Published in final edited form as:

Neuroscience. 2007 March 16; 145(2): 438–452.

FOS AND GLUTAMATE AMPA RECEPTOR SUBUNIT COEXPRESSION ASSOCIATED WITH CUE-ELICITED COCAINE- SEEKING BEHAVIOR IN ABSTINENT RATS

Arturo R. Zavala¹, Sudipta Biswas¹, Richard E. Harlan², and Janet L. Neisewander¹

1 Department of Psychology, Arizona State University, Box 871104, Tempe, AZ 85287-1104

2 Department of Structural and Cellular Biology, Tulane University School of Medicine, Box SL49, 1430 Tulane Avenue, New Orleans, LA 70112

Abstract

Cocaine-associated cues acquire incentive motivational effects that manifest as craving in humans and cocaine-seeking behavior in rats. We have reported an increase in neuronal activation in rats, measured by Fos protein expression, in various limbic and cortical regions following exposure to cocaine-associated cues. This study examined whether the conditioned neuronal activation involves glutamate AMPA receptors by measuring coexpression of Fos and AMPA glutamate receptor subunits (GluR1, GluR2/3, or GluR4). Rats trained to self-administer cocaine subsequently underwent 22 days of abstinence, during which they were exposed daily to either the self-administration environment with presentations of the light/tone cues previously paired with cocaine infusions (Extinction group) or an alternate environment (No Extinction group). All rats were then tested for cocaine-seeking behavior (i.e., responses without cocaine reinforcement) and Fos and AMPA glutamate receptor subunits were measured post-mortem using immunocytochemistry. The No Extinction group exhibited increases in cocaine-seeking behavior and Fos expression in limbic and cortical regions relative to the Extinction group. A large number of Fos immunoreactive cells coexpressed GluR1, GluR2/3, and GluR4, suggesting that an action of glutamate at AMPA receptors may in part drive cue-elicited Fos expression. Importantly, there was an increase in the percentage of cells colabeled with Fos and GluR1 in the anterior cingulate and nucleus accumbens shell and cells colabeled with Fos and GluR4 in the infralimbic cortex, suggesting that within these regions, a greater, and perhaps even different, population of AMPA receptor subunit-expressing neurons is activated in rats engaged in cocaine-seeking behavior.

Keywords

reinstatement; drug conditioning; immediate early gene; incentive motivation; c-fos

Glutamate mechanisms are involved in incentive motivation for cocaine that is triggered by either cocaine-associated cues or drug priming (Kalivas and McFarland, 2003, Tzschentke and Schmidt, 2003). In particular, AMPA glutamate receptor activation is critical because infusions

Corresponding Author: Dr. Janet L. Neisewander, Professor, Department of Psychology, Arizona State University, Box 871104, Tempe, AZ 85287-1104, Phone: (480) 965-0209, Fax: (480) 965-8544, Email: janet.neisewander@asu.edu

Section Editor: Dr. Gregory J. Quirk, Ponce School of Medicine, Department of Physiology, Dr. Ana Marchand Perez Street, Urb. Industrial Reparada, Ponce, 00731, Puerto Rico

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of AMPA into the nucleus accumbens (NAC) reinstates cocaine-seeking behavior (Cornish et al., 1999, Suto et al., 2004); whereas antagonism of AMPA receptors in this region attenuates reinstatement of cocaine-seeking behavior by either a cocaine priming injection, intra-accumbens AMPA infusions, or intra-medial prefrontal cortex infusions of cocaine (Cornish and Kalivas, 2000, Park et al., 2002). These effects are region-specific within the NAC because infusions of an AMPA receptor antagonist into the NAC core (NACc), but not the NAC shell (NACs), attenuate cocaine-seeking behavior maintained by a second-order schedule of reinforcement (Di Ciano and Everitt, 2001). Consistent with these findings, AMPA receptor antagonists given systemically attenuate cocaine-seeking behavior maintained by cues under a second-order schedule of reinforcement (Backstrom and Hyttia, 2003), as well as reinstatement of extinguished cocaine-seeking behavior by cocaine-paired cues (Backstrom and Hyttia, 2006). Recently, AMPA receptors in the dorsal striatum have also been implicated in cocaine-seeking behavior maintained under a second-order schedule of reinforcement (Vanderschuren et al., 2005), suggesting a more extensive involvement of AMPA receptors.

AMPA receptors are composed of four individual subunits (GluR1-GluR4) that combine to form functionally different receptors (Hollmann and Heinemann, 1994). There is considerable evidence that AMPA receptor subunits are altered in response to repeated cocaine administration (Fitzgerald et al., 1996, Churchill et al., 1999) and withdrawal from cocaine self-administration (Lu et al., 2003, Tang et al., 2004, Hemby et al., 2005, Lu et al., 2005). Moreover, an increase in the surface expression of GluR1 in the NAC is evident in rats exhibiting a sensitized behavioral response to cocaine (Boudreau and Wolf, 2005), suggesting an involvement of GluR1 trafficking in cocaine-induced neuroplasticity (Wolf et al., 2004). Similar mechanisms may underlie extinction-induced up-regulation of GluR1 protein levels in the NACs (Sutton et al., 2003, Self et al., 2004). Collectively, these findings suggest a critical role of AMPA receptor subunits in behavioral changes resulting from cocaine.

Research using expression of the immediate early gene (IEG) product, Fos, as a marker of neuronal activation has implicated a neural circuit involving the NAC, the anterior cingulate (ACg) and prelimbic (PrL) subregions of the prefrontal cortex, the dentate gyrus (DG) and CA1 regions of the hippocampal formation, and the basolateral amygdala (BLA) in the incentive motivational effects of cocaine cues (Crawford et al., 1995, Neisewander et al., 2000, Ciccocioppo et al., 2001). Furthermore, we have shown that Fos expression associated with cue-elicited cocaine-seeking behavior is not due to motor performance of the operant response per se (Neisewander et al., 2000). The potential role of AMPA receptors within several regions of this circuit has not been investigated. In the present study, we characterized whether cells that exhibit cue-elicited Fos expression in these regions coexpress AMPA receptor subunits (GluR1-GluR4), which would suggest a role of AMPA receptor mechanisms in cue-conditioned Fos expression.

EXPERIMENTAL PROCEDURES

Subjects

Male Sprague Dawley rats weighing 225–250 g at the start of the experiment were housed individually and kept in climate-controlled colony rooms under a 12-h reverse light:dark cycle (lights off at 6:00 am). Rats were acclimated to handling for 5 days prior to surgery. Housing facilities were accredited through the Association for Assessment and Accreditation of Laboratory Animal Care International and care of the animals was in accordance with the conditions set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, 1996). Experimental procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University.

Surgery

Implantation of intravenous catheters was performed under deep anesthesia with Nembutal (50 mg/kg, i.p., Abbott Laboratories, Chicago, IL). To prevent bronchial secretions, rats were pretreated with atropine sulfate (10 mg/kg, i.p.; Sigma) prior to the anesthetic. The catheters were constructed from silastic tubing (10 cm in length, inner diameter 0.012, outer diameter 0.025 inches, Dow Corning, Midland, MI) connected to a bent 22 gauge metal cannula encased within a plastic screw connector (Plastics One, Roanoke, VA) at one end and affixed with a small ball of aquarium sealant approximately 2.7 cm from the outer end. Catheters were inserted into a small incision in the jugular vein and secured with sutures on either side of the ball. The other end of the catheter ran subcutaneously along the neck and exited through an incision across the skull. The metal cannula end was secured to the top of the skull using dental acrylic cement and small anchor screws drilled into the skull. Throughout the experiment, catheter patency was maintained by flushing daily with 0.1 ml bacteriostatic saline containing heparin sodium (10 U/ml; Elkins-Sinn Inc., Cherry Hill, NJ), streptokinase (0.67 mg/ml; Astra USA, Inc., Westborough, MA), and ticarcillin disodium (SmithKline Beecham Pharmaceuticals, Philadelphia, PA). Catheter patency was confirmed periodically by infusions of the rapid, short-acting anesthetic methohexital sodium (16.67 mg/ml, i.v.), which produces a brief loss of muscle tone only when administered i.v.

