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Facilitative effects of environmental enrichment for cocaine relapse prevention are dependent on extinction training context and involve increased TrkB signaling in dorsal hippocampus and ventromedial prefrontal cortex

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Abstract

Cocaine-cue extinction training combined with brief interventions of environmental enrichment (EE) was shown previously to facilitate extinction and attenuate reacquisition of cocaine self-administration in rats. It is unknown whether or not the usefulness of this approach would be undermined if extinction training took place in a novel rather than familiar context. Drawing on previous studies involving pharmacological interventions, we hypothesized that the facilitative effects of EE for cocaine relapse prevention would be independent of the context used for extinction training. Rats trained to self-administer cocaine underwent cocaine-cue extinction training in either the familiar self-administration context or a novel context, with or without EE. Rats then were tested for reacquisition of cocaine self-administration in the familiar context. Target brain regions were lysed and probed for memory-related changes in receptors for glutamate and BDNF by western blotting. Contrary to our hypothesis, the facilitative effects of EE for cocaine relapse prevention were dependent on the context used for extinction training. While EE facilitated extinction regardless of context used, it inhibited cocaine relapse only after extinction training in the familiar context. EE was associated with increased GluA2 in nucleus accumbens, TrkB in dorsal hippocampus and activated TrkB in ventromedial prefrontal cortex. Of these, the

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Author Contributions

MHH and JMG contributed equally to this research and are co-first authors.

KMK and HM conceived and designed the experiments, MHH acquired and analyzed molecular data with technical support from AT, and JMG acquired and analyzed behavioral data with technical support from KM. All authors were involved in interpreting results, drafting the manuscript and approving the final version.

Declaration of competing interest

All authors declare no competing financial interests.

changes in dorsal hippocampus and ventromedial prefrontal cortex mirrored outcomes of the cocaine relapse tests in that these changes were specific to rats receiving EE plus extinction training in the familiar context. These findings support a role for hippocampal-prefrontal BDNF-TrkB signaling in extinction-based relapse prevention strategies involving EE.

Keywords

Cocaine; Context-dependent; Cue extinction; Environmental enrichment; Relapse; Self-administration

1. Introduction

Through associative learning, cues that co-occur with drug taking can come to elicit cravings and potentially lead to relapse [1]. Cue exposure therapy, although effective for reducing reactivity to anxiety-related cues [2,3], has proven ineffective as a standalone treatment for drug addiction [4,5]. However, preclinical studies suggest that exposure therapy for drug addiction may be more effective when combined with plasticity-promoting interventions, and work with an animal model of cue exposure therapy (cocaine-cue extinction) supports the therapeutic potential of this combined treatment approach [6]. In cocaine-cue extinction, an established association between a discrete visual cue and cocaine self-administration is “extinguished” by replacing the cocaine with a saline solution while maintaining response-contingent visual cue presentations. While this paradigm is not sufficient as a stand-alone treatment to inhibit reacquisition of cocaine self-administration, it does effectively reduce reacquisition of cocaine self-administration when paired with the NMDA receptor glycine site partial agonist D-cycloserine [7] or the Glycine Transporter-I (GlyT-I) inhibitor Org24598 or RO4543338 [8,9]. These observations suggest that the combination of cue exposure therapy with plasticity-promoting drugs may have therapeutic value.

Similar results were obtained when cocaine-cue extinction was combined with environmental enrichment (EE), a cognitive enhancing behavioral strategy. EE typically consists of physical (exercise), social (presence of conspecifics) and cognitive (novel objects) components, as this combination is thought to provide the best outcome for improving learning and memory [10]. Cocaine-trained rats that received brief 4 h EE sessions scheduled 24 h before and immediately after each weekly cocaine-cue extinction session showed facilitated extinction and reduced reacquisition of cocaine self-administration relative to a control group that did not receive EE [11]. Furthermore, the protective effects of EE were lost if cocaine-trained rats did not receive extinction training or if cocaine-trained rats received extinction training but with EE sessions scheduled outside the memory consolidation window [11]. Similarly, a transient elimination of cocaine seeking behavior was observed following prolonged EE treatment paired with cocaine context extinction [12]. This is consistent with a large body of literature suggesting that EE has cognitive enhancing and neuroprotective effects, although the molecular basis for these effects is not clear [13].

A potential limitation to the usefulness of extinction training in a clinic setting is that the retrieval of extinction memory is context-dependent [14]. Drug studies demonstrated that

rats with a history of cocaine self-administration followed by cocaine-cue extinction training in a novel context robustly reinstated cocaine seeking after reexposure to the familiar drug self-administration context [15]. However, pairing extinction training with certain pharmacological agents, including D-cycloserine, can overcome context-dependency [16,17,18,19]. This raises the question of whether the facilitative effects of EE on cocaine relapse prevention also would be independent of the context used for extinction training. In the present study, we addressed this question by subjecting cocaine-trained rats to cocaine-cue extinction training in either the familiar self-administration context or a novel context, with or without EE, and testing for reacquisition of cocaine self-administration in the familiar context.

A secondary aim of this study was to explore the neuroanatomical and molecular correlates of this behavioral intervention. To accomplish this, we used immunoblotting to probe for plasticity-related proteins in brain tissue collected from the experimental animals upon completion of the behavioral experiment. We focused on four brain regions that have been implicated in cocaine-cue extinction: the ventromedial prefrontal cortex, nucleus accumbens, dorsal hippocampus and basolateral amygdala [6]. Within these regions we probed for changes in BDNF-TrkB signaling, which is thought to mediate some of the effects of EE [13], and is also known to play a role in synaptic plasticity and learning [20,21,22], including extinction of conditioned fear [23,24] and cocaine associations [25,26]. We also probed for changes in AMPA receptor expression, modulation of which is thought to underlie memory-related plasticity, including the formation and extinction of cocaine associations [27,28]. We probed for activated and total TrkB using a phospho-specific antibody against the autophosphorylation site Tyrosine 816 and a total TrkB antibody. To probe for changes in expression of AMPA receptor expression we used antibodies against subunits GluA1 and GluA2, and to probe for changes in AMPA receptor phosphorylation we used a phospho-specific antibody against the PKA site Serine 845 (Ser845) on GluA1. Phosphorylation of GluA1 at this site is associated with AMPA receptor trafficking and regulation of channel conductance. Levels of Ser845-phosphorylated GluA1 have previously been shown to increase in the basolateral amygdala, along with total levels of GluA1, following cocaine-cue extinction training [11].

2. Materials and methods

2.1 Animals

Adult male Wistar rats (WIS/Crl; 275–300g upon arrival) were obtained from Charles River Laboratories, USA and housed individually in standard cages in a temperature-controlled (21–23°C) facility under a 12 h light/dark cycle (08:00 h on, 22:00 h off). After a 72-h acclimation period, food was restricted to 16–20g per day to maintain animals at 90% of growth-adjusted free-feeding weight. Water was available *ad libitum*. All procedures complied with the 8th edition of the NIH Guide for Care and Use of Laboratory Animals and were approved by the Boston University Institutional Animal Care and Use Committee.

