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Relapse to cocaine seeking increases activity-regulated gene expression differentially in the prefrontal cortex of abstinent rats

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Abstract

Rationale—Alterations in the activity of the prefrontal and orbitofrontal cortices of cocaine addicts have been linked with re-exposure to cocaine-associated stimuli. *Objectives* Using an animal model of relapse to cocaine seeking, the present study investigated the expression patterns of four different activity-regulated genes within prefrontal cortical brain regions after 22 h or 15 days of abstinence during context-induced relapse.

Materials and methods—Rats self-administered cocaine or received yoked-saline for 2 h/day for 10 days followed by 22 h or 2 weeks of abstinence when they were re-exposed to the self-administration chamber with or without levers available to press for 1 h. Brains were harvested and sections through the prefrontal cortex were processed for in situ hybridization using radioactive oligonucleotide probes encoding c-fos, zif/268, arc, and bdnf.

Results—Re-exposure to the chamber in which rats previously self-administered cocaine but not saline, regardless of lever availability, increased the expression of all genes in the medial prefrontal and orbitofrontal cortices at both time points with one exception: *bdnf* mRNA was significantly increased in the medial prefrontal cortex at 22 h only if levers previously associated with cocaine delivery were available to press. Furthermore, re-exposure of rats to the chambers in which they received yoked saline enhanced both *zif/268* and *arc* expression selectively in the orbito-frontal cortex after 15 days of abstinence.

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Conclusions—These results support convergent evidence that cocaine-induced changes in the prefrontal cortex are important in regulating drug seeking following abstinence and may provide additional insight into the molecular mechanisms involved in these processes.

Keywords

Addiction; Arc; BDNF; c-fos; Relapse; zif/268; Self-administration; Abstinence; Extinction

Relapse to drug seeking and taking in addicted humans is often triggered by re-exposure to environmental cues previously associated with the drug (Ehrman et al. 1992). Similarly, in animal studies, during chronic drug self-administration, the drug-paired environment acquires conditioned reinforcing properties that trigger drug-seeking behavior upon re-exposure to the context following forced abstinence (Crombag and Shaham 2002; Fuchs et al. 2005, 2006). Thus, like other forms of learning, relapse to drug-seeking involves the formation and retrieval of instrumental memories (Hyman 2005). A major question in addiction research is whether the neural substrates underlying drug-seeking involve the same cell signaling and synaptic plasticity processes within the same brain regions as those implicated in other forms of reward learning and memory (Berke and Hyman 2000; Hyman and Malenka 2001; Nestler 2001).

The inhibitory control functions of the prefrontal cortical system modulate reward-related behaviors and become impaired with chronic drug use (Jentsch and Taylor 1999). For example, cerebral blood flow and glucose metabolism are decreased in the prefrontal cortices of cocaine addicts (Volkow and Fowler 2000; Franklin et al. 2002; Goldstein and Volkow 2002; Bolla et al. 2003; Matochik et al. 2003). In contrast, the orbitofrontal cortex (OFC) and the dorsolateral prefrontal cortex (PFC) become activated in cocaine abusers during exposure to visual cues depicting drug-related stimuli, and this activity is correlated with self-reports of craving (Grant et al. 1996; Maas et al. 1998; Childress et al. 1999; Wang et al. 1999; London et al. 2000; Volkow and Fowler 2000; Bonson et al. 2002). Homologous brain regions in rats are implicated in drug seeking (Porrino and Lyons 2000); however, a growing body of evidence suggests that distinct and overlapping neural substrates are important following abstinence versus extinction training. Following chronic cocaine self-administration and subsequent extinction of operant responding, inactivation of the anterior cingulate (specifically Cg1 using the Paxinos and Watson 1998 nomenclature), the prelimbic (PrL) or the OFC, but not the infralimbic (IL) cortex suppressed cue-induced (McLaughlin and See 2003; Fuchs et al. 2004; Di Pietro et al. 2006), cocaine prime-induced (McFarland and Kalivas 2001), or contextual (Fuchs et al. 2005) reinstatement of cocaine seeking. However, inactiva-tion of the PrL/Cg1 cortex failed to alter cocaine seeking evoked by re-exposure to the self-administration chamber following forced abstinence without extinction training (Fuchs et al. 2006). Together, these reports suggest that the dorsomedial (dm) PFC is differentially involved in drug seeking depending on the environmental circumstances or model used.

Exposure to drugs of abuse leads to short- and long-term neuroadaptive changes in the brain, many of which involve the regulation of plasticity-related gene expression. These experience-dependent, long-term modifications are thought to be responsible for the

regulation of habitual drug-seeking behavior by incentive associations (Bliss and Collingridge 1993; Robinson and Berridge 1993, 2001). Cocaine induces the activation of immediate early genes (IEGs) like the transcription factors, *c-fos* and *zif/268*, and effector genes like the activity-regulated cytoskeleton-associated gene (*arc*) and brain-derived neurotrophic factor (*bdnf*) that is highly correlated with neuronal activity (Graybiel et al. 1990; Young et al . 1991; Moratalla et al. 1992; Bhat and Baraban 1993; Daunais and McGinty 1994, 1995; Fosnaugh et al. 1995; Tan et al. 2000; Grimm et al. 2003; Le Foll et al. 2005; Fumagalli et al. 2006). Furthermore, cocaine-associated cues activate IEGs (Brown et al. 1992; Crawford et al. 1995; Everitt and Robbins 2000; Ciccocioppo et al. 2001; Thomas et al. 2003). Most relevant to the present study, re-exposure to an environment previously associated with cocaine self-administration, with or without a priming injection following prolonged cocaine abstinence, resulted in enhanced Fos protein expression within the anterior cingu- late, nucleus accumbens, basolateral amygdala, hippocampal formation, and central grey (Neisewander et al. 2000). In a recent study, greater Fos immunoreactivity was also detected in the medial PFC, OFC, and striatum of abstinent rats that were re-exposed to the cocaine-associated chamber when compared to rats that underwent explicit extinction training (Zavala et al. 2007).

Activity-regulated genes contribute to the promotion and maintenance of synaptic plasticity (Lu 2003, Rial Verde et al. 2006), associative learning (Bozon et al. 2003, Davis et al. 2003, Liu et al. 2004, Malkani et al. 2004, Plath et al. 2006), and long-term storage and retrieval of memories (Guzowski et al. 2000; Hall et al. 2001; Thomas et al. 2002; Bozon et al. 2003; Davis et al. 2003; Liu et al. 2004; Plath et al. 2006). Furthermore, manipulation of BDNF levels in the brain alters cocaine seeking (Horger et al. 1999; Lu et al. 2004; Berglind et al. 2007; Graham et al. 2007). Additionally, early phases of instrumental learning (Hernandez et al. 2006; Kelly and Deadwyler 2003) elicit widespread *zif/268* and *arc* induction throughout an extensive corticostriatal network in rats. Furthermore, knockdown of *c-fos*, *zif/268*, *arc*, or tissue plasminogen activator (tPA) that cleaves proBDNF to mature BDNF impairs long-term memory consolidation (Calabresi et al. 2000, Guzowski et al. 2000; Fleischmann et al. 2003; Jones et al. 2001, Plath et al. 2006) and produces deficits in drug-induced conditioned place preference (Valjent et al. 2006).