Apparatus

Training took place in operant conditioning chambers (20 × 28 × 20 cm) equipped with two levers mounted on the front wall (Med Associates, St. Albans, VT), a cue light above one lever, a tone generator, and a house light mounted on the center of the back wall. The lever with the cue light was designated as the active lever and the other as the inactive lever. Each chamber was contained inside a sound attenuating chamber. A 10 ml syringe housed in an infusion pump was located outside of the chamber and was attached via Tygon tubing to a liquid swivel (Instech, Plymouth Meeting, PA). The outlet of the swivel was fastened to the catheters via Tygon tubing that ran through a metal spring leash (Plastics One).

Self-administration training

After at least 5 days of recovery from surgery, rats were randomly divided into groups that were either trained to press a lever reinforced by cocaine infusions (Cocaine group; $n = 16$) or to receive an equal volume of saline (Control group; $n = 9$) yoked to the schedule completions made by a rat in the Cocaine group. Training sessions occurred daily for 2 h, at the same time of day across 28 consecutive days. Schedule completions by a Cocaine rat resulted in simultaneous presentation of a tone (500 Hz, 10 db above background), cue light, and house light, followed one second later by a cocaine infusion (0.75 mg/kg/0.1 ml, i.v.). Control yoked rats were simultaneously presented the same stimulus complex when they received the saline infusion (0.1 ml, i.v.) contingent upon responses of their Cocaine rat counterpart. Upon completion of the 6 s infusion, the cue light, tone, and infusion pump were inactivated simultaneously. The house light remained on for an additional 20 s signaling a timeout period during which lever presses had no scheduled consequences. Responses on the inactive lever were recorded but had no scheduled consequences. Based on their individual self-administration performance, rats progressed from a fixed ratio (FR) 1 schedule to a variable ratio (VR) 2, VR 3, and finally to a VR 5 schedule of reinforcement. Lever presses by Control rats had no programmed consequences. Beginning two days prior to self-administration training, rats were restricted to 18 g of rat chow/day to facilitate acquisition of cocaine self-administration (Carroll et al., 1981). The rats remained food restricted until a criterion of 7 cocaine infusions/h was achieved on 2 consecutive days, after which they were given access

to food *ad libitum* throughout the remainder of the experiment. Rats were maintained on a VR 5 schedule of reinforcement with no food restrictions for at least the last 5 days of training.

Extinction training

Extinction training began the day after self-administration training was completed and involved daily 90-min sessions for 22 consecutive days. Prior to the start of this phase, rats were further assigned to Extinction or No Extinction conditions. Assignment to these conditions was counterbalanced for total number of cocaine infusions obtained during cocaine self-administration. Control rats were assigned to the same condition as their Cocaine rat counterpart. Rats in the Cocaine-Extinction group ($n = 8$) received response-contingent presentations of the stimulus complex previously paired with cocaine infusions on an FR 1 schedule during the extinction sessions. The infusion pumps were activated during extinction training, however, no infusions were delivered. Extinction training was designed to decrease the incentive motivational and conditioned reinforcing effects of the cocaine cues. Rats in the Control-Extinction group ($n = 4$) were presented with the same stimulus complex contingent upon the responses of their Cocaine rat counterpart. Rats in the Cocaine-No Extinction ($n = 8$) and Control-No Extinction ($n = 5$) groups were placed in gray plastic holding cages that were of equal size as the operant chambers, but with different bedding and visual cues. Rats in the No Extinction condition were ran at the same time as rats in the Extinction condition, although the holding cages were located in a separate room from where self-administration took place. Thus, the incentive motivational/conditioned reinforcing effects of the cocaine-associated stimuli remained intact in the Cocaine-No Extinction group.

Test for cocaine-seeking behavior

Cocaine-seeking behavior was operationally defined as responses on the active lever in the absence of cocaine reinforcement or on the corresponding lever in saline-yoked controls. Testing occurred the day after the last extinction session. All rats were placed back into their self-administration chambers, and a non-contingent presentation of the cocaine-paired stimulus complex (i.e., cue light, tone, house light, and pump motor) was delivered. Thereafter FR1 schedule completions by a Cocaine rat activated this stimulus complex for both the Cocaine rat and his Control rat counterpart. The test for cocaine-seeking behavior lasted 90 min, during which responses by all rats on both the active and inactive lever were recorded. Lever presses by Control rats had no consequences.

Tissue preparation

Immediately after the 90-min test for cocaine-seeking behavior, rats were deeply anesthetized using Nembutal (100 mg/kg, i.p.) and intracardially perfused with ice cold 0.1 M phosphate buffered saline (PBS), pH 7.4, followed by ice cold 4% paraformaldehyde (PFA) dissolved in 0.1 M PBS, pH 7.4. Brains were then removed and post-fixed in PFA for 24 h and stored in 30% sucrose at 4°C. Serial brain sections (40 μ m) were then cut using a freezing microtome from different levels corresponding to +3.2, +1.6, and -2.56 mm from bregma (Paxinos and Watson, 1998). Sections were stored in 0.1 M PBS containing 0.1% sodium azide at 4°C until processed for immunolabeling.

Immunocytochemistry

Free floating sections from rats in each group were processed simultaneously for Fos protein expression. Initially, sections were washed extensively in 0.1 M PBS (6 times for 10 min each), and incubated for 1 h in 0.2% Triton X-100 in 0.1 M PBS containing 5% normal horse serum (Vector Laboratories, Burlingame, CA). Sections were then incubated for 48 h at 4°C with the anti-c-fos goat polyclonal primary antibody (1:20,000; Santa Cruz Biotechnology, Santa

Cruz, CA, sc-52-G) containing 0.1 M PBS, 0.2% Triton X-100, and 1.5% normal horse serum. The tissue was then washed in 0.1 M PBS (6 times for 5 min each) and incubated for 1 h in 0.1 M PBS containing biotinylated horse anti-goat antibody IgG (1:200; Vector Laboratories) and 1% normal horse serum. The tissue was then given additional washes in 0.1 M PBS (3 times for 10 min each) and incubated for 1 h in avidin-biotinylated horse-radish peroxidase complex (ABC Elite kit, Vector Laboratories) diluted in 0.1 M PBS. The sections were then washed twice in 0.1 M PBS, followed by a wash in 0.1 M Tris buffer, pH 7.6. The tissue was then incubated in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 2.5% nickel ammonium sulfate (Sigma) and 0.005% hydrogen peroxidase (Sigma) for 3–5 min. This reaction was terminated by rinsing the tissue in 0.1 M PBS (4 times for 10 min each). Sections were then processed for double-immunocytochemistry using the following primary antibodies: rabbit anti-GluR1 polyclonal antibody (1:1000; Chemicon, Temecula, CA, AB 1504), rabbit anti-GluR2/3 polyclonal antibody (1:1000; Chemicon, Temecula, CA, AB 1506), or rabbit anti-GluR4 polyclonal antibody (1:500; Chemicon, Temecula, CA, AB 1508). Sections were washed extensively in 0.1 M PBS (6 times for 10 min each), incubated for 1 h in 0.2% Triton X-100 in 0.1 M PBS containing 5% normal goat serum (Vector Laboratories, Burlingame, CA), and incubated for 48 h at 4°C in the primary antibody containing 0.1 M PBS, 0.2% Triton X-100, and 1.5% normal goat serum. The tissue was then washed in 0.1 M PBS (6 times for 5 min each), incubated for 1 h in 0.1 M PBS containing biotinylated goat anti-rabbit antibody IgG (1:200; Vector Laboratories) and 1% normal goat serum, washed in 0.1 M PBS (3 times for 10 min each) and incubated for 1 h in avidin-biotinylated horse-radish peroxidase complex diluted in 0.1 M PBS. Next, sections were washed twice in 0.1 M PBS, followed by a wash in 0.1 M Tris buffer, pH 7.6, and incubated in 0.05% DAB in 0.005% hydrogen peroxidase for 3–5 min. This reaction was terminated by rinsing the tissue in 0.1 M PBS (4 times for 10 min each). Finally, sections were mounted onto slides coated with gelatin chrom-alum, dried, and dehydrated before coverslipping.

