Behavioral/Systems/Cognitive

Cocaine-Induced Intracellular Signaling and Gene Expression Are Oppositely Regulated by the Dopamine D1 and D3 Receptors

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Repeated exposure to cocaine can induce neuroadaptations in the brain. One mechanism by which persistent changes occur involves alterations in gene expression mediated by the dopamine receptors. Both the dopamine D1 and D3 receptors have been shown to mediate gene expression changes. Moreover, the D1 and D3 receptors are also coexpressed in the same neurons, particularly in the nucleus accumbens and also caudoputamen (CPu). Little is known however, whether these two receptors coordinately regulate gene expression after cocaine administration and the underlying mechanisms. We have used various gene mutant mice to address this issue. We show that extracellular signal-regulated kinase (ERK) activation and c-*fos* induction in the CPu in response to acute cocaine administration is mediated by the D1 receptor and inhibited by the D3 receptor. Moreover, ERK activation mediates acute cocaine-induced expression of Fos family genes, including c-*fos*, *fosB* and *fra*2. Interestingly, dynorphin, neogenin, and synaptotagmin VII, genes that possess cAMP-response element binding protein and AP-1 transcription complex-binding consensus sequences in their promoters, are also oppositely regulated by the D1 and D3 receptors after repeated exposure to cocaine. Furthermore, such regulation depends on proper ERK activation and c-*fos* function. These results suggest that the D1 and D3 receptors elicit opposite regulation of target gene expression by regulating ERK activation and c-*fos* induction after acute and chronic cocaine treatment.

Key words: **cocaine; dopamine receptors; signal transduction; mitogen-activated protein kinases; c-Fos; gene expression**

Introduction

Drug addiction is a long-lasting condition involving persistent neuroadaptations in the brain (Koob et al., 1998; White and Kalivas, 1998; Nestler, 2001; Hyman and Malenka, 2001; Laakso et al., 2002). The brain dopamine (DA) system that originates from the midbrain and projects to the nucleus accumbens (NAc), caudoputamen (CPu), and other forebrain structures is a key neural substrate for mediating persistent changes induced by repeated exposure to abused drugs (Koob, 1992). The DA receptors identified so far can be grouped into two classes, the D1 class (D1 and D5) and the D2 class (D2, D3 and D4) receptors (Civelli et al., 1993; Sibley et al., 1993; Missale et al., 1998). The D1 class receptors couple to Gs G-proteins and activate adenylyl cyclases, whereas the D2 class receptors couple to G_i or G_0 G-proteins and inhibit adenylyl cyclases. The D1 receptor is widely expressed in the brain, including the NAc, CPu, and other forebrain structures (Civelli et al., 1993; Sibley et al., 1993; Missale et al., 1998). The D3 receptor is primarily expressed in the ventral striatum, a region where abundant D1 receptor also exists (Civelli et al., 1993; Sibley et al., 1993; Missale et al., 1998; Schwartz et al., 1998). Using mice deficient in either the D1 or D3 receptors, we and others have shown that the D1 and D3 receptors are involved in mediating the locomotor-stimulant and the rewarding effects of psychostimulants (Xu et al., 1994a,b; Drago et al., 1994, 1996; Accili et al., 1996; Xu et al., 1997, 2000; Carta et al., 2000; Caine et al., 2002).

The signaling pathways and gene expression changes associated with various DA receptors have been suggested to play a critical role in drug-induced neuroadaptations in the brain. Cocaine induces the phosphorylation of cAMP-response element binding protein (CREB) and expression of the immediate early genes (IEGs) including c-*fos* and Δ*fosB,* mostly in D1 receptor-expressing neurons in both the NAc and CPu (Graybiel et al., 1990; Cenci et al., 1992; Hope et al., 1994; Konradi et al., 1994; Kosofsky et al., 1995; Moratalla et al., 1996a; Zhang et al., 2002a). The induction of CREB and ΔFosB oppositely modulates the rewarding behavioral effects of cocaine (Carlezon et al., 1998; Kelz et al., 1999; Colby et al., 2003). Moreover, gene expression after a brief exposure to cocaine is more dependent on CREB, whereas gene expression becomes increasingly ΔFosB-dependent after repeated cocaine administration (McClung and Nestler, 2003). The D3 receptors also regulate gene expression after cocaine challenges (Carta et al., 2000).

