Journal of Neurochemistry, 2002, **82**, 1453–1464

The dopamine D1 receptor is a critical mediator for cocaine-induced gene expression

Dongsheng Zhang,* Lu Zhang,* Dan Wen Lou,* Yusaku Nakabeppu,† Jianhua Zhang* and Ming Xu*

**Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati Medical Center, Cincinnati, Ohio, USA*
†Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

Abstract

The dopamine D1 receptor plays a major role in mediating behavioral responses to cocaine administration. The time course for the acquisition and the relative stability for the expression of behavioral responses suggest the involvement of enduring neuroadaptations in response to repeated cocaine exposure. Changes in gene expression through the D1 receptors may accompany and mediate the development of such neuroadaptations to repeated cocaine stimulation. To test this possibility, we systematically compared the expression of the *fos* and *Jun* family immediate early genes in the nucleus accumbens and caudoputamen in D1 receptor mutant and wild-type control mice after acute and repeated cocaine exposure. Moreover, we compared the expression of three molecules that have been implicated in mediating the actions of cocaine, Gαolf, β-catenin and brain-derived neurotrophic factor, in the two groups of mice before and after cocaine administration. We found that there is a lack of induction of c-Fos, FosB, Fra-2 and JunB by acute cocaine exposure, and of ΔFosB by repeated cocaine administration in both the NAc and CPu of D1 receptor mutant mice compared with wild-type control mice. Moreover, the D1 receptor is differentially required for mediating Gαolf, β-catenin and BDNF expression in the NAc and CPu upon cocaine exposure. These results suggest that the D1 receptor is a critical mediator for cocaine-induced expression of these genes.

Keywords: cocaine, D1 receptor, dopamine, gene regulation.

J. Neurochem. (2002) **82,** 1453–1464.

Drug addiction is a brain disease with complex psychological and social factors (Leshner 1997; Gawin 1991). A central feature of drug addiction is compulsive drug-taking (Koob *et al*. 1998; Berke and Hyman 2000; Wise 2000). Elucidating the cellular and molecular mechanisms underlying the transition between acute, occasional drug use and the loss of behavioral control over repeated drug-taking is critical for understanding drug addiction (Koob *et al*. 1998; Berke and Hyman 2000; Wise 2000). The neurobiological mechanisms mediating both the acute and chronic behavioral actions of cocaine are primarily associated with the brain dopamine (DA) pathways originating in the midbrain ventral tegmental area (VTA) and substantia nigra area and project to the nucleus accumbens (NAc), caudoputamen (CPu), amygdala and frontal cortex (Koob 1992; Nestler 1993; Self and Nestler 1995; Nestler and Aghajanian 1997; Koob *et al*. 1998; White and Kalivas 1998; Wise 1998; Berke and Hyman 2000). Lesioning the mesolimbic DA pathway abolishes cocaine self-administration (Roberts *et al*. 1977, 1980). Cocaine binds to the DA transporter to block DA reuptake (Ritz *et al*. 1987). Increased extracellular DA can be detected in the NAc during cocaine self-administration (Pettit and Justice 1989). This evidence suggests that the behavioral effects of cocaine are mediated by its ability to potentiate DA neurotransmission (Kuhar *et al*. 1991).

The DA D1 receptor plays a major role in mediating behavioral responses to cocaine administration (White and Kalivas 1998). D1 receptor agonists and antagonists can influence locomotor and stereotyped responses to cocaine and cocaine self-administration (Koob *et al*. 1987; Cabib *et al*. 1991; Caine and Koob 1994; Tella 1994; Self *et al*.

Received May 6, 2002; revised manuscript received June 10, 2002; accepted June 12, 2002.

Address correspondence and reprint requests to Dr Ming Xu, Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati Medical Center, Cincinnati, OH 45267–0521, USA. E-mail: Ming.Xu@uc.edu

Abbreviations used: BDNF, brain-derived neurotrophic factor; CPu, caudoputamen; DA, dopamine; IEGs, immediate early genes; NAc, nucleus accumbens.

1996). Moreover, D1 receptor antagonists can prevent or attenuate the development of stimulant-induced behavioral sensitization (Mattingly *et al.* 1996; White and Kalivas 1998). Repeated cocaine administration also leads to persistent increases in D1 receptor sensitivity within the NAc (Henry and White 1991, 1995). Using mutant mice deficient in the D1 receptor, we also showed that D1 receptors play a critical role in mediating both the acute and the chronic locomotor-stimulating effects of cocaine (Xu *et al*. 1994a; Xu *et al*. 2000). However, the D1 receptor-mediated molecular events that accompany the acute and chronic behavioral changes remain largely unknown.

The time course for the acquisition and the relative stability for the expression of behavioral responses suggest the involvement of enduring neuroadaptations, such as changes in gene expression, in response to repeated cocaine exposure (Nestler 1993; Self and Nestler 1995; Nestler and Aghajanian 1997; Koob *et al*. 1998; White and Kalivas 1998; Berke and Hyman 2000). The *fos* and *jun* immediate early gene (IEG) families encode transcription factors. The Fos family proteins form heterodimers with the Jun family proteins and the resulting AP-1 transcription complexes bind to the AP-1 site that is a consensus regulatory sequence found in a large number of cellular genes (Chiu *et al*. 1988; Halazonetis *et al*. 1988; Kouzarides and Ziff 1988). c-*fos* expression can be rapidly induced by many stimuli, ranging from neurotransmitters to neurotrophins (Sheng and Greenberg 1990). These unique properties of the Fos and Jun family proteins, being transcription factors and being able to be rapidly turned on, make them attractive candidates for mediating some of the neuroadaptations to repeated cocaine exposure. It has long been hypothesized that the Fos and Jun family proteins can couple acute stimuli received at the cell surface to long-term neuroplastic changes via regulating gene expression (Sheng and Greenberg 1990; Morgan and Curran 1991).

Much evidence suggests that members of the *fos* family IEGs are involved in both acute and chronic behavioral effects of cocaine through the D1 receptors (Graybiel *et al*. 1990; Nestler 2000). Fos expression is induced through D1 receptors by acute cocaine injections (Robertson *et al*. 1990; Young *et al*. 1991; Cole *et al*. 1992). Bilateral infusion of an antisense oligonucleotide to c-*fos* in the NAc blocks the acute locomotor-stimulating effects of cocaine without affecting spontaneous exploratory activity (Heilig *et al*. 1993). Repeated cocaine injections induce long lasting AP-1 transcription complexes consisting of ΔFosB (Hope *et al*. 1994; Nye *et al*. 1995; Nestler *et al*. 1999; Nestler *et al*. 2001). Chronic cocaine exposure also leads to network level changes in IEG expression (Moratalla *et al*. 1996a). Overexpression of ΔFosB in mice increases the rewarding and locomotor-stimulating effects of cocaine (Kelz *et al*. 1999). c-Fos expression is also induced in the NAc, CPu, amygdala and medial prefrontal cortex by cocaine self-administration (Daunais *et al*. 1995; Pich *et al*. 1997; Kuzmin and Johansson 1999; Howes *et al*. 2000). However, the significance of the c-Fos induction by cocaine and the molecular targets of c-Fos remain poorly defined.

We previously showed that the D1 receptor is crucial for c-Fos and JunB induction by acute cocaine administration (Moratalla *et al*. 1996c). However, the role of the D1 receptor in mediating the induction of other IEGs by cocaine is not firmly established. Moreover, repeated cocaine exposure elicits complex physiological changes that likely involve changes in the expression of other cellular genes through the D1 receptors. To understand the molecular mechanisms underlying the development of such neuroadaptations, we compared the expression of the *fos* and *Jun* family IEGs, and the expression of three molecules that have been implicated in the actions of cocaine, Gαolf, β-catenin and brain-derived neurotrophic factor (BDNF) in the NAc and CPu in D1 receptor mutant and wild-type control mice after acute and repeated cocaine exposure.

Experimental procedures

Mice

The D1 receptor mutant mice were previously generated by Xu *et al*. as described (1994b). All mice were housed in a pathogen-free animal facility in the University of Cincinnati College of Medicine. The D1 receptor mutant mice have been back-crossed from the initial C57BL/6Jx129Sv genetic background with wild-type C57BL/6J mice for three generations. Homozygous mutant and wild-type littermates were derived from heterozygous breeding. Genotypes of both mutant and wild-type mice were determined by Southern blotting as described (Xu *et al*. 1994b). D1 receptor mutant and wild-type control littermates 5–10 weeks of age (mean age was 7 weeks) were group housed in an animal housing room on a 12-h light/dark cycle with food and water available *ad libitum*. The temperature and humidity of the room were controlled. An animal use protocol was approved by the Institutional Animal Care and Use Committee, and we followed the guidelines of the NIH for the care and use of laboratory animals.

Drugs

Cocaine hydrochloride (Sigma, St Louis, MO, USA) was dissolved in saline and doses injected were 20 and 30 mg/kg. Saline was injected as 0 dose controls. All injections were administered intraperitoneally (i.p.) in 1 mL/(100 g body weight) volumes. Injections were performed during the light phase of the light/dark cycle.

Treatment paradigms

We used the same acute and repeated injection paradigms as described by Xu *et al*. (1994a, 2000). Briefly, for the acute injections, D1 receptor mutant and wild-type control mice were injected i.p. with 30 mg/kg of cocaine ($n = 24$ each) or saline ($n = 20$ each). For repeated injections, both the mutant and wild-type mice ($n = 32$ each) were injected twice daily i.p. at 11.00 and

16.00 h for seven consecutive days with 20 mg/kg of cocaine. Roughly equal numbers of male and female mice were used for each genotype. D1 receptor mutant mice exhibited attenuated locomotor responses compared with wild-type control mice upon either acute or repeated cocaine exposure as previously reported (Xu *et al*. 1994a, 2000).

Nuclear extract preparation

Two hours after acute saline or cocaine injections, or 24 h after the last chronic cocaine injection, mice were decapitated and intact brains rapidly removed. Both the NAc and CPu tissues were isolated by gross dissection, respectively. For the NAc, tissues from two to three mice were pooled during nuclear extract isolation. For the CPu, extracts were prepared from individual mouse brains. The samples were homogenized in a buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 20% glycerol, 5 mM MgCl, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 5 mM DTT and protease inhibitor cocktail as described (Hope *et al*. 1994). Homogenates were incubated on ice for 15–20 min and centrifuged at 12 000 *g* for 15 min at 4°C. Brain micropunches from the NAc and CPu from a ΔFosB-expressing mouse and a non-ΔFosB-expressing mouse, respectively, were kindly provided by Drs Nestler and Ulery (Kelz *et al*. 1999). Protein extracts were similarly isolated. Protein concentrations were determined by the Bradford method as described (Zhang *et al*. 2002).

Western blotting

Ten to twenty micrograms of boiled protein samples were resolved on 10% or 12% SDS-polyacrylamide gels, electrophoresed and transferred electrophoretically onto PVDF membranes as previously described (Zhang *et al*. 2002). The membranes were then blocked with non-fat dry milk for each antibody, incubated in primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactivity were visualized using enhanced chemiluminescence. Western blots for each protein was repeated at least three times and NAc and CPu samples from multiple mice were used.

We used primary antibodies against c-Fos (K25, sc253X; 1 : 10 000), FosB (H75, sc-7203; 1 : 1000), Fra-1 (N17, sc-183; 1 : 1000), Fra-2 (Q20, sc-604; 1 : 1000), c-Jun (H79, sc-1694; 1 : 1000), JunB (N17, sc-46; 1 : 1000), JunD (329, sc-74; 1 : 1000), BDNF (N20, sc-546; 1 : 1000), Gαolf (K19, sc-385; 1: 2000), β-actin (I19, sc-1616; 1 : 5000), and secondary HRP-conjugated antirabbit, antigoat and antimouse IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-β-catenin antibody was from BD Transduction Laboratories, Lexington, KY, USA (C19220, KY; 1 : 3000). The anti-C-terminus FosB antibody that is-non-cross-reactive with ΔFosB was raised as described (Nakabeppu and Nathans 1991; 0.5 mg/mL at 1 : 2000 dilution). Blocking peptides were used to verify the specificities of the antibodies for all the IEG products and for BDNF. A positive control for the anti-β-catenin antibody was also used (Transduction Laboratories).

Immunohistochemistry

We processed four each D1 receptor mutant and wild-type mice 24 h after the last chronic cocaine injection as described (Zhang *et al*. 2002). Adult D1 receptor mutant and wild-type mice were anesthetized with Nembutal and were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (pH 7.3). Brains were postfixed in 4% paraformaldehyde for 2 h and were cryoprotected in 20% sucrose overnight. Freshly frozen coronal sections were cut on a cryostat at 25 μm. Free-floating sections were blocked with 0.4% Triton-100 and 0.1% BSA in PBS for 1 h and were incubated at 4°C overnight with either an anti-N-terminus FosB antibody that recognizes both FosB and ΔFosB (Santa Cruz Biotechnology) or an anti-C-terminus FosB that detects only FosB (Nakabeppu and Nathans 1991), respectively. Brain sections stained with the anti-N-terminus FosB antibody were incubated with a goat Alexa-488-conjugated secondary antibody and Hoechst33258 to visualize cells containing both FosB and ΔFosB. Brain sections stained with the anti-C-terminus FosB antibody were incubated with a rabbit Alexa-546-conjugated secondary antibody and Hoechst33258 to detect cells immunopositive for FosB specifically.

Quantification

We scanned in all western blotting results for each sample from different mice and the relative band intensity defined as density readings subtracted by background for each signal was quantified using Metamorph program (Universal Imaging Corp., West Chester, PA, USA). Equal amounts of protein were used for expression comparisons of each individual protein at the 0 and 2 h and 7 d time points. Thus, the expression level for each protein can be compared with one another at these time points. However, the relative exposure time is different from one protein to another, therefore the expression levels of different proteins cannot be directly compared.

A two-tailed student's *t*-test was used to compare the expression of various genes between wild-type and D1 receptor mutant mice, and between saline- and cocaine- treated mice within each genotype. Images of immunostained NAc brain sections were captured by SpotCam. Cells containing both FosB and ΔFosB or only FosB were identified by computer-assisted determination of fluorescence using the Metamorph program. The percentage of FosB and ΔFosB expression was calculated from the number of neurons exhibiting immunoreactive signals for both FosB and ΔFosB, and for FosB only in each NAc section. Data were obtained from four non-serial sections per mouse. A two-tailed student's *t*-test was used to compare the expression of FosB and ΔFosB between wild-type and D1 receptor mutant mice (Zhang *et al*. 2002). Significant levels were set at 0.05.

Results

A lack of induction of the *fos* family and *Jun*B IEGs by cocaine exposure in the NAc of D1 receptor mutant mice compared with wild-type control mice

We previously demonstrated that the behavioral responses of the D1 receptor mutant mice are attenuated compared with wild-type control littermates after either acute or repeated cocaine exposure (Xu *et al*. 1994a, 2000). We also showed that the D1 receptor is critical for the induction of c-Fos and JunB by acute cocaine exposure (Moratalla *et al*. 1996c). To further understand D1 receptor-mediated molecular changes accompanying cocaine-induced behavioral changes, we

systematically compared the expression of seven *fos* and *Jun* family IEGs in the NAc induced by acute and repeated cocaine exposure in D1 receptor mutant and wild-type mice. We treated both groups of mice with cocaine either for 2 h or twice daily for seven consecutive days. We also injected both groups of mice with saline as controls.

Western blot analyses indicate that basal expression of all the IEGs was similar in both groups of mice (Figs 1a and b). Upon acute cocaine exposure, c-Fos, FosB, Fra-2 and JunB were induced in wild-type mice. In contrast, only Fra-2 was induced in D1 receptor mutant mice, and levels of c-Fos, FosB, Fra-2 and JunB were significantly lower in D1 receptor mutant mice compared with wild-type mice (Figs 1a and b). The expression of Fra-1, c-Jun and JunD did not appear to be different after acute cocaine administration compared with those of the basal levels in either group of mice (Figs 1a and b).

Twenty-four hours after seven consecutive days of repeated cocaine exposure, levels of c-Fos, FosB, Fra-2 and JunB returned to basal levels in wild-type mice (Figs 1a and b). In D1 receptor mutant mice, levels of c-Fos, FosB, Fra-2 and JunB remained similar to both basal levels and levels after acute cocaine administration (Figs 1a and b). Moreover, JunB levels in D1 receptor mutant mice were lower than those in wild-type mice (Figs 1a and b). The expression of Fra-1, c-Jun and JunD did not appear to be different after repeated cocaine administration compared with those of the basal levels in both D1 receptor mutant and wild-type mice (Figs 1a and b). Together, these results suggest that the D1 receptor is critical for mediating the induction of c-Fos, FosB, Fra-2 and JunB by acute cocaine administration. Moreover, the expression of all seven Fos family and Jun family proteins are similar to baseline levels 24 h after repeated cocaine exposure.

A lack of induction of the *fos* family and *Jun*B IEGs by cocaine exposure in the CPu of D1 receptor mutant mice compared with wild-type control mice

To further investigate the role of the D1 receptor in mediating IEG induction by both acute and repeated cocaine exposure, we performed western blot analyses using extracts prepared from the CPu. Baseline expression of all the IEGs was similar between wild-type and D1 receptor mutant mice (Figs 2a and b). Acute cocaine administration induced c-Fos, FosB, Fra-2 and JunB expression in wild-type mice. In contrast, acute cocaine treatment did not induce significant IEG expression in D1 receptor mutant mice (Figs 2a and b). Consequently, c-Fos, FosB, Fra-2 and JunB levels were significant lower in the D1 receptor mutant mice compared with wild-type mice (Figs 2a and b). Fra-1, c-Jun and JunD levels were unchanged in response to cocaine administration and were not different between D1 receptor mutant and wild-type control mice (Figs 2a and b).

Twenty-four hours after seven consecutive days of repeated cocaine exposure, the expression of all *fos* and *Jun* family proteins were similar to baseline levels and there was no obvious difference between the D1 receptor mutant and wild-type control mice (Figs 2a and b). These results indicate that the D1 receptor is critical for mediating the induction of c-Fos, FosB, Fra-2 and JunB by acute cocaine administration in the CPu. Moreover, there is a striking parallel in the overall dynamic regulation of *fos* family and *Jun*B IEGs in the NAc and CPu by particularly acute cocaine exposure.

Reduced ΔFosB induction in both NAc and CPu of D1 receptor mutant mice compared with wild-type control mice by repeated cocaine exposure

ΔFosB proteins are splice variants encoded by the *fosB* gene (Nestler *et al*. 2001). ΔFosB proteins are up-regulated in the NAc and CPu upon repeated cocaine exposure and the induction of ΔFosB may represent one mechanism by which cocaine produces enduring neuroadaptations in the brain that contribute to the addicted state (Nestler *et al*. 2001). However, pharmacological studies were inconclusive regarding the critical role of the D1 receptor in mediating ΔFosB induction. On the one hand, Nestler and colleagues reported that the induction of ΔFosB can be blocked by pretreatment of a D1 receptor antagonist (Nye *et al*. 1995). On the other hand, Graybiel and colleagues showed that repeated cocaine exposure induces both D1 receptor-depedent and independent ΔFosB (Moratalla *et al*. 1996b). To resolve this issue, we compared the induction of ΔFosB expression in both NAc and CPu of D1 receptor mutant mice and wild-type control mice upon acute and repeated cocaine exposure.

Western blot analyses indicate that there was no baseline difference in ΔFosB expression in the NAc and CPu in wild-type and D1 receptor mutant mice (Figs 3a and b). Moreover, ΔFosB was not induced in the NAc and was weakly induced in the CPu in both groups of mice by acute cocaine administration compared with saline-injected controls (Figs 3a and b). There was no obvious difference in ΔFosB expression in the CPu induced by the acute cocaine treatment between D1 receptor mutant and wild-type mice (Figs 3a and b). In contrast, repeated cocaine exposure induced abundant ΔFosB expression in both the NAc and CPu in wild-type mice but not in D1 receptor mutant mice (Figs 3a and b). This result suggests that ΔFosB induction in the NAc and CPu by repeated cocaine exposure depends on a functional D1 receptor. Moreover, the requirement for the D1 receptor for cocaine-induced ΔFosB expression is similar in the NAc and CPu.

To further verify the differential ΔFosB expression induced by repeated cocaine exposure in D1 receptor mutant and wild-type mice, we compared ΔFosB and FosB expression in the NAc by immunostaining. We used an anti-N-terminus FosB antibody that recognizes both FosB and ΔFosB, and an anti-C-terminus FosB antibody that recognizes only the full length FosB but not ΔFosB (Nakabeppu and

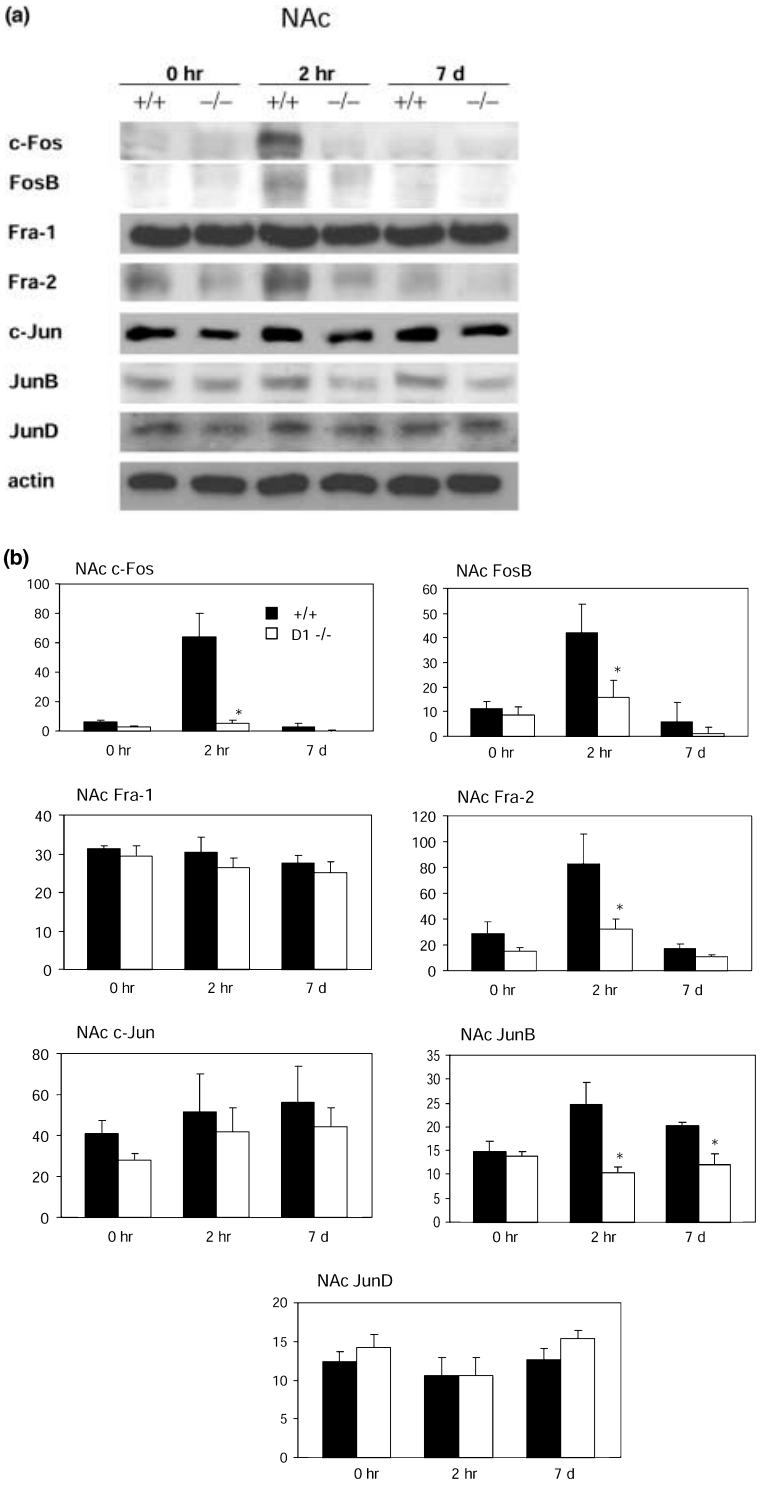

Fig. 1 A lack of induction of the *fos* family and *Jun*B IEGs by cocaine exposure in the NAc of D1 receptor mutant mice compared with wild-type control mice. Both D1 receptor mutant (–/–) and wild-type (+/+) mice were treated with cocaine for 2 h (2 h, $n = 16$ mice each) or twice daily for seven consecutive days (7 d, $n = 24$ mice each) or with saline (0 h, $n = 12$ mice each). (a) Nuclear extracts were isolated from the NAc of these mice and western blotting was performed for the indicated IEG products. Equal amounts of protein were loaded in each lane. (b) Mean + SEM of the expression of the various proteins expressed as relative band intensity in the NAc of D1 receptor mutant and wild-type mice. *$p < 0.05$ between the two groups of mice.

Nathans 1991). Cocaine induced more robust immunopositive signals that include both FosB and ΔFosB in the NAc in wild-type mice compared with D1 receptor mutant mice (Figs 4a and b), whereas FosB expression was roughly equal in the wild-type and D1 receptor mutant NAc (Figs 4c and d). A subtraction of FosB signals from the total FosB and ΔFosB indicate that repeated cocaine exposure induced a higher level of ΔFosB in the NAc of wild-type mice compared with the D1 receptor mutant mice (Fig. 4e). These results paralleled with those from our western blot analyses. Thus, the D1 receptor is critical for ΔFosB induction in response to repeated cocaine exposure in the NAc.

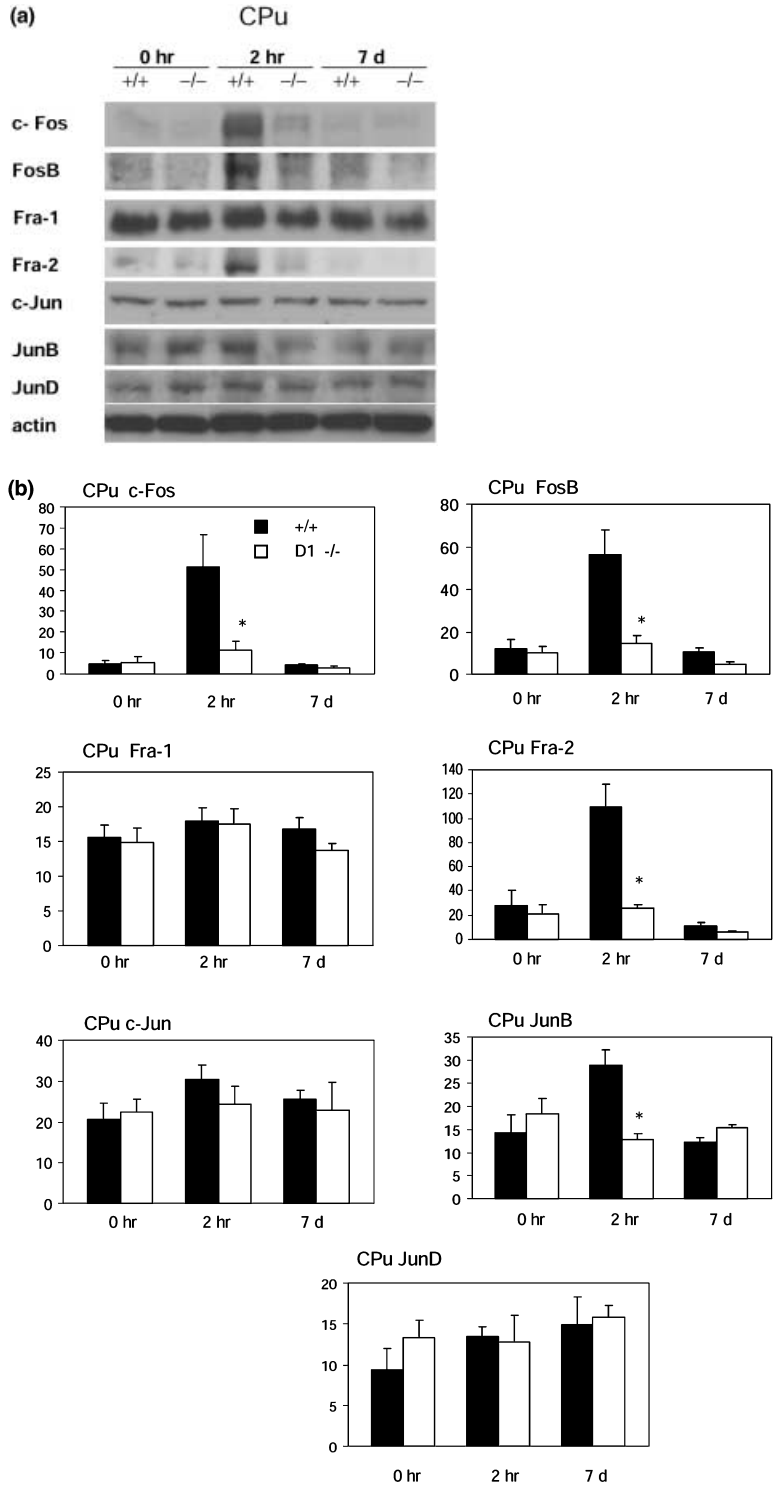

Fig. 2 A lack of induction of the *fos* family and *Jun*B IEGs by cocaine exposure in the CPu of D1 receptor mutant mice compared with wild-type control mice. Both D1 receptor mutant (–/–) and wild-type (+/+) mice ($n = 8$ mice each at each time point) were treated with cocaine for 2 h or twice daily for seven consecutive days or with saline as before. (a) Nuclear extracts were isolated from the CPu and western blotting was similarly performed for the indicated IEG products. Equal amounts of protein were loaded in each lane. (b) Mean + SEM of the expression of the various proteins expressed as relative band intensity in the CPu of D1 receptor mutant and wild-type mice. *$p < 0.05$ between the two groups of mice.

Requirement of the D1 receptor for mediating Gαolf, β-catenin and BDNF expression in the NAc and CPu upon cocaine exposure

Repeated cocaine exposure elicits a very complex physiological response that likely involves changes in expression of cellular effector genes in addition to the IEGs. To start investigating the role of the D1 receptor in mediating cocaine-induced gene expression, we selected three candidate genes involved in signal transduction and neuronal plasticity and compared their expression in the NAc and CPu

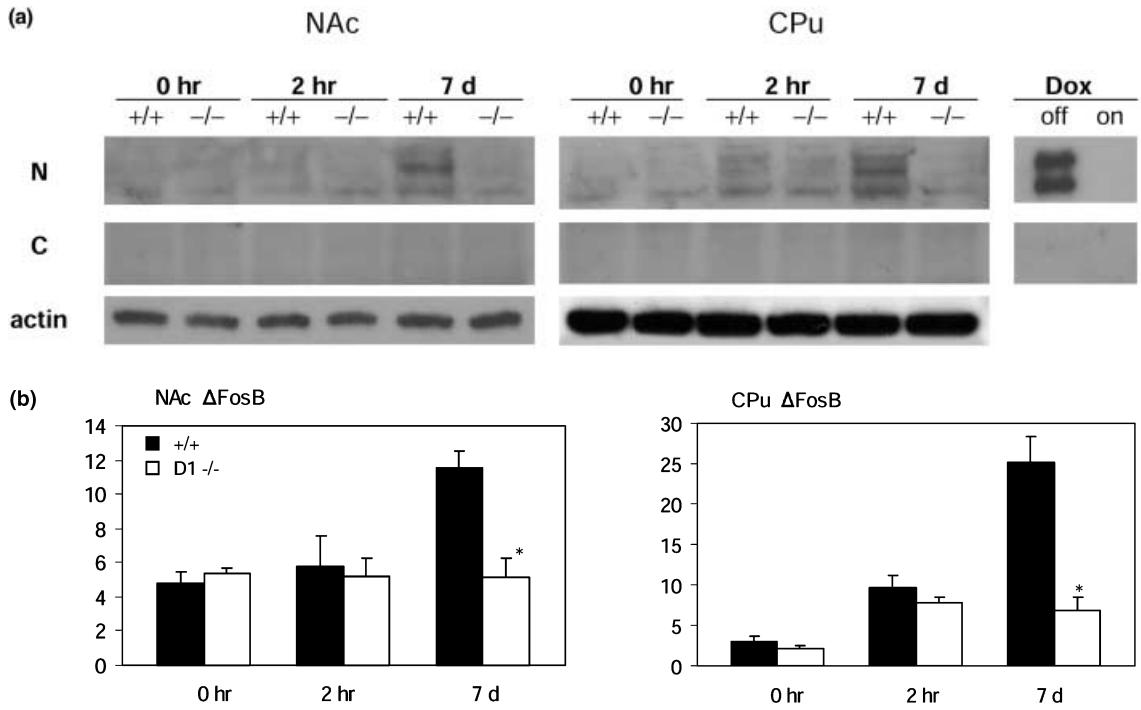

Fig. 3 Reduced ΔFosB induction in both the NAc and CPu of D1 receptor mutant mice compared with wild-type control mice by repeated cocaine exposure. Both D1 receptor mutant (–/–) and wild-type (+/+) mice were treated with cocaine for 2 h ($n = 24$ mice each) or twice daily for seven consecutive days ($n = 32$ mice each) or with saline ($n = 20$ mice each). (a) Nuclear extracts were isolated from the NAc and CPu and western blotting was similarly performed using either an anti-N-terminus FosB antibody (N) or an anti-C-terminus FosB antibody (C). Equal amounts of protein were loaded in each lane. We used extracts from a ΔFosB expressing transgenic mouse brain (Dox off) as a positive control, and extracts from a non-ΔFosB expressing transgenic mouse brain (Dox on) as a negative control. (b) Mean + SEM of ΔFosB expression expressed as relative band intensity in the NAc and CPu of D1 receptor mutant and wild-type mice. *$p < 0.05$ between the two groups of mice.

of D1 receptor mutant and wild-type mice before and after cocaine exposure. Gαolf is the α subunit of the heterotrimeric GTP-binding protein and it mediates D1 receptor signaling by coupling the D1 receptor with intracellular cAMP production (Zhuang *et al*. 2000; Corval *et al*. 2001). β-Catenin functions both in intercellular junctions and in the Wnt growth factor signaling pathway for transcriptional activation (Daniels *et al*. 2001). BDNF plays a key role in neuronal development and synaptic plasticity (Casaccia-Bonnefil *et al*. 1998).

Western blot analyses indicate that basal Gαolf levels were increased in D1 receptor mutant mice compared with the wild-type mice in NAc and CPu (Figs 5a–c). Moreover, there was a significant Gαolf induction in the CPu in D1 receptor mutant mice but not in wild-type mice after both acute and repeated cocaine exposure (Figs 5a–c). Basal β-catenin levels were similar in the NAc and CPu in D1 receptor mutant mice compared with wild-type mice (Figs 5a–c). Moreover, β-catenin was induced by acute cocaine exposure in the NAc and by repeated cocaine treatment in both the NAc and CPu in wild-type mice (Figs 5a–c). In contrast, acute cocaine exposure reduced β-catenin expression in the CPu and chronic cocaine injections decreased β-catenin expression in the NAc in the D1 receptor mutant mice (Figs 5a–c). There were lower basal levels of BDNF in the NAc and CPu in the D1 receptor mutant mice compared with wild-type mice (Figs 5a–c). Moreover, BDNF was slightly induced by acute cocaine treatment in the NAc and CPu of wild-type mice. In contrast, BDNF remained at basal levels in NAc and was reduced in CPu after acute cocaine exposure in D1 receptor mutant mice (Figs 5a–c). There were similar levels of BDNF after repeated cocaine exposure in the two groups of mice (Figs 5a–c). Together, these results suggest that the D1 receptor is critical in differentially regulating the expression of Gαolf, β-catenin and BDNF. Moreover, similar to that for the *fos* and *Jun* family IEGs, there is also a parallel in the overall dynamic regulation of Gαolf, β-catenin and BDNF in the NAc and CPu by acute and repeated cocaine exposure.

Discussion

The D1 receptor plays a major role in mediating behavioral responses to both acute and repeated cocaine exposure.

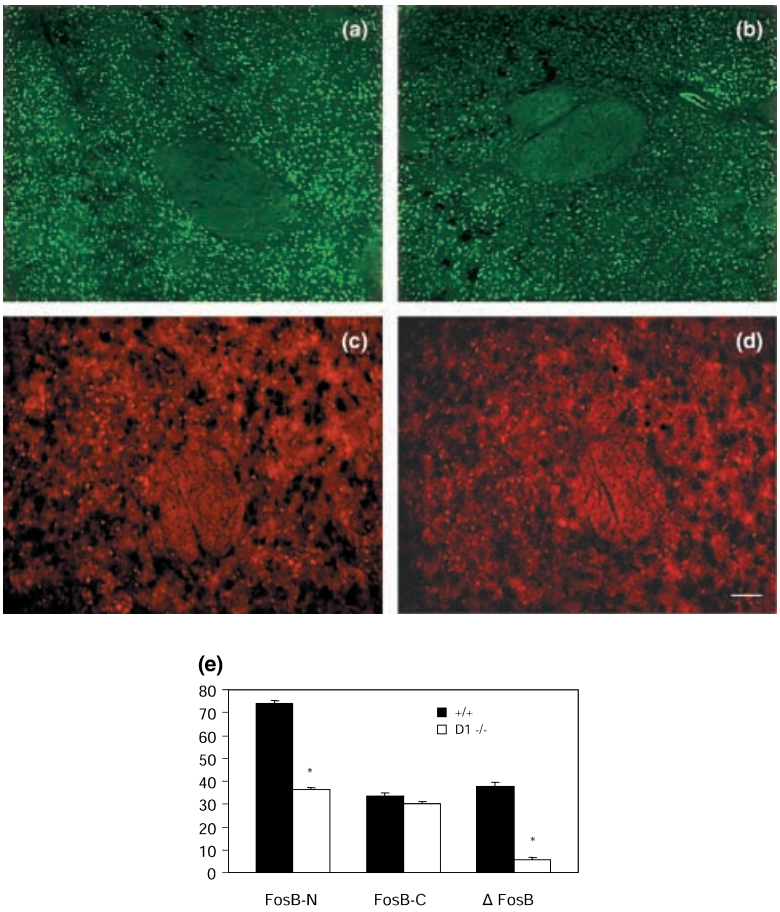

Fig. 4 Reduced ΔFosB induction in the NAc of D1 receptor mutant mice compared with wild-type mice by repeated cocaine exposure. D1 receptor mutant and wild-type mice ($n = 4$ each) were treated with cocaine twice daily for seven consecutive days. Coronal sections ($n = 4$ for each mouse) through the NAc of wild-type (a and c) and D1 receptor mutant (b and d) mice were stained with antibodies that recognize both FosB and ΔFosB (a and b), and only FosB (c and d). The scale bar is 80 μm. (e) Mean + SEM of percent FosB and ΔFosB, FosB and ΔFosB (FosB- and ΔFosB-positive cells subtracted by FosB-positive cells) expressing cells in the NAc of the two groups of mice. There was no significant difference in FosB/ΔFosB or FosB expression among brain sections from the same genotype. $^*p < 0.05$ between the two groups of mice.

Changes in gene expression through the D1 receptors may mediate the development of enduring neuroadaptations to repeated cocaine stimulation. To test this possibility, we systematically compared the expression of the *fos* and *Jun* family IEGs in the NAc and CPu in D1 receptor mutant and wild-type mice after acute and repeated cocaine exposure. We also compared the expression of three additional molecules that have been implicated in mediating the actions of cocaine, Gαolf, β-catenin and BDNF, before and after cocaine administration.

Our systematic comparison revealed that FosB and Fra-2 induction depends on a functional D1 receptor in both the NAc and CPu, whereas Fra-1, c-Jun and JunD are not significantly induced by the acute cocaine exposure. We also confirmed our previous observation that acute c-Fos and JunB induction by cocaine in the NAc and CPu requires a D1 receptor (Moratalla *et al*. 1996c). After repeated cocaine exposure, ΔFosB induction is significantly attenuated in both the NAc and CPu in D1 receptor mutant mice compared with wild-type mice, whereas the expression of the rest of the *fos* family and *Jun* family IEGs are similar to the baseline levels in the two groups of mice. These findings establish that the D1 receptor is a critical upstream mediator for the induction of c-Fos, FosB, Fra-2 and JunB by acute cocaine administration, and for ΔFosB induction upon repeated cocaine exposure in both the NAc and CPu in D1 receptor mutant mice.

We found that acute and repeated cocaine exposure increased Gαolf expression in the CPu in D1 receptor mutant but not wild-type mice. β-catenin was induced by acute cocaine exposure in the NAc and by repeated cocaine treatment in both the NAc and CPu in wild-type mice. In contrast, β-catenin expression was reduced in the CPu and NAc in D1 receptor mutant mice after acute and repeated cocaine exposure, respectively. BDNF was induced by acute cocaine treatment in the NAc and CPu of wild-type mice, whereas BDNF remained at basal levels in the NAc and was reduced in the CPu after acute cocaine exposure in D1 receptor mutant mice. These results indicate that the induction of Gαolf, β-catenin and BDNF by cocaine depends on a functional D1 receptor. Moreover, the differential requirement of the D1 receptor suggests that additional regulatory mechanisms exist for the regulation of these genes. As Gαolf mediates D1 receptor signaling by coupling the D1 receptor with intracellular cAMP production and mutation of Gαolf in mice abolishes acute c-*fos* induction by cocaine (Zhuang *et al*. 2000; Corval *et al*. 2001), Gαolf is possibly also a key mediator downstream of the D1 receptor

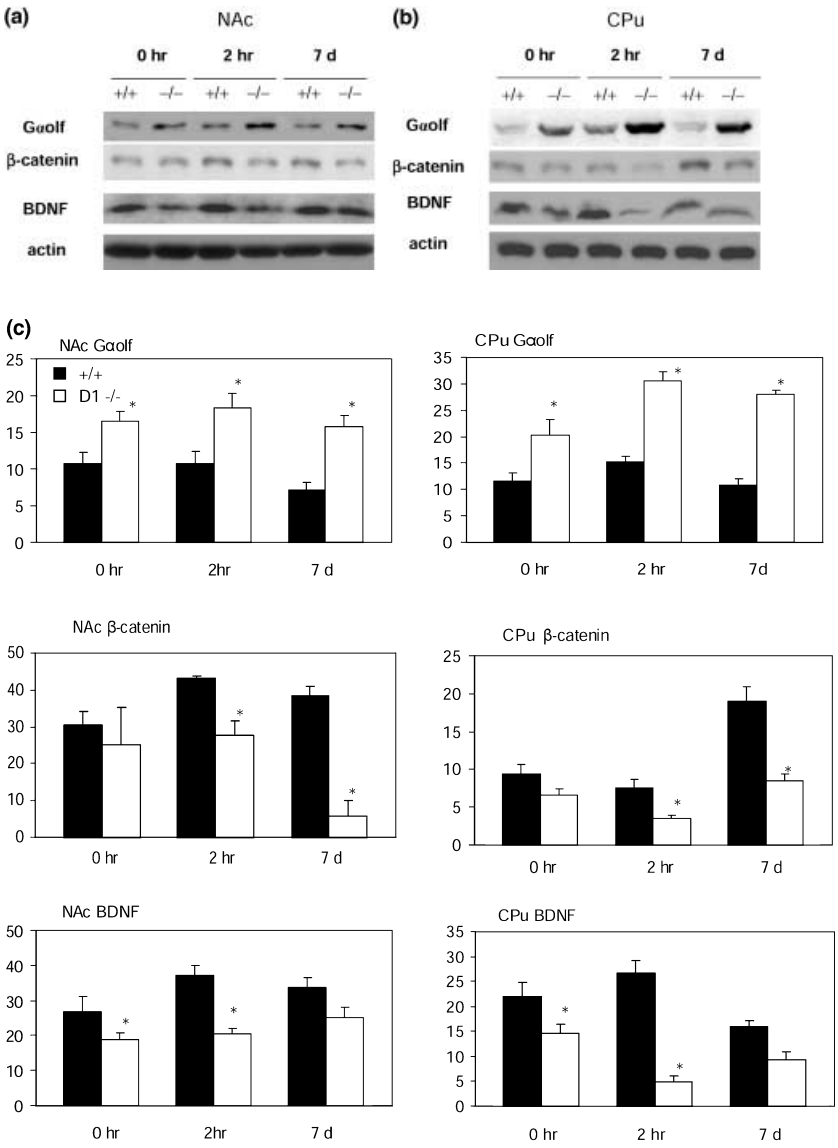

Fig. 5 The D1 receptor is critical for mediating Gαolf, β-catenin and BDNF expression in the NAc and CPu upon cocaine exposure. Both D1 receptor mutant (–/–) and wild-type (+/+) mice were treated with cocaine for 2 h ($n = 24$ mice each) or twice daily for seven consecutive days ($n = 32$ mice each) or with saline ($n = 20$ mice each). Nuclear extracts were isolated from both the NAc (a) and CPu (b) and western blotting was performed for the indicated proteins. Equal amounts of protein were loaded in each lane. (c) Mean + SEM of the expression of Gαolf, β-catenin and BDNF expressed as relative band intensity in the NAc and CPu of D1 receptor mutant and wild-type mice. *$p < 0.05$ between the two groups of mice.

for FosB, ΔFosB, Fra-2 and JunB induction by cocaine. β-Catenin is up-regulated by cocaine self-administration in the NAc and has been suggested to mediate aspects of cocaine actions (Freeman *et al*. 2001). Whether expression of the Gαolf and β-catenin genes are regulated by the AP-1 transcription complex or by other D1 receptor-mediated signaling events is still unclear. BDNF can enhance cocaine-induced locomotor activity and conditioned reward (Horger *et al*. 1999). We have shown that c-Fos can regulate basal and induced BDNF expression both *in vivo* and *in vitro* (Zhang *et al*. 2002). Moreover, BDNF expression can be regulated by CREB which is another key intracellular mediator of cocaine actions (Carlezon *et al*. 1998; Shieh *et al*. 1998; Tao *et al*. 1998).

Based on these findings, we propose a testable model for the development of neuroadptations that underlie cocaine-induced enduring behavioral changes. Acute cocaine exposure activates the D1 receptor and the Gαolf protein transduces signals from the activated D1 receptor, resulting in increased levels of cAMP and induction of c-Fos, FosB, Fra-2 and JunB proteins. These second and third intracellular messengers act on additional cellular target genes to initiate further molecular changes in the brain. For example, increased β-catenin expression can initiate changes in both intercellular interactions and gene expression through the Wnt signaling pathway. Increased BDNF expression can initiate changes in synaptic strength and neuronal signaling. Importantly, most of the molecular changes may not be permanent because of the acute nature of the cocaine exposure. Repeated cocaine exposure reinforces and strengthens molecular changes mediated by Gαolf and β-catenin, and induces additional molecular changes mediated by

ΔFosB that may be different from those mediated by c-Fos, FosB and Fra-2. Because of the persistent nature of the stimuli, the cocaine-induced molecular changes through the D1 receptors can lead to more permanent neuroadaptations that contribute to an addicted state that is fundamentally different from the state after the acute or an occasional cocaine exposure.

We found that basal expression of all the *fos* and *Jun* IEGs and β-catenin was similar in the NAc and CPu in D1 receptor mutant and wild-type mice. Basal expression of Gαolf is higher whereas basal BDNF expression is lower in the NAc and CPu in D1 receptor mutant compared with wild-type mice. Herve *et al*. also reported an increased basal Gαolf expression in the striatum of drug-naive D1 receptor mutant mouse compared with normal control mouse (Herve *et al*. 2001). The changed basal expression of Gαolf and BDNF in D1 receptor mutant mice suggests that there are developmental effects to compensate for the loss of the D1 receptor. Whether such developmental compensations also affect cocaine-induced gene expression through the D1 receptors remains unknown. We used mice with a mixed genetic background in our study. Whereas future studies using mice with a congenit background is necessary, the fact that acute cocaine was no longer able to induce c-*fos* expression in D1 receptor mutant mice that have been crossed with the C57BL/6 mice either for one (Moratalla *et al*. 1996c) or three generations argues that variation in genetic background may not be a major contributing factor in the observed cocaine-induced gene expression difference in D1 receptor mutant and normal control mice.

In the current study, we tested the *fos* and *Jun* family IEGs and three candidate genes whose induction by cocaine exposure depends on a functional D1 receptor. The D1 receptor has been shown to mediate the expression of many cellular genes (Berke *et al*. 1998). To fully understand the molecular events accompanying cocaine-induced acute and chronic behavioral effects, we will need to use the microarray method that will allow us to identify global gene expression changes through the D1 receptor upon cocaine exposure. Moreover, we will need to functionally perturb the expression of the candidate genes identified by the microarrays *in vivo* and to investigate whether such perturbations affect behavioral changes in response to cocaine exposure. Elucidating the mechanisms underlying the initiation and expression of enduring neuroadaptations to repeated cocaine exposure may provide insights into the molecular basis of compulsive drug-taking behaviors and new strategies for the treatment of drug abuse.

Acknowledgements

We thank Ryan Walsh for discussion and helping with immunostaining, Drs E. Nestler and P. Ulery for kindly providing the ΔFosB brain tissues. This work was supported by grants from the NIH (DA11284 to JZ, and DA11005 and DA13786 to MX) and the Epilepsy Foundation of America (MX).

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