Immunoreactivity analysis

Fos and GluR specific immunoreactivity were examined using a Nikon Eclipse E600 (Nikon Instruments, Melville, NY) microscope set at 40× magnification and counted by an observer blind to treatment conditions using the Image tool software package (Version 3.0, University of Texas Health Sciences Center, San Antonio, TX). Fig. 1 illustrates the specific subregions analyzed. Sections taken at +3.2 mm from bregma contained the PrL, infralimbic (IL), and orbitofrontal (OF) cortex; sections taken at +1.6 mm from bregma contained the Cg2 region of the ACg cortex, NACs, NACc, and dorsal caudate-putamen (dCPu); sections taken at –2.56 mm from bregma contained the somatosensory (SS) cortex, lateral amygdala (LA), BIA, central amygdala (CeA), dorsal hippocampal CA1, CA3, and DG. Images were captured from each hemisphere of three sections double-labeled for Fos and one of the AMPA receptor subunits (GluR1, GluR2/3, or GluR4). These sections were taken such that the rostral-caudal extent of each region was sampled (340µm). The area of each sample measure was 0.065 mm², but the number of samples varied by region. For all regions except the NACs, NACc, and OF, 12 sample areas were counted (i.e., 2 sample areas/hemisphere/section). For each of the NAC regions, 6 sample areas were counted (i.e., 1 sample area/hemisphere/section), and for the OF, 18 sample areas were counted (i.e., 3 sample areas/hemisphere/section). The counts from all of the sample areas of a given region were averaged to give a mean number of immunoreactive cells/0.065 mm². Colocalization of Fos plus a given GluR subunit was also counted and identified as a blue-black nucleus (indicating Fos immunoreactivity) surrounded by a brown cytoplasm (indicating GluR subunit immunoreactivity). Fig. 2 illustrates representative images, taken at 60× to enhance the visualization of the double labeling, of a small part of representative regions of cortex double-labeled for Fos and GluR subunits in all groups.

Statistics

Separate factorial ANOVAs were used to analyze active lever responses during extinction and active and inactive lever responses on the test day with self-administration history and extinction condition as between subjects factors and day as a repeated measure for extinction data. Separate repeated measures ANOVAs were used to analyze number of Fos, GluR1, GluR2/3, and GluR4 labeled immunoreactive cells with group as a between subjects factor and brain region as a repeated measures factor. Two dependent measures were analyzed to assess colocalization: 1) the number of cells colabeled with Fos plus a given GluR subunit and 2) the percentage of Fos cells colabeled with a given GluR subunit. The percentage of Fos cells colabeled with a given GluR subunit was calculated by dividing the number of Fos plus GluR subunit colabeled cells by the number of Fos immunoreactive cells in a given region for each individual rat. These data are presented and analyzed separately for cortex, striatum, amygdala, or hippocampal subregions using ANOVAs with group as a between subjects factor and GluR subunit and subregion as repeated measures. Sources of main effects and interactions were further analyzed using tests of simple effects and post-hoc Tukey HSD tests. In addition to comparing group differences, the correlation between lever presses on the test day and the number of Fos cells double labeled with GluR subunits within the areas where significant effects were found was determined using the Pearson product-moment correlation (r).

RESULTS

Acquisition of cocaine self-administration

Rats exhibited a mean ($\pm$ SEM) total cocaine intake of 388.08 ± 28.70 mg/kg throughout the 28 days of training. Cocaine intake was stable (variation of $< 18\%$) during the last 10 days of training and did not vary between Cocaine groups. Specifically, rats self-administered a mean ($\pm$ SEM) of 17.35 ± 1.42 mg/kg/day of cocaine and exhibited a mean ($\pm$ SEM) response rate of 78.69 ± 13.52 active lever presses/2 h during the last 10 days of training.

Cocaine-seeking behavior

Extinction training was used to devalue the motivational significance of the cocaine cues and self-administration environment. Responses on the active lever by the Cocaine-Extinction group were significantly greater compared to the Control-Extinction group on the first day, as indicated by a significant self-administration history $\times$ extinction training day interaction ($F_{21,210} = 2.67$, $P < 0.001$) and a test of simple effects ($P < 0.05$). Response rates declined across days in the former group, such that there were no group differences on days 10, 12, 16, and 17 (see Fig. 3). Furthermore, lever presses by the Cocaine-Extinction group were significantly lower on the last day of extinction training (day 22) than on the first day of extinction ($t_7 = 3.56$, $P < 0.05$).

The test day results indicated that rats in the Cocaine-No extinction group exhibited significantly more responses on the active lever than rats in all other groups (see Fig. 3), whereas there were no differences observed among the other groups, as indicated by a significant self-administration history $\times$ extinction training interaction ($F_{1,21} = 22.43$, $P < 0.001$) and subsequent Tukey tests, ($P < 0.05$). In contrast, lever presses on the inactive lever during the test day by rats in the No Extinction groups were significantly higher than rats assigned to the Extinction groups, regardless of self-administration history, as indicated by a main effect of extinction training ($F_{1,21} = 5.25$, $P < 0.05$). Specifically, rats in the No Extinction condition had a mean ($\pm$ SEM) response rate of 12.85 ± 3.03 lever presses; whereas the rats in the Extinction condition had a mean ($\pm$ SEM) response rate of 4.00 ± 1.19 lever presses on the test day.

A comparison of cocaine-seeking behavior on the first day of extinction between the Cocaine-Extinction group (i.e., extinction training day 1) and the Cocaine-No Extinction group (i.e., test day) failed to show a significant difference, although there was a trend for a difference ($t_{14} = 1.41$, $P = 0.09$). Specifically, rats in the Cocaine-No Extinction group tested after a 22-day abstinence period had a mean ($\pm$ SEM) response rate of 112.38 ± 15.05 , whereas the rats in the Cocaine-Extinction group had a mean ($\pm$ SEM) response rate of 78.50 ± 18.64 .

Fos protein immunoreactivity

As expected, Controls that were assigned to Extinction and No Extinction groups did not differ in any of the dependent measures, indicating that their history of exposure to different environments during the 22-day extinction phase had no bearing on Fos expression. Thus, immunohistochemistry data from these two groups were combined to form a single Control group. Fig. 4 presents the results of the Fos protein expression for each group in all of the regions examined. Exposure to response-contingent cues and the environment associated with cocaine self-administration increased Fos protein expression in the Cocaine-No Extinction group relative to all other groups in all regions except the SS. The overall ANOVA indicated a significant group $\times$ brain region interaction ($F_{26,286} = 4.612$, $P < 0.05$). Subsequent one-way ANOVAs of group for each region were also significant ($F_{2,22} = 8.72$ – 22.06 , $P < 0.05$) in each region except the SS cortex, and in each case the Cocaine-No Extinction group exhibited significantly greater Fos protein expression relative to all other groups, Tukey HSD tests, $P < 0.05$. The lack of group differences in the SS cortex, a region that is not considered part of the neural circuit involved in incentive motivational effects of cocaine cues, demonstrates region specificity of conditioned Fos expression.

AMPA glutamate receptor subunit immunoreactivity

Fig. 5 presents the results of the AMPA GluR1, GluR2/3, and GluR4 expression for each group in all regions examined. Expression of these subunit proteins did not differ across groups in any region analyzed.

Fos and AMPA subunit coexpression in the cortex

Fig. 6 presents the results of the double labeling of Fos protein and AMPA GluR1, GluR2/3, and GluR4 coexpression for each group in the PrL, IL, OF, ACg and SS regions of cortex. There was an increase in cells coexpressing Fos and GluR1, GluR2/3, or GluR4 in the Cocaine-No Extinction group relative to all other groups in all regions, except for the SS cortex for all subunits and the ACg for the GluR2/3 subunit, evident as a significant group $\times$ GluR subunit $\times$ brain region interaction ($F_{16,176} = 2.495$, $P < 0.05$), and subsequent one-way ANOVAs of group for each region followed by Tukey tests ($P < 0.05$). Examination of the percentage of Fos cells exhibiting coexpression with GluR subunits revealed that the Cocaine-No Extinction group, relative to all other groups, exhibited a greater percentage of cells that were colabeled with Fos and GluR1 and Fos and GluR4 in the ACg and IL cortex, respectively, indicated by a significant group $\times$ GluR subunit $\times$ brain region interaction ($F_{16,176} = 3.327$, $P < 0.05$), and subsequent one-way ANOVAs of group and Tukey tests ($P < 0.05$). In addition, there was a greater percentage of Fos cells coexpressing Fos and GluR4 in the SS cortex in the Cocaine-Extinction group relative to the Cocaine-No Extinction group only.

Fos and AMPA subunit coexpression in the striatum

Fig. 7 presents the results of the double labeling of Fos protein and AMPA GluR1, GluR2/3, and GluR4 coexpression for each group in the NACs, NACc, and dCPu regions. There was an increase in cells coexpressing Fos and GluR1 or GluR2/3 in the Cocaine-No Extinction group relative to all other groups in all regions examined, evident by a significant group $\times$ GluR

subunit $\times$ brain region interaction ($F_{8,88} = 6.712$, $P < 0.05$), and subsequent one-way ANOVAs of group and Tukey tests ($P < 0.05$). In contrast, there was only a small increase in Fos and GluR4 coexpression in the NACc in the Cocaine-No Extinction group relative to the Control group, but no changes relative to the Cocaine-Extinction group and no group differences in either the NACs or dCPu. Analysis of the percentage of Fos cells exhibiting coexpression revealed that there was a greater percentage of cells in the NACs that were colabeled with Fos and GluR1 in the Cocaine-No Extinction group relative to all other groups, indicated by a significant group $\times$ GluR subunit $\times$ brain region interaction ($F_{8,88} = 2.047$, $P < 0.05$), and subsequent one-way ANOVAs of group and Tukey tests ($P < 0.05$). In addition, the Cocaine-No Extinction group exhibited a greater percentage of Fos cells coexpressing GluR2/3 in the dCPu relative only to the Control group, and a decrease percentage of Fos cells coexpressing GluR4 in the NACc relative to the Cocaine-Extinction group.

Fos and AMPA subunit coexpression in the amygdala

Fig. 8 presents the results of the double labeling of Fos protein and AMPA GluR1, GluR2/3, and GluR4 subunit coexpression for each group in the LA, BIA, and CeA regions of the amygdala. There was an increase in cells coexpressing Fos and each of the GluR subunits by the Cocaine-No Extinction group relative to the all other groups in the BIA, evident by a significant group $\times$ GluR subunit $\times$ brain region interaction ($F_{8,88} = 2.789$, $P < 0.05$), and subsequent one-way ANOVAs of group and Tukey tests ($P < 0.05$). In addition, in the CeA, the Cocaine-No Extinction group exhibited an increase in cells coexpressing Fos and GluR2/3 and GluR4 relative to all other groups, but when examining coexpression of Fos and GluR1, there was only an increase relative to the Cocaine-Extinction group. In the LA, the Cocaine-No Extinction group exhibited an increase coexpression of Fos and GluR1, but only a modest increase relative to the Control group when examining Fos and GluR2/3 coexpression, and no differences when examining Fos and GluR4 coexpression. In contrast, there was no evidence for a greater percentage of Fos cells coexpressed with any of the GluR subunits examined across any region of the amygdala analyzed.

Fos and AMPA subunit coexpression in the hippocampus

Fig. 9 presents the results of the double labeling of Fos protein and AMPA GluR1, GluR2/3, and GluR4 subunit coexpression for each group in the CA1, CA3, and DG regions of the hippocampus. The Cocaine-No Extinction group exhibited an increase in Fos and GluR colabeling compared to all other groups regardless of the region and across all GluR subunits considered, indicated by a main effect of group ($F_{2,22} = 14.579$, $P < 0.05$) and Tukey test ($P < 0.05$). In contrast, there was no indication of a greater percentage of Fos cells exhibiting coexpression of Fos and any of the GluR subunits in any hippocampal region examined.

Correlation between responding and double labeling of Fos and GluR subunits

Fig. 10 illustrates the scatterplots depicting the correlation between responding and double labeling of Fos and a GluR subunit in the IL, ACg, and NACs, which were the regions in which an increase percentage of Fos-labeled neurons coexpressed a particular GluR subunit. There was a significant positive correlation between the lever presses exhibited on the test day and the number of cells colabeled for Fos and GluR4 in the IL cortex ($r_{24} = 0.913$, $P < 0.05$), as well as the number of cells colabeled for Fos and GluR1 in the ACg and NACs ($r_{24} = 0.882$, $P < 0.05$ and $r_{24} = 0.814$, $P < 0.05$), respectively). For comparison, in the SS control region in which no conditioned Fos expression was observed, there was no relationship between responding and coexpression of Fos and GluR1.

DISCUSSION

The present findings suggest AMPA glutamate receptors likely play a role in c-Fos induction in the neural circuitry activated by cocaine cues. Specifically, a large number of Fos-labeled neurons coexpressed the AMPA receptor subunits GluR1, GluR2/3, and GluR4 (see Figs. 6–9), consistent with evidence suggesting an involvement of AMPA receptors in the induction of Fos (Mead et al., 1999, Garcia et al., 2003, Fowler et al., 2004, Soyguder, 2005). The analysis of the percentage of Fos-labeled cells exhibiting coexpression with each of the AMPA receptor subunits indicated that in most regions, group differences in the number of colabeled cells were proportional to group differences in Fos labeling in that region, such that no difference in the percentage of Fos cells exhibiting AMPA receptor subunit was observed in these regions, even though absolute numbers of cells colabeled were higher in cocaine-seeking rats. These findings suggest that for the most part, all rats exhibited activation of corticolimbic circuits, but the degree of neuronal activation of the circuit was stronger in cocaine-seeking rats. In contrast to this general rule, important region-specific increases in the percentage of Fos cells coexpressing particular AMPA receptor subunits were also observed. Specifically, there was an increase in the percentage of Fos cells expressing GluR1 in the NACs and Cg2 subregion of the ACg cortex and an increase in the percentage of Fos cells expressing GluR4 in the IL cortex of rats exhibiting cue-elicited cocaine-seeking behavior relative to the control groups (See Figs. 6–7). These findings could reflect up-regulation of AMPA receptors, synaptogenesis, or recruitment of additional neurons. Further research is needed to investigate these hypotheses. In any case, it appears that within the NACs, ACg and IL, a greater, and perhaps even different, population of AMPA receptor subunit-expressing neurons is activated in rats engaged in cocaine-seeking behavior. Consistent with this interpretation, we found that a greater level of responding on the test day was associated with a greater number of Fos cells that exhibited colocalization with a given GluR subunit within the NACs, ACg, and IL (see Fig. 10).

The conditioned Fos expression in the present study extends, and is in agreement with, our previous research demonstrating an increase in Fos expression in the same limbic and cortical regions of cue-conditioned rats relative to controls (Neisewander et al., 2000). Importantly, in our previous study we showed that the enhanced Fos expression was not caused by lever pressing behavior, because the increase in Fos expression was evident in cue-conditioned rats regardless of whether or not they could engage in cocaine-seeking behavior during the test (i.e., regardless of whether the lever was present or not). In addition, our previous results found no increase in Fos expression in the motor cortex of rats engaged in cocaine-seeking behavior, mitigating the idea that motor behavior differences underlie the effects observed in cocaine-seeking rats. Moreover, our previous findings suggests Fos activation on the test day was not due to extinction learning, because rats that had no opportunity to perform the operant showed the same pattern of Fos induction. Finally, in the present study there were no group differences in Fos expression in the SS cortex, suggesting enhanced Fos expression in cocaine-seeking rats was not likely due to increase sensory processing.

The conditioned increases in Fos expression in the CeA, LA, and dCPu in the present study were not observed previously, nor did we examine the dorsal hippocampus, which also exhibited increased Fos expression in the present study. It is possible that the neuronal activation evident in these regions may involve processes discussed above, because we did not have a conditioned, nonresponding group in the present study to rule out these factors (i.e., sensory, motor, extinction learning). Regarding the discrepancy in neuronal activation in the amygdala and dCPu across our studies, we suggest two additional possibilities. First, the availability of response-contingent cues during the test for cocaine-seeking behavior in the present study may have resulted in recruitment of additional circuits that include the CeA, LA,

and dCPu, compared to the non-contingent cue presentations used previously. Second, the use of different antibodies (i.e., the Santa Cruz Biotechnology versus the Oncogene Science antibody used previously) may account for the divergent patterns of Fos expression, although both are selective and specific for Fos. In either case, the present findings are consistent with evidence that has accumulated since our original report suggesting that cue-elicited cocaine-seeking behavior is dependent on a limbic-cortical circuitry that includes the nucleus accumbens (McFarland and Kalivas, 2001, Anderson et al., 2003, Fuchs et al., 2004a, Ito et al., 2004, Bachtell et al., 2005, Schmidt et al., 2006), amygdala (Whitelaw et al., 1996, Meil and See, 1997, Kruzich and See, 2001, Fuchs et al., 2002, Alleweireldt et al., 2006), dorsal striatum (Vanderschuren et al., 2005, Fuchs et al., 2006), dorsal hippocampus (Vorel et al., 2001, Meyers et al., 2003, Fuchs et al., 2005), prefrontal cortex (Weissenborn et al., 1997, Park et al., 2002, McLaughlin and See, 2003, Fuchs et al., 2005) and orbitofrontal cortex (McFarland and Kalivas, 2001, Fuchs et al., 2004b).

We hypothesize that the changes in percentage of Fos-labeled cells coexpressing AMPA receptor subunits observed in the NACs, ACg, and IL suggest unique neuroadaptations related to incentive motivational effects of cocaine cues expressed via cocaine seeking-behavior. The specific processes affected by the observed neuroadaptations will require further investigation. However, based on previous research, some lead hypotheses can be suggested. For instance, prior research suggests extinction training induces up-regulation of AMPA GluR1 and GluR2/3 subunits in the NACs and GluR1 or GluR2 overexpression in the NACs facilitates extinction of cocaine-seeking behavior (Sutton et al., 2003), which may reflect enhanced learning or decreased motivation. In contrast, the NACc, and not the NACs, is critical for expression of cue-elicited cocaine-seeking behavior (Fuchs et al., 2004a, Ito et al., 2004), and in particular, involves AMPA/Kainate receptors (Di Ciano and Everitt, 2001). Taken together, neuronal activity in neurons expressing the GluR1 subunit in the NACs likely reflects neuroadaptations involved in cue-elicited motivation for cocaine and/or extinction learning. A more detail examination of the neuronal pattern in the NACs should also be considered in future studies, given the heterogeneity of connectivity within the NACs (Wright and Groenewegen, 1995, Wright et al., 1996, Groenewegen et al., 1999), and evidence demonstrating that cocaine differentially affects IEG expression within subdivisions of the NACs (Todtenkopf et al., 2002, Brenhouse and Stellar, 2006).

The ACg is part of the circuitry activated by cocaine-conditioned stimuli in both animals (Crawford et al., 1995, Neisewander et al., 2000, McLaughlin and See, 2003) and humans (Childress et al., 1999, Garavan et al., 2000, Wexler et al., 2001). Moreover, we have argued that the ACg may be part of a final common pathway for all stimuli that trigger incentive motivation for cocaine based on our finding that either cocaine-associated cues or cocaine priming increased Fos expression in this region in animals with a history of cocaine self-administration, whereas in contrast, there was no effect of cocaine priming in saline-yoked controls (Neisewander et al., 2000). Thus, Fos expression in the ACg was specific to processes related to evoked incentive motivation for cocaine. One functional role attributed to the ACg is decision-making about goal-directed behavior based on reward value expectations (Rushworth et al., 2004). For instance, rats with lesions of the ACg cease preference for an effortful high reward and instead prefer a less effortful low reward compared to controls (Walton et al., 2003, Schweimer and Hauber, 2005). Thus, one possible explanation for the increase in percentage of cells coexpressing Fos and GluR1 subunits observed in the ACg cortex may reflect neuroadaptations involved in the decision to engage in cocaine-seeking behavior based on the motivational value of cocaine-conditioned stimuli.

The neuronal activation associated with cue-elicited cocaine-seeking behavior observed in the IL is somewhat surprising, given that neither tetrodotoxin inactivation or excitotoxic lesions

of the IL affects expression of cocaine-conditioned place preference (Tzschentke and Schmidt, 1999) or reinstatement of extinguished cocaine-seeking behavior by cocaine cues, footshock stress, or priming injections of cocaine (Capriles et al., 2003, McLaughlin and See, 2003). A critical difference between the present and previous findings on operant cocaine-seeking behavior is the lack of extinction of cocaine-seeking behavior in the present study prior to cue presentation. Thus, in the present study, rats in the Cocaine-No Extinction group were experiencing extinction for the first time on the test day. Consistent with this explanation, the IL has been implicated in extinction learning of both aversive and appetitive conditioned responses (Quirk et al., 2000, Milad and Quirk, 2002, Milad et al., 2004, Rhodes and Killcross, 2004). Thus, one possible explanation for the increase in percentage of cells coexpressing Fos and GluR4 subunits observed in the IL cortex may reflect neuroadaptations involved in extinction of cocaine-seeking behavior.

There were no group differences in total immunolabeling of any AMPA receptor subunits in any region examined. Similar lack of alterations in AMPA receptor subunit expression has been reported in the ventral tegmental area and substantia nigra of rats given repeated systemic injections of cocaine (Lu et al., 2002), although repeated amphetamine administration alters AMPA receptor subunit expression in NAC and prefrontal cortex (Lu and Wolf, 1999). It is possible that immunolabeling is not sensitive enough to detect small group differences since changes in protein and mRNA levels of AMPA receptor subunits have been reported using western blotting and *in situ* hybridization, respectively, after systemic injections of cocaine or withdrawal from cocaine self-administration (Fitzgerald et al., 1996, Churchill et al., 1999, Ghasemzadeh et al., 1999, Lu et al., 2003, Grignaschi et al., 2004, Tang et al., 2004, Hemby et al., 2005, Lu et al., 2005).

It was surprising that such a small proportion of Fos positive neurons coexpressed AMPA receptor subunits. A noteworthy methodological issue is that only a small sample area of each brain region was analyzed due to the high magnification needed to identify colabeling. The data are presented as immunoreactive cells/0.065 mm², which represents the average of small areas throughout a region, consequently yielding a small, but reliable, measure of colabeling. The percentage of Fos neurons exhibiting coexpression across all subunits ranged from 17% in the dCPu to 45% in the CA3. These percentages indicate that not all neurons activated by cocaine cues contain AMPA receptors. Indeed, induction of IEGs has been characterized for numerous neurotransmitter systems (reviewed in Harlan and Garcia, 1998). Nevertheless, the presence of colabeled Fos and GluR subunit cells suggests AMPA receptor mechanisms are involved throughout the limbic and cortical circuitry involved in cocaine-seeking behavior. Importantly, the small proportion of colabeled cells suggests a highly selective recruitment of AMPA receptor expressing cells within regions of the circuit that are involved in cue-elicited cocaine-seeking behavior, rather than a generalized activation.

We expected Fos coexpression of glutamate AMPA receptor subunits based on previous research demonstrating AMPA receptor involvement in Fos expression (Fowler et al., 2004, Soyguder, 2005), including Fos expression elicited by drugs of abuse (Mead et al., 1999, Garcia et al., 2003). The role of AMPA receptors in IEG induction likely involves Ca⁺⁺-mediated receptor transduction, given AMPA receptors containing GluR1, GluR3 and GluR4 subunits are permeable to Ca⁺⁺ (Hollmann and Heinemann, 1994) and the extensive role of Ca⁺⁺ in intracellular signaling cascades and regulation of gene expression (Hughes and Dragunow, 1995, Poser and Storm, 2001). It is also possible that other neurotransmitters initiate the signaling cascade leading to increase Fos expression in cells coexpressing AMPA receptors. For instance, dopamine actions at dopamine D1 receptors may lead to increased Fos (Robertson et al., 1989, Berretta et al., 1992, Wirtshafter and Osborn, 2005), as well as increased surface

expression of GluR1-containing AMPA receptors (Mangiavacchi and Wolf, 2004, Sun et al., 2005).

In summary, the present study suggests pervasive involvement of AMPA receptor subunits in the neuronal circuitry activated by cocaine cues, and has identified the ACg and IL cortices and NACs as regions in which unique neural adaptations occur due to cue-elicited cocaine-seeking behavior. Further research using procedures that directly manipulate AMPA receptor mechanisms within these brain regions, particularly in the ACg and IL, is needed to examine their precise involvement in the incentive motivational effects of cocaine predictive stimuli.

Acknowledgements

The authors wish to thank Laura Muhammad, Rebecca Hobbs, Lucinda Young, Lisa Schell, and Ryan Meyers for their assistance in the collection of the data, Dr. Tom Shepherd for his expert technical assistance, and Dr. Peter Kufahl for comments on an earlier version of this manuscript. This research was supported by NIDA Grant DA 13649 (JLN), the American Psychological Association Diversity Program in Neuroscience (ARZ) and the Minority Access to Research Careers at Arizona State University.

References

- Alleweireldt AT, Hobbs RJ, Taylor AR, Neisewander JL. Effects of SCH-23390 infused into the amygdala or adjacent cortex and basal ganglia on cocaine seeking and self-administration in rats. *Neuropsychopharmacology* 2006;31:363–374. [PubMed: 15957007]
- Anderson SM, Bari AA, Pierce RC. Administration of the D1-like dopamine receptor antagonist SCH-23390 into the medial nucleus accumbens shell attenuates cocaine priming-induced reinstatement of drug-seeking behavior in rats. *Psychopharmacology (Berl)* 2003;168:132–138. [PubMed: 12491029]
- Bachtell RK, Whisler K, Karanian D, Self DW. Effects of intra-nucleus accumbens shell administration of dopamine agonists and antagonists on cocaine-taking and cocaine-seeking behaviors in the rat. *Psychopharmacology (Berl)* 2005;183:41–53. [PubMed: 16163523]
- Backstrom P, Hyytia P. Attenuation of cocaine-seeking behaviour by the AMPA/kainate receptor antagonist CNQX in rats. *Psychopharmacology (Berl)* 2003;166:69–76. [PubMed: 12525959]
- Backstrom P, Hyytia P. Ionotropic and metabotropic glutamate receptor antagonism attenuates cue-induced cocaine seeking. *Neuropsychopharmacology* 2006;31:778–786.
- Berretta S, Robertson HA, Graybiel AM. Dopamine and glutamate agonists stimulate neuron-specific expression of Fos-like protein in the striatum. *J Neurophysiol* 1992;68:767–777. [PubMed: 1359024]
- Boudreau AC, Wolf ME. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci* 2005;25:9144–9151. [PubMed: 16207873]
- Brenhouse HC, Stellar JR. c-Fos and deltaFosB expression are differentially altered in distinct subregions of the nucleus accumbens shell in cocaine-sensitized rats. *Neuroscience* 2006;137:773–780. [PubMed: 16337088]
- Capriles N, Rodaros D, Sorge RE, Stewart J. A role for the prefrontal cortex in stress- and cocaine-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl)* 2003;168:66–74. [PubMed: 12442201]
- Carroll ME, France CP, Meisch RA. Intravenous self-administration of etonitazene, cocaine and phencyclidine in rats during food deprivation and satiation. *J Pharmacol Exp Ther* 1981;217:241–247. [PubMed: 6112257]
- Childress AR, Mozley PD, McElgin W, Fitzgerald J, Reivich M, O'Brien CP. Limbic activation during cue-induced cocaine craving. *Am J Psychiatry* 1999;156:11–18. [PubMed: 9892292]
- Churchill L, Swanson CJ, Urbina M, Kalivas PW. Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. *J Neurochem* 1999;72:2397–2403. [PubMed: 10349849]

- Ciccocioppo R, Sanna PP, Weiss F. Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: reversal by D(1) antagonists. *Proc Natl Acad Sci U S A* 2001;98:1976–1981. [PubMed: 11172061]
- Cornish JL, Duffy P, Kalivas PW. A role for nucleus accumbens glutamate transmission in the relapse to cocaine-seeking behavior. *Neuroscience* 1999;93:1359–1367. [PubMed: 10501460]
- Cornish JL, Kalivas PW. Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. *J Neurosci* 2000;20:1–5. [PubMed: 10627575]
- Crawford CA, McDougall SA, Bolanos CA, Hall S, Berger SP. The effects of the kappa agonist U-50,488 on cocaine-induced conditioned and unconditioned behaviors and Fos immunoreactivity. *Psychopharmacology (Berl)* 1995;120:392–399. [PubMed: 8539319]
- Di Ciano P, Everitt BJ. Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior. *Neuropsychopharmacology* 2001;25:341–360. [PubMed: 11522463]
- Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ. Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J Neurosci* 1996;16:274–282. [PubMed: 8613793]
- Fowler JH, Whalley K, Murray T, O'Neill MJ, McCulloch J. The AMPA receptor potentiator LY404187 increases cerebral glucose utilization and c-fos expression in the rat. *J Cereb Blood Flow Metab* 2004;24:1098–1109. [PubMed: 15529010]
- Fuchs RA, Branham RK, See RE. Different neural substrates mediate cocaine seeking after abstinence versus extinction training: a critical role for the dorsolateral caudate-putamen. *J Neurosci* 2006;26:3584–3588. [PubMed: 16571766]
- Fuchs RA, Evans KA, Ledford CC, Parker MP, Case JM, Mehta RH, See RE. The role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. *Neuropsychopharmacology* 2005;30:296–309. [PubMed: 15483559]
- Fuchs RA, Evans KA, Parker MC, See RE. Differential involvement of the core and shell subregions of the nucleus accumbens in conditioned cue-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl)* 2004a;176:459–465. [PubMed: 15138757]
- Fuchs RA, Evans KA, Parker MP, See RE. Differential involvement of orbitofrontal cortex subregions in conditioned cue-induced and cocaine-primed reinstatement of cocaine seeking in rats. *J Neurosci* 2004b;24:6600–6610. [PubMed: 15269272]
- Fuchs RA, Weber SM, Rice HJ, Neisewander JL. Effects of excitotoxic lesions of the basolateral amygdala on cocaine-seeking behavior and cocaine conditioned place preference in rats. *Brain Res* 2002;929:15–25. [PubMed: 11852027]
- Garavan H, Pankiewicz J, Bloom A, Cho JK, Sperry L, Ross TJ, Salmeron BJ, Risinger R, Kelley D, Stein EA. Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. *Am J Psychiatry* 2000;157:1789–1798. [PubMed: 11058476]
- Garcia MM, Anderson AT, Edwards R, Harlan RE. Morphine induction of c-fos expression in the rat forebrain through glutamatergic mechanisms: role of non-n-methyl-D-aspartate receptors. *Neuroscience* 2003;119:787–794. [PubMed: 12809699]
- Ghasemzadeh MB, Nelson LC, Lu XY, Kalivas PW. Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J Neurochem* 1999;72:157–165. [PubMed: 9886066]
- Grignaschi G, Burbassi S, Zennaro E, Bendotti C, Cervo L. A single high dose of cocaine induces behavioural sensitization and modifies mRNA encoding GluR1 and GAP-43 in rats. *Eur J Neurosci* 2004;20:2833–2837. [PubMed: 15548228]
- Groenewegen HJ, Wright CI, Beijer AV, Voorn P. Convergence and segregation of ventral striatal inputs and outputs. *Ann N Y Acad Sci* 1999;877:49–63. [PubMed: 10415642]
- Harlan RE, Garcia MM. Drugs of abuse and immediate-early genes in the forebrain. *Mol Neurobiol* 1998;16:221–267. [PubMed: 9626665]
- Hemby SE, Horman B, Tang W. Differential regulation of ionotropic glutamate receptor subunits following cocaine self-administration. *Brain Res* 2005;1064:75–82. [PubMed: 16277980]

- Hollmann M, Heinemann S. Cloned glutamate receptors. *Annu Rev Neurosci* 1994;17:31–108. [PubMed: 8210177]
- Hughes P, Dragunow M. Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacol Rev* 1995;47:133–178. [PubMed: 7784478]
- Ito R, Robbins TW, Everitt BJ. Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. *Nat Neurosci* 2004;7:389–397. [PubMed: 15034590]
- Kalivas PW, McFarland K. Brain circuitry and the reinstatement of cocaine-seeking behavior. *Psychopharmacology (Berl)* 2003;168:44–56. [PubMed: 12652346]
- Kruzich PJ, See RE. Differential contributions of the basolateral and central amygdala in the acquisition and expression of conditioned relapse to cocaine-seeking behavior. *J Neurosci* 2001;21:RC155. [PubMed: 11425882]
- Lu L, Dempsey J, Shaham Y, Hope BT. Differential long-term neuroadaptations of glutamate receptors in the basolateral and central amygdala after withdrawal from cocaine self-administration in rats. *J Neurochem* 2005;94:161–168. [PubMed: 15953359]
- Lu L, Grimm JW, Shaham Y, Hope BT. Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. *J Neurochem* 2003;85:1604–1613. [PubMed: 12787079]
- Lu W, Monteggia LM, Wolf ME. Repeated administration of amphetamine or cocaine does not alter AMPA receptor subunit expression in the rat midbrain. *Neuropsychopharmacology* 2002;26:1–13. [PubMed: 11751027]
- Lu W, Wolf ME. Repeated amphetamine administration alters AMPA receptor subunit expression in rat nucleus accumbens and medial prefrontal cortex. *Synapse* 1999;32:119–131. [PubMed: 10231131]
- Mangiavacchi S, Wolf ME. D1 dopamine receptor stimulation increases the rate of AMPA receptor insertion onto the surface of cultured nucleus accumbens neurons through a pathway dependent on protein kinase A. *J Neurochem* 2004;88:1261–1271. [PubMed: 15009682]
- McFarland K, Kalivas PW. The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. *J Neurosci* 2001;21:8655–8663. [PubMed: 11606653]
- McLaughlin J, See RE. Selective inactivation of the dorsomedial prefrontal cortex and the basolateral amygdala attenuates conditioned-cued reinstatement of extinguished cocaine-seeking behavior in rats. *Psychopharmacology (Berl)* 2003;168:57–65. [PubMed: 12845418]
- Mead AN, Vasilaki A, Spyrali C, Duka T, Stephens DN. AMPA-receptor involvement in c-fos expression in the medial prefrontal cortex and amygdala dissociates neural substrates of conditioned activity and conditioned reward. *Eur J Neurosci* 1999;11:4089–4098. [PubMed: 10583497]
- Meil WM, See RE. Lesions of the basolateral amygdala abolish the ability of drug associated cues to reinstate responding during withdrawal from self-administered cocaine. *Behav Brain Res* 1997;87:139–148. [PubMed: 9331482]
- Meyers RA, Zavala AR, Neisewander JL. Dorsal, but not ventral, hippocampal lesions disrupt cocaine place conditioning. *Neuroreport* 2003;14:2127–2131. [PubMed: 14600510]
- Milad MR, Quirk GJ. Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature* 2002;420:70–74. [PubMed: 12422216]
- Milad MR, Vidal-Gonzalez I, Quirk GJ. Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner. *Behav Neurosci* 2004;118:389–394. [PubMed: 15113265]
- Neisewander JL, Baker DA, Fuchs RA, Tran-Nguyen LT, Palmer A, Marshall JF. Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. *J Neurosci* 2000;20:798–805. [PubMed: 10632609]
- Park WK, Bari AA, Jey AR, Anderson SM, Spealman RD, Rowlett JK, Pierce RC. Cocaine administered into the medial prefrontal cortex reinstates cocaine-seeking behavior by increasing AMPA receptor-mediated glutamate transmission in the nucleus accumbens. *J Neurosci* 2002;22:2916–2925. [PubMed: 11923456]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 1998.

- Poser S, Storm DR. Role of Ca²⁺-stimulated adenylyl cyclases in LTP and memory formation. *Int J Dev Neurosci* 2001;19:387–394. [PubMed: 11378299]
- Quirk GJ, Russo GK, Barron JL, Lebron K. The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *J Neurosci* 2000;20:6225–6231. [PubMed: 10934272]
- Rhodes SE, Killcross S. Lesions of rat infralimbic cortex enhance recovery and reinstatement of an appetitive Pavlovian response. *Learn Mem* 2004;11:611–616. [PubMed: 15466316]
- Robertson HA, Peterson MR, Murphy K, Robertson GS. D1-dopamine receptor agonists selectively activate striatal c-fos independent of rotational behaviour. *Brain Res* 1989;503:346–349. [PubMed: 2532558]
- Rushworth MF, Walton ME, Kennerley SW, Bannerman DM. Action sets and decisions in the medial frontal cortex. *Trends Cogn Sci* 2004;8:410–417. [PubMed: 15350242]
- Schmidt HD, Anderson SM, Pierce RC. Stimulation of D1-like or D2 dopamine receptors in the shell, but not the core, of the nucleus accumbens reinstates cocaine-seeking behaviour in the rat. *Eur J Neurosci* 2006;23:219–228. [PubMed: 16420431]
- Schweimer J, Hauber W. Involvement of the rat anterior cingulate cortex in control of instrumental responses guided by reward expectancy. *Learn Mem* 2005;12:334–342. [PubMed: 15930509]
- Self DW, Choi KH, Simmons D, Walker JR, Smagula CS. Extinction training regulates neuroadaptive responses to withdrawal from chronic cocaine self-administration. *Learn Mem* 2004;11:648–657. [PubMed: 15466321]
- Soyguder Z. Multiple neurotransmitter receptors contribute to the spinal Fos expression. *Brain Res* 2005;1033:202–209. [PubMed: 15694925]
- Sun X, Zhao Y, Wolf ME. Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. *J Neurosci* 2005;25:7342–7351. [PubMed: 16093384]
- Suto N, Tanabe LM, Austin JD, Creekmore E, Pham CT, Vezina P. Previous exposure to psychostimulants enhances the reinstatement of cocaine seeking by nucleus accumbens AMPA. *Neuropsychopharmacology* 2004;29:2149–2159. [PubMed: 15266353]
- Sutton MA, Schmidt EF, Choi KH, Schad CA, Whisler K, Simmons D, Karanian DA, Monteggia LM, Neve RL, Self DW. Extinction-induced upregulation in AMPA receptors reduces cocaine-seeking behaviour. *Nature* 2003;421:70–75. [PubMed: 12511956]
- Tang W, Wesley M, Freeman WM, Liang B, Hemby SE. Alterations in ionotropic glutamate receptor subunits during binge cocaine self-administration and withdrawal in rats. *J Neurochem* 2004;89:1021–1033. [PubMed: 15140200]
- Todtenkopf MS, Mihalakopoulos A, Stellar JR. Withdrawal duration differentially affects c-fos expression in the medial prefrontal cortex and discrete subregions of the nucleus accumbens in cocaine-sensitized rats. *Neuroscience* 2002;114:1061–1069. [PubMed: 12379259]
- Tzschentke TM, Schmidt WJ. Functional heterogeneity of the rat medial prefrontal cortex: effects of discrete subarea-specific lesions on drug-induced conditioned place preference and behavioural sensitization. *Eur J Neurosci* 1999;11:4099–4109. [PubMed: 10583498]
- Tzschentke TM, Schmidt WJ. Glutamatergic mechanisms in addiction. *Mol Psychiatry* 2003;8:373–382. [PubMed: 12740594]
- Vanderschuren LJ, Di Ciano P, Everitt BJ. Involvement of the dorsal striatum in cue-controlled cocaine seeking. *J Neurosci* 2005;25:8665–8670. [PubMed: 16177034]
- Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL. Relapse to cocaine-seeking after hippocampal theta burst stimulation. *Science* 2001;292:1175–1178. [PubMed: 11349151]
- Walton ME, Bannerman DM, Alterescu K, Rushworth MF. Functional specialization within medial frontal cortex of the anterior cingulate for evaluating effort-related decisions. *J Neurosci* 2003;23:6475–6479. [PubMed: 12878688]
- Weissenborn R, Robbins TW, Everitt BJ. Effects of medial prefrontal or anterior cingulate cortex lesions on responding for cocaine under fixed-ratio and second-order schedules of reinforcement in rats. *Psychopharmacology (Berl)* 1997;134:242–257. [PubMed: 9438674]
- Wexler BE, Gottschalk CH, Fulbright RK, Prohovnik I, Lacadie CM, Rounsaville BJ, Gore JC. Functional magnetic resonance imaging of cocaine craving. *Am J Psychiatry* 2001;158:86–95. [PubMed: 11136638]

- Whitelaw RB, Markou A, Robbins TW, Everitt BJ. Excitotoxic lesions of the basolateral amygdala impair the acquisition of cocaine-seeking behaviour under a second-order schedule of reinforcement. *Psychopharmacology (Berl)* 1996;127:213–224. [PubMed: 8912399]
- Wirtshafter D, Osborn CV. The atypical dopamine D1 receptor agonist SKF 83959 induces striatal Fos expression in rats. *Eur J Pharmacol* 2005;528:88–94. [PubMed: 16324697]
- Wolf ME, Sun X, Mangiavacchi S, Chao SZ. Psychomotor stimulants and neuronal plasticity. *Neuropharmacology* 2004;47(Suppl 1):61–79. [PubMed: 15464126]
- Wright CI, Beijer AV, Groenewegen HJ. Basal amygdaloid complex afferents to the rat nucleus accumbens are compartmentally organized. *J Neurosci* 1996;16:1877–1893. [PubMed: 8774456]
- Wright CI, Groenewegen HJ. Patterns of convergence and segregation in the medial nucleus accumbens of the rat: relationships of prefrontal cortical, midline thalamic, and basal amygdaloid afferents. *The Journal of comparative neurology* 1995;361:383–403. [PubMed: 8550887]

Abbreviations

ACg	anterior cingulate
AMPA	L-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BIA	basolateral amygdala
CeA	central amygdala
DAB	3,3'-diaminobenzidine tetrahydrochloride
dCPu	dorsal caudate-putamen
DG	dentate gyrus
FR	fixed ratio
IEG	immediate early gene
IL	infralimbic
LA	lateral amygdala
NAC	nucleus accumbens
NACc	nucleus accumbens core
NACs	nucleus accumbens shell

OF	orbitofrontal
PBS	phosphate buffered saline
PFA	paraformaldehyde
PrL	prelimbic
SS	somatosensory
VR	variable ratio

Figure 1.

Schematic representation of regions analyzed. Numbers on the bottom right of each section represents distance from bregma. Numbers in the sections represent the regions analyzed as follows: 1) prelimbic cortex; 2) infralimbic cortex; 3) orbitofrontal cortex; 4) anterior cingulate cortex; 5) dorsal caudate-putamen; 6) nucleus accumbens shell; 7) nucleus accumbens core; 8) hippocampal CA1 region; 9) hippocampal dentate gyrus; 10) hippocampal CA3 region; 11) somatosensory cortex; 12) lateral amygdala; 13) central amygdala; and 14) basolateral amygdala. Schematic figures were adapted from Paxinos and Watson (1998).

Figure 2.

Photomicrographs of coronal sections taken at 60 $\times$ to enhance the visibility of the labeling for Fos-immunoreactivity (green arrows), GluR-immunoreactivity, and Fos and GluR-immunoreactivity (red arrows) in representative rats in the Control, Cocaine-Extinction, and Cocaine-No Extinction groups are shown. GluR1, GluR2/3, and GluR4 colabeling with Fos is shown in the ACg, OF, and IL regions of the cortex, respectively, to demonstrate labeling across several areas.

Figure 3.

Effect of extinction training on cocaine-seeking behavior in rats with a history of cocaine self-administration (Cocaine-Extinction) or saline-yoked (Control-Extinction). Cocaine-seeking behavior is illustrated as nonreinforced lever presses ($\pm$ SEM) during 90-min extinction sessions and on the test day. *represents a difference from the Control-Extinction group, Tukey HSD test ($P < 0.05$). +represents a difference from all other groups, Tukey HSD test ($P < 0.05$).

Figure 4. Number of Fos-immunoreactive labeled cells ($\pm$ SEM) in cortical and limbic regions of rats following the test for cocaine-seeking behavior. ^arepresents a difference from all other groups; Tukey HSD test ($P < 0.05$).

Figure 5. Number of GluR1- (**A**), GluR2/3- (**B**), GluR4-immunoreactive (**C**) labeled cells ($\pm$ SEM) in cortical and limbic regions of rats following the test for cocaine-seeking behavior.

Figure 6.

Number of cells double-labeled for Fos protein and GluR1 (A), GluR2/3 (B), and GluR4 (C) subunits and percentage of Fos cells colabeled with GluR1 (D), GluR2/3 (E), and GluR4 (F) subunits in the prelimbic (PrL), infralimbic (IL), orbitofrontal (OF), anterior cingulate (ACg), and somatosensory (SS) cortex regions. ^arepresents a difference from all other groups, Tukey HSD test ($P < 0.05$). ^erepresents a difference from the Cocaine-No Extinction group, Tukey HSD test ($P < 0.05$).

Figure 7.

Number of cells double-labeled for Fos protein and GluR1 (**A**), GluR2/3 (**B**), and GluR4 (**C**) subunits and percentage of Fos cells colabeled with GluR1 (**D**), GluR2/3 (**E**), and GluR4 (**F**) subunits in the nucleus accumbens shell (NACs), nucleus accumbens core (NACc), and dorsal caudate putamen (dCPu) regions. ^arepresents a difference from all other groups, Tukey HSD test ($P < 0.05$). ^brepresents a difference from the Cocaine-Extinction group, Tukey HSD test ($P < 0.05$). ^crepresents a difference from the Control group, Tukey HSD test ($P < 0.05$).

Figure 8.

Number of cells double-labeled for Fos protein and GluR1 (**A**), GluR2/3 (**B**), and GluR4 (**C**) subunits and percentage of Fos cells colabeled with GluR1 (**D**), GluR2/3 (**E**), and GluR4 (**F**) subunits in the lateral (LA), basolateral (BIA), and central (CeA) amygdala regions. ^arepresents a difference from all other groups, Tukey HSD test ($P < 0.05$). ^brepresents a difference from the Cocaine-Extinction group, Tukey HSD test ($P < 0.05$). ^crepresents a difference from the Control group, Tukey HSD test ($P < 0.05$).

Figure 9.

Number of cells double-labeled for Fos protein and GluR1 (**A**), GluR2/3 (**B**), and GluR4 (**C**) subunits and percentage of Fos cells colabeled with GluR1 (**D**), GluR2/3 (**E**), and GluR4 (**F**) subunits in the CA1, CA3, and dentate gyrus (DG) hippocampal regions. ^drepresents a main effect of group ($P<0.05$).

Figure 10.

Scatterplot of the relationship between lever pressing behavior on the test day and the number of cells double-labeled for Fos protein and GluR4 in the infralimbic cortex (A), or the number of cells double-labeled for Fos protein and GluR1 in the anterior cingulate cortex (B), nucleus accumbens shell (C), and somatosensory cortex (D). *represents a significant correlation ($P < 0.05$).