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Research report

Changes in expression of c-Fos protein following cocaine-cue extinction learning

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HIGHLIGHTS

- Cocaine-cue extinction increased c-Fos expression in BLA and PL.
- Cocaine-cue and saline-cue extinction increased c-Fos expression in dSUB and IL.
- Number of lever responses correlated with c-Fos expression in NAc core and CPu.
- GluR2 expression was not altered after cocaine-cue extinction or control training.
- Understanding extinction mechanisms may improve exposure therapy in cocaine addicts.

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ABSTRACT

Extinguishing abnormally strengthened learned responses to cues associated with drugs of abuse remains a key tactic for alleviating addiction. To assist in developing pharmacotherapies to augment exposure therapy for relapse prevention, investigation into neurobiological underpinnings of drug-cue extinction learning is needed. We used regional analyses of c-Fos and GluR2 protein expression to delineate neural activity and plasticity that may be associated with cocaine-cue extinction learning. Rats were trained to self-administer cocaine paired with a light cue, and later underwent a single 2 h extinction session for which cocaine was withheld but response-contingent cues were presented (cocaine-cue extinction). Control groups consisted of rats yoked to animals self-administering cocaine and receiving saline non-contingently followed by an extinction session, or rats trained to self-administer cocaine followed by a no-extinction session for which levers were retracted, and cocaine and cues were withheld. Among 11 brain sites examined, extinction training increased c-Fos expression in basolateral amygdala and prelimbic prefrontal cortex of cocaine-cue extinguished rats relative to both control conditions. In dorsal subiculum and infralimbic prefrontal cortex, extinction training increased c-Fos expression in both cocaine-cue and saline-cue extinguished rats relative to the no-extinction control condition. GluR2 protein expression was not altered in any site examined after extinction or control training. Findings suggest that basolateral amygdala and prelimbic prefrontal cortex neurons are activated during acquisition of cocaine-cue extinction learning, a process that is independent of changes in GluR2 abundance. Other sites are implicated in processing the significance of cues that are present early in extinction training.

1. Introduction

Through the process of associative learning, cues paired with drugs of abuse gain enhanced salience to exert long-lasting and powerful influences over the behavior of individuals abusing drugs [1]. Extinguishing abnormally strengthened learned responses to cues associated with drugs of abuse remains a key tactic for alleviating addiction. Exposure-based behavioral therapies make use of an extinction strategy whereby individuals are confronted repeatedly with cues in a controlled setting in an effort to reduce cue salience [2]. In animal models of exposure therapy for cocaine addiction, cocaine-paired stimuli are presented repeatedly during extinction training without the delivery of cocaine [3–6]. These studies suggest that the combination of extinction training with cognitive enhancing pharmacotherapy facilitates extinction learning and deters relapse behavior in rats and monkeys trained to

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self-administer cocaine. These encouraging preclinical findings prompt investigation into the neurobiological underpinnings of cocaine-cue extinction learning. This knowledge may assist in the development of improved pharmacotherapies to use in conjunction with exposure therapy for relapse prevention in addicts. While current knowledge of substrates and mechanisms of cocaine-cue extinction learning is limited [7], it is likely that this form of extinction learning involves neurosubstrates that are similar to those involved in extinction of conditioned fear [8]. Brain regions such as the ventromedial prefrontal cortex (vmPFC) and basolateral amygdala (BLA) may have a prominent role in this regard.

In the present investigation, expression of c-Fos protein, a member of the Fos family of proteins and a product of the immediate early gene *c-fos*, was measured to determine which brain sites may become acutely active during acquisition of cocaine-cue extinction learning. Previous studies in rats have shown increased c-Fos protein expression within striatum, hippocampus, prefrontal cortex and/or amygdala after cocaine-cue reinstatement tests that followed a period of abstinence [9,10] or response extinction training for which both cocaine and response-contingent cocaine-paired cues were withheld [11,12]. Our cue exposure paradigm, in which response-contingent cues are presented in the absence of cocaine injections, differs significantly from other extinction training paradigms in which both cocaine and cues are withheld. This is an important distinction because clinical treatment protocols utilize cue exposure as a means to extinguish conditioned responses [13].

While the design of the present investigation was optimized for measuring c-Fos protein acutely expressed in response to cocaine-cue extinction learning (see Section 2), the expression of GluR2-containing AMPA receptors also was evaluated. AMPA receptors containing the GluR2 subunit predominate in principal neurons throughout the adult brain [14] and co-express with c-Fos protein in larger numbers than other subunits [10]. GluR2-containing AMPA receptors within the amygdala may play a crucial role in the formation of extinction memory that leads to reduction in previously established conditioned fear responses [15]. Previous studies in cocaine-trained rats have shown that intracellular, surface and/or total GluR2 subunit expression is not altered in nucleus accumbens (NAc) after response extinction training or cocaine-cue reinstatement tests following a period of abstinence [16,17]. In the present study, we determined if GluR2 subunit expression in several brain sites was altered after cocaine-cue extinction training.

2. Material and methods

2.1. Subjects

Male Wistar rats (Crl(WI)BR; 275–300 g) were housed individually in a temperature- (21–23 °C) and light- (08:00 h on, 20:00 h off) controlled facility. Rats were maintained in accordance with the 1996 NIH Guide for Care and Use of Laboratory Animals. The Boston University Institutional Animal Care and Use Committee approved research protocols. Animals were implanted with indwelling venous catheters using the surgical procedures described previously [3].

2.2. Drugs

Cocaine HCl (gift from NIDA, Bethesda, MD) was dissolved in sterile 0.9% saline containing 3 IU heparin/ml to a concentration of 1.6 mg/ml. For intravenous (i.v.) self-administration of cocaine, a 0.3 mg/kg unit dose was delivered at a rate of 0.03 ml/s. For yoked-control rats, saline vehicle was infused at the same rate as cocaine.

2.3. Behavioral procedures

The experimental design is outlined in Table 1. During daily 1 h cocaine self-administration sessions, rats that later underwent cocaine-cue extinction training (Group 1) were initially trained to press a lever designated as active to obtain 0.3 mg/kg i.v. injections of cocaine paired with the simultaneous presentation of a distinctive visual stimulus (2-s light) under a fixed-ratio (FR) 1 schedule of reinforcement. Responses on a second inactive lever had no scheduled consequences. Training continued until rats self-administered cocaine under a second-order reinforcement schedule. A second-order schedule was used because it exposes animals to a greater number of response-contingent drug-paired cues during sessions compared to a fixed ratio schedule. A high cue exposure baseline provides a stringent test of how well drug-seeking responses are reduced in later extinction tests. For this schedule, the 2-s cue light was presented under an FR5 contingency during the entire session. The delivery of cocaine co-occurred with cue light presentation upon completion of the first FR5 after each 5-min fixed interval (FI) of time elapsed. After cocaine was delivered, the FI component was again in effect. This schedule is specified as an FI 5-min [FR5:S] second-order schedule, where the S refers to the 2-s light cue. During 1 h daily sessions, rats could self-administer a maximum 11 cocaine injections.

Training continued for a minimum of 30 sessions until rats self-administered cocaine reliably. After baseline cocaine self-administration was stable (i.e., the absence of increasing or decreasing trends and $\leq$10% individual variability day-to-day), rats underwent 2 weeks of abstinence prior to the extinction test session. During the abstinence period, rats received ten 1 h sessions in operant chambers for which levers were retracted, and without presentation of either the cocaine-paired stimulus or the delivery of cocaine. The multiple abstinence sessions were included to dampen the ability of the contextual cues in the test environment to acutely express c-Fos protein on test day [18–20]. In addition, multiple abstinence sessions were used to restore the ability of discrete cue presentations and lever pressing to acutely express c-Fos protein on test day [11] as well as to reveal neural activation patterns associated with the novel cocaine-cue extinction learning. Following the abstinence period, rats underwent a single 2 h cocaine-cue extinction test session. During the extinction test, an FI 5-min [FR5:S] second-order schedule also was used whereby the 2-s cue light was presented under an FR5 contingency during the entire session. In addition, saline was substituted for cocaine and the delivery of saline co-occurred with cue light presentation upon completion of the first FR5 after each FI 5-min elapsed.

Several control groups were included in this experiment to evaluate the extent to which the molecular changes were selectively associated with cocaine-cue extinction learning (Table 1). Rats receiving saline (Groups 2 and 3) were yoked to rats self-administering cocaine (Group 1) and received saline infusions non-contingently. One group had infusions paired with the 2-s light cue (Group 2), whereas the other group did not (Group 3). Following the 2-week abstinence period as described above, rats in Groups 2 and 3 were given a yoked-extinction test, which was identical to their yoked-training sessions. Additional control groups were trained to self-administer cocaine. One group was trained to self-administer cocaine in the usual manner (Group 4) and the other group was trained to self-administer cocaine with cues presented in a random non-contingent manner (Group 5). Following the 2-week abstinence period as described above, rats in Groups 4 and 5 were given an additional abstinence session rather than an extinction session as the test (No-EXT session). With this design, it was possible to determine if changes in c-Fos or GluR2 protein expression on test day were associated specifically with cocaine-cue extinction learning (Group 1), or if changes also were evident simply as a matter of lever pressing and receiving an i.v. injection of saline following paired or unpaired discrete cue presentation (Groups 2 and 3) or of chronic exposure to the cocaine context following a history of paired or unpaired discrete cue presentation during cocaine self-administration training (Groups 4 and 5). A home cage control group was not evaluated because our primary interest was in determining which brain sites exhibited enhanced neural activity while rats underwent extinction to the discrete cues paired with cocaine. The appropriate controls for this precise question were Groups 2–5.

2.4. Neurochemical procedures

Expression of c-Fos and GluR2 proteins were measured immunohistochemically. As c-Fos protein has been shown to peak in neural expression 60–120 min after exposure to an acute stimulus or novel event [21], rats were sacrificed by an overdose of sodium pentobarbital 60 min following completion of the 2 h test session. This time-point was selected for sacrifice because it corresponds to the time c-Fos protein would hypothetically reach peak expression following extinction learning, an event that occurred during the last 30 min of the 2 h extinction session (90 min before sacrifice). Rats were perfused with 4% paraformaldehyde solution and the brains were extracted, flash frozen in isopentane and stored at −80 °C. Different sets of 40 μm coronal brain slices were collected and stored in ethylene glycol solution at −20 °C for the assays. One set of these brain slices was used for c-Fos detection and another set for GluR2 detection. Sections taken at +3.72 to +2.52 mm contained the prelimbic and infralimbic prefrontal cortices (PL and IL), the anterior cingulate (ACC) and M1 motor cortices (M1); sections taken at +2.76 to −1.20 mm contained the nucleus accumbens core and shell (NAc core and NAc shell); sections taken at +2.50 to −0.48 mm contained the caudate putamen (CPu); sections taken at −1.5 to −3.48 mm contained the BlA; sections taken at −3.00 to −5.50 mm contained dorsal hippocampal subiculum (dSUB); sections taken at −4.50 to −6.50 mm contained the ventral hippocampal subiculum (vSUB) and paraventricular thalamic nucleus (PVN). These sites previously have been shown to be relevant for extinction learning and addiction [22–24]. The M1 motor cortex (M1) served as a control site.