2.2 Apparatus

The drug self-administration chambers (model ENV-008CT; Med Associates, St Albans, VT, USA) were configured and outfitted as previously described in detail [29]. Briefly, each chamber was enclosed in a ventilated sound-attenuating cubicle and was equipped with 2 levers, 2 cue lights, a house light, a single-channel fluid swivel, a spring leash and counterbalanced arm assembly, a syringe pump, an 8-ohn speaker, and a food trough and pellet dispenser. The environmental enrichment chambers also were as previously described in detail [11]. Briefly, each powder-coated wire cage (76 × 46 × 74 cm; Super Pet Inc., Walnut Creek, CA, USA) was equipped with two running wheels, three levels of ramps and platforms, movable tunnel structures, and numerous manipulable items and chew toys. Items were changed weekly to maintain novelty. Commercial pulp fiber bedding was used as nesting material on the cage bottom and pieces of sweetened cereal were hidden in various locations to encourage foraging.

2.3 Surgery and Catheter Maintenance

Prior to surgery, rats were trained to lever press under a fixed-ratio (FR) 1 schedule of food delivery (45 mg pellets; Bio-Serv, Frenchtown, NJ, USA). After rats learned to rapidly press a lever for 50 food pellets, catheters were implanted into the right femoral vein as described previously [29]. Rats received subcutaneous Buprenorphine (0.05 mg/kg; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA) as a preemptive analgesic on the day of surgery and twice the following day. Rats also received a subcutaneous injection of the NSAID Flunixin, (1.0 mg/kg; Merck & Co., Inc., Whitehouse Station, NJ, USA) immediately after surgery and once daily for the next two days. The antibiotic Baytril (5 mg; Bayer Health Supply, Kansas City, KS, USA) was administered i.v. for 3 days post-surgery to reduce risk of systemic infection. To maintain patency, catheters were locked daily (Monday-Thursday) with 0.2 ml of a 0.9% saline solution containing 30.0 IU/ml heparin, an anticoagulant (SAGENT Pharmaceuticals, Schaumburg, IL, USA) and 100 mg/ml Cefazolin, an antibiotic (Apotex Corporation, Weston, FL, USA). Over weekends and holidays, a solution consisting of glycerol (Sigma-Aldrich, St. Louis, MO, USA) and 1000 IU/ml heparin (3:1) was used to lock the catheter. Prior to the start of the next behavioral session, the glycerol solution was removed and the catheter flushed with 0.2 ml of a dilute heparinized 0.9% saline solution (3.0 IU/ml). Catheters were checked daily for leaks and tested periodically for patency by infusing 1.0 mg/0.1 ml of methohexital sodium (JHP Pharmaceuticals, Rochester, MI, USA), which induces rapid transient loss of muscle tone. Improperly functioning catheters were surgically removed and replaced by a catheter implanted into the left femoral vein.

2.4 Cocaine Self-Administration

2.4.1 Baseline Training—Daily 1 h cocaine self-administration sessions (Monday-Friday) began one week after surgery in Context A (Table 1). Cocaine hydrochloride (National Institute on Drug Abuse Drug Supply, Bethesda, MD, USA) was dissolved in a dilute heparinized 0.9% saline solution (3.0 IU/ml). The cocaine training dose was 0.3 mg/kg (a 1.6 mg/ml concentration infused at a rate of 1.8 ml/min for a duration of 0.6 s/100 g body weight). All rats were trained to self-administer i.v. cocaine first under an FR 1 schedule and then under an FR 5 schedule, with a 2-sec cue light paired with each cocaine

infusion. Once FR 5 responding was reliably maintained, cocaine delivery and cue light presentation transitioned to a second-order schedule that incorporated FR and fixed-interval (FI) parameters (FI 2 min [FR 5: S]). Under this schedule, every fifth press of the active lever (FR 5) produced a 2-sec cue light (S), and the first FR 5 response unit completed after the 2-min FI elapsed produced the 2-sec cue light paired with an i.v. cocaine infusion. Responses on the inactive lever (right or left, counterbalanced across subjects) were counted separately, but had no scheduled consequences. During 1 h sessions, rats could earn a maximum of 30 infusions. In addition, white noise (70-db) was presented in the background for the duration of each session. During this latter phase of self-administration training, all rats were acclimated in weekly 15 min periods to the EE chamber and companion animals to ensure compatibility and an identical handling history. After cocaine self-administration under the second-order schedule was well established (25–35 sessions), animals were assigned randomly to one of three treatment groups, as described below in section 2.4.2.

2.4.2 Extinction Training with and without Environmental Enrichment— Beginning 48 h after the last baseline cocaine self-administration session, lever pressing was extinguished in either the familiar self-administration context (Context A) or a novel context (Context B). The background stimuli used for Context B (Table 1) were shown previously to be discriminable from those used for Context A [30]. During extinction sessions, completion of every fifth response on the active lever produced the 2-sec cue light, and the first FR 5 completed after the FI 2-min elapsed produced the 2-sec cue light and an i.v. saline infusion. Three 1 h extinction training sessions were conducted weekly, analogous to clinical protocols utilizing 1 h sessions spaced at weekly intervals [31,32]. During the six intervening days between successive extinction training sessions, subjects remained in their home environments, unless designated to receive EE.

EE consisted of 4 h periods in the enrichment chamber. EE periods were provided both 24 h before and immediately after each of the 3-weekly extinction training sessions. Four compatible rats were placed together during each 4 h EE period. We previously established this EE protocol as optimal for facilitating extinction in Context A and inhibiting subsequent reacquisition of cocaine self-administration [11]. To determine whether the facilitative effects of EE for cocaine relapse prevention were independent of the context used for extinction training, one treatment group underwent extinction training in familiar Context A in conjunction with EE (AAA + EE; n=8), while a second treatment group underwent extinction training in novel Context B in conjunction with EE (ABA + EE; n=8). The third treatment group (control) underwent extinction in familiar context A, but did not receive EE, and remained in the homecage instead during these periods (AAA + No EE; n=8).

2.4.3 Reacquisition of Cocaine Self-Administration—Reacquisition of cocaine self-administration was evaluated beginning 1 week after the last extinction training session. All reacquisition test sessions took place in the familiar self-administration context (Context A), and all conditions and the second-order schedule parameters were identical to those established during the baseline cocaine self-administration phase, as described in section 2.4.1. The reacquisition phase lasted for 15 consecutive daily sessions (Monday-Friday).

2.5 Molecular Analysis of Brain Tissue

2.5.1 Tissue Processing—Rats were decapitated 24 h following the last cocaine self-administration reacquisition session and the brain extracted. Target brain regions (ventromedial prefrontal cortex, basolateral amygdala, dorsal hippocampus, and nucleus accumbens) were quickly isolated using a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI) and razor blades according to published methods (Heffner et al., 1980). Tissue was transferred to a plastic microtube and flash frozen by immersion in a beaker of 2-methylbutane on dry ice. After storage at −80°C, samples were homogenized using a disposable tissue grinder in 200ul of homogenization buffer (10mM Tris-HCl pH 7.2, 150mM NaCl, 0.1% SDS, 0.1% Triton x-100, 1% sodium deoxycholate, 5mM EDTA) with Roche complete protease inhibitor cocktail (Millipore Sigma 4693116001) and phosphatase inhibitor cocktail (Bimake B15001) added. Homogenate was sonicated for 5 seconds, centrifuged at 17 000xg for 10min and the supernatant was collected and subjected to Pierce BCA protein assay according to the manufacturer's instructions (Thermo Scientific 23225). Samples were diluted with 2X Laemmli sample buffer and frozen at −20°C until immunoblotting.

2.5.2 Immunoblotting—Individual animal samples, each containing 40ug total protein, were subjected to SDS-PAGE. The 24 samples from each brain region were distributed across two 12-lane blots such that all three experimental groups were equally represented in each blot (i.e., of the 8 animals in each experimental condition, lysates from 4 animals were loaded onto one blot and lysates from the other 4 animals were loaded onto another blot). Triplicates of each blot were run to permit probing with multiple sets of antibodies. After transfer to PVDF membrane, one blot was probed with antibodies against Ser845-phosphorylated rat GluA1 (Millipore-Sigma AB5849), the human GluA1 cytoplasmic domain (Millipore Sigma AB1504) and human Tubulin βIII C-terminal amino acids 442–446 (Millipore Sigma SAB4300623) sequentially to produce estimates of total and Ser845-phosphorylated GluA1. An identical blot was probed with antibodies against rat Tyr816-phosphorylated TrkB (Millipore Sigma ABN1381), human TrkB N-terminal amino acids 156–322 (BD Biosciences 610101), rat GluA2 N-terminal amino acids 175–430 (Millipore Sigma MAB397) and Tubulin beta III to produce an estimate of total GluA2, total TrkB and Tyr816-phosphorylated TrkB. A third identical blot was sequentially probed with the antibodies against the GluA1 cytoplasmic domain and GluA2 N-terminal region to produce and estimate of the GluA1/2 ratio. Blots were stripped between probing with different antibodies. For phospho-specific antibodies, 5% BSA TBST (20mM Tris, 150mM NaCl, 0.1% Tween 20) was used as the blocking solution and blots were incubated in primary antibody overnight at 4°C. For non-phospho-specific antibodies, 4% skim milk TBST was used as the blocking solution and primary antibody incubation was for 2 h at room temperature. Goat anti-rabbit-HRP (Millipore Sigma A4914) and goat anti-mouse-HRP (Millipore Sigma A4416) secondary antibodies were used. Blots were quantified using the Gel Analyzer feature of ImageJ.

2.5.3 Antibody validation—To validate the phospho-specificity of the TrkB Tyr816 and GluA1 pSer845 antibodies, cultured cortical neurons (DIV 17) were lysed in a buffer containing 50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM DTT, 1% Triton x-100 and Roche

EDTA-free protease inhibitor cocktail, with either 1.5mM MgCl2 (CIP lysis buffer) or 2mM MnCl2 (lambda lysis buffer), and incubated at 37°C for 30 minutes with or without added phosphatase inhibitor cocktail (Bimake B15001), calf intestinal phosphatase (CIP) (New England Biolabs M0290) and lambda phosphatase (Sigma P9614–2KU). Lysates were then denatured with Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting with against TrkB Tyr816 or GluA1 Ser845 phospho-specific antibodies. Blots were then stripped and re-probed to detect total levels of the target proteins. To confirm specificity of the total GluA1 and TrkB antibodies, an adult rat hippocampus was isolated, rapidly homogenized in ice-cold homogenization buffer with Roche complete protease inhibitor cocktail added, and run alongside cultured neurons (DIV 15) lysed in Laemmli sample buffer. As a negative control, HEK293T cells were also lysed in Laemmli sample buffer and run on the gel.

2.6 Statistical Analysis

To compare baseline cocaine self-administration behavior in rats subsequently assigned to the three treatment groups, the last 5 sessions under the second-order schedule were averaged in individual animals prior to analysis. The number of responses on the active and inactive levers were analyzed by a two-factor repeated measures ANOVA (Group x Lever) and the average daily mg/kg cocaine intake at baseline was analyzed by a one-factor ANOVA (Group). Due to 2- to 4-fold differences in baseline response rates among individual rats within each treatment group (see individual data points in Fig. 1A), active lever responses during the 3-weekly extinction sessions and 15-daily reacquisition sessions were expressed as percent of baseline responses prior to analysis (see raw data in Appendix A. Supplementary Data, Table S1). The percentages of baseline active lever responses were analyzed by a two-factor repeated measures ANOVA (Group x Session) for the extinction and reacquisition phases. The average daily mg/kg cocaine intake across the 15 reacquisition sessions was analyzed by a one-factor ANOVA (Group). The Tukey test was used for all post-hoc comparisons in these analyses.

For immunoblot data, band optical densities were normalized to tubulin to control for variability in loading, and then all values were normalized to the average of the AAA No EE controls from the same blot to permit analysis of group data distributed across blots. Outlying values within each group for each molecular target and brain region were identified by the Grubbs test and excluded from the analyses. The remaining values for each molecular target and brain region were subjected to one-factor ANOVA (Group). The Holm-Sidak method was used for post-hoc comparisons.

3. Results

3.1. Cocaine Self-Administration

3.1.1. Baseline Training—Baseline cocaine self-administration behavior was comparable across groups before treatment, with inactive lever responses <25% of active lever responses for each group (Fig. 1A). A 2-factor RM ANOVA revealed a significant main effect of lever ($F[1,21] = 164.320$, $p=0.001$) for active vs. inactive lever responses, but failed to detect a significant main effect of group ($p=0.45$) or a group x lever interaction

(p=0.64). The 1-factor ANOVA for the average daily mg/kg cocaine intake at baseline (Fig. 1B) revealed no significant differences between groups as well (p=0.33).

3.1.2. Extinction Training with and without Environmental Enrichment—

Baseline-normalized active lever response rates differed by group over the course of extinction training (Fig. 2). A 2-factor RM ANOVA revealed a significant main effect of group ($F[2,21]=6.0$, $p=0.01$), but not of session number ($p=0.07$) or the group x session number interaction ($p=0.14$). Post-hoc tests showed that response rates across the 3-weekly sessions were significantly lower in groups that underwent extinction training in either the familiar context or the novel context paired with EE (AAA + EE, ABA + EE) compared to the control group that underwent extinction training in the familiar context without EE (AAA + No EE; $p=0.01$ and 0.03, respectively).

3.1.3 Reacquisition of Cocaine Self-Administration—

During the cocaine self-administration reacquisition phase, baseline-normalized active lever responses across the 15 daily sessions differed by group as well (Fig. 3A). A 2-factor RM ANOVA revealed a significant main effect of group ($F[2,21]=8.1$, $p=0.002$), but not of session number ($p=0.08$) or the group x session number interaction ($p=0.69$). Post-hoc tests showed that responses were significantly lower in the group that underwent extinction in the familiar context paired with EE (AAA + EE) compared to the group that underwent extinction training in the novel context paired with EE (ABA + EE; $p=0.002$) and the control group that underwent extinction training in the familiar context without EE (AAA + No EE; $p=0.03$). The 1-factor ANOVA for the average daily mg/kg cocaine intake across the 15 reacquisition sessions revealed a significant difference between groups ($F[2,21] = 21.4$, $p<0.001$), with significantly lower cocaine intake in the AAA + EE group relative to the ABA + EE ($p=0.001$) and AAA + No EE ($p=0.040$) groups (Fig. 3B).

3.2. Molecular Analysis of Brain Tissue

3.2.1. Antibody validation—

Prior to probing brain tissue samples, we used cultured cortical neurons to validate the specificity of TrkB Tyr816 (Fig. 4A) and GluA1 Ser845 (Fig. 4B) phospho-specific antibodies. Both antibodies detected a band of the expected molecular weight in lysate (Fig. 4A, B upper panels). The intensity of the bands was reduced by 30min incubation at 37°C in the absence of phosphatase inhibitors, consistent with dephosphorylation by endogenous phosphatases present in the lysate (Fig. 4A, B upper panels). A similar decrease in band intensity could be induced in the presence of phosphatase inhibitors by adding sufficient exogenous lambda phosphatase or calf intestinal phosphatase to exceed the capacity of the phosphatase inhibitor (Fig. 4A, B upper panels). To rule out protein degradation as the cause of the decrease, the blots were stripped and re-probed for total levels of the corresponding protein (Fig. 4A, B lower panels). The TrkB and GluA1 antibodies were validated by demonstrating that they recognize the corresponding overexpressed recombinant protein, but not a negative control protein, in HEK293T cell lysate, and that they recognize bands of the appropriate size in rat brain lysate and cultured neuron lysate, but not in negative control (HEK293T cell) lysate (Fig. 4C, D).

3.2.2. Regional Changes in TrkB, GluA1 and GluA2—Upon completion of behavioral experimentation, animals were sacrificed and their brains were dissected for molecular studies. The expression levels of molecular targets for which there were no significant differences between the AAA + No EE, AAA + EE, and ABA + EE test groups by 1-factor ANOVA are shown in Appendix A. Supplementary Data, Table S2. For the remaining targets, we observed 3 main patterns of group differences. The first pattern consisted of molecular changes related specifically to a history of EE exposure combined with extinction training in either familiar context A or novel context B. This pattern was most pronounced for GluA2 in the nucleus accumbens ($F[2, 21] = 4.4$, $p=0.03$; 1-factor ANOVA), which showed increased expression in the AAA + EE ($p=0.04$) and ABA + EE ($p=0.05$) groups relative to the AAA + No EE control (Fig. 5A). A somewhat similar pattern was observed for total GluA1 in the nucleus accumbens ($F[2, 21] = 4.1$, $p=0.03$; 1-factor ANOVA). The ABA + EE group had increased expression relative to the AAA + No EE control ($p=0.05$), whereas expression in the AAA + EE group differed neither from the AAA + No EE ($p=0.07$) nor from the ABA + EE ($p=0.68$) groups (Fig. 5B).

The second pattern consisted of molecular changes specifically related to a history of EE exposure combined with extinction training in novel context B. This pattern was most pronounced for Serine 845-phosphorylated GluA1 in the ventromedial prefrontal cortex ($F[2, 20] = 12.5$, $p=0.001$; 1-factor ANOVA), which showed increased expression in the ABA + EE group relative to the AAA + EE ($p=0.01$) and AAA + No EE ($p<0.001$) groups (Fig. 5C). A somewhat similar pattern was observed for total GluA1 in this same brain region ($F[2, 21]=5.0$, $p=0.02$; 1-factor ANOVA). The ABA + EE group had increased expression relative to the AAA + No EE group ($p=0.01$), whereas expression in the AAA + EE group differed neither from the AAA + No EE ($p=0.14$) nor from the ABA + EE ($p=0.23$) groups (Fig. 5D). There were no between-group differences in the ratio of GluA1-pSer845 to total GluA1 in this brain region (Table S2).

The third pattern consisted of molecular changes specifically related to a history of EE exposure combined with extinction training in familiar context A, thus mirroring the behavioral outcome for relapse prevention. This pattern was most pronounced for total TrkB (Fig. 6A) in the dorsal hippocampus ($F[2,19]=9.2$, $p=0.002$; 1-factor ANOVA) and for the proportion of autophosphorylated TrkB (Fig. 6B) in the ventromedial prefrontal cortex ($F[2,20]=6.4$, $p=0.01$; 1-factor ANOVA). In the dorsal hippocampus, total TrkB levels were elevated in the AAA + EE group relative to the AAA + No EE ($p=0.002$) and ABA + EE ($p=0.02$) groups, while the ratio of autophosphorylated/total TrkB was not significantly different (Table S2). In the ventromedial prefrontal cortex, the ratio of autophosphorylated/total TrkB was increased in the AAA + EE group relative to the AAA + No EE ($p=0.03$) and ABA + EE ($p=0.01$) groups, with no significant difference in total TrkB (Table S2). A somewhat similar pattern was observed for absolute levels of phosphorylated TrkB (Fig. 6C) in the nucleus accumbens ($F[2,20]=4.8$, $p=0.02$; 1-factor ANOVA), and phosphorylated GluA1 (Fig. 6D) in the nucleus accumbens ($F[2,20]=5.1$, $p=0.02$; 1-factor ANOVA). These phosphorylated proteins were elevated in the AAA + EE group relative to the AAA + No EE control ($p=0.02$ for p-GluA1; $p=0.02$ for p-TrkB), but in both cases, the increase in the AAA

+ EE group relative to the ABA + EE group was not significant (p=0.09 for p-GluA1; p=0.15 for p-TrkB).

Representative blots are shown at the top of each panel in Figure 5 and Figure 6 for 4 of the 8 animals tested in each of the three experimental groups. The band for the protein target of interest is marked by an arrow and the nearest point on the molecular weight ladder is depicted.

4. Discussion

The results of this study show that brief interventions of EE combined with cocaine-cue extinction training inhibited subsequent reacquisition of cocaine self-administration when all training and testing occurred in the familiar self-administration context under the AAA + EE condition. While these findings replicate our earlier observations [11], the present study indicates that the facilitative effects of EE for cocaine relapse prevention depended on the context used for extinction training. Rats in the ABA + EE condition relapsed immediately to baseline levels of cocaine self-administration, as did rats in the AAA + No EE control condition. It should be noted that we elected not to test an ABA + No EE condition due to limited resources available at the time of behavioral testing. If we had discovered that the facilitative effects of EE for cocaine relapse prevention were *independent* of the extinction training context, then it would have been necessary to test an ABA + No EE group to confirm that these rats relapsed immediately to baseline levels of cocaine self-administration, as would be expected after cocaine-cue extinction training in a novel context [15]. Since animals relapsed immediately to baseline levels in the ABA + EE group, the inclusion of an ABA + No EE group was not necessary to determine whether or not the facilitative effects of EE for cocaine relapse prevention depended on the extinction training context and to discern the molecular differences between the two EE exposed groups (AAA + EE and ABA + EE) that could account for their behavioral differences. Given that behavioral therapy is generally provided in a clinic setting and not in a user's usual drug-taking environment, our findings suggest that brief interventions of EE alone may have limited applicability as an adjunct to cue exposure therapy for relapse prevention in individuals addicted to cocaine. However, the combination of EE with cue exposure therapy and certain circumscribed pharmacotherapies may nevertheless have promise.

Preclinical studies have obtained context-independent extinction memory retrieval in a number of paradigms by pairing extinction training with cognitive-enhancing pharmacological agents such as D-cycloserine, atomoxetine, l-DOPA, and histone deacetylase inhibitors [16,17,19,33]. Of direct relevance to the present study, extinction memory retrieval during reinstatement testing was found to be context-independent in rats trained to self-administer cocaine prior to receiving systemic administration or infusions of D-cycloserine into the nucleus accumbens during cocaine-cue extinction training sessions in context A or B [18]. Clinical studies also have paired cue exposure therapy with D-cycloserine and have obtained some promising results for anxiety disorders [2,3,34] and alcohol use disorder [35,36]. Although clinical investigations of cocaine dependence suggest that combining D-cycloserine with cue exposure therapy in a clinic setting [37] or a naturalistic setting [38] is not sufficient to reduce cocaine craving or inhibit relapse, the use

of a more optimized cue exposure therapy procedure [34,39] coupled with a more efficacious cognitive-enhancer such as a GlyT-1 inhibitor [8,9] may lessen the constraint of context for extinction memory retrieval and effectively inhibit cocaine relapse in people. Determining whether a GlyT-1 inhibitor can produce context-independent effects on extinction memory retrieval and whether brief interventions of EE can amplify the generalizing effects of a GlyT-1 inhibitor on extinction memory retrieval are important directions for our future preclinical investigations.

Mechanistically, numerous reports suggest that fear extinction relies on BDNF-dependent plasticity within the ventromedial prefrontal cortex [24,40,41,42], and extinction of cocaine associations has also been found to depend on BDNF signaling within the ventromedial prefrontal cortex [26]. Consistent with this, one of the strongest molecular correlates of successful relapse prevention by EE in our assay was TrkB activation in the ventromedial prefrontal cortex. A number of studies have pointed to hippocampal inputs to the ventromedial prefrontal cortex as the probable source of BDNF driving fear extinction [23,24,41,42], and *de novo* protein synthesis within the dorsal hippocampus has also been shown to be necessary for consolidation of cocaine-cue extinction [29]. Furthermore, some of the developmental and cognitive-enhancing effects of EE have been ascribed to BDNF. Most notable is one report in which an EE-induced reduction in cocaine-seeking activity was associated with increased BDNF expression in the hippocampus [43]. The increased hippocampal TrkB expression associated with reduced relapse in our study may be related to these known roles for hippocampal BDNF in extinction and EE. In addition to increased TrkB signaling and expression in the ventromedial prefrontal cortex and dorsal hippocampus, we also detected an increase in the absolute levels of TrkB-pTyr816 and GluA1-pSer845 in the nucleus accumbens in the AAA + EE group relative to the AAA + No EE group. This suggests that facilitation of extinction memory retrieval by EE may also involve increased TrkB- and AMPA receptor-mediated signaling within this region, although no changes in total protein levels or phospho-to-total ratio were observed, limiting the interpretation of this observation. Moreover, as these molecular changes associated with inhibition of cocaine relapse are correlative measures, a future direction would be to establish a causal link between these neuroadaptations and the facilitative effects of EE for cocaine relapse prevention.

Further insight into the neurobiological correlates of EE comes from the observation that total levels of GluA2 were increased in the nucleus accumbens in both groups that underwent EE relative to the group that did not. While a similar trend was observed for total GluA1 in this region, the crucial comparison of AAA + EE vs. AAA + No EE did not reach statistical significance. Together these observations suggest that brief interventions of EE may increase AMPA receptor expression in the nucleus accumbens, in particular through a GluA2-dependent mechanism. These EE-related changes in AMPA receptor expression may contribute to the reduction in cocaine-seeking responses produced by the EE intervention during extinction training, regardless of context used and whether or not cocaine relapse was subsequently inhibited. Others reported a similar reduction in cocaine-seeking responses after viral-mediated overexpression of GluA1 or GluA2 in nucleus accumbens shell during extinction training in a familiar context [28]. The overexpression of GluA1 or GluA2 in the nucleus accumbens shell did not protect against cocaine relapse in tests conducted 1 week

after overexpression declined. Together, these findings suggest that elevated GluA1 or GluA2 within the nucleus accumbens may relate to reduced motivation for cocaine rather than facilitated extinction learning during extinction training.

Lastly, the failure of EE to prevent the context dependency of extinction memory retrieval during cocaine reacquisition test sessions may be related to the lack of increase in TrkB signaling in the ventromedial prefrontal cortex of the ABA + EE group. Previous pharmacological investigations utilizing a AAA design demonstrated that BDNF infusion into medial prefrontal cortex prior to extinction attenuated relapse in rats trained to self-administer cocaine, whereas blockade of BDNF activity in medial prefrontal cortex counteracted the cocaine relapse prevention effects of BDNF [44,45]. Importantly, after pharmacological inactivation of the prelimbic region of the ventromedial prefrontal cortex, ABA renewal of extinguished cocaine seeking was attenuated [46], consistent with the view that the ventromedial prefrontal cortex is involved in the recall of contexts in which extinction takes place [47]. It also is possible that the failure of EE to prevent the context dependency of extinction memory retrieval during cocaine reacquisition test sessions may be related to the increase in absolute levels of Ser845-phosphorylated GluA1 found selectively in ventromedial prefrontal cortex of the ABA + EE group. Protein Kinase A-mediated phosphorylation of GluA1 at Ser845 drives activity-dependent increases in membrane expression of GluA1-containing AMPA receptors and is required for long-term potentiation of synaptic strength [48,49,50]. Of relevance, a prior study demonstrated increased c-Fos protein expression in the infralimbic region of the ventromedial prefrontal cortex during ABA renewal of extinguished cocaine seeking, suggesting that neuronal activity within this region may contribute to context-discrimination [51].

It is important to note that several studies have pointed to plasticity in the basolateral amygdala as playing a key role in fear [25,52] and cocaine-cue [25,29,52,53] extinction learning. Our inability to detect any EE-related plasticity changes in the basolateral amygdala 24 h after the last cocaine self-administration reacquisition session may indicate that any molecular changes in this region are transient, and do not play an enduring role in the maintenance of extinction memory. Alternatively, we previously reported that expression of total GluA1 and GluA1 pSer845 in basolateral amygdala was increased, regardless of EE exposure, in animals that received extinction vs. no-extinction training [11]. These changes were detected 24 h after the last cocaine self-administration reacquisition session and were dependent on extinction training and independent of EE exposure. It is possible that the lack of molecular changes in the basolateral amygdala across the AAA + No EE, AAA + EE, and ABA + EE conditions in the present study represent a generalized extinction-induced increase in all three groups, but the lack of a no-extinction control group in the present study for comparison purposes renders this possibility speculative at this time, but this is another important direction for our future preclinical investigations.

5. Conclusions

The results of this study provide new insights into the mechanisms of relapse prevention by a combined EE/cocaine-cue extinction intervention in rats trained to self-administer cocaine. The beneficial effects of this treatment strategy appear to be mediated by increased TrkB

signaling in hippocampal-prefrontal circuits (also see [26]). TrkB activation by BDNF, which is increased by EE [54,55,56], modulates trafficking and synaptic insertion of AMPA receptors [21], and plays an important role in learning and memory, including extinction memory [57]. Consistent with this, we found that relapse prevention by EE combined with extinction in the familiar self-administration context was associated with increased TrkB expression/signaling in the dorsal hippocampus and ventromedial prefrontal cortex, two regions strongly implicated in extinction [58]. In contrast, EE combined with extinction training in a novel context failed to induce these regionally selective molecular changes, and failed to prevent relapse. More research is needed to reduce or eliminate the context dependency of extinction memory retrieval in order to improve cocaine relapse prevention efforts with cue exposure therapy in a clinic setting. If extinction context-independent effects cannot be achieved with a combination of pharmacological (e.g., a GlyT-1 inhibitor) and behavioral (e.g., EE) cognitive-enhancing interventions, then an alternative approach in a clinic setting may be to use virtual reality during cue exposure therapy sessions to recreate the drug-taking environment while extinguishing reactivity to drug-related cues (see [59] for review).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Brief EE sessions combined with extinction training inhibit cocaine relapse
- Beneficial effects of EE depend on extinction training in a familiar context
- TrkB signaling in prefrontal cortex and hippocampus may underlie the benefits of EE
- Molecular correlates of EE differ for extinction in a novel vs. familiar context

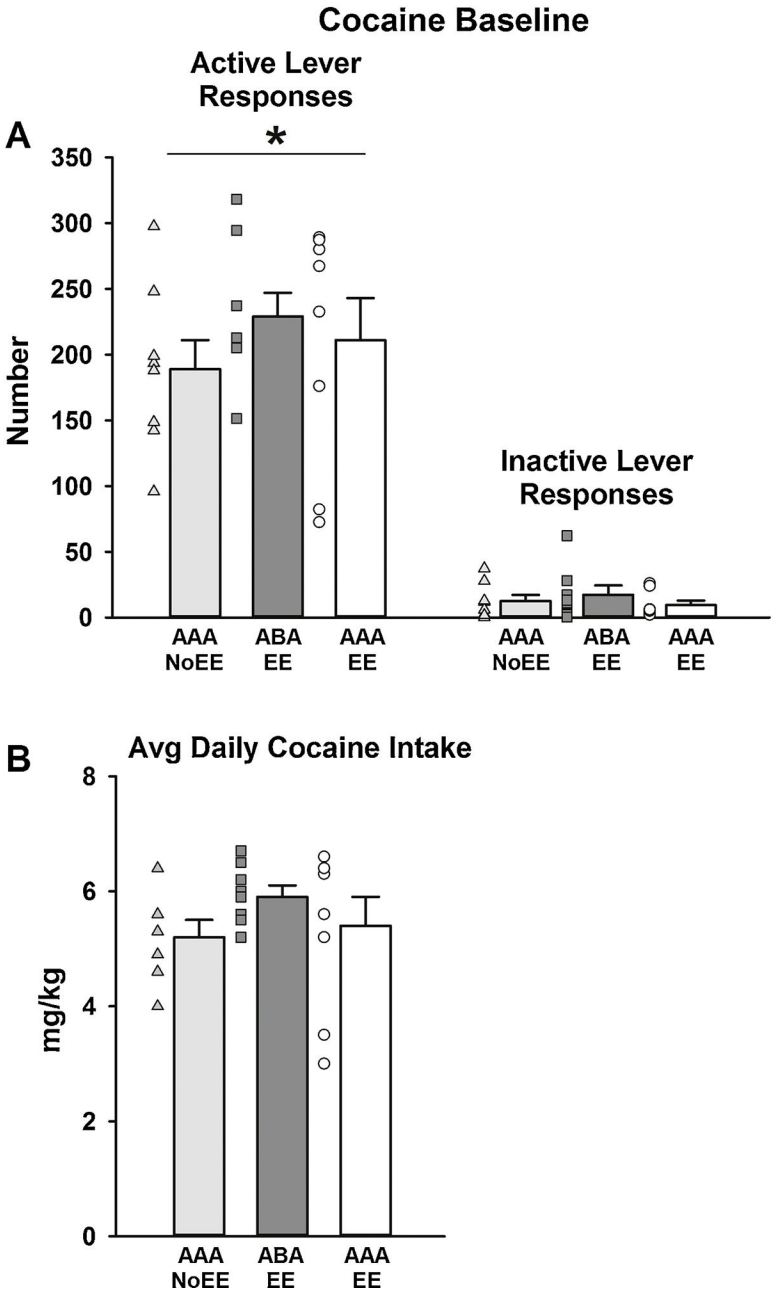

Figure 1.

Cocaine self-administration at baseline in groups of rats prior to extinction training in context A without the environmental enrichment intervention (AAA + No EE group), extinction training in context A with the environmental enrichment intervention (AAA + EE group), and extinction training in context B with the environmental enrichment intervention (ABA + EE group). N = 8 rats per group. Values are the mean ± S.E.M. and the individual data points for (A) active and inactive lever responses averaged over the last 5 sessions of self-administration training (* p=0.001 comparing active to inactive lever responses); and (B) daily mg/kg cocaine intake averaged over the last 5 sessions of self-administration training.

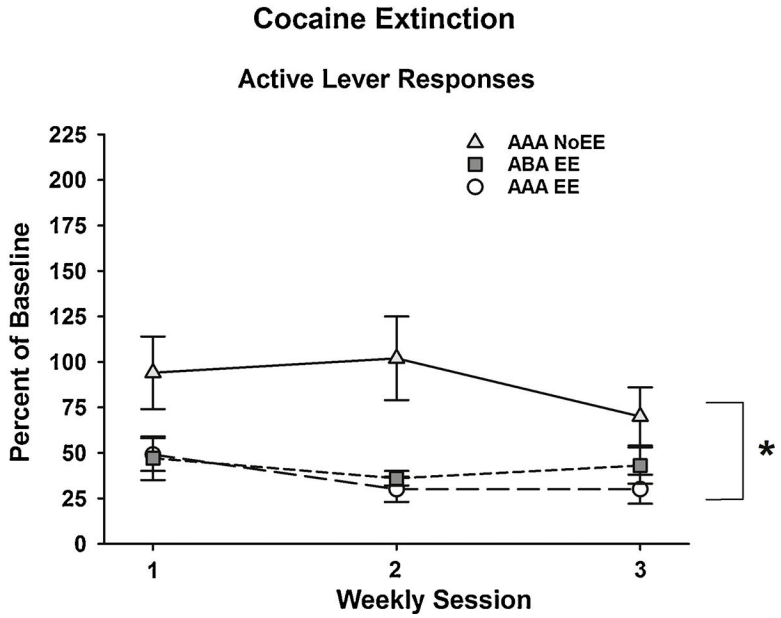

Figure 2.

Responding during the 3 weekly extinction training sessions in the 3 groups of rats as defined in the Figure 1 legend. N = 8 rats per group. Values are the mean ± S.E.M. for active lever responses expressed as percent of baseline (* $p \leq 0.03$ comparing the AAA + EE and ABA + EE groups to the AAA + No EE group).

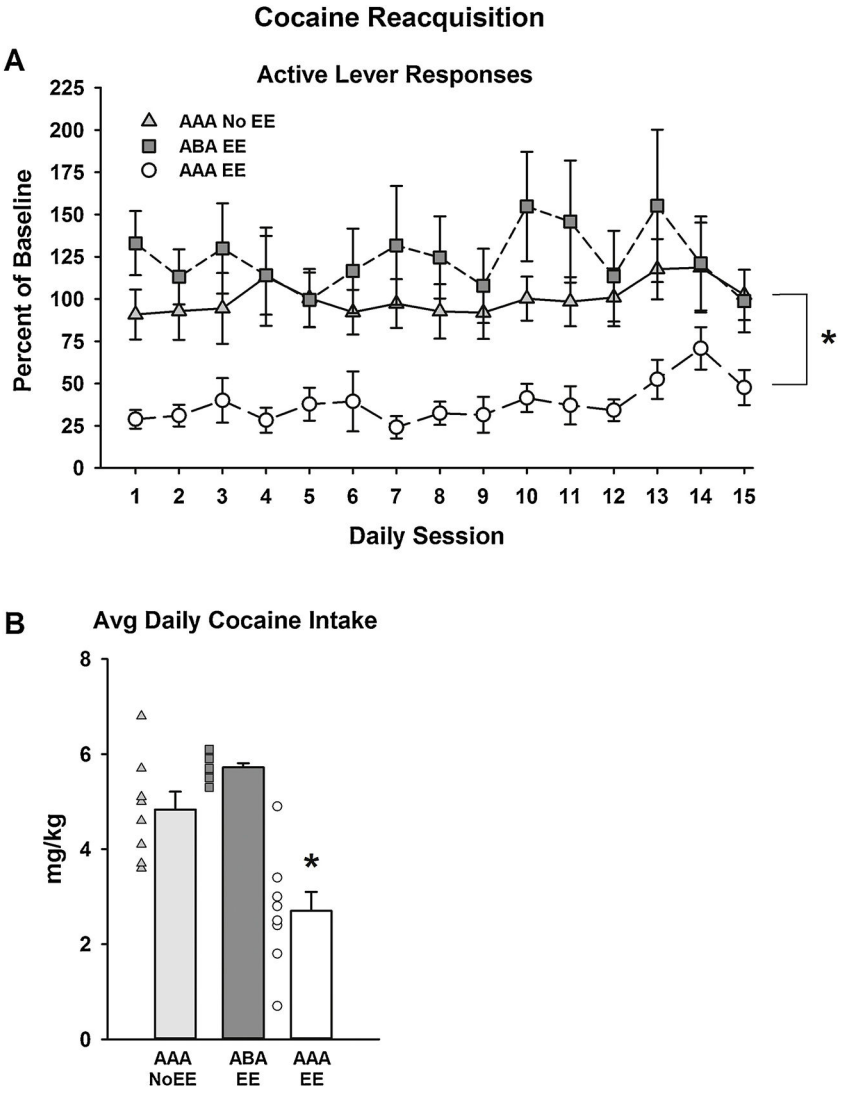

Figure 3.

Reacquisition of cocaine self-administration during the 15 daily sessions in the 3 groups of rats as defined in the Figure 1 legend. N = 8 rats per group. Values are the mean ± S.E.M. for (A) active lever responses expressed as percent of baseline (* $p \leq 0.03$ comparing the AAA + EE group to the ABA + EE and AAA + No EE groups); and (B) daily mg/kg cocaine intake and individual data points averaged over the 15 self-administration reacquisition sessions (* $p \leq 0.04$ comparing the AAA + EE group to the ABA + EE and AAA + No EE groups).

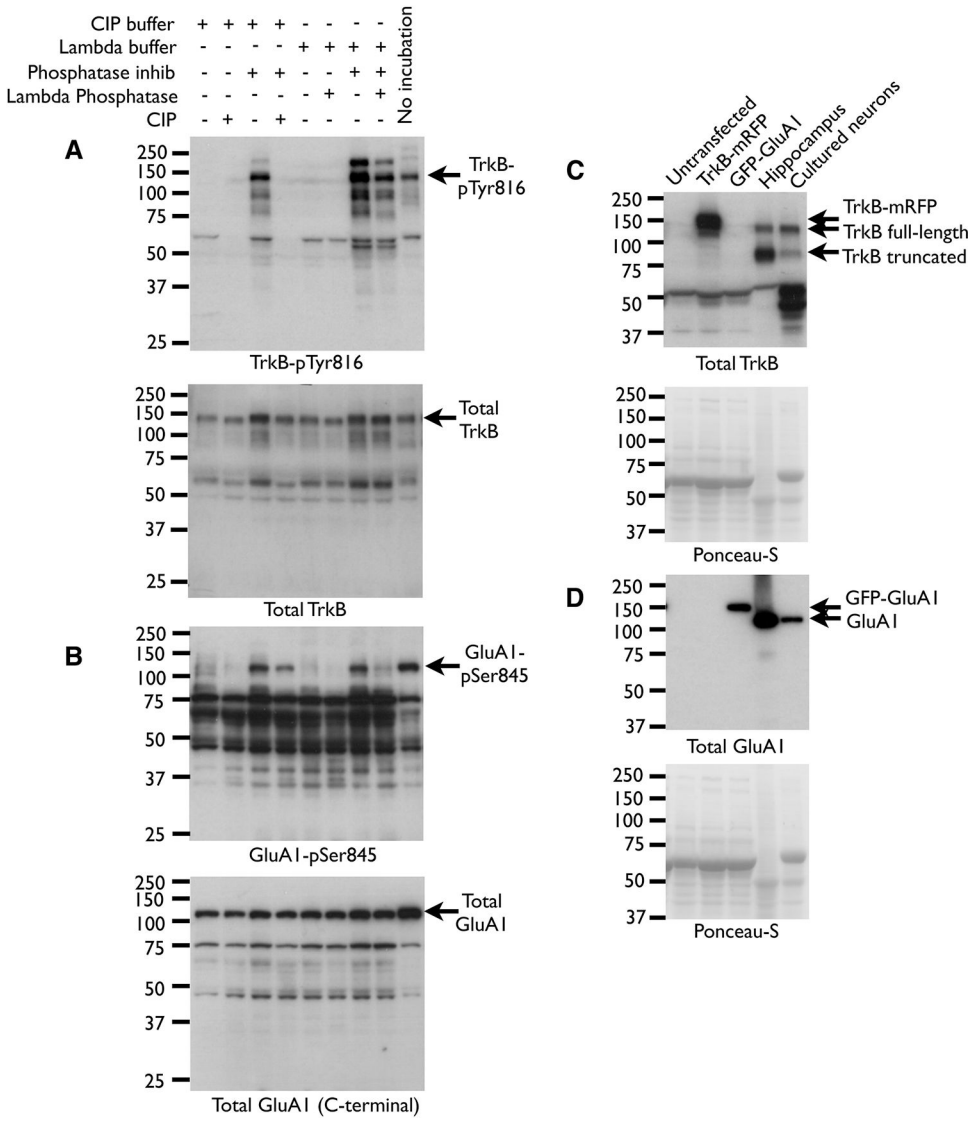

Figure 4.

Validation of phospho-specific and total antibodies against TrkB and GluA1. Lysate from cultured neurons (DIV 17) was incubated with or without phosphatase inhibitor, lambda phosphatase or calf intestinal phosphatase (CIP), then lysed and subjected to immunoblot with antibodies directed against TrkB-pTyr816 (A, upper panel) or GluA1-pSer845 (B, upper panel). The blots were stripped and reprobed with antibodies directed against TrkB N-terminal region (A, lower panel) and the GluA1 cytoplasmic domain (B, lower panel). Total TrkB (C) and GluA1 (D) antibodies were validated by probing lysate from adult rat hippocampus, cultured rat cortical neurons (DIV 15), and HEK cells that were either not transfected or transfected with expression vectors encoding TrkB-mRFP or GFP-GluA1 fusion proteins. Before probing, membranes were ponceau-stained to confirm equal loading.

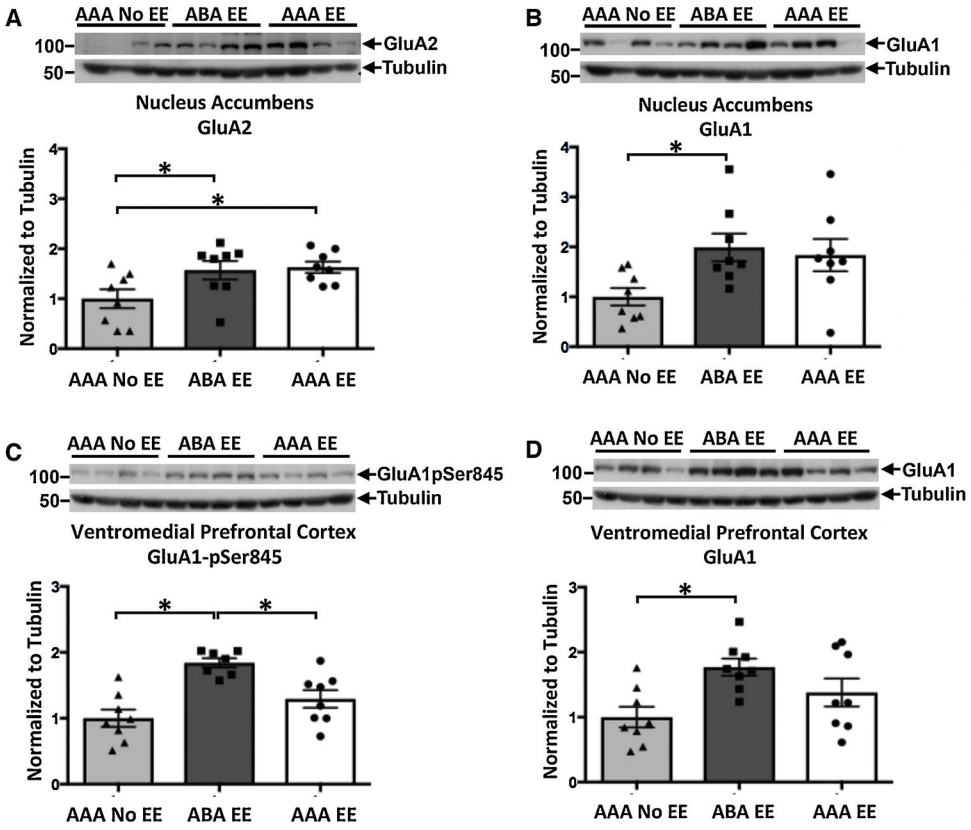

Figure 5.

Molecular correlates of EE exposure and of extinction training in the novel context in the 3 groups of rats as defined in the Figure 1 legend. Tissue harvesting was 24 hr after the last cocaine reacquisition session. Values are the mean ± S.E.M. and the individual data points of normalized band intensity for (A) nucleus accumbens total GluA2 (N= 8 rats per group, * $p \leq 0.05$ comparing AAA + EE and ABA + EE groups to the AAA + No EE group,); (B) nucleus accumbens total GluA1 (N = 8 rats per group, * $p=0.05$ comparing the ABA + EE group to the AAA + No EE group); (C) ventromedial prefrontal cortex GluA1-pSer845 (N=7–8 rats per group, * $p \leq 0.01$ comparing the ABA + EE group to the AAA + EE and AAA + No EE groups); and (D) ventromedial prefrontal cortex total GluA1 (N= 8 rats per group, * $p=0.01$ comparing the ABA + EE group to the AAA + No EE group). Representative blots are shown at the top of each panel.

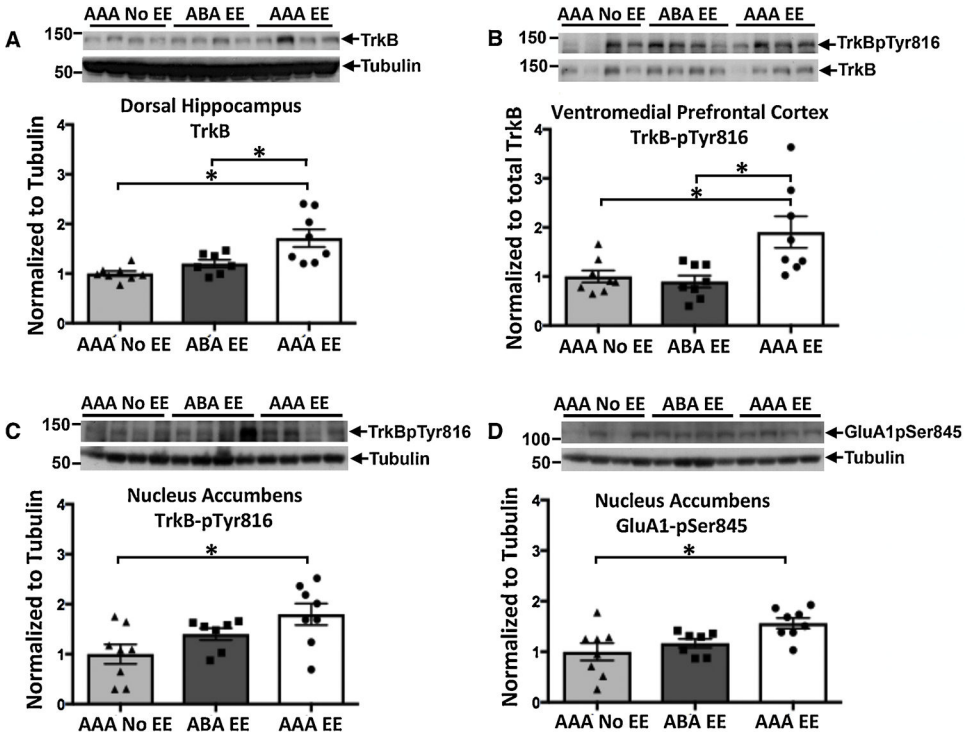

Figure 6.

Molecular correlates of cocaine relapse prevention in the 3 groups of rats as defined in the Figure 1 legend. Tissue harvesting was 24 hr after the last cocaine reacquisition session. Values are the mean ± S.E.M. and the individual data points of normalized band intensity for (A) dorsal hippocampus total TrkB (N=7–8 rats per group, * $p \leq 0.02$ comparing the AAA + EE group to the ABA + EE and AAA + No EE groups); (B) ventromedial prefrontal cortex TrkB-pTyr816, (N = 7–8 rats per group, * $p \leq 0.03$ comparing the AAA + EE group to the ABA + EE and AAA + No EE groups); (C) nucleus accumbens TrkB-pTyr816 (N = 7–8 rats per group, * p=0.02 comparing the AAA + EE group to the AAA No EE group); and (D) nucleus accumbens GluA1-pSer845 (N = 7–8 rats per group, * p=0.02 comparing the AAA + EE group to the AAA + No EE group). Representative blots are shown at the top of each panel.

Table 1.

Background stimuli used in operant chambers for familiar Context A and novel Context B

	Olfactory Contextual Cue	**Auditory Contextual Cue**	**Visual Contextual Cue**
Context A	Aspen bedding	Continuous white noise (70 db)	Clear panel on rear wall of chamber
Context B	Cedar bedding	Intermittent tone (70 db; 7 kH; 0.5 s duration every second)	Black panel on rear wall of chamber