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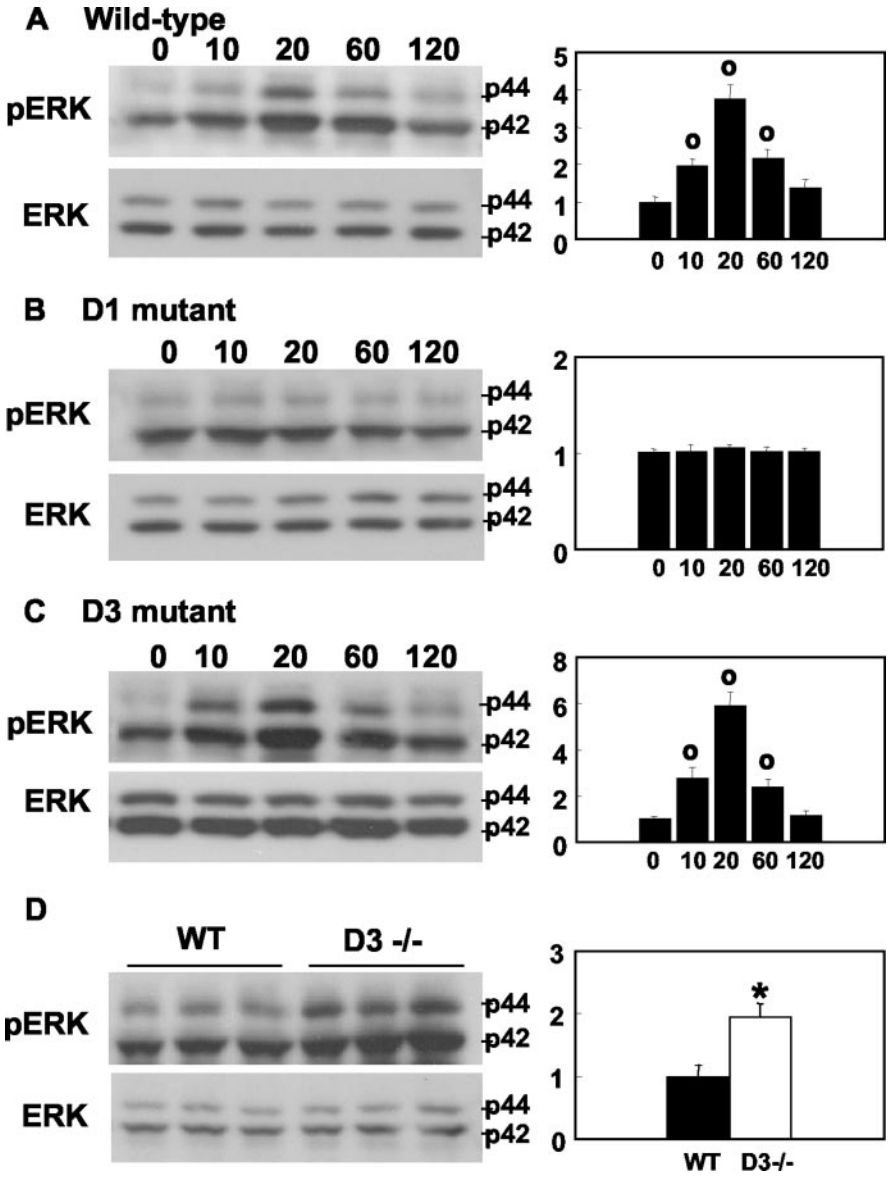

Figure 1. Opposite regulation of ERK activation in the CPu of D1 and D3 receptor mutant mice compared with wild-type mice after acute cocaine treatment. Time course (left) and quantification (right) of cocaine-induced ERK phosphorylation in the CPu in wild-type (*A*), D1 (*B*), and D3 receptor mutant (*C*) mice. Western blot analyses were performed 10 ($n = 3$ mice each), 20 ($n = 8$ mice each), 60 ($n = 3$ mice each), and 120 min ($n = 3$ mice each) after a cocaine injection at the 30 mg/kg dose. Antibodies against dually phosphorylated (Thr^{202} and Tyr^{204}) ERK (pERK) or total ERK were used. Data represent the mean + SEM pERK levels over the WT basal levels ($n = 3$ mice each). °$p < 0.05$ compared with basal levels. *D,* Comparison of the ERK phosphorylation in the CPu in D3 receptor mutant (D3−/−) and wild-type (WT) mice ($n = 8$ mice each) 20 min after a cocaine injection at the 30 mg/kg dose. *$p < 0.05$ compared with wild-type mice.

The mitogen-activated protein kinase pathway (MAPK) has been implicated in mediating the rewarding effects of abused drugs. MAPKs are critical for cells to respond to changes in the physical and chemical properties of their environment (Schaeffer and Weber, 1999; Davis, 2000). Three major groups of MAPKs exist in mammals, including the ERKs, the c-Jun N-terminal kinases (JNKs), and the p38 (Davis, 2000). Once activated, MAPKs translocate into the cell nucleus and activate transcription factors, such as c-Fos and CREB (Adams and Sweatt, 2002). These kinases are activated by distinct stimuli through independent signaling pathways and serve a variety of cell regulatory functions, including synaptic plasticity and learning and memory (Schaeffer and Weber, 1999; Davis, 2000; Adams and Sweatt, 2002; Morozov et al., 2003). Acute and chronic exposure to abused drugs, such as cocaine and morphine, can also induce MAPK activation in the DA system, and such activation may contribute to the development of drug-induced persistent changes in the brain (Berhow et al., 1996; Valjent et al., 2000; Choe et al., 2002; Choe and Wang, 2002; Mazzucchelli et al., 2002).

