavioral effects were mirrored by a decrease in the number of WFA/c-Fos

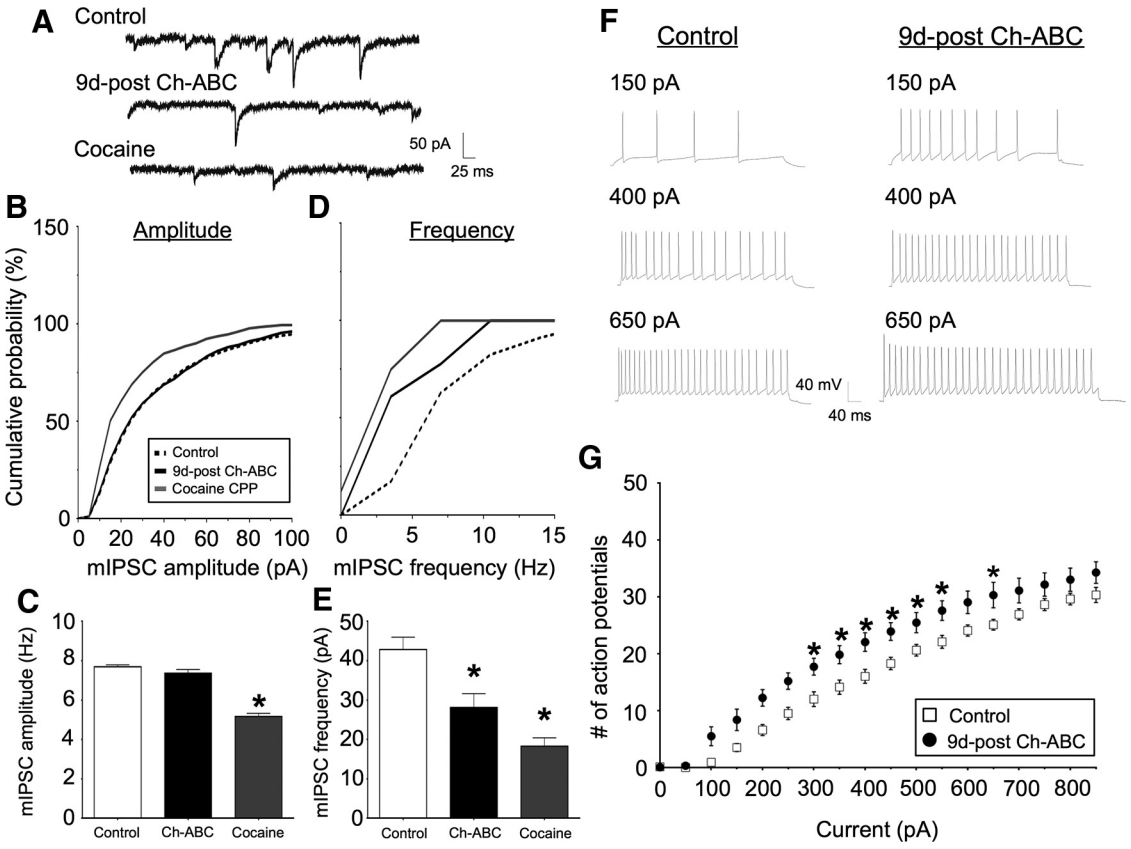

Figure 7. Infusion of Ch-ABC into the mPFC increases the excitability of mPFC pyramidal neurons, and mIPSC amplitude and frequency are decreased in mPFC pyramidal neurons following cocaine-induced CPP. ***A***, Example traces of mIPSC recordings from mPFC pyramidal neurons from slices extracted from control ($n = 11$ rats, 46 neurons), cocaine-induced CPP trained rats ($n = 7$ rats, 16 neurons), or rats infused with Ch-ABC into the mPFC 9 d earlier ($n = 4$ rats, 18 neurons). Control slices are pooled data from intra-mPFC vehicle-injected animals and naive animals. Control experiments were interleaved with all other experimental groups. Holding potential, 70 mV. Calibration: 50 pA, 25 ms. ***B***, ***C***, Pyramidal neurons from slices where animals were previously exposed to cocaine-induced CPP training showed significantly attenuated mIPSC amplitudes compared with cells from control slices (Kolmogorov–Smirnov test, $p < 0.001$). However, mIPSC amplitude was unaltered in brain slices previously microinjected with Ch-ABC (Kolmogorov–Smirnov test, $p < 0.51$). ***D***, ***E***, Cocaine exposure or infusion of Ch-ABC 9 d before slice preparation significantly reduced mIPSC frequency compared with control slices (ANOVA, $p < 0.05$). ***F***, Example traces of action potential firing from control rats ($n = 4$ rats, 10 neurons) and rats infused with Ch-ABC ($n = 3$ rats, 6 neurons) into the mPFC 9 d before slice preparation at various holding currents: 150, 400, and 650 pA. Calibration: 40 mV, 40 ms. ***G***, Microinjection of Ch-ABC into the mPFC 9 d before slice preparation significantly increased the number of action potentials elicited at various holding currents (two-way ANOVA with Sidak's *post hoc*, $p < 0.01$). Data are mean ± SEM. *$p < 0.05$, compared with control slices.