Because cortical activation in response to cocaine-paired cues and/or context is most frequently observed under abstinence conditions (Breiter and Rosen 1999; Childress et al. 1999; Volkow and Fowler 2000) and IEGs display different induction thresholds (Worley et al. 1993) and functions, in the current study, alterations in the expression patterns of two IEGS that encode transcription factors, *c-fos* and *zif/268*, and two genes that encode synaptic proteins, *arc* and *bdnf*, were investigated in the medial PFC and OFC after extinction responding following 22 h or 15 days of abstinence.

Experimental procedures

Animals

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–325 g at the start of the experiment, were individually housed in a temperature- and humidity-controlled vivarium on a reversed light/dark cycle. The rats received 20–25 g of rat

chow per day, which maintained them at approximately 90% of free feeding body weight, and were allowed water ad libitum. The housing and treatment of the rats were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). Formal approval to conduct the experiments was obtained from the MUSC IACUC. All efforts were made to minimize the number of animals used and their suffering. The rats were given 5 days for adaptation before the start of the experiment.

Lever response training

The rats were trained to lever press on a fixed ratio 1 (FR1) schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH) in self-administration chambers (30×20× 24 cm high; Med Associates, St. Albans, VT) during a 16-h overnight training session. The chambers were equipped with two retractable levers, a food pellet dispenser, and a house light on the wall opposite to the levers. During the session, each active lever press resulted in delivery of a food pellet only. Lever presses on the inactive lever had no programmed consequences. After lever training, pellet dispensers were removed from the chambers.

Surgery

Forty-eight hours after lever training, the rats were anesthetized using ketamine and xylazine (66.6 and 1.3 mg/kg, i.p., respectively) followed by Equithesin (0.5 ml/kg, i.p.). Indwelling catheters were constructed as described previously (Fuchs et al. 2004). The end of the catheter was inserted into the right jugular vein. The catheter ran subcutaneously and exited on the rat’s back, posterior to the shoulder blades.

To maintain catheter patency, the catheters were flushed daily for 5 days after surgery with 0.1 ml of cefazolin (10.0 mg/ml; Schein Pharmaceutical, Florham Park, NJ) dissolved in heparinized saline (70 U/ml; Elkins-Sinn, Cherry Hill, NJ). The catheters were then flushed with 0.1 ml of heparinized saline (10 U/ml) before each self-administration session and with 0.1 ml of cefazolin solution and 0.1 ml of heparinized saline after each session. Catheter patency was periodically checked by infusing 0.1 ml of methohexital sodium (20 mg/ml, i.v.; Eli Lilly & Co., Indianapolis, IN), resulting in a rapid and short-term loss of muscle tone.

Cocaine self-administration

The experimental design is illustrated in Fig. 1. The rats were randomly assigned to cocaine or yoked-saline treatment. Self-administration was conducted in 2 h sessions on consecutive days during the rats’ dark cycle and continued until the rats self-administered a minimum of ten infusions per session for a minimum of 10 days (maintenance criteria). The rats responded on an FR1 schedule of cocaine reinforcement (cocaine hydrochloride; 0.6 mg/kg in 50 μl of sterile saline; National Institute on Drug Abuse, Research Triangle Park, NC). Active lever pressing resulted in a 2-s activation of the infusion pump only. After each infusion, responses on the active lever had no consequences during a 20-s timeout period. Responses on the inactive lever had no programmed consequences. Yoked rats received infusions of saline (50 μl) contingent upon the cocaine infusions received by the animal in the adjacent chamber. Data collection and reinforcer delivery were controlled using MedPC software version IV (Med Associates).

Experiment 1 Extinction responding 22 h following the end of cocaine self-administration—The rats (n=32) were randomly assigned to either cocaine (n=16) or yoked-saline (n=16) treatment groups and trained to self-administer cocaine as described above. After the last self-administration day, the rats remained in the colony room for a 22-h period. Then, they were placed back into the self-administration chamber with no levers available (NL; n=8/group for Coc and Sal) or with the levers available (LA; n=8/group for Coc and Sal). The number of lever presses was recorded for 1 h but had no scheduled consequences. Following the 1-h test, the rats were anesthetized with Equithesin and decapitated.

Experiment 2 Extinction responding 15 days following the end of cocaine self-administration—In experiment 2, the rats (n=30) were randomly assigned to either cocaine or yoked-saline treatment groups and trained to self-administer cocaine as described above. After the tenth day of self-administration, the rats were returned to the colony room for post-cocaine days 1–7. On post-cocaine days 8–14, all the rats were transported to a separate procedure room (alternate environment) that was distinctly different from the self-administration room and were placed into clear plastic holding chambers for 2 h/day. On post-cocaine day 15, one third of the rats were returned to the alternate environment (ALT; n=5/group for Coc and Sal), one third to the self-administration chamber with no levers available (NL; n=5/group for Coc and Sal), and one third to the self-administration chamber with the levers available (LA; n=5/group for Coc and Sal) for 1 h. The number of lever presses for the latter group was recorded but had no scheduled consequences. We included the ALT environment group in order to distinguish the impact of recent contextual familiarity from remote familiarity (dishabituation) when rats were re-exposed to the self-administration chamber without extinguishing the motivational effects of the cocaine-paired environment (Fuchs et al. 2006). The similar transport procedures and temporal pattern used for the ALT environment and the self-administration environment also controlled for general handling and arousal. Following the 1-h test, the rats were anesthetized with Equithesin and decapitated. The brains were rapidly frozen in dry ice-cooled isopentane (−40°C) and stored at −80°C until sectioning.

In situ hybridization histochemistry

In situ hybridization histochemistry was performed as described previously (Gonzalez-Nicolini and McGinty 2002; Schwendt et al. 2006) using antisense ^{35}S-labelled oligodeoxynucleotide probes complementary to rat *c-fos* (GCA GCG GGA GGA TGA CGC CTC GTA GTC -CGC GTT GAA ACC CGA GAA CAT), zif/268 (CC GTT GCT CAG CAG CAT CAT CTC CTC CAG TTT GGG GTA GTT GTC C), *arc* (GGC AGC TTC AAG AGA GGA GGG GAC GGT GCT GGT GCT GGG GTG GTA), or *bdnf* (GCA TTG CGA GTT CCA GTG CCT TTT GTC TAT GCC CCT GCA GCC TTC CTT) mRNA [Integrated DNA Technologies (IDT), Coralville, IN]. The 45–48mer oligodeoxynucleotide probes (IDT) were labeled at the 3′ end using alpha-[^{35}S]-dATP (Amersham Biosciences, Piscataway, NJ) and terminal deoxynucleotidyl transferase (Roche Diagnostics, Indianapolis, IN). Frozen 12-μm sections from decapitated rats were cut in a cryostat and thaw-mounted onto charged slides. The sections were fixed with 4% paraformaldehyde, pretreated with 0.25% acetic anhydride in sterile 0.1 M triethanolamine/0.9% NaCl pH 8.0 to reduce

nonspecific binding and defatted in an ascending alcohol series followed by chloroform and a descending alcohol series, then hybridized with 1×10^6 dpm of one of the probes in hybridization buffer at 37°C for 20 h. After stringent washes (4× sterile saline citrate (SSC), 1× SSC, 2× SSC/50%formamide at 40°C, 1× SSC, water), the slides were dried and placed in an X-ray film cassette along with ^{14}C standards (American Radiolabeled Chemicals, St Louis, MO) and Biomax MR film (Eastman Kodak, Rochester, NY). The films were developed at several time intervals in order to establish linearity and an optimal signal/noise ratio.

Image analysis

Quantitation of film autoradiograms was performed using the Macintosh-based NIH Image program as previously described (Wang and McGinty 1995). The ^{14}C standards were measured, plotted against known dpm/mg, and a calibration curve was generated. The mean density and number of pixels per area were measured in the selected PFC areas (Fig. 2) that included PrL and Cg1 together and OFC [ventral (VO) and lateral orbital (LO) cortex; Paxinos and Watson 1998] independently from three adjacent sections per brain. These particular prefrontal regions were selected for measurement because they have been previ ously implicated in studies of reinstatement of cocaine seeking (McFarland and Kalivas 2001; McLaughlin and See 2003; Fuchs et al. 2004, 2005, 2006; Berglind et al. 2007). The measurements were expressed as integrated density (no. of pixels per area × mean density).

Statistical analysis

Operant responding data were analyzed for total active and inactive lever responses using a one-way ANOVA. Since the measurements of the hybridization signals were strongly correlated and could not be treated independently, a nested repeated measures one-way (experiment 1) or two-way (experiment 2) ANOVA was used to analyze the hybridization data (mixed model SAS 9.1) followed by a Tukey-Kramer (for unequal group numbers) honestly significant difference (HSD) test when an interaction was found or to further analyze the source of main effects. Correlation coefficients between the number of lever presses during the 1-h test and gene expression in the Coc-LA groups in experiments 1 and 2 were determined by a simple regression analysis using GraphPad Prism 4.

Results

Cocaine self-administration and cocaine-seeking behavior 22 h and 15 days after the end of self-administration

Experiment 1—The mean (±SEM) cocaine intake for the last 3 days of self-administration was 21.4±2.3 mg/kg/day. The mean (±SEM) number of active lever responses (40.87± 4.0) was significantly different from yoked-saline active lever responses (7.53±1.5; $F_{(1,28)}$=66.79, $p<0.0001$) during self-administration (Fig. 3a, left). Re-exposure to the drug-associated context (self-administration chamber) after a 22-h abstinence period revealed a significant difference in active and inactive lever presses in rats with a history of cocaine self-administration compared to yoked-saline controls (Fig. 3a, right). The mean (±SEM) number of active lever responses during the 1-h test period for rats with a cocaine history (49±6.4) was significantly greater than yoked-saline (6.8±2.3) animals ($F_{(1,13)}$=43.04,

p<0.0001). The mean number of inactive lever presses during the 1-h test period for cocaine and yoked-saline animals was 14± 4.7 and 4.6±1.1, respectively ($F_{(1,13)}$=4.22, p=0.061).

Experiment 2—The mean (±SEM) cocaine intake for the last 3 days of self-administration (Fig. 3b, left) was 17.4± 1.4 mg/kg/day. The mean (±SEM) number of active lever responses (46.13±5.4) was significantly different from yoked-saline active lever responses (5.10±0.84; $F_{(1,28)}$= 41.03, p<0.0001). Inactive lever responding was not different between cocaine (3.4±0.74) and yoked-saline (5.5±1.2) rats ($F_{(1,28)}$=0.96, p=0.33). Re-exposure to the drug-associated context (self-administration chamber) after a 2-week abstinence period revealed a significant difference in active and inactive lever presses in rats with a history of cocaine self-administration compared to yoked-saline controls (Fig. 3b, right). The mean (±SEM) number of active lever responses during the 1 h test period for cocaine (87± 29.6) was significantly greater than yoked-saline (15±5.7) animals ($F_{(1,8)}$ =5.83, p=0.04). The mean number of inactive lever presses during the 1-h test period for cocaine and yoked-saline animals was 28.6±6.9 and 7.8±2.5, respectively ($F_{(1,8)}$=7.84, p=0.023). As described in previous reports (Berglind et al. 2007; Fuchs et al. 2006), this increase in inactive lever pressing after 15 days of abstinence likely represents an alternative cocaine-seeking strategy in rats with heightened motivation to seek the drug when it is no longer available on the previously cocaine-paired lever.

Gene expression

Experiment 1

Dorsomedial PFC: Twenty-two hours after the end of cocaine or yoked-saline administration, IEG expression was significantly greater in the dmPFC of rats that were re-exposed to the operant chamber previously associated with cocaine availability, whether levers were available, than in rats re-exposed to the operant chamber previously associated with yoked-saline infusions (Fig. 4). However, *bdnf* mRNA was only elevated when levers previously associated with cocaine were available to press (Fig. 4d). Statistical analysis of gene expression changes revealed no significant drug treatment (cocaine or saline) by lever effect (LA or NL) interactions for any of the genes. A significant main effect of drug treatment was observed for *c-fos* ($F_{(1,27)}$= 37.48, p<0.0001), *zif/268* ($F_{(1,26)}$=87.12, p<0.0001), *arc* ($F_{(1,27)}$=186.19, p<0.0001), and *bdnf* ($F_{(1,25)}$=9.69, p= 0.0046). A significant main effect of lever availability was found for bdnf ($F_{(1,25)}$=9.03, p=0.006). Multiple comparison tests revealed significantly higher levels of mRNA expression for IEGs in the dmPFC of Coc-NL vs Sal-NL rats and for all genes in the dmPFC of Coc-LA vs. Sal-LA rats.

OFC: Twenty-two hours after the end of cocaine or yoked-saline administration, gene expression was greater in the OFC of rats re-exposed to the operant chamber previously associated with cocaine self-administration than in the OFC of rats re-exposed to the operant chamber previously associated with yoked-saline infusions, regardless of the availability of levers (Fig. 4). Statistical analysis revealed a significant drug treatment (cocaine or saline) by lever (LA or NL) interaction for *zif/268* ($F_{(1,26)}$=4.40, p=0.046). A significant drug treatment main effect was found for *c-fos* ($F_{(1,27)}$=28.49, p<0.0001), *zif/268* ($F_{(1,26)}$ =34.96, p< 0.0001), *arc* ($F_{(1,26)}$=114.31, p<0.0001), and bdnf ($F_{(1,24)}$ =18.76 p=0.0002). Multiple

comparison tests revealed significantly higher expression levels of mRNA for all genes in the OFC of Coc-NL vs Sal-NL rats and in the OFC of Coc-LA vs. Sal-LA rats.

Experiment 2

Dorsomedial PFC: Following 15 days of abstinence, activity-regulated gene expression was significantly greater in the dmPFC of rats that were re-exposed to the operant chamber previously associated with cocaine availability, whether or not levers were available, than in rats re-exposed to the operant chamber previously associated with yoked-saline infusions or in the dmPFC of rats with a cocaine history that were placed in an alternate environment on the test day (Fig. 5). Statistical analysis of gene expression revealed significant drug treatment (cocaine or saline) by environment (alternate room or operant chamber with or without levers available) interactions for c-fos ($F_{(2,22)}$= 4.00, p=0.03), zif/268 ($F_{(2,22)}$=3.71, p=0.04), and arc ($F_{(2,22)}$ =3.74, p=0.04), and significant drug-treatment ($F_{(1,23)}$=39.16, p<0.0001) and environment ($F_{(1,23)}$=7.30, p=0.004) main effects for bdnf mRNA expression. Multiple comparison tests revealed significantly higher levels of all genes within the dmPFC in rats previously treated with cocaine and re-exposed to the self-administration chamber with (Coc-LA group) or without (Coc-NL group) the levers available as compared to rats that never returned to the cocaine self-administration context (Coc-ALT group). Additionally, mRNA levels of all genes were significantly greater in cocaine-treated rats re-exposed to the self-administration chamber than in their yoked-saline counterparts (Fig. 5a–d).

OFC: Following 15 days of abstinence, activity-regulated gene expression was differentially affected in the OFC of rats that were re-exposed to the operant chamber previously associated with cocaine delivery, whether levers were available, as compared to rats re-exposed to the operant chamber previously associated with yoked-saline infusions or rats with a cocaine history that were placed in an alternate environment on the test day (Fig. 5). There were no significant drug treatment by test environment interactions for any of the genes. A significant main effect of drug treatment was observed for *c-fos* ($F_{(1,23)}$=14.34, p= 0.001), *zif/268* ($F_{(1,21)}$=17.04, p=0.0005), *arc* ($F_{(1,22)}$= 52.43, p<0.0001), and *bdnf* ($F_{(1,20)}$=17.02, p=0.0006). Additionally, a significant main effect of test environment (ALT, NL, or LA) was seen for *c-fos* ($F_{(2,23)}$=15.63, p< 0.0001), *zif/268* ($F_{(2,21)}$=11.37, p=0.0005), *arc* ($F_{(2,22)}$= 17.37, p<0.0001), and *bdnf* ($F_{(2,20)}$=16.26, p<0.0001). Pairwise comparisons demonstrated significantly greater levels of mRNA in Coc-LA and Coc-NL rats for *c-fos*, *arc*, *zif/268*, and *bdnf* vs. Coc-ALT rats. Additionally, Coc-ALT rats displayed significantly greater mRNA levels than Sal-ALT rats for *c-fos*, *arc*, and *bdnf*.

In contrast to the dmPFC, an impact of environmental familiarity was apparent in OFC gene expression. Tukey-Kramer HSD tests revealed that *c-fos* mRNA levels were significantly higher in Sal-NL rats vs Sal-ALT rats, whereas *zif/268* mRNA levels were significantly higher in Coc-LA rats than in Sal-LA rats, as well as in Sal-LA and Sal-NL rats vs. Sal-ALT rats. *Arc* mRNA was significantly higher in Coc-LA and Coc-NL rats than in Sal-LA and Sal-NL rats, respectively. In addition, *arc* mRNA was greater in Sal-LA and Sal-NL rats than in Sal-ALT rats, and *bdnf* levels were significantly higher in Sal-LA and Sal-NL rats than in Sal-ALT rats.

Behavioral correlations with gene expression

In order to determine if the intensity of gene expression positively correlated with active lever pressing as a measure of drug seeking, a simple regression analysis was used to obtain correlation coefficients for gene expression and total lever presses during the 1-h test day session. No significant correlation was found between the number of active lever presses and any of the genes in the dmPFC and OFC of 22 h abstinence animals (Fig. 6). However, following 15 days of abstinence, lever pressing was significantly correlated with dmPFC increases in *c-fos*, *zif/268*, and *arc* mRNA (Fig. 7). Additionally, a significant correlation was found between lever pressing and *c-fos* and *arc* expressions in the OFC. No significant correlation was found between *bdnf* expression and lever pressing following 15 days abstinence in the dmPFC or the OFC.

Discussion

In the present study, IEG and *bdnf* mRNA was greater in the dmPFC and OFC of rats re-exposed to an environment previously associated with cocaine but not an environment previously associated with yoked-saline infusions. Moreover, the increased gene expression was independent of lever availability in the operant chamber, with the exception of bdnf mRNA in the dmPFC at 22 h, which was only elevated when levers previously associated with cocaine were available to press. These data suggest that drug-associated activity in the cerebral cortex stimulates IEG and *bdnf* gene expression in a way that is consistent with alterations induced by other activating stimuli (Bramham and Messaoudi 2005; Rocamora et al. 1996). Although ALT environment and home-cage control groups were not included in experiment one, evidence in the literature and from our own studies indicates that *bdnf* and IEG expression is not increased 1 day after the end of repeated cocaine exposure. IEG expression, particularly, *c-fos* and *zif268*, returns to basal levels in the striatum during the first day of withdrawal after repeated, non-contingent cocaine exposure (Hope et al. 1994; Moratalla et al. 1994) although *arc* expression has been reported to endure for 72 h in striatum and medial PFC (Fumagalli et al. 2006). However, 1 day after the end of cocaine self-administration, the level of AP-1 binding in the PFC was not different than saline controls (Pich et al. 1997), although delta FosB immuno-reactivity was elevated in PrL and OFC 24 h after repeated contingent or non-cotingent cocaine exposure (Winstanley et al. 2007). In contrast, using a self-administration paradigm similar to the one described herein (except that active lever pressing activated tone and light cues), the level of arc mRNA was not different from saline controls, but *bdnf* and *zif/268* mRNAs were downregulated in mPFC (McGinty et al., in preparation).

Following 15 days of abstinence, only rats with a cocaine history that were placed into the self-administration chamber during testing exhibited increases in gene expression in the dmPFC, suggesting that recognition of a previously drug-paired context, independent of instrumental responding, induced robust dmPFC activation. Thus, alterations in transcriptional and effector gene expression within the dmPFC are selective for drug history associations and are not merely induced by a recently or remotely familiar environment in the absence of drug associations. In contrast, the interpretation of changes in gene expression seen in the OFC is more complex because of drug-dependent, as well as drug-

independent, contributions to gene activation. Only *arc* and *zif268* mRNA in the OFC of Coc-NL and Coc-LA rats were significantly greater than that in Sal-NL and Sal-LA rats, reflecting cocaine seeking independent of instrumental responding. These data are consistent with, and extend previous work showing, IEG mRNA induction in the mPFC and OFC upon re-exposure of rats to a context previously paired with cocaine (Zavala et al. 2007), high-dose nicotine cues (Schiltz et al. 2005), or stress in combination with low-dose nicotine cues (Schiltz et al. 2007).

A number of studies have demonstrated a time-dependent increase in drug-seeking behavior with more prolonged periods of withdrawal (Neisewander et al. 2000; Shaham et al. 2003) and that drug seeking becomes more prominent with the passage of time. In the present study, there was no association between lever pressing and gene expression 1 day after cocaine self-administration ended. In contrast, after 15 days of abstinence, there was a robust correlation between the total number of lever presses during testing and increased *c-fos*, *zif/268*, and *arc* mRNA in the mPFC and *c-fos* and *arc* mRNA levels in the OFC. This correlation suggests that the number of active lever presses more selectively reflects drug salience after prolonged abstinence than after brief abstinence and, further, that the intensity of IEG expression, particularly in the mPFC, encodes this salience. These results are also congruent with the idea that choice responding (in this case, lever pressing to obtain a drug reward) over time is mediated by frontal association cortices (Miller and Cohen 2001).

Functional significance of activation in dmPFC

Recently, increased *c-fos* and *zif/268* gene expression in the PFC has been implicated in the storage and retrieval of spatial and fear-conditioned memories. For example, gene expression induced by memory processing of a previously learned spatial location produced increases in these transcription factors within the PFC of mice following both a recent (1 day later) or a remote (30 days later) memory test. However, inactivating the PFC with a lidocaine infusion selectively disrupted spatial memory retrieval of remote, but not recent, memory (Maviel et al. 2004). Similarly, Frankland et al. (2004) found that retrieval of remote, but not recent, contextual fear memories increased IEG expression in the PFC and that inactivation of the anterior cingulate cortex selectively prevented remote memory retrieval. Thus, increased gene expression in the PFC upon re-exposure to a drug-associated context following short and long periods of abstinence may reflect activation of common neural substrates important in retrieving memories of drug availability.

Alternatively, dmPFC activation in the present study may reflect a function other than recognition of a drug-paired context. For example, inactivation of the dmPFC does not prevent relapse to cocaine seeking in rats after 2 weeks (Fuchs et al. 2006) or 1 day of abstinence (Jamie Peters and Peter Kalivas, personal communication). Because re-exposure to the conditioned environment occurred in the absence of a drug-reinforcer, the test session acted effectively as an extinction trial at both time points. Thus, increases in gene expression within the dmPFC may reflect changes necessary to consolidate information that a conditioned context previously paired with cocaine no longer predicts cocaine availability. With regard to drug addiction and dmPFC function, this possibility is of particular interest because it pertains to decision making, reversal learning, and extinction of a conditioned

response. Thus, the PFC may not only play a role in conditioned responses, but also in the unconditioned response that promotes the extinction of the previously learned response to the context.

Functional significance of activation in OFC

In human and animal studies, OFC neurons not only encode reward valence (Gottfried et al. 2003; Schoenbaum et al. 2003; Roesch and Olson 2004), but also display state-dependent responding to reward presentation and to stimuli that signify reward (Rosenkilde et al. 1981; Tremblay and Schultz 1999; Garavan et al. 2000; Hollerman et al. 2000; Schultz et al. 2000; Schoenbaum et al. 2003; Roesch and Olson 2004). In addition, increases in OFC activity in response to drug-associated stimuli are correlated with compulsive drug intake in drug addicts (Volkow and Fowler 2000; Volkow et al. 2004). Therefore, the increased expression of activity-related genes in the OFC upon returning to a drug-paired context may be, in part, a reflection of anticipated drug reward and/or compulsive drug-seeking behavior.

Although both the dmPFC and OFC were activated by drug seeking, the OFC alone was activated by other stimuli. Contextual activation in the OFC was evident after 15 days of abstinence in saline-treated (Sal-NL and Sal-LA) rats returned to the operant chamber vs. Sal-ALT rats. The OFC of Sal-ALT rats exhibited the least amount of activation when rats were placed in a context that they were habituated to, whereas the OFC of Sal-NL and Sal-LA rats was more activated in the chamber that they had not experienced for 2 weeks. Finally, the OFC of rats with a cocaine history that were placed in the alternative environment on the test day following 15 days of abstinence exhibited significantly higher levels of c-fos, arc, and bdnf than saline-treated rats. These data suggest that the OFC of rats with a cocaine history encoded the experience of being transferred out of the homeroom to the alternate environment differently than did the saline-treated rats. These data suggest that gene expression is altered differentially by environmental stimuli in the dmPFC and the OFC.

Functional significance of gene expression changes

Fos and Zif/268 are nuclear transcription factors that regulate the expression of late-target genes whereas the protein products of the effector genes, *arc* and *bdnf*, are rapidly expressed and delivered to activated synapses (Link et al. 1995; Steward and Worley 2001; Ying et al. 2002; Bozon et al. 2003; Tongiorgi et al. 2004). In addition, early phases of instrumental learning (Hernandez et al. 2006; Kelly and Deadwyler 2003) elicit widespread *zif/268* and *arc* induction throughout an extensive corticostriatal network in rats, whereas manipulation of BDNF levels in the brain alters cocaine-seeking in a regionally selective manner (Horger et al. 1999; Lu et al. 2004; Berglind et al. 2007; Graham et al. 2007).

In addition to individual changes in each of these genes, clear evidence indicates that these genes show unique interactions, especially *arc* and *bdnf*. BDNF promotes synaptic plasticity and synaptic consolidation that are dependent upon the transcription and translation of *arc* (Kang and Schuman 1996; Guzowski et al. 2001; Messaoudi et al. 2002; Bramham and Messaoudi 2005; Soule et al. 2006; Wibrand et al. 2006). Thus, an increase in *bdnf* mRNA in the cortex may drive the *arc* response by initiating translation of newly synthesized *arc*

mRNA in activated dendritic spines (Yin et al. 2002; Ying et al. 2002). In addition, BDNF-induced *arc* synthesis during extinction learning may help drive synaptic consolidation to completion (Bramham and Messaoudi 2005; Haubensak et al. 1998; Lessmann 1998), a mechanism likely necessary to consolidate learned associations into long-term memory. Recently, Shepherd et al. (2006) extended this concept by demonstrating that increased expression of *arc* mRNA in the hippocampus increases endocytosis of AMPA receptors. As dendritic mRNAs that are transported to active synapses, *arc* and *bdnf* may play an important role in the AMPA receptor turnover that is necessary for both activity-induced and homeostatic forms of plasticity (Sutton and Schuman 2006).

Conclusion

The induction of activity-regulated genes in drug-seeking rats suggests that prefrontal cortical regions participate in the associative processing of complex drug-associated, contextual stimuli. These data indicate that transcriptional and effector genes within the dorsomedial PFC are generally responsive when animals are re-exposed to a drug-associated context immediately or temporally remote from the drug experience. In contrast, gene expression in the OFC is sensitive to the drug-associated context soon after drug exposure, but only *arc* and *zif/268* in OFC are responsive to interactions between the environment and drug history when the re-exposure is temporally remote. Based on their anticipated role in stabilization of long-term memories, it is likely that these four genes are regulating different molecular processes that together orchestrate how the PFC responds to the incentive motivational effects of a cocaine-paired context. Mapping the selective regional patterns of activity-regulated genes under specific environmental conditions may provide a clearer understanding of the neurobiological substrates of cocaine addiction.

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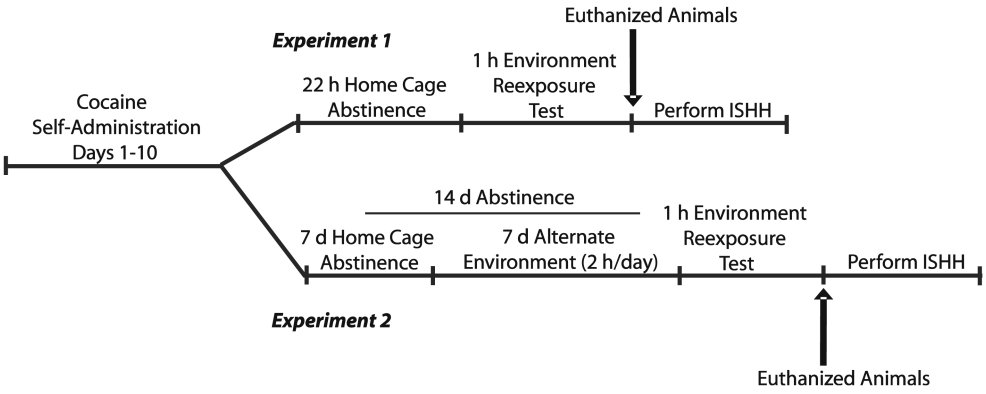

Fig. 1.

Schematic representing the design used in experiments 1 and 2. Sal-ALT and Coc-ALT rats were not included in the design of experiment 1 because exposure to an alternate environment on day 1 of abstinence would have constituted a novel experience. In experiment 2, all rats were habituated to the ALT environment before the test day

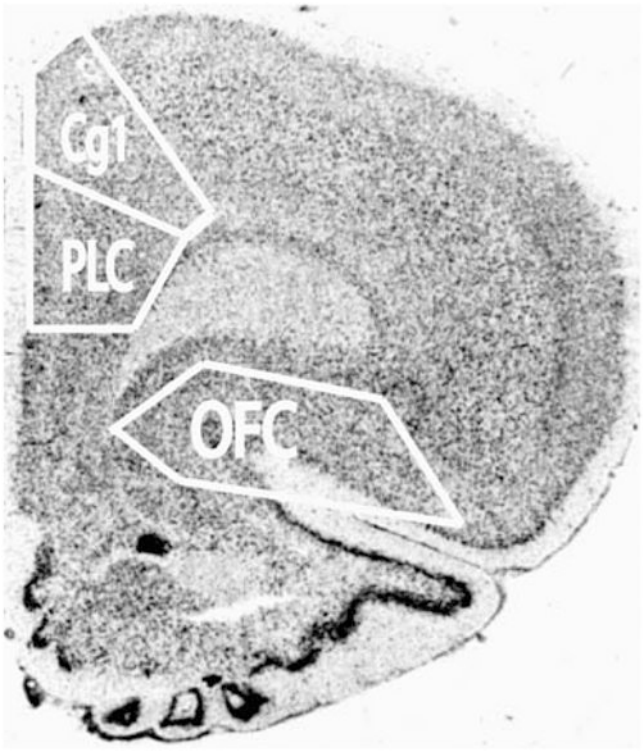

Fig. 2.

Regions of interest selected for measurement (*white outlines*) in medial *PFC* and *OFC* depicted on an image of a Nissl-stained section at Bregma 2.70 according to Paxinos and Watson 1998. Note that the OFC selection area does not include the claustrum and under density slicing conditions, the threshold for detection does not include white matter

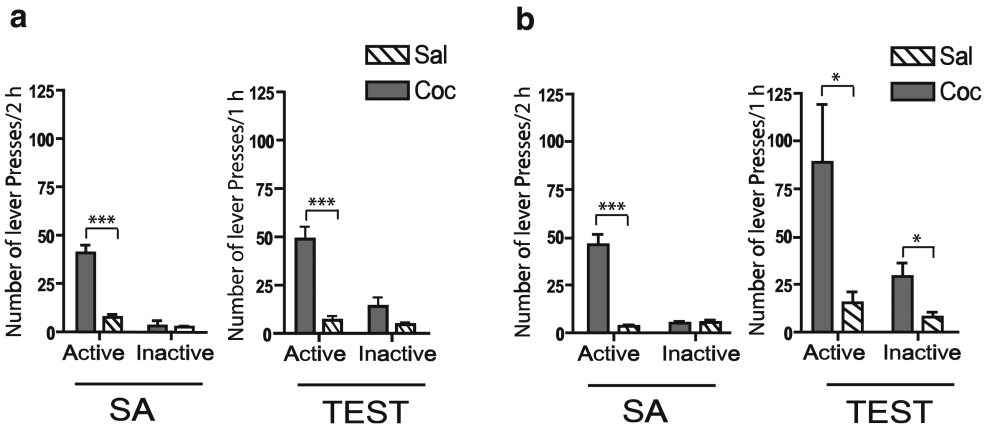

Fig. 3.

The average number of active and inactive lever presses from the last 3 days of 2-h self-administration (*SA*) sessions and the 1-h test session (*TEST*) of cocaine (*grey*) and yoked-saline (*white-striped*) after 22 h (**a**) and 15 days of abstinence (**b**). *p< 0.05,***p<0.001; cocaine vs. saline

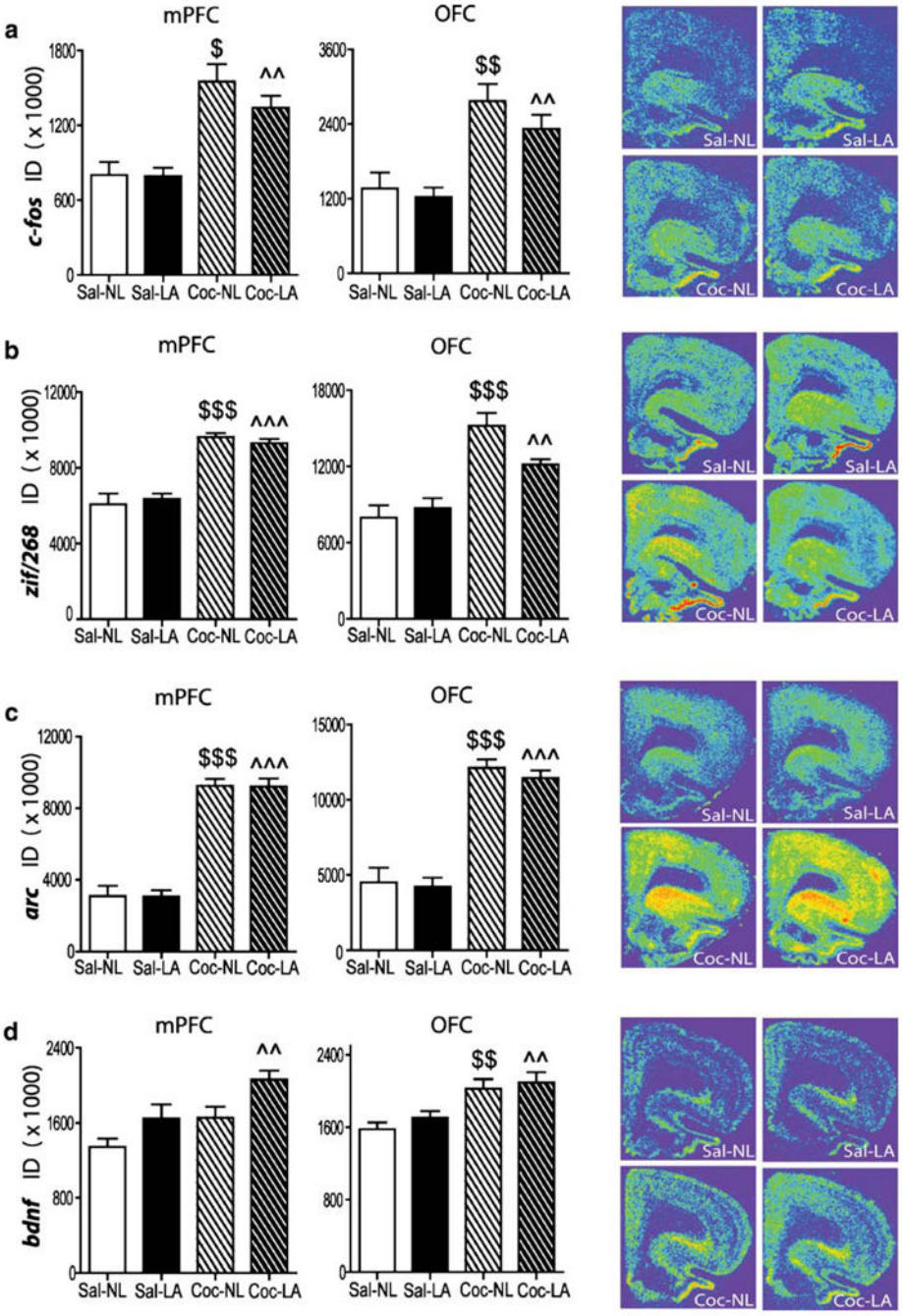

Fig. 4.

After 22 h of abstinence, rats with a cocaine history had greater *c-fos*, *zif268*, *arc*, and *bdnf*, *mRNA* in the dmPFC and OFC than rats with a saline history when re-exposed to the operant chamber. *c-fos* (a), *zif268* (b), *arc* (c), and *bdnf* (d) hybridization signals. *Right*, representative coronal hemi-sections illustrating the expression pattern of each gene. *Left and middle*: quantitative analysis of the integrated density of the hybridization signal in dmPFC (*left*) and OFC (*middle*). $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$}p<0.001$ vs. Sal-NL; $^{\wedge}p<0.05$,$^{\wedge\wedge}p<0.01$, $^{\wedge\wedge\wedge}p<0.001$ vs. Sal-LA. *ID* integrated density

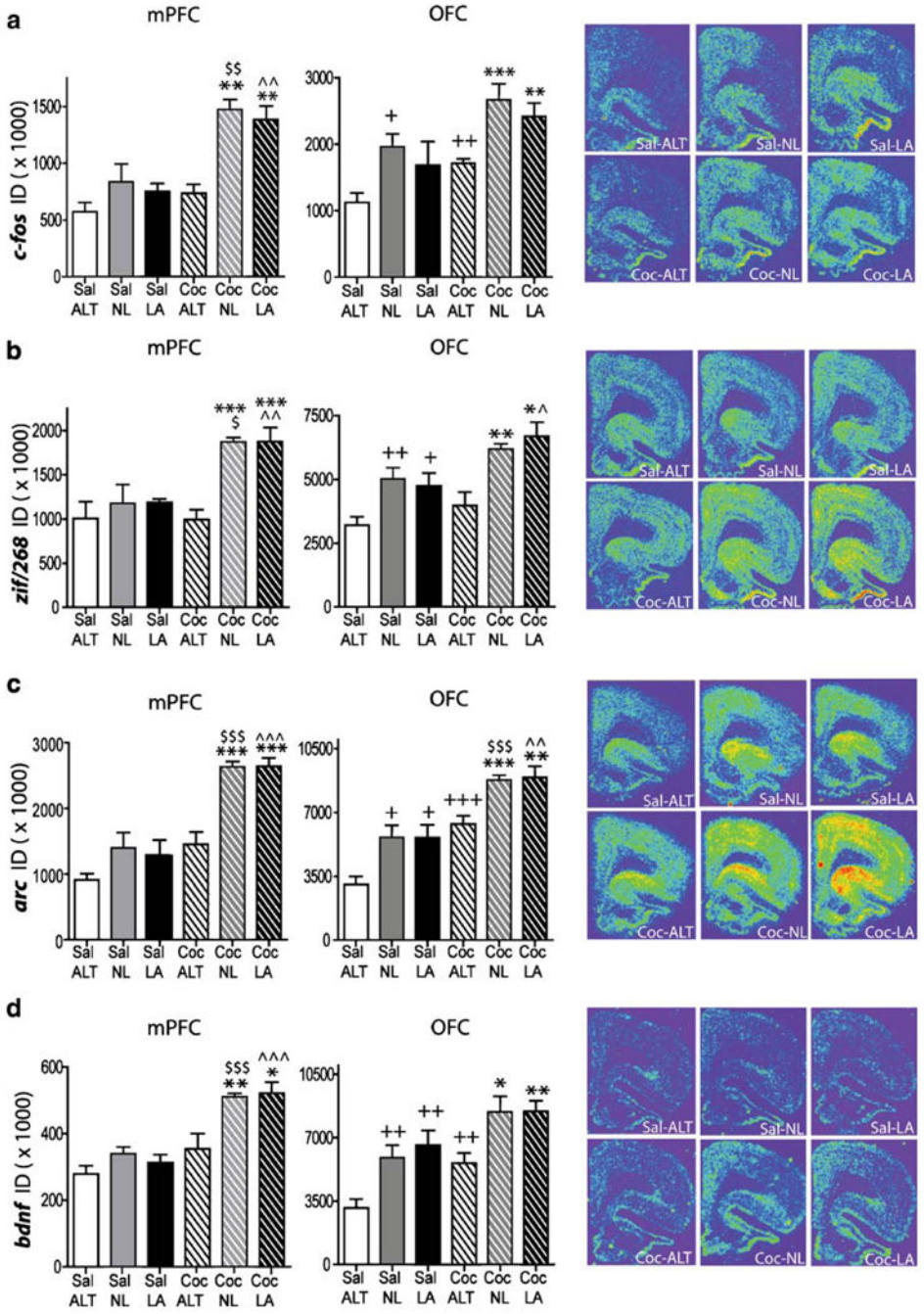

Fig. 5.

After 15 days of abstinence, rats with a cocaine history had greater increases in *c-fos*, *zif268*, *arc*, and *bdnf* mRNA in the dmPFC and OFC than rats with a saline history when re-exposed to the operant chamber. *c-fos* (**a**), *zif268* (**b**), *arc* (**c**), and *bdnf* (**d**) hybridization signals. *Right*, representative coronal hemi-sections illustrating the expression pattern of each gene. *Left and middle*: quantitative analysis of the integrated density of the hybridization signals in dorsomedial PFC *(left)* and OFC *(middle). *p<0.05, **p*<0.01,

***$p<0.001$ vs. Coc-ALT; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ vs. Sal-ALT; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs. Sal-LA; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$}p<.001$ vs. Sal-NL. *ID* integrated density

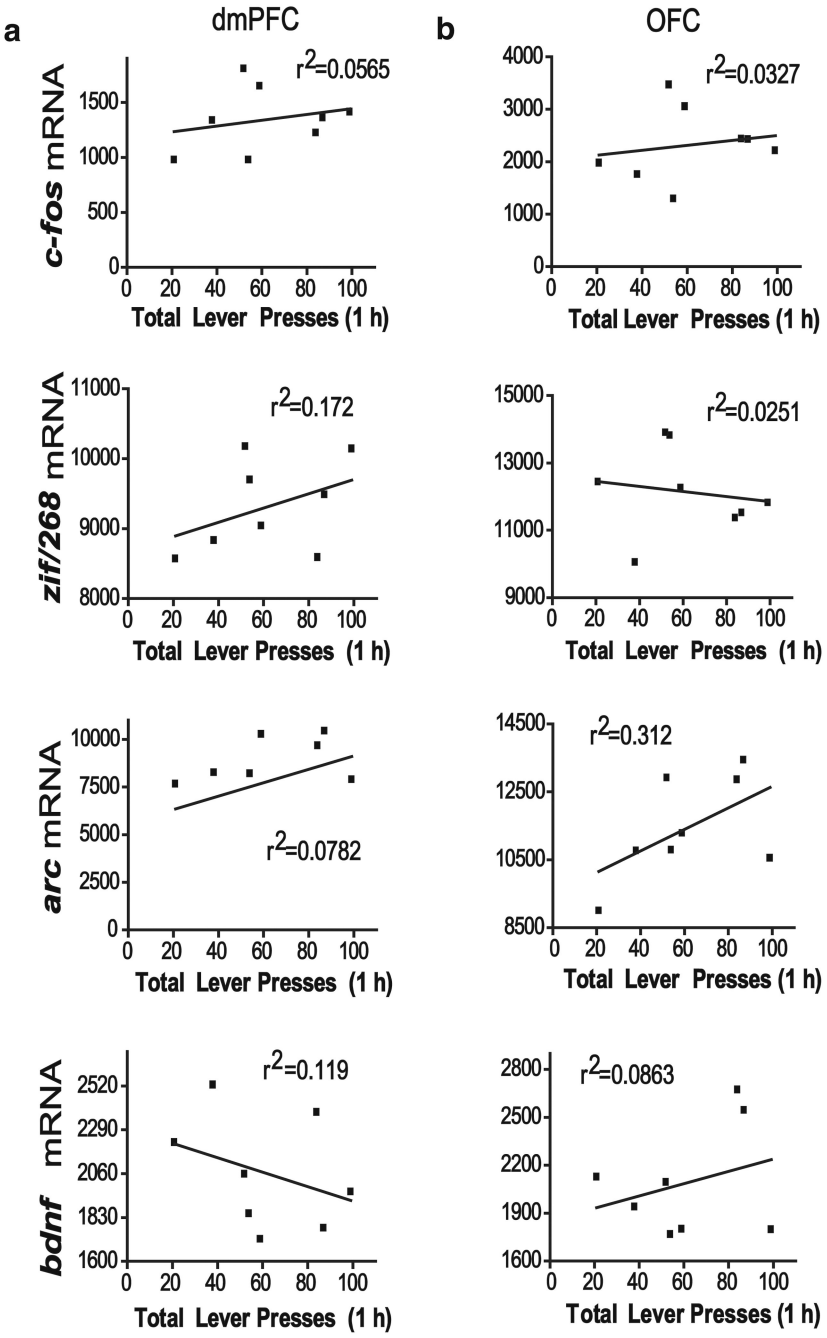

Fig. 6.

Correlation of total lever pressing and gene expression (ID×1,000) in the dmPFC (**a**) and OFC (**b**) of 22 h abstinence COC-LA rats. *$p<0.05$, **$p<0.01$. *ID* integrated density

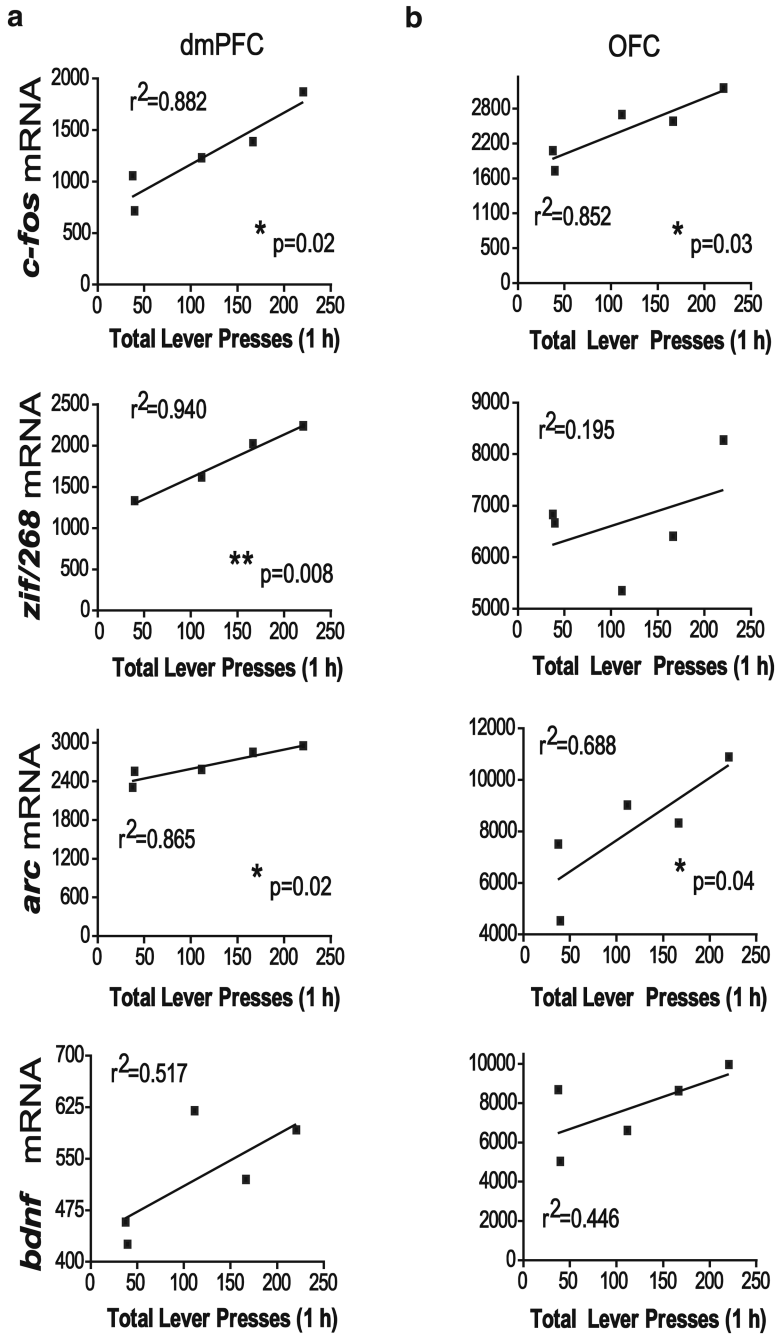

Fig. 7.

Correlation of total lever pressing and gene expression (ID×1,000) in the dmPFC (**a**) and OFC (**b**) of 15 d abstinence COC-LA rats. *p<0.05, **p< 0.01. *ID* integrated density