The D1 and D3 receptors are coexpressed in neurons, particularly in the NAc and also CPu (Surmeier et al., 1996, 1998; Le Moine and Bloch, 1996; Ridray et al., 1998). D1 and D3 receptors can exert both opposite and synergistic interactions in the brain. For example, we previously found that D1 and D3 receptor mutant mice exhibit attenuated and enhanced locomotor activity, respectively, compared with wild-type mice after acute cocaine treatment (Xu et al., 1994b, 1997). Moreover, the D1 and D3 receptors exhibit synergistic effects on substance P expression in the NAc, yet they exert opposite regulatory effects on c-Fos expression in the islands of Calleja (Schwartz et al., 1998; Ridray et al., 1998). Although the D1 receptor has been implicated in mediating the acute cocaine-induced ERK activation (Valjent et al., 2000), whether the D1 and D3 receptors coordinately regulate MAPK signal transduction and gene expression after cocaine exposure remains poorly understood. We have used various gene mutant mice to address this issue. Our results suggest that the D1 and D3 receptors elicit opposite regulation of target gene expression by influencing ERK activation and c-*fos* induction after acute and chronic cocaine treatment.

Materials and Methods

Mice. The DA D1 and D3 receptor mutant mice were previously generated by Xu et al. (1994a, 1997) as described. Homozygous mutant and wild-type littermates were produced from heterozygous breeding. The D1 receptor neuron-specific c-*fos* mutant mice (DC) were generated by crossing a transgenic mouse carrying a D1 receptor gene promoter driving a *cre* recombinase gene with a floxed c-*fos* mouse generated previously (Zhang et al., 2002b) (J. Zhang, L. Zhang, H. Jiao, D. Zhang, D. Lou, J. Katz, and M. Xu, unpublished observations). The genetic background of all the mice was 129Sv/C57BL6J. Genotypes of the various mutant and wild-type mice were determined by genomic Southern blotting as described (Xu et al., 1994a). D1 and D3 receptor mutant, DC, and wild-type control littermates seven to 10 weeks of age (mean age was 8 weeks) were group-housed in an animal housing room on a 12 hr light/dark cycle with food and water available *ad libitum*. Both the temperature and humidity of the room were controlled. We strictly followed the guidelines for the care and use of laboratory animals.

Drugs. Cocaine hydrochloride, the D1 receptor agonist SKF81297 (Sigma, St. Louis, MO) and the D2 receptor agonist PD128907 (Tocris Cookson, Ballwin, MO) were dissolved in saline as described (Xu et al., 1997). Saline was used as 0 dose controls. SL327 (Bristol-Myers Squibb,

Princeton, NJ) was dissolved in dimethylsulfoxide and then diluted in saline. SL327 is a potent and selective small-molecule MEK inhibitor. Systemic administration of SL327 has been shown to selectively inhibit ERK activation in the brain (Wang et al., 2003). All injections were administered intraperitoneally in volumes of 10 ml/kg. Injections were performed during the light phase of the light/dark cycle.

Drug treatment. For the acute treatment, D1, D3 receptor mutant and wild-type mice were injected intraperitoneally with 30 mg/kg of cocaine or saline. Mice were killed 10 ($n = 3$ each), 20 ($n = 8$ each), 60 ($n = 3$ each), and 120 min ($n = 3$ each) after the injections. For repeated injections, the various mutant and wild-type mice ($n = 8$ each) were injected with 20 mg/kg of cocaine twice daily intraperitoneally for 7 consecutive days. D1 receptor mutant mice exhibited attenuated locomotor responses compared with wild-type control mice after either acute or repeated exposure to cocaine, and D3 receptor mutant mice exhibited enhanced locomotor responses to a novel environment compared with wild-type mice, as previously reported (Xu et al., 1994b, 1997, 2000). SL327 (50 mg/kg) was injected intraperitoneally ($n = 4$ mice) 15 min before both acute and repeated cocaine administration. The choice of the timing and dose of SL327 was based on our previous concentration-dependent studies for the effect of SL327 on phospho-ERK expression in the brain tissue (Wang et al., 2003). For the DA receptor agonist injections, mice were grouped into three groups ($n = 3$ mice in each group), and mice in each group received SKF81297 at 3.0 mg/kg, PD128907 at 1.5 mg/kg, and both SKF81297 (3.0 mg/kg) and PD128907 (1.5 mg/kg), respectively, as described (Xu et al., 1997). Approximately equal numbers of male and female mice were used for each genotype.