double-labeled neurons within the PL mPFC, without altering the number of WFA- or c-Fos-labeled neurons. Decreased CPP behavior and the corresponding decrease in WFA/c-Fos double-labeled cells suggest that acquisition and reconsolidation processes were impaired. Finally, exposure to cocaine decreased both the frequency and amplitude of mIPSCs on pyramidal neurons within the mPFC, whereas administration of Ch-ABC decreased only the frequency of mIPSCs on pyramidal neurons within the mPFC.

The present study is the first to show that removal of PNNs impairs the acquisition and reconsolidation of a cocaine-associated memory. Treatment with Ch-ABC before training for cocaine CPP may have impaired either or both the acquisition and consolidation of memory. Acquisition and consolidation of memory are traditionally manipulated immediately before or following memory training (for review, see Abel and Lattal, 2001); however, in our study, we treated animals with Ch-ABC 3 d before memory training and observed a disrupted cocaine-induced CPP memory and thus cannot conclude definitively whether initial learning or consolidation of memory was impaired.

The mechanism by which PNN removal disrupts memory stabilization remains unknown. Recent work has shown that removal of PNNs within the anterior cingulate region of the mPFC increases fast rhythmic activity of GABAergic interneurons when tested 3 d following treatment, which is hypothesized to be influenced by a larger cation pool surrounding PNN-surrounded neurons because PNNs are thought to act as a cation buffer (Brückner et al., 1993; Steullet et al., 2014). However, we showed that, in naive rats, Ch-ABC treatment decreased the frequency, but not amplitude, of mIPSCs onto pyramidal neurons when tested 9 d after treatment, suggesting altered presynaptic signaling (Augustine and Kasai, 2007). These studies yield conflicting results, which could be due to differences in brain region and timing following PNN removal. Although our results do not indicate a mechanism for altered presynaptic signaling, it is hypothesized that PNNs play a role in regulating neural plasticity via three possible mechanisms: (1) altering the formation of new neuronal contacts (Corvetti and Rossi, 2005; Barritt et al., 2006); (2) acting as a scaffold for molecules that can inhibit synaptic formation (Deepa et al., 2002); and (3) limiting receptor motility at synapses (Frischknecht et al., 2009). Further work is needed to address the mechanism responsible for the decrease in mIPSC frequency that we observed. In contrast to Ch-ABC-induced effects, cocaine-induced CPP produced decreases in both the frequency and amplitude of mIPSCs when tested 2 d after the last

cocaine exposure and 15 min after reexposure to the cocaine-paired chamber. This latter finding with cocaine is consistent with previous work demonstrating increased excitability in pyramidal neurons in the mPFC following repeated, noncontingent cocaine administration (Dong et al., 2005; Nasif et al., 2005a, b; Huang et al., 2007a, b; Ford et al., 2009; Hearing et al., 2013). Interestingly, we also found increased excitability of mPFC pyramidal neurons 9 d following Ch-ABC administration. Collectively, these studies suggest that Ch-ABC may occlude cocaine-induced adaptations in the mPFC such that it decreases the ability of cocaine to fully modify reward-associated circuitry, leading to attenuation of cocaine-induced CPP behavior. Future studies will be needed to examine changes within PNN-surrounded neurons following cocaine-induced CPP and Ch-ABC administration.

A second possible explanation for disrupted memory following PNN removal is that PNNs are involved in memory stabilization. It has been hypothesized that the memory engram may reside within the pattern of the holes within PNNs (Tsien, 2013). Upon removal of PNNs with Ch-ABC, the pattern of the holes would be disrupted, which may then disrupt memory stabilization and maintenance.

Previous studies examining the role of PNNs in memory demonstrate the importance of timing of Ch-ABC administration relative to behavioral training and/or extinction learning. For example, Gogolla et al. (2009) demonstrated that removal of PNNs within the BLA decreased a conditioned fear memory only when given before fear conditioning and extinction training. When PNNs were removed after fear conditioning, behavior was similar to that of controls. In contrast, impairment of drug memory following removal of PNNs within the BLA required different timing (Xue et al., 2014). Removal of PNNs within the BLA decreased a conditioned drug memory only when Ch-ABC was given before extinction, after the drug memory had been formed. If PNN removal occurred before training, no decrease was observed in the conditioned memory. In contrast, we did not observe differences in the rate of extinction between treatment groups when Ch-ABC was given after training but before extinction, suggesting that PNNs in the PL mPFC do not contribute to extinction learning, in concordance with earlier work in cocaine self-administering rats demonstrating a role for the IL mPFC, but not PL mPFC, in extinction consolidation (LaLumiere et al., 2010). Additionally, removal of PNNs following training and extinction did not alter behavior during a cocaine-induced memory reactivation, whether it was given 3 d or 11–13 d after treatment. This set of findings suggests that the acquisition and maintenance of cocaine CPP memory require intact PNNs within the PL mPFC. This finding also indicates that PNN removal alone is not sufficient to impair cocaine-seeking behavior once the memory is formed but that memory reactivation is required to dampen subsequent cocaine-primed reinstatement. Therefore, removal of PNNs in the PL mPFC may alter mPFC output at the time of memory reactivation, which then blunts subsequent expression of the cocaine-associated memory.

The mechanism by which removal of PNNs alters mPFC output remains unknown. Previous studies have demonstrated a role for GABAergic interneurons within the mPFC in regulating cocaine-seeking behavior (Kalivas and McFarland, 2003; Miller and Marshall, 2004). Administration of GABA agonists impairs cocaine-seeking behavior when given systemically (Heinrichs et al., 2010; Robinson and Franklin, 2010; Robinson et al., 2011), and GABA agonists administered into the mPFC decrease reinstatement when given before the reinstatement test (Fuchs et al., 2005). We show that following confinement in the cocaine-paired chamber, c-Fos levels are increased within PNN-surrounded neurons, of which ~80% are PV-containing, GABAergic interneurons (Table 1). Based on these studies, one possible explanation for our finding is that this increase may be a compensatory response to heightened firing of pyramidal output neurons after exposure to the cocaine-paired chamber. Given that this same level of heightened firing of pyramidal neurons may not occur in Ch-ABC-treated rats, the reduction in c-Fos labeling in Ch-ABC-treated rats may reflect a reduced negative feedback necessary to suppress pyramidal output. A second explanation is that the dose of Ch-ABC was toxic; however, the number of PNNs at behaviorally relevant time points was not decreased, and NeuN staining was not different between treatment groups, suggesting that the dose was not toxic. A more likely explanation for our findings is that PNN-surrounded GABAergic interneurons in the mPFC are involved in establishing and maintaining memories associated with cocaine-seeking behavior. This explanation is more likely because the decrease in c-Fos labeling within PNN-surrounded neurons is observed only in rats displaying impaired memory.

As we and others have shown, PNNs primarily surround PV-containing, GABAergic interneurons (Härtig et al., 1992; Schüppel et al., 2002; Dityatev et al., 2007). PV-containing interneurons have been identified as fast spiking and generate and maintain gamma oscillations necessary for attention, cognitive flexibility, and performance (Sparta et al., 2014). Acute cocaine exposure increases gamma oscillations in layer V of the mPFC (Dilgen et al., 2013); however, the impact of more prolonged cocaine use on gamma oscillations in the mPFC is unknown. Following extended withdrawal from cocaine, the intrinsic excitability of fast-spiking interneurons is increased, which may contribute to abnormal gamma oscillations (Campanac and Hoffman, 2013). Additionally, abnormal gamma activity occurs following relapse to alcohol (De Ridder et al., 2011). PV-containing, fast-spiking, GABAergic interneurons in the mPFC have also been implicated in the pathology of schizophrenia (Costa et al., 2004), and PNN expression is decreased in the mPFC of human subjects diagnosed with schizophrenia (Mauney et al., 2013). Future studies are needed to evaluate the contribution of PNNs to gamma oscillations and the resulting role in addiction.

In conclusion, this study is the first to demonstrate a contribution of mPFC PNNs to drug-associated memories. Here we show that removal of PNNs disrupts acquisition and reconsolidation of cocaine-induced CPP, which corresponds to a decrease in the number of cells expressing c-Fos surrounded by PNNs. In addition, we show that Ch-ABC administration decreases the frequency of mIPSCs, whereas cocaine administration decreases both the frequency and amplitude of mIPSCs in mPFC pyramidal neurons. Together, our results suggest that removal of PNNs within the mPFC impairs cocaine-induced behavioral plasticity during acquisition and reconsolidation of a cocaine-reward memory and implicate PNNs as therapeutic targets for disruption of cocaine-associated memories.

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