Behavioral/Systems/Cognitive

Extinction Training after Cocaine Self-Administration Induces Glutamatergic Plasticity to Inhibit Cocaine Seeking

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Learning to inhibit drug seeking can be an important strategy for inhibiting relapse, and this can be modeled by extinguishing drug seeking in response to a drug-paired context. Rats were either extinguished or withdrawn without extinction training (abstinence) from cocaine self-administration, and measurements of postsynaptic density proteins in the core and shell subcompartments of the nucleus accumbens were compared with yoked-saline controls. Only extinguished rats had elevations of PSD-95, Homer1b/c, and Narp in the postsynaptic density of the core, whereas no proteins measured were altered in the postsynaptic density of the shell in either extinguished or abstinent rats. Using a biotinylation strategy, it was found that surface expression of mGluR5 was reduced only in the core of extinguished animals. Although both extinguished and abstinent animals showed a reduction in long-term potentiation elicited in the core by stimulating prefrontal cortex, blunted long-term depression was observed only in extinguished rats. These data indicate that the elevation in Homer1b/c in the core may have sequestered mGluR5 away from the membrane surface and that the loss of surface mGluR5 inhibits long-term depression. Accordingly, when Homer1c was overexpressed in the core of cocaine-naive rats with an adenoassociated virus, long-term depression was inhibited. This mechanism may contribute to the inhibition of cocaine seeking by extinction training because overexpression of Homer1c in the core also inhibited cue-induced reinstatement of cocaine seeking. These data identify a cellular mechanism that may contribute to extinction-induced inhibition of cocaine seeking.

Introduction

Cocaine addiction is a disease characterized by relapse to drug taking, even after long periods of drug abstinence (O'Brien, 2001). One strategy for inhibiting relapse is psychosocial interventions promoting behavioral self-regulation that can include training to extinguish responding to drug-associated environmental stimuli (Conklin and Tiffany, 2002; Havermans and Jansen, 2003). A widely used animal model of relapse is the extinction–reinstatement paradigm. This model combines training animals to self-administer drug with extinction training to inhibit responding in the drug-associated context. Once behavioral responding is inhibited, the drug-seeking response is reinstated with stimuli known to cause relapse in humans, including stress, cues previously associated with drug delivery, and/or the drug itself (Epstein et al., 2006). Using this protocol to model cocaine addiction has revealed enduring neuroplasticity in glutamatergic synapses in the nucleus accumbens (Kauer and Malenka, 2007; Kalivas et al., 2009), a key brain nucleus mediating cocaine seeking (Koob et al., 2004). However, it remains to be determined which of these adaptations result from cocaine self-administration and which may be associated with the learning and memory consolidation elicited by extinction training (Quirk and Mueller, 2008).

There is reason to suspect that adaptations in the nucleus accumbens may be induced by extinction training. For example, inactivation of the projection from the prefrontal cortex (PFC) to the core compartment of the nucleus accumbens (NAcore) blocks the reinstatement of cocaine seeking in extinguished animals (Cornish et al., 1999; Park et al., 2002; Di Ciano et al., 2008). However, inactivation of neither the prefrontal cortex (Fuchs et al., 2006) nor ventral striatum (See et al., 2007) inhibits relapse in animals undergoing self-administration followed by 2–3 weeks of abstinence without extinction training (referred to herein as "abstinent relapse"), indicating that extinction training may recruit this projection to regulate cocaine seeking. Also, extinction training upregulates GluR1 and/or GluR2 subunits of the AMPA receptor in the shell subcompartment (NAshell) (Sutton et al., 2003; Ghasemzadeh et al., 2009b). Correspondingly, viral overexpression of GluR1 in the NAshell facilitates extinction learning and attenuates reinstated cocaine seeking, whereas overexpression of "pore-dead" GluR1 potentiates cocaine-induced reinstatement (Sutton et al., 2003; Bachtell et al., 2008). Finally, inactivation of the infralimbic PFC projection to the NAshell reverses extinction-induced inhibition of cocaine seeking (Peters et al., 2008).

Based on these data, it has been proposed that extinction training induces glutamatergic plasticity in the projection from

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the PFC to NAshell that reduces the reinstatement of cocaine seeking (Self et al., 2004; Peters et al., 2009). To expand on this hypothesis, we determined the levels of expression for a number of glutamate receptor and postsynaptic density (PSD) scaffolding proteins in the NAcore and NAshell after cocaine self-administration and 3 weeks of either abstinence or extinction training. Surprisingly, the strongest pattern of extinction training-associated protein changes was in NAcore, not NAshell, and was in part related to mGluR5 and its signaling/scaffolding protein Homer (Xiao et al., 1998; Fagni et al., 2002). Correspondingly, extinction training was shown to inhibit mGluR5-dependent long-term depression (LTD) in the NAcore and Homer1c overexpression inhibited both long-term depression and cue-induced reinstatement of cocaine seeking.

Materials and Methods

Subjects. Male Sprague Dawley rats (270–300 g at the time of surgery; Charles River) were used in this study. They were individually housed in a temperature-controlled vivarium (22°C) on a 12 h light/dark cycle (lights on at 7:00 A.M.) with food and water *ad libitum*. All methods used were in compliance with National Institutes of Health guidelines for care of laboratory animals and were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Surgery. For the implantation of catheters, rats were anesthetized with ketamine HCl (87.5 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.). Ketorolac (3 mg/kg, i.p.) was administered before surgery to provide analgesia. Catheter construction and surgical implantation has been described in detail previously (Moussawi et al., 2009). For experiments requiring intraaccumbal virus infusions, the catheter implantation was followed immediately sterotaxic implantation of guide cannula over the NAcore (anteroposterior, ±1.7 mm; mediolateral, ±2.5 mm at 6° angle; dorsoventral, −5.5 mm) (Paxinos and Watson, 1986). Rats were allowed to recover 6–10 d after surgery before the start of the experimental protocol.

Cocaine self-administration, extinction, and abstinence procedures. All self-administration procedures occurred in standard operant chambers with two retractable levers, a house light, a cue light, and tone generator (MED Associates). Before the initiation of cocaine self-administration training, all animals were food deprived for 24 h and then underwent a single 15 h session in which presses on the active lever (the right lever) resulted in the delivery of a single food pellet (45 mg; Noyes). Subsequent to food training, animals were food restricted for the remainder of the experiment and were given 20 g of food immediately after each daily drug self-administration session. One day later, subjects ($n = 114$) began cocaine self-administration on an FR1 schedule with a 20 s time-out. Active lever presses made during the time-out were recorded but did not result in drug delivery. Each active lever press produced a 0.05 ml infusion of 0.20 mg of cocaine (dissolved in 0.9% sterile saline; kindly provided by National Institute on Drug Abuse) and the presentation of drug-paired cues (illumination of the light over the active lever and the presentation of a 2900 Hz tone). A subset of animals ($n = 73$) served as yoked-saline controls and received a saline infusion each time their cocaine counterpart received a cocaine infusion. Each cocaine self-session lasted 2 h or until the rats had taken a maximum of 200 infusions. Self-administration continued until subjects had attained 12 d with a minimum of 10 cocaine infusions. Subjects then were divided into two groups: one group undergoing extinction training and another undergoing home cage abstinence. During extinction training, presses on the previously active lever were recorded but no longer produced drug or presentation of the drug-paired cues. Animals in the abstinence group remained in their home cages with the exception of a 2 h period during which they were removed and placed into an alternative environment (standard housing Plexiglas cage) to control for the effects of handling and daily removal from the home environment. Extinction or abstinence procedures continued for 3 weeks, at which point animals were decapitated and NAcore and NAshell tissue was dissected. In a subset of animals ($n = 18$), extinction training only lasted for 2 d and animals were decapitated 24 h after the last extinction session.

Food self-administration procedures. To control for the general effect of extinction training, one group of animals ($n = 9$) self-administered sucrose pellets for 12 d and then underwent 3 weeks of extinction training. Control animals ($n = 9$) were placed in the self-administration chambers daily but did not receive food reinforcement.

Subfractionation and Western blotting methods. Dissected NAshell and NAcore were homogenized in sucrose buffer containing protease inhibitors. After spinning at 1000 × *g*, the supernatant was retrieved and spun at 12,000 × *g*. The resulting pellet was resuspended in 1 mM EDTA, 4 mM HEPES, and protease inhibitors, and spun at 12,000 × *g*. The supernatant was discarded and the pellet was resuspended in 0.5% Triton X in PBS and spun at 12,000 × *g*. The resulting pellet contained the PSD (Triton X-insoluble) fraction, and the supernatant was retained as the non-postsynaptic membrane fraction (non-PSD) (Triton X-soluble). The pellet containing the PSD fraction was suspended in 1% SDS in radioimmunoprecipitation assay buffer. Protein content was measured using the Bradford assay, and two samples were pooled to generate sufficient tissue for immunoblotting. Proteins were separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blocked in 3% milk and probed overnight at 4°C with primary antibody against GluR1 (1:200; Millipore Bioscience Research Reagents), washed with TBS-Tween, and incubated with secondary antibody at room temperature. After visualization (Pierce Western Mouse Pico kit) of GluR1, membranes were reprobed with Homer1b/c antibody (1:5000; Millipore Bioscience Research Reagents), then neuronal activity regulated pentraxin (NARP) (1:3000; Paul Worley, Baltimore, MD), and subsequently with PSD-95 (1:2500; Sigma-Aldrich). Separate sets of membranes was used to probe for GluR2 (1:300; BD Biosciences), mGluR1 (1:1000; Millipore), mGluR5 (1:20,000; Millipore), and mGluR2/3 (1:5000; Millipore). Both mGluR1 and mGluR5 are present in monomer and dimer forms, and we report dimer expression because this is the active form of group I mGluRs (Pin et al., 2003; Kniazeff et al., 2004; Tateyama et al., 2004). Figure 1*B* illustrates the relative capacity of this subfractionation procedure to separate PSD from non-PSD proteins.

Biochemical measurement of surface-expressed mGluR5. A subset of animals that underwent cocaine self-administration and either 3 weeks of extinction training ($n = 9$) or abstinence ($n = 7$) and their yoked-saline counterparts (extinction controls, $n = 9$; abstinence controls, $n = 7$) were killed via rapid decapitation. The NAcore was dissected and sliced into prism-shaped sections (200 μm) with a McIlwain tissue chopper. The tissue was incubated for 1 h in artificial CSF (aCSF) containing 1 mg/ml sulfo-NHS-SS-biotin (Pierce) at 4°C with gentle shaking. Unreacted biotinylation reagent was removed by two 10 min washes in ice-cold aCSF and quenched by two 20 min washes in ice-cold aCSF containing 100 mM glycine. The sections were then homogenized by sonication in 150 μl of lysis buffer containing 25 mM HEPES, 150 mM NaCl, 1% Triton X-100, and Complete Mini protease inhibitors (Roche). Homogenates were subsequently centrifuged (10,000 × *g* at 4°C for 10 min) to remove insoluble material. A total of 250 μg of each sample lysate was incubated overnight at 4°C with 50 μl of streptavidin agarose beads (Sigma-Aldrich) and the remainder of the sample was stored at −80°C as the total protein fraction (T). Biotinylated proteins attached to streptavidin-coated beads were separated by centrifugation from the nonbiotinylated proteins (NB) in the supernatant. The beads were washed three times at 4°C in lysis buffer and once in 50 mM Tris-HCl, pH 7.4. Biotinylated proteins (B) were eluted with Laemmli sample buffer containing 100 mM dithiothreitol. The amount of mGluR5 protein in the total and biotinylation fractions was analyzed by quantitative Western blotting using anti-mGluR5.

Electrophysiological recordings. Extracellular field potentials were recorded after 2–3 weeks of extinction ($n = 7$) or abstinence ($n = 11$) in urethane-anesthetized rats that were mounted in a stereotaxic apparatus. Detailed methods describing this experiment have been published previously (Moussawi et al., 2009). Data were collected every 30 s at a 10 kHz sampling frequency, and then averaged every 1 min. Pulse width was set to 0.3 ms and basal stimulation intensity corresponded to 30–40% of minimum current intensity that evoked a maximum field response for long-term potentiation (LTP) experiments, and 40–50% for LTD exper-

iments. Baseline data were collected for 20–30 min before the induction protocol (LTP or LTD). Field potential amplitude was measured as the difference between the mean of a 2–4 ms window before the stimulation artifact and the mean of a 1 ms window around 15 ms after the stimulation artifact (corresponding to the negative peak of the field potential). Data were then normalized to baseline. The LTP protocol involved tetanic stimulation at the minimum current intensity that evoked a maximum field response (from an input–output curve) using two bursts of 100 pulses at 50 Hz (2 s), with 20 s interburst interval. The LTD protocol involved stimulation at the minimum current intensity that evoked a maximum response using three trains of 900 pulses at 5 Hz (3 min), with a 5 min intertrain interval.

Homer1c overexpression. Recombinant adenoassociated viral vectors with equal ratios of serotype 1 and 2 capsid proteins (rAAV1/2) carrying the cDNA for full-length hemagglutinin (HA)-tagged rat Homer1c ($n = 18$) or enhanced green fluorescent protein (GFP) ($n = 16$) as a control were constructed and infused into the NAcore as described previously (Szumlinski et al., 2006). AAV1/2 vectors specifically transduce neurons (Klugmann et al., 2005) and transgene expression is controlled by the strong CBA (chicken-β-actin) promoter. For the measurement of long-term depression, five animals were infused with adenoassociated virus (AAV) vectors 2 weeks before electrophysiolgical recording. For the reinstatement experiment, microinjections occurred 24 h after the final self-administration session and animals either began extinction training on the day after the viral infusion ($n = 21$) or experienced abstinence in the home cage for 2 weeks before beginning extinction training ($n = 13$). Animals in both groups underwent extinction training for a minimum of 2 weeks or until the extinction criterion was achieved (25% of active lever pressing during self-administration), and cue-induced reinstatement tests were conducted during which the cues previously paired with cocaine (light plus tone) were again presented on active lever pressing. Responses on the previously active lever were recorded but did not result in drug infusions. For the measurement of LTD, five cocaine-naive animals were injected with Homer1c or GFP AAV into the contralateral NAcore as described above. Two to 3 weeks later, the rats underwent the induction of LTD as described above by recording at the AAV infusion site and stimulating the prelimbic region of the prefrontal cortex. Each hemisphere of the brain was examined for the induction of LTD, with the Homer1c side examined first in three of the five animals.

Histology. Rats were overdosed with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused with 0.9% saline. The brains were removed and stored in 10% formalin for at least 24 h. Coronal sections (150 μm thick) were mounted on slides and stained with cresyl violet. Sites of injector needles or recording electrodes were verified with a light microscope. To verify overexpression of Homer AAV, slides were incubated with mouse anti-HA primary antibody (Covance; 1:1000), and labeled proteins were detected using a biotinylated anti-mouse IgG and visualized with diaminobenzidine (Szumlinski et al., 2006).

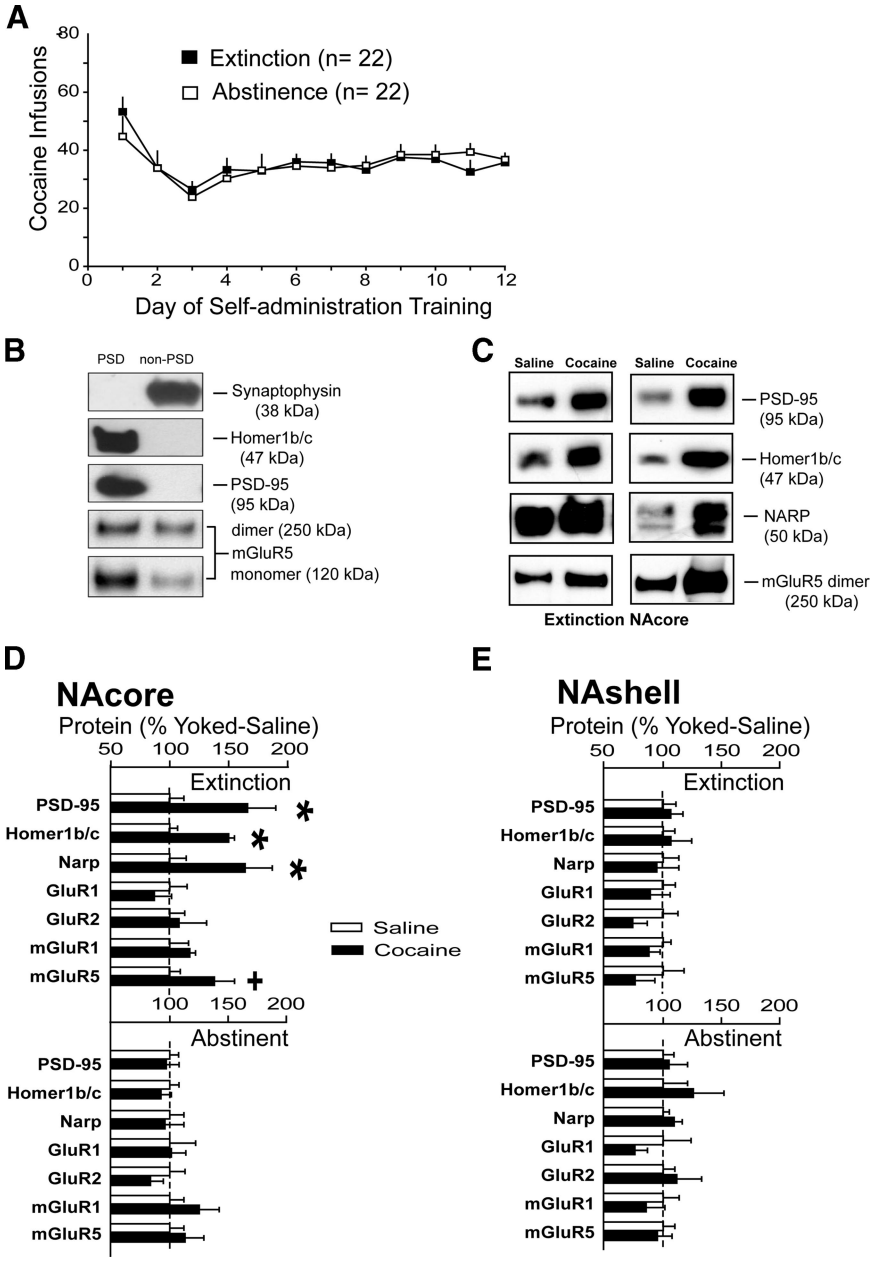

Figure 1. Extinction training (Ex) and abstinence (Ab) after cocaine self-administration produced different patterns of changes in protein expression in the PSD-enriched fraction. ***A***, The mean number of infusions did not differ between Ab and Ex groups. ***B***, Representative Western blots demonstrating the composition of PSD and non-PSD fractions based on the presence of characteristic proteins. ***C***, Sample Western blots showing protein upregulation in the Ex group. ***D***, NAcore PSD-enriched fraction protein expression. Two-tailed Student's t test revealed that PSD-95 ($t_{(13)} = 2.50$; $p = 0.027$), Homer1b/c ($t_{(15)} = 3.15$; $p = 0.007$), NARP ($t_{(14)} = 2.22$; $p = 0.043$), and mGluR5 ($t_{(20)} = 2.00$; $p = 0.059$) were increased after extinction training but not abstinence. ***E***, Nucleus accumbens shell PSD-enriched fraction protein expression showed no differences after extinction training or abstinence. $N = 6–12$ for all groups. $^{*}p < 0.05$; $^{+}0.05 > p < 0.06$. Error bars indicate SEM.

Results

Increased protein expression in NAcore after extinction training but not abstinence

Animals underwent 2 weeks of cocaine self-administration and 3 weeks of either extinction training or abstinence in the home cage with daily handling. Control animals received saline infusions when their yoked cocaine counterpart self-administered cocaine. Akin to treatment animals, yoked control animals were divided into abstinent and extinction treatment groups. Figure 1*A* shows

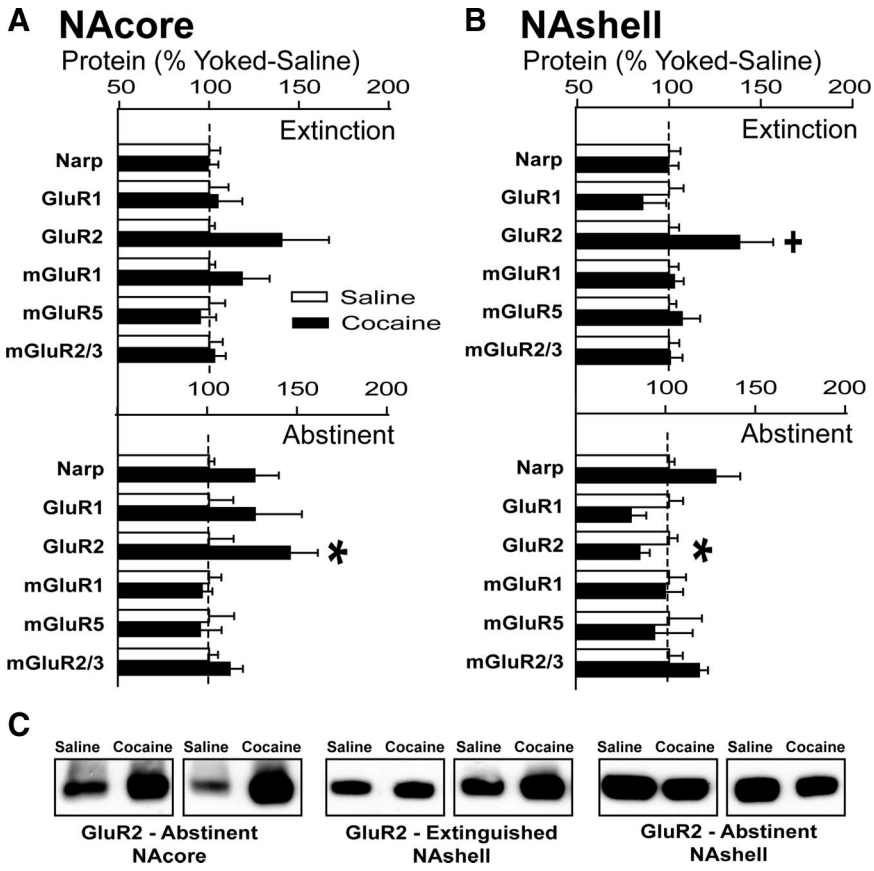

Figure 2. Extinction training and abstinence after cocaine self-administration produced different patterns of changes in protein expression in the non-PSD subfraction (Triton X-100-soluble fraction). ***A***, No change was measured in the NAcore in extinguished subjects, whereas changes after abstinence were observed in levels of GluR2 ($t_{(12)} = 2.18$; $p = 0.050$). ***B***, In the NAshell non-PSD fraction, GluR2 ($t_{(11)} = 2.13$; $p = 0.057$) was changed in extinguished animals, and in abstinent animals changes were measured in GluR2 ($t_{(12)} = 2.38$; $p = 0.035$). $N = 6–12$ for all groups. ***C***, Representative immunoblots of significant changes in protein level. $^{*}p < 0.05$; $^{+}0.05 > p < 0.06$. Error bars indicate SEM.

no differences in the mean number of cocaine infusions self-administered by the abstinent and extinction groups (active lever presses are not shown but were not different between groups). Stable amounts of extinguished active lever pressing were achieved within 1 week of training, in rats undergoing extinction training (data not shown). Figure 1, *C* and *D*, shows that, although no changes were measured in the PSD-enriched subfraction of the NAcore in abstinent subjects, expression of PSD-95, Homer1b/c, and Narp were increased in cocaine animals after extinction training. In addition, mGluR5 showed a trend toward an increase ($p < 0.06$).

In contrast to the changes observed in the NAcore after extinction training, there was no change in NAshell PSD protein expression in either the abstinent or extinction groups (Fig. 1*E*). In the non-PSD fraction (Triton X-100-soluble fraction), Figure 2*A* reveals only GluR2 expression was increased in the NAcore of cocaine self-administering animals after abstinence. Figure 2*B* shows a trend ($p < 0.06$) toward increased GluR2 in extinguished subjects and reduced GluR2 in the non-PSD subfraction of the NAshell in abstinent animals.

Control extinction protocols do not affect protein expression

To ensure that the increase in glutamatergic protein expression in the PSD-enriched fraction of the NAcore (Fig. 1*D*) was not a consequence of extinction training regardless of the original learned response, a group of animals was trained to self-administer sucrose pellets. Figure 3 shows that animals rapidly extinguished their responding on the previously active lever, and after 3 weeks of extinction from sucrose self-administration, the expression of PSD-95, Homer1b/c, NARP, and mGluR5 was unchanged relative to control rats in the PSD subfraction of the NAcore (Fig. 3*B*). To investigate the amount of post-cocaine extinction training necessary to elicit the protein upregulation, we examined animals with only 2 d of extinction training after 2 weeks of cocaine self-administration (Fig. 3*C*). After 2 d of extinction training, the levels of PSD-95, Homer1b/c, Narp, and mGluR5 were unchanged in the PSD fraction of the NAcore (Fig. 3*D*) or NAshell (data not shown). Thus, the protein upregulation elicited in the NAcore after 3 weeks of extinction from cocaine self-administration was not a consequence of either cocaine self-administration or extinction learning alone, but rather a combination of these two training procedures.

Extinction training alters the surface expression of mGluR5

Given that one function of Homer1b/c is the reduction of mGluR1/5 membrane surface expression through intracellular retention (Fagni et al., 2002; Kammermeier, 2006), we hypothesized that the upregulated Homer1b/c (Fig. 1*D*) promotes the internalization and sequestering of mGluR5 into the PSD subfraction, thereby accounting for both the loss of LTD (Moussawi et al., 2009) and increase in PSD levels of mGluR5 (Xiao et al., 1998; Fagni et al., 2002). To examine this possibility, a biotinylation strategy was used to measure the surface expression of mGluR5 in the NAcore after 3 weeks of extinction training or abstinence in rats trained to self-administer cocaine. Figure 4 shows that the surface expression of mGluR5 dimer was reduced after extinction training, but not abstinence, supporting the hypothesis that upregulated Homer1b/c in the NAcore after extinction training serves to internalize and sequester mGluR5 in the PSD.

Extinction training is required for cocaine-induced loss of long-term depression

Two well characterized forms of neuroplasticity at glutamatergic synapses in the CNS are LTP and LTD (Malenka and Bear, 2004), and the proteins found to be upregulated in the NAcore by extinction training have been linked to LTP and/or LTD. Narp multimerizes with neuronal pentraxin1 to promote clustering of AMPA receptors and synaptogenesis (Xu et al., 2003). Since AMPA receptor clustering promotes LTP (Andrásfalvy and Magee, 2004) and Narp mRNA has been shown to be upregulated after LTP induction (Wibrand et al., 2006), it follows that upregulating Narp by extinction training might lead to enhanced LTP. Conversely, targeted disruption of PSD-95 in the hippocampus enhances LTP induction (Migaud et al., 1998) and nucleus accumbens LTP is augmented as a consequence of re-

duced PSD-95 levels (Yao et al., 2004), supporting a reduction in LTP by the upregulated PSD-95 in extinguished animals. The changes in PSD proteins also provide a mixed expectation regarding LTD. Homer1b/c binds mGluR5 and can thereby promote either retention of mGluR5 on the surface or internalization, depending on the *in vitro* experimental conditions (Xiao et al., 1998; Fagni et al., 2002). Endocannabinoid-mediated LTD in the accumbens is mGluR5 dependent (Robbe et al., 2002). Accordingly, surface retention or internalization of mGluR5 via increased Homer1b/c binding would be expected to promote or inhibit LTD, respectively.

Impaired LTP and LTD was recently recorded in field potentials measured in the NAcore after prefrontal cortex stimulation in animals extinguished from cocaine self-administration (Moussawi et al., 2009). Using this model, we compared animals undergoing extinction training or abstinence from 2 weeks of cocaine self-administration for the capacity to induce LTP and LTD in the NAcore. Figure 5*A* shows that LTP was impaired in both extinguished and abstinent animals. However, LTD was impaired only after extinction training (Fig. 5*B*). This implies that the selective upregulation in Narp and PSD-95 in the NAcore of extinguished animals does not confer differences in the ability to induce LTP. However, upregulation of Homer1b/c and internalization of mGluR5 after extinction training may be linked to a loss of LTD. To verify this hypothesis, we used an AAV infection strategy to induce bilateral overexpression of HA-tagged Homer1c in the NAcore of cocaine-naive animals and measured LTD (for an example of Homer1c-overexpressing neurons in the NAcore, see Fig. 6*E*). We found that Homer1c overexpression impaired the induction of LTD by low-frequency stimulation, indicating that, in extinguished animals, the increase of Homer1b/c expression could be mediating the reduced capacity to induce LTD.

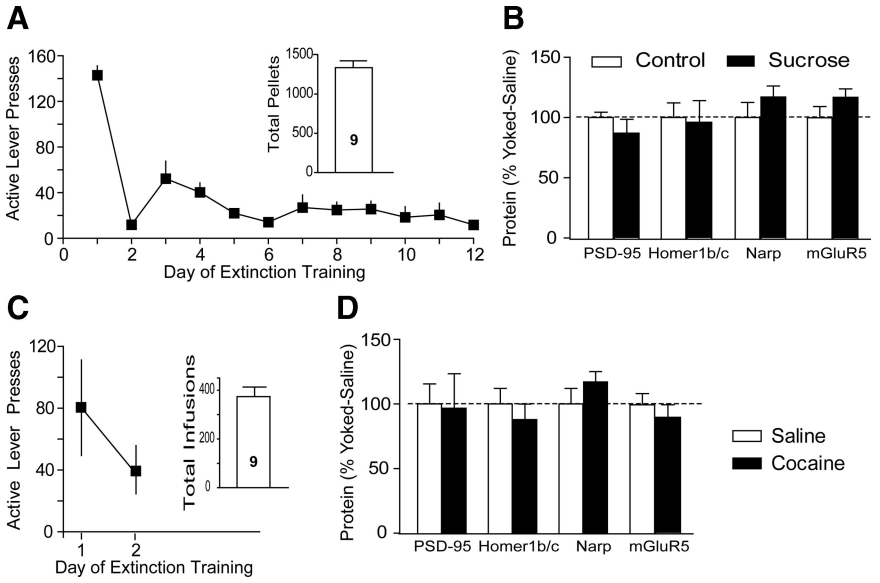

Figure 3. Extinction-associated changes in protein require both extinction training and cocaine self-administration. ***A***, Mean active lever responses during the first 12 d of extinction training after sucrose pellet self-administration. Inset, Total number of sucrose pellets earned during self-administration training. $N = 9$. ***B***, There were no changes in protein expression of PSD-95, Homer1b/c, and NARP in the NAcore after 2 weeks extinction of sucrose-reinforced responding. $N = 6–12$ in each group. ***C***, Mean active lever responses during the 2 d after cocaine self-administration. Inset, Total number of cocaine infusions earned during self-administration training. $N = 9$. ***D***, Nucleus accumbens core PSD-fraction protein expression. There were no changes in protein expression of PSD-95, Homer1b/c, and NARP after 2 d of extinction training of cocaine-reinforced responding. $N = 6–12$ in each group. Error bars indicate SEM.

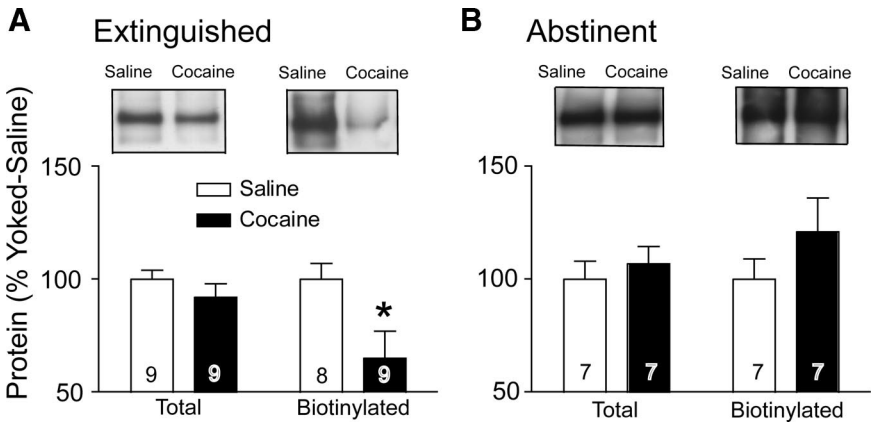

Figure 4. Surface expression of mGluR5 is reduced after extinction training but not abstinence from cocaine self-administration. ***A***, Surface expression of mGluR5 was significantly decreased after cocaine self-administration and 3 weeks of extinction ($t_{(1,14)} = 3.31$; $p = 0.006$), whereas expression was unaltered in a whole-cell fraction. ***B***, Surface expression of mGluR5 was not altered after abstinence from cocaine self-administration. *N* is shown in bars. Error bars indicate SEM.

Overexpression of Homer1b/c in the NAcore inhibits cue-induced reinstatement

Upregulating Homer1b/c and reducing the surface expression of mGluR5 and ability to induce LTD could be either a pathologic consequence of cocaine self-administration and extinction training or a compensatory mechanism serving to reduce additional cocaine seeking. Based on literature showing that virally mediated overexpression of Homer1 long forms prevents behavioral sensitization induced by chronic cocaine (Szumlinski et al., 2006) and that Homer1 gene deletion produces a cocaine-sensitized phenotype (Szumlinski et al., 2004), we predicted that overexpression of Homer1c would attenuate the reinstatement of cocaine seeking. We infused Homer1c AAV into the NAcore after cocaine self-administration. Figure 6*E* shows that this procedure induced robust and localized expression of HA-tagged Homer1c in soma and fibers selectively in the NAcore. This AAV requires 2 weeks to achieve maximum expression (Szumlinski et al., 2006), allowing us to examine two conditions of Homer1c overexpression: first, virus infusion followed immediately by 3 weeks of extinction training (Fig. 6*A*,*C*), and second, virus infusion followed by a 2 week abstinence period before initiating extinction training (Fig. 6*B*,*D*). Using either protocol, there was no effect of Homer1c overexpression on extinction responding compared with animals bilaterally infected in the NAcore with GFP control

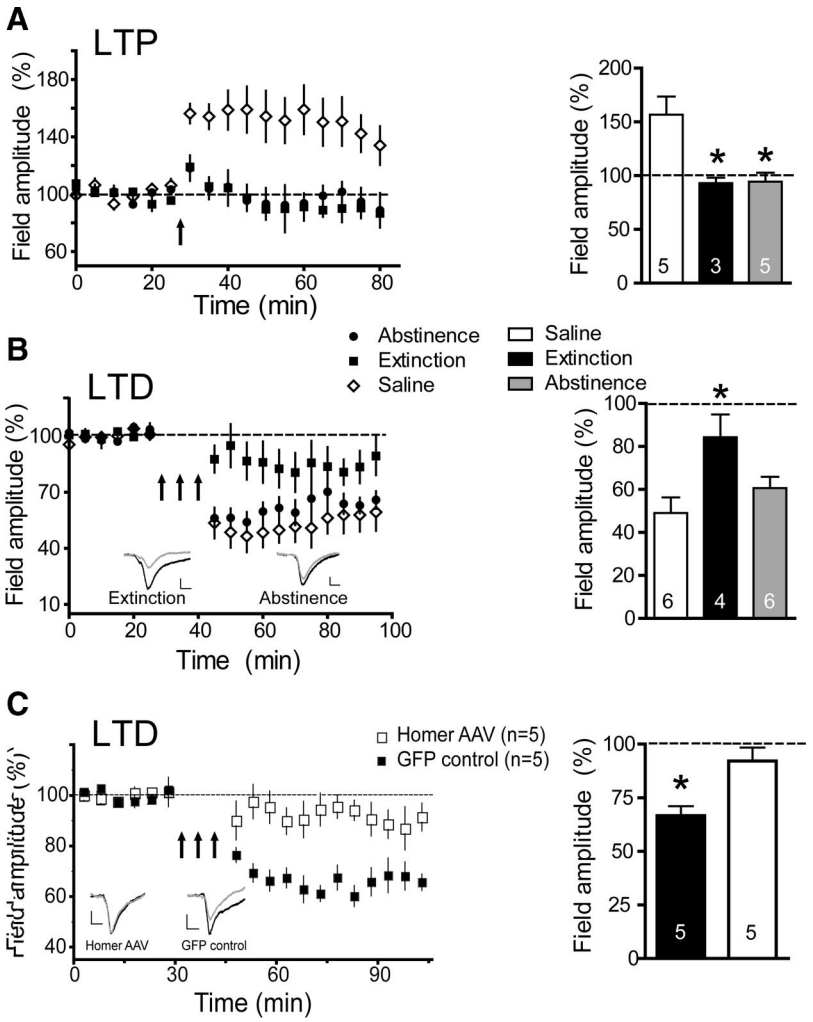

Figure 5. Extinction training inhibits the induction of LTD in the NAcore elicited by PFC stimulation. ***A***, Rats trained to self-administer cocaine show blunted LTP regardless of abstinence or extinction training (time, $F_{(16,32)} = 36.94, p < 0.001$; treatment, $F_{(2,32)} = 4.67, p = 0.030$; interaction, $F_{(32,208)} = 3.24, p < 0.001$). The right panel shows the average field amplitude measured between 15 and 30 min after high-frequency stimulation ($F_{(2,12)} = 8.30; p = 0.008$). ***B***, Only rats extinguished from cocaine self-administration show blunted LTD (time, $F_{(16,32)} = 5.26, p < 0.001$; treatment, $F_{(2,32)} = 8.90, p = 0.006$; interaction, $F_{(32,160)} = 5.63, p < 0.001$). The right panel shows the average field amplitude measured between 15 and 30 min after low-frequency stimulation ($F_{(2,13)} = 5.22; p = 0.022$). ***C***, When Homer1c was overexpressed in the NAcore of cocaine-naive animals, LTD was blunted relative to GFP-infused controls (time, $F_{(17,136)} = 13.37, p < 0.001$; treatment, $F_{(1,136)} = 17.49, p = 0.003$; interaction, $F_{(17,136)} = 5.658, p < 0.001$). The right panel shows the average field amplitude measured between 15 and 30 min after low-frequency stimulation ($t_{(1,9)} = 17.483; p = 0.003$). *N* is shown in bars. $^*p < 0.05$, using Dunnett's *t* test to compare extinction and abstinent groups to yoked-saline. Error bars indicate SEM.

virus (Fig. 6*A*,*B*). Notably, in the experiment in which a 2 week abstinence phase was imposed before the initiation of extinction training, exposure to the operant chamber on the first day of extinction served as an abstinent-relapse test in the presence of full Homer1c overexpression. The lack of difference between GFP- and Homer1c-infected animals on day 1 of extinction in Figure 6*B* revealed that Homer1c overexpression in the NAcore did not inhibit abstinent relapse. In contrast, Figure 6, *C* and *D*, reveals that, when using either infection protocol, Homer1c overexpression attenuated cue-induced reinstatement of cocaine seeking.

Discussion

Extinction training and abstinence after cocaine self-administration produced different patterns of protein expression in the NAcore and NAshell. Notably, extinction training upregulated the level of proteins that modulate the expression and clustering of glutamate receptors in the PSD, including PSD-95, Narp, and Homer1b/c. The extinction-induced increase in Homer1b/c was associated with enhanced internalization and sequestering of mGluR5 in the PSD subfraction, which likely accounted for the selective loss of mGluR5-dependent LTD in the extinguished, but not abstinent animals. Supporting this connection, overexpression of Homer1c promotes intracellular retention of mGluR5 (Kammermeier, 2006) and resulted in a loss of the ability to elicit LTD in the NAcore and also inhibited cue-induced reinstatement.

Altered protein expression in the NAcore by extinction training inhibits reinstatement

The possibility that upregulated Homer1b/c in the NAcore may be a neuroadaptation produced by extinction training to inhibit cocaine seeking is consistent with the literature. Other than the immediate-early gene-like product Homer1a, all forms of Homer are constitutively expressed in the PSD and possess a coiled-coil motif at the C terminal that permits multimerization between Homer proteins and mGluR1/5, forming a signaling and trafficking complex (Xiao et al., 1998; Fagni et al., 2002). The interaction with Homer1b/c includes reducing mGluR1/5 membrane surface expression through intracellular retention (Fagni et al., 2002; Kammermeier, 2006). Accordingly, we found that mGluR5 surface expression was reduced in the presence of extinction training-induced increases in Homer1b/c. The fact that mGluR5 levels were also marginally increased (Fig. 1*D*) indicated not only internalization, but sequestration of the receptor in the PSD subfraction. Ghasemzadeh et al. (2009a) reported no change in mGluR5 monomer subunit expression after extinction and an increase after abstinence; however, the dimer is the active form of the receptor (Pin et al., 2003; Kniazeff et al., 2004; Tateyama et al., 2004).

The reduced surface expression of mGluR5 in extinguished animals was associated with a loss of LTD and is consistent with the mGluR5-dependent LTD shown previously in the nucleus accumbens (Robbe et al., 2002; Moussawi et al., 2009). Moreover, inhibition of mGluR5 binding to Homer proteins prevents mGluR5-dependent LTD in the hippocampus (Ronesi and Huber, 2008). Also, a loss of mGluR5-dependent LTD in the accumbens after a single noncontingent injection of cocaine is accompanied by an increase in the expression of long-form Homer proteins (which includes Homer1b/c), and decreased surface expression of mGluR5 (Fourgeaud et al., 2004). The preservation of LTD in abstinent animals contrasts a previous study showing a loss of LTD in the NAcore after 21 d of abstinence in rats trained to self-administer cocaine (Martin et al., 2006). Ex-

planations for this discrepancy include the fact that Martin et al. conducted *in vitro* whole-cell recordings while we measured *in vivo* field potentials. *In vivo* recordings permit selective stimulation of PFC afferents into the NAcore, whereas *in vitro* electrical stimulation may activate multiple glutamatergic afferents to accumbens spiny cells. Supporting stimulating multiple afferents as a potentially important distinction, withdrawal from noncontingent chronic cocaine administration differentially alters the capacity of *in vivo* hippocampal versus prefrontal cortex stimulation to induce LTP or LTD in the NAshell (Goto and Grace, 2005).

The present data indicate that the upregulated Homer1b/c-induced internalization of mGluR5 and loss of LTD may contribute to the inhibition of cocaine seeking produced by extinction training. Thus, overexpression of Homer1c in the NAcore inhibited cue-induced reinstatement in extinguished animals but did not alter abstinent relapse. Previous studies also show that Homer1 negatively regulates cocaine reward learning and behavioral plasticity. Homer1 gene deletion predisposes mice to cocaine conditioned place preference and locomotor sensitization (Szumlinski et al., 2004), and viral overexpression of Homer1c in the accumbens prevents both locomotor sensitization and the reductions in extracellular glutamate in the NAcore elicited by chronic noncontingent cocaine administration (Szumlinski et al., 2006). In addition, mGluR5 regulates the expression of cocaine behavior and the development of behavioral plasticity to chronic cocaine in a manner consistent with the reduction in surface expression of mGluR5 observed in the present study. For example, constitutive deletion of the mGluR5 gene renders mice incapable of developing cocaine-induced locomotor sensitization or reward learning (Chiamulera et al., 2001), and pharmacological antagonism of mGluR5 attenuates cue- and cocaine-induced reinstatement of drug seeking (Bäckström and Hyytiä, 2006; Kumaresan et al., 2009). Conversely, glutamatergic tone on mGluR5 in the accumbens is reduced after chronic cocaine administration because of downregulated cystine–glutamate exchange (Madayag et al., 2007; Moussawi et al., 2009), and restoring glutamatergic tone onto mGluR5 with *N*-acetylcysteine after withdrawal from cocaine self-administration not only potentiates cocaine-induced reinstatement in the presence of an allosteric mGluR5 agonist but also restores the capacity to induce LTD in the NAcore (Moussawi et al., 2009). Considered together, the present and previous studies support a conclusion that extinction training is upregulating Homer1b/c levels in the NAcore causing internalization of mGluR5 and loss of LTD, thereby inhibiting cocaine seeking.

Although the loss of the ability to elicit LTD in the NAcore can be induced by increasing Homer1c expression (Fig. 5*C*), it is possible that the loss of LTD is an epiphenomenon of decreased mGluR5 surface expression that is unrelated to the attenuation of reinstatement. Stimulation of mGluR5 receptors in the accumbens has many intracellular consequences in addition to being essential for the induction of mGluR5-dependent LTD. For example, mGluR5 stimulation leads to the appearance of NMDA receptor-dependent slow inward currents in medium spiny neurons (D'Ascenzo et al., 2007), increased ERK (extracellular signal-regulated kinase) activation (Mao et al., 2005), and increased phosphorylation of protein kinase Cε (Olive et al., 2005). Thus, the loss of LTD is just one of many possible mechanisms by which decreased mGluR5 signaling in the accumbens could lead to attenuated reinstatement of cocaine seeking. However, preventing the induction of LTD in the nucleus accumbens has been shown to prevent the expression of behavioral sensitization to amphetamine (Brebner et al., 2005), indicating that there are addiction-related behaviors that are directly related to the ability to induce LTD.

The fact that both abstinent and extinguished animals demonstrated loss of LTP indicates that the extinction-selective

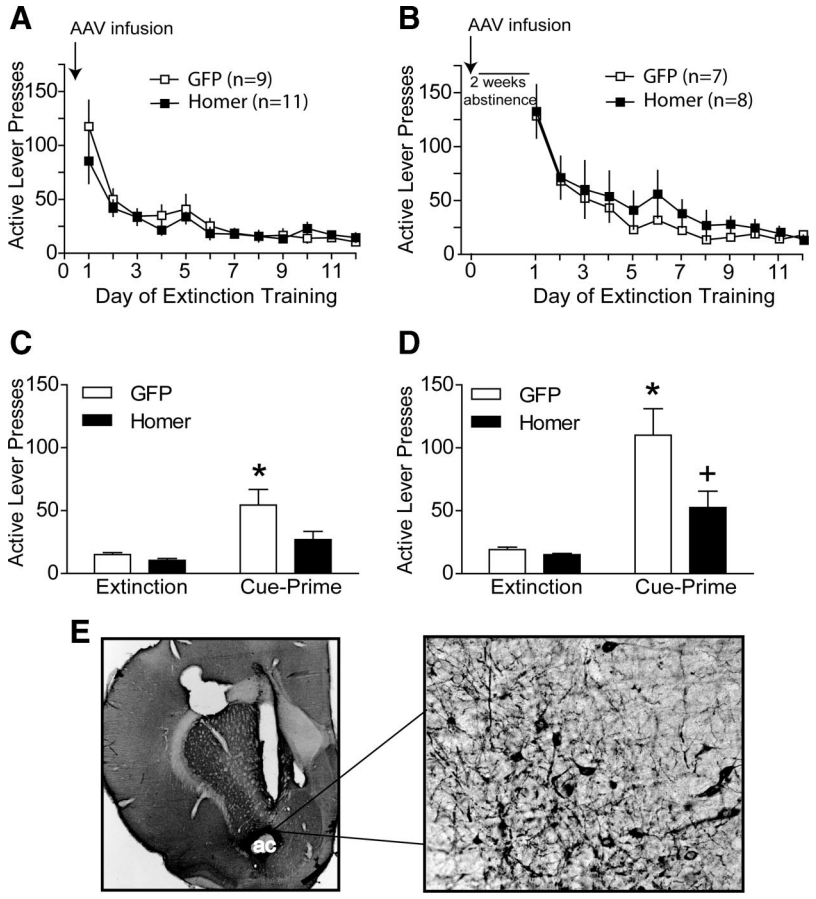

Figure 6. Overexpression of Homer1c in the NAcore attenuated reinstatement of cocaine seeking. ***A***, There was no difference in extinction learning between Homer1c-AAV- and GFP-AAV-infected animals when the AAVs were administered 1 d before the initiation of extinction training. ***B***, There was no difference in extinction learning between Homer1c- and GFP-infected animals when the AAVs were infused 2 weeks before initiating extinction training. ***C***, Homer1c-AAV infection immediately before extinction training attenuated cue-primed reinstatement. A two-way ANOVA with repeated measures over trial (i.e., extinction and cue-prime) confirms significant main effects of virus ($F_{(1,18)} = 5.68$; $p = 0.028$) and trial ($F_{(1,18)} = 20.00$; $p < 0.001$), as well as interaction ($F_{(1,18)} = 4.22$; $p = 0.054$). ***D***, Homer1c-AAV infection followed by 2 weeks of abstinence before extinction training attenuated cue-primed reinstatement. A two-way ANOVA revealed significant main effects of virus ($F_{(1,13)} = 7.43$; $p = 0.017$) and trial ($F_{(1,13)} = 28.90$; $p < 0.001$), as well as a significant interaction ($F_{(1,13)} = 6.37$; $p = 0.025$). ***E***, Immune staining of HA-tagged Homer1c reveals cellular labeling 5 weeks after virus infusion into the NAcore. ac, Anterior commissure. $^{*}p < 0.05$, compared with extinction; $^{+}p < 0.05$, comparing Homer-AAV to GFP-AAV, using a least significant difference *post hoc* test (Milliken and Johnson, 1984). Error bars indicate SEM.

changes in protein expression are not significant mediators of LTP. The increase in PSD-95 after extinction training has been observed previously in NAcore whole-cell lysates (Ghasemzadeh et al., 2009b); however, in the same extinguished animals, neither Homer1b/c nor PSD-95 were upregulated in the NAcore PSD-enriched fraction. These authors used 10 d of extinction training, whereas we used 21 d, and we showed that the upregulation of PSD-95 and Homer1b/c does not occur after 2 d of extinction training (Fig. 3*D*). Other studies also find progressive increases or decreases in protein content at increasing durations of withdrawal from chronic cocaine administration (Bowers et al., 2004; Lu et al., 2005; Conrad et al., 2008).

Consequences of protein changes in the NAshell

In addition to protein changes in the NAcore, we found decreased GluR2 expression in the NAshell after abstinence but not extinction. Similarly, Sutton et al. (2003) found an increase in GluR2 expression in extinguished animals relative to abstinent animals. We did not observe the significant decrease in GluR1 in the NAshell that has been previously reported (Ghasemzadeh et al., 2009b); however, in both the PSD-enriched (Fig. 1*E*) and non-PSD (Fig. 2*B*) fractions of NAshell tissue from abstinent animals, there was a trend toward a decrease in GluR1 expression. Akin to the upregulation of Homer1b/c in the NAcore PSD subfraction, a viral overexpression strategy has been used to show that the ability of extinction training to prevent the reduction GluR1 and GluR2 associated with abstinence contributes to the capacity of extinction learning to suppress the reinstatement of cocaine seeking (Sutton et al., 2003). Sutton et al. also reported that overexpressing GluR1 and GluR2 facilitated extinction of the cocaine-seeking response. Here, we found that, although overexpression of Homer1c was able to attenuate reinstatement, it was not able to facilitate extinction learning or prevent abstinent relapse (Fig. 6*B*). Thus, increased Homer1b/c expression seems to be a consequence of extinction learning, but does not promote it. The absence of an effect of Homer1c overexpression on abstinent relapse may stem from the fact that the NAcore does not seem to be an essential brain region for the expression of abstinent relapse (See et al., 2007). Extinction training is required to bring this area into the reinstatement neurocircuitry (Peters et al., 2008).

Conclusions

The present results demonstrate that extinction training after cocaine self-administration induces changes in the expression and/or distribution of Homer1b/c and mGluR5 in the PSD of the NAcore, and the increase in Homer1b/c expression by extinction training inhibited the induction of LTD. Importantly, these changes were associated with the capacity of extinction training to inhibit cue-induced cocaine seeking. Thus, rather than contributing to cocaine seeking, the upregulated Homer1b/c and the associated reduced surface expression of mGluR5 and loss of LTD are compensatory adaptations induced by extinction training that reduce cocaine seeking. This marks the Homer–mGluR5 signaling complex as a potential therapeutic target for reducing relapse in cocaine addiction by potentiating learning to inhibit drug seeking.

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