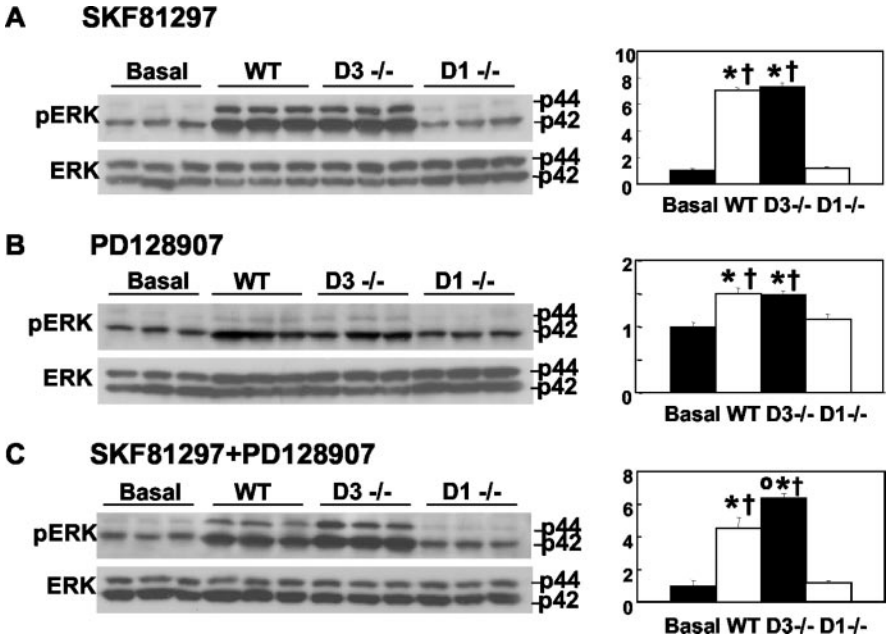

Figure 2. Effects of D1 and D2 class receptor agonists on ERK activation in the CPu in D1, D3 receptor mutant, and wild-type mice. D1 (D1−/−), D3 (D3−/−) receptor mutant, and wild-type (WT) mice ($n = 3$ each) received injections of SKF81297 (*A*), PD128907 (*B*), or both (*C*). Western blot analyses were performed using CPu extracts prepared 20 min after the injections. Antibodies against dually phosphorylated ERK (pERK) or total ERK were used. Data represent the mean + SEM pERK levels over the WT basal levels ($n = 3$ mice each). $^{*}p < 0.05$ treated groups versus WT basal levels. $^{\dagger}p < 0.05$ treated groups versus treated D1 receptor mutant mice. $^{\circ}p < 0.05$ treated D3 receptor mutant mice versus treated WT mice.

Protein extract preparation. The CPu and NAc tissues were isolated by gross dissection, and extracts were prepared from individual mouse brains as described (Zhang et al., 2002a). The samples were homogenized in 300 μl of buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 10 mM EGTA, 2 mM sodium pyrophosphate, 4 mM paranitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotintin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. Protein concentrations were determined by the Bradford method as before (Zhang et al., 2002a).

Western blotting. Twenty micrograms of total protein were separated by 10% SDS-PAGE for the ERK, JNK, p38, c-Fos, neogenin, and synaptotagmin VII detection as described (Zhang et al., 2002a). The resolved proteins were transferred onto polyvinylidene difluoride membranes, the blots were blocked in 5% nonfat dry milk, 10 mM Tris-HCl, pH 7.5, and 0.1% Tween 20 and were incubated in primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were visualized using enhanced chemiluminescence. Antibodies against ERK, JNK, p38 and phospho-ERK, phospho-JNK, phospho-p38 (Cell Signaling Technology, Beverly, MA) and c-Fos, neogenin and synaptotagmin VII (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution, and antibodies against actin (Santa Cruz Biotechnology) were used at the 1:3000 dilution. HRP–anti-rabbit conjugate (Santa Cruz Biotechnology) was used at 1:5000 dilutions for ERK, JNK, p38, c-Fos, and HRP–anti-goat conjugate (Santa Cruz Biotechnology) at 1:5000 dilutions for neogenin and synaptotagmin VII. Blocking peptides were used to verify the expression of the various forms of synaptotagmin VII. All Western blot analyses were performed at least three times, and parallel results were obtained.

Immunohistochemistry. For dynorphin immunostaining, four each D1, D3 receptor mutant, and wild-type mice were used 24 hr after the last chronic cocaine injection. Mice were anesthetized and perfused with PBS followed by 4% paraformaldehyde in PBS as before (Zhang et al., 2002b). The brains were removed and postfixed in 4% paraformaldehyde for 2 hr and were cryoprotected in 20% sucrose overnight. Freshly frozen coronal sections (25 μm) were cut using a cryostat. Free-floating sections were blocked with 0.4% Triton X-100 and 0.1% BSA in PBS for 1 hr and were incubated at 4°C overnight in PBS, 4% Triton X-100 and 0.1% BSA containing a polyclonal antibody against Dynorphin B1–13 (Serotec, Oxford, UK) at 1:100 dilution. Sections were then incubated for 1 hr at room temperature with a biotin-conjugated secondary antibody. Then, sections were incubated for 1 hr in an avidin–biotin-peroxidase complex (ABC) solution (Vector Laboratories, Burlingame, CA). The sections were subjected to DAB and Nickel staining. The experiment was repeated three times using multiple brain sections.

For immunocolocalization of phospho-ERK and dynorphin, four wild-type mice were perfused 20 min after an acute cocaine treatment at the 30 mg/kg dose. We performed two sets of controls to confirm the specificity of double immunostaining. First, omission of the primary antibodies resulted in no staining, suggesting that the secondary antibodies lack nonspecific binding to sections under the conditions used. Second, omission of one of the two primary antibodies abolished staining of the omitted antibody without affecting the other, indicating that the secondary did not show any significant cross-reactivity and labeling one antibody did not affect labeling the other.

Brain sections were then incubated in a mixture of two antibodies: one raised in mouse recognizing the amino acid sequence containing phospho-Tyr-204 of ERK (1:100 dilution; Santa Cruz Biotechnology) and the other raised in rabbit recognizing the Dynorphin B1–13 (1:100 dilution; Serotec) overnight at 4°C (Moratalla et al., 1996b). The sections were then incubated with Alexa Fluor 488-conjugated (1:250 dilution; Molecular Probes, Eugene, OR) and TRITC-conjugated secondary antibodies to visualize cells immunopositive for both phospho-ERK and dynorphin. Immunofluorescent images of each section were captured by Spotcam program. The experiment was repeated at least three times using multiple brain sections.

RNA isolation and microarray analyses. Six each D1, D3 receptor mutant, and wild-type mice were decapitated 24 hr after the last chronic

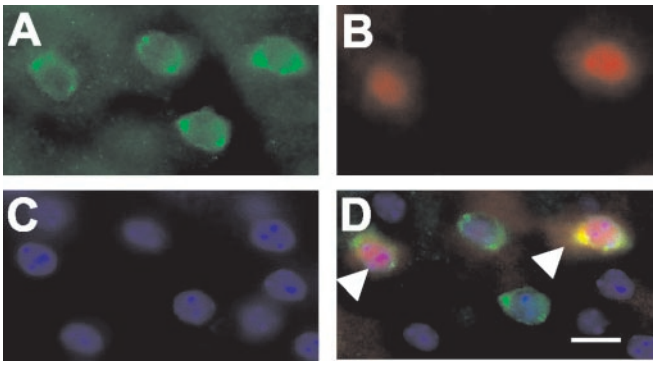

Figure 3. Phospho-ERK is induced by acute cocaine administration mostly in dynorphin-expressing neurons in the NAc in wild-type mice. Staining for dynorphin (*A*), phospho-ERK (*B*), and nuclear DNA (*C*, DAPI) show that phospho-ERK is expressed in dynorphin-expressing neurons (*D*). Double-labeled cells are indicated by white arrows. The results are representative of results of multiple sections from four wild-type mice. Sections were obtained 20 min after an intraperitoneal cocaine treatment. Scale bar, 10 μm.

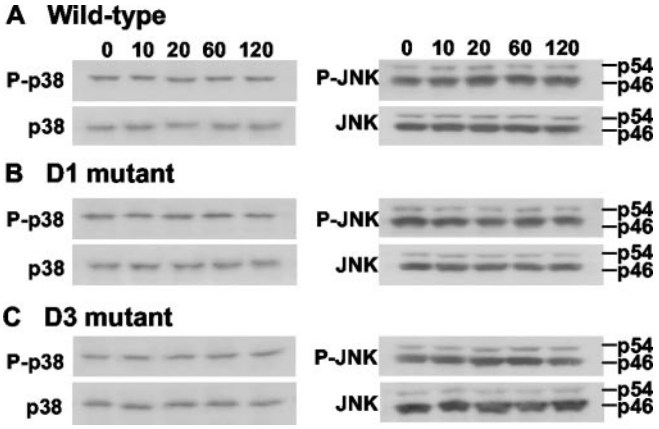

Figure 4. p38 and JNK phosphorylation in the CPu in D1, D3 receptor mutant, and wild-type mice is not obviously changed after acute cocaine treatment. Time course of cocaine-induced phosphorylation of p38 and JNK in the CPu in wild-type (*A*), D1 (*B*), and D3 receptor mutant (*C*) mice. The phosphorylation status of p38 and JNK was determined 10, 20, 60, and 120 min after a cocaine injection (30 mg/kg, i.p.). Total cell extracts were analyzed by Western blotting using antibodies against dually phosphorylated-p38 (Thr ^{180} and Tyr ^{182}), dually phosphorylated-JNK (Thr ^{183} and Tyr ^{185}), total p38, or JNK, respectively. The same set of mice used in probing ERK activation was used in analyzing p38 and JNK activation after cocaine injections.

cocaine or saline injection as described (Zhang et al., 2002a). Total RNA was isolated with TRIZOL Reagent (Invitrogen, Gaithersburg, MD) according to manufacturer's protocol. After extraction with chloroform, RNA was precipitated by isopropyl alcohol. The RNA precipitate was washed with 70% ethanol and dissolved in DEPC-treated water. Both the quality and quantity of the total RNA were examined by gel electrophoresis and by an Agilent 2001 Bioanalyzer (Agilent).

Sample labeling, microarray hybridization, and image scanning were all performed according to the Affymetrix Expression Analysis Technical Manual at the Microarray Core in the Children's Hospital Research Foundation in Cincinnati. Ten micrograms of total RNA was used to synthesize cDNA that were subsequently used as templates to generate cRNA. Biotinylated cRNA was fragmented and hybridized to Affymetrix mouse U74Av2 genechips that contain probe sets for >12,000 genes and expressed sequence tags. The genechips were washed and scanned with a laser scanner (Agilent). The Affymetrix GENECHIP software MAS 5.0 was used to calculate the raw expression value of each gene from the scanned image. Samples were excluded if the ratio was >2, or if there were visible defects on the arrays, or if the hybridization was much weaker or stronger than other arrays. Hybridizations were performed twice using RNA isolated from independent mice.

A linear scaling procedure was performed so that signal intensities for all genes on an array are multiplied by a scaling factor that makes the average intensity value for each array equal to a preset value of 1500 (Tang et al., 2002). This procedure scaled the average intensity of all the arrays to the same level and made the comparison among different samples possible.

Quantification. For phosphoproteins, after Western blots, membranes were stripped and reprobed with antibodies against total ERK, total JNK, and total p38. X-ray films were scanned. For each sample, the intensity of phospho-ERK (both bands), phospho-JNK, and phospho-p38 bands was divided by the intensity of total ERK, total JNK, and total p38 bands, respectively. The Metamorph software was used. For c-Fos, neogenin, and synaptotagmin VII, Western blotting bands for each sample from different mice were scanned, and the density of each signal per fixed band area was also quantified using Metamorph program. Actin expression was used as an internal control for protein expression at different time points. The expression level for each protein is an average of densities per band area from different mice and the number of Western blots performed for each protein. A two-way ANOVA was used to compare the expression of various genes under different treatment conditions between wild-type, D1, D3 receptor mutant, or DC mice, and between saline- and cocaine-treated mice within each genotype. In all cases, significant levels were set at $p < 0.05$.

Results

ERK activation in the CPu in response to acute cocaine administration is mediated by the D1 receptors and inhibited by the D3 receptors

Previous studies demonstrated that cocaine could induce ERK activation in the striatum (Valjent et al., 2000). To determine the role of DA receptors in acute cocaine-induced MAPK activation and gene expression, we evaluated how the D1 and D3 receptors are involved in the activation of ERK1 (44 kDa) and ERK2 (42 kDa) after acute cocaine administration. We prepared extracts from the CPu from the D1 and D3 receptor mutant mice and wild-type control littermates at different time points after cocaine injections. We then performed Western blot analyses of ERK activation using anti-phospho-ERK1/2 antibodies that recognize only the activated forms of ERK1/2. As shown in Figure 1, *A* and *C*, cocaine produced a similar time course in ERK phosphorylation in wild-type and D3 receptor mutant mice with a maximal effect observed 20 min after the cocaine injection compared with that in saline-treated control mice. Interestingly, the degree of ERK activation was higher in the CPu in D3 receptor mutant mice than in wild-type mice (Fig. 1*D*) ($^*p < 0.05$). Specifically, the level of activated ERK is 1.5 fold higher in the CPu in D3 receptor mutant mice than that in wild-type mice 20 min after the cocaine injection, indicating that ERK activation is enhanced in D3 receptor mutant than in wild-type mice. The levels of phospho-ERK returned to baselines 120 min after the cocaine injection in the CPu in both wild-type and D3 receptor mutant mice (Fig. 1*A*,*C*). In contrast to that in wild-type and D3 receptor mutant mice, ERK activation was completely abolished in D1 receptor mutant mice (Fig. 1*B*). Basal phosphorylation levels of ERK are similar in wild-type, D1, and D3 receptor mutant mice ($n = 2$–3 mice each, data not shown). These results indicate that the D1 and D3 receptors are oppositely involved in the acute cocaine-induced ERK activation in the CPu.

The D3 receptor can modulate D1 and D2 class receptor interactions at the level of ERK activation in the CPu

We previously reported that the D3 receptor mutation causes enhanced locomotor activation in response to a combined stim-

ulation by the D1 and D2 class agonists (Xu et al., 1997). To determine the molecular mechanism of how D3 receptors modulate D1 and D2 class receptor interactions, we investigated how the D3 receptor modulates ERK activation in response to D1 and D2 costimulation. We injected D1, D3 receptor mutant, and wild-type mice with the D1 class agonist SKF81297, the D2 class agonist PD 128907, or both. We found that SKF81297 produced a marked increase in ERK phosphorylation that was identical in D3 receptor mutant and wild-type mice, but was totally ineffective in D1 receptor mutant mice (Fig. 2*A*). Similarly, PD128907 induced ERK activation equally well in wild-type and D3 receptor mutant mice but not in D1 receptor mutant mice (Fig. 2*B*). When the two agonists were coadministered, the ERK phosphorylation was also increased (Fig. 2*C*). Interestingly, similar to those observed after acute cocaine treatment, ERK activation was significantly higher in D3 receptor mutant mice than in wild-type when the two agonists were coadministered ($^{\circ}p < 0.05$). This finding indicates that during coactivation of the D1 and D2 class receptors, stimulation of D3 receptors causes a suppression of ERK activation.

ERK is activated by acute cocaine administration in D1 receptor-expressing neurons in the striatum

To determine whether cocaine-induced ERK activation occurs primarily in DA D1 receptor-expressing neurons, we analyzed the distribution of phospho-ERK and dynorphin in the striatum by double-immunostaining (Moratalla et al., 1996b). We evaluated the induction of phospho-ERK at the 20 min time point at which cocaine induces maximal ERK activation (Fig. 3*A*) and dynorphin expression (Fig. 3*B*). As shown in Figure 3, *C* and *D*, phospho-ERK immunofluorescence mostly colocalizes with that for dynorphin in the cytoplasm in the NAc and CPu (data not shown). Additionally, a subset of dynorphin-positive neurons did not have phospho-ERK immunoreactivity. These results indicate that ERK is activated in some but not all the dynorphin-positive neurons in wild-type mice and suggest that the activation of ERK is mostly in D1 receptor-expressing neurons in the striatum.

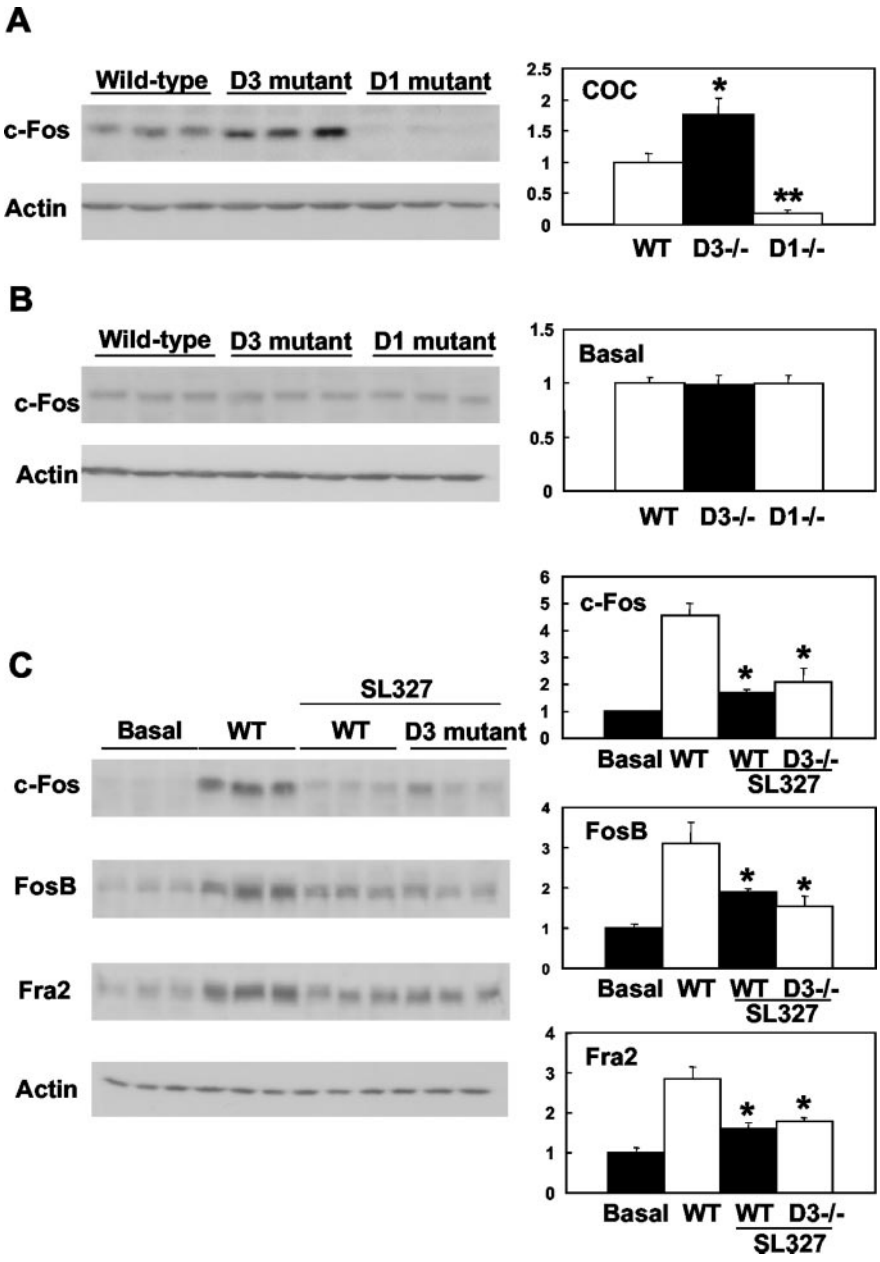

Figure 5. Opposite regulation of c-*fos* induction in the CPu by the D1 and D3 receptors after cocaine treatment is dependent on ERK activation. *A,* Opposite regulation of c-*fos* induction by cocaine in the CPu in D1 and D3 receptor mutant mice. D1 (D1−/−), D3 (D3−/−) receptor mutant, and wild-type (WT) mice ($n = 3$ mice each) were treated with cocaine at the 30 mg/kg dose for 2 hr. Protein extracts were isolated from the CPu of individual mouse, and Western blotting was performed. c-Fos levels in the wild-type mice were set at 1 for quantifications. *B*, The basal c-*fos* expression in the CPu of D1, D3 receptor mutant, and wild-type mice. c-Fos levels in the wild-type mice were set at 1 for quantitative comparisons. *C,* The induction of several Fos family proteins, including c-Fos, FosB, and Fra2, depends on ERK activation. Western blot analyses were performed in the absence or presence of SL327. SL327 was injected 15 min before cocaine treatment, and protein extracts from the CPu ($n = 4$ mice each) were isolated 2 hr after cocaine injections. Equal amounts of protein were loaded in each lane. Data represent mean + SEM of c-Fos, FosB, and Fra2 expression in the CPu of D3 receptor mutant and wild-type mice. Saline-injected WT levels were set at 1 for quantifications. Treated mice versus cocaine-treated WT mice $^{*}p < 0.05$; $^{**}p < 0.01$.

Cocaine-induced MAPK activation is specific for ERK

To determine whether cocaine-induced activation of MAPK is specific for ERK, we measured p38 and JNK activation in the CPu by an acute cocaine administration. We performed Western blot analyses of p38 and JNK using anti-phospho-p38 and anti-phospho-JNK antibodies that recognize only the activated forms of p38 and JNK. As shown in Figure 4*A–C*, there was no obvious p38 activation in the CPu in the three groups of mice within 2 hr of the acute cocaine injection. Similarly, JNK activation in the CPu could not be detected within the same time span after cocaine injection in the three groups of mice (Fig. 4*A–C*). Furthermore, basal phosphorylation levels of phospho-p38 and phospho-JNK were similar in wild-type, D1 and D3 receptor mutant mice ($n = 2$–3 mice each, data not shown), indicating that p38 and JNK activation by acute cocaine treatment is not obviously modulated by the D1 and D3 receptors.

The opposite regulation of ERK by the D1 and D3 receptors results in opposite regulation of c-*fos* induction by acute cocaine administration

c-Fos is induced by acute cocaine administration and ERK has been implicated in the c-Fos induction (Moratalla et al., 1996a; Valjent et al., 2000). We investigated whether the opposite regulatory roles of the D1 and D3 receptors on ERK activation extends into c-*fos* induction by acute cocaine administration. Similar to previous reports (Moratalla et al., 1996a; Drago et al., 1996; Zhang et al., 2002a), c-*fos* was not induced in the CPu in D1 receptor mutant mice compared with that in wild-type mice 2 hr after the cocaine injection (Fig. 5*A*). In contrast, c-Fos levels were increased by 1.7-fold in the CPu in D3 receptor mutant mice compared with that in wild-type mice after cocaine injections (Fig. 5*A*) (Carta et al., 2000). Basal c-Fos levels were similar in the CPu in wild-type, D1, and D3 receptor mutant mice (Fig. 5*B*). These results further suggest that the D1 and D3 receptors play opposite regulatory roles in c-Fos induction in the CPu by acute cocaine injections.

To determine whether the opposite regulation of the D1 and D3 receptors on c-Fos depends on the ERK pathway, we treated the mice with a MEK-selective inhibitor SL327 before the acute cocaine injections. SL327 inhibited the c-Fos induction by acute cocaine administration in the CPu in both wild-type and D3 receptor mutant mice (Fig. 5*C*). Our previous studies demonstrated that the D1 receptor is critical for mediating the induction of FosB and Fra2 by acute cocaine administration (Zhang et al., 2002a). We found that SL327 also attenuated the induction of FosB and Fra2 in the CPu in wild-type and D3 receptor mutant mice (Fig. 5*C*). These results indicate that the induction of several Fos family proteins by acute cocaine administration depends on the ERK signaling pathway.

Opposite regulation of target gene expression by the D1 and D3 receptors after chronic cocaine treatment

We previously showed that the D1 receptor is critical in mediating gene expression in the NAc and CPu after repeated exposure to cocaine (Zhang et al., 2002a). To investigate the role of D1 and D3 receptor on gene expression after chronic cocaine administration, we previously performed a systematic microarray analysis. We focused on the CPu where more robust cocaine-induced molecular changes occur (Berke et al., 1998). Analyses of the microarray results indicated that there are a number of potential cocaine-responsive genes in D1 and D3 receptor mutant mice that show opposite expression patterns compare to wild-type mice after repeated cocaine exposure (data not shown). These genes included those encoding dynorphin, neogenin, and synaptotagmin VII. We further verified their differential expression in the striatum after repeated cocaine administration.

Immunohistochemistry analysis showed that dynorphin levels were decreased in the CPu and NAc in D1 receptor mutant mice and increased in the CPu and NAc in D3 receptor mutant mice compared with those in wild-type mice, respectively, after repeated cocaine treatment (Fig. 6*A–F*). In the cortex, dynorphin levels were similar in D1, D3 receptor mutant, and wild-type mice (Fig. 6*G–I*). Western blot analysis showed that neogenin expression was decreased by repeated cocaine treatment in both the NAc and CPu in wild-type mice (Fig. 7*A*). Moreover, neogenin expression was less reduced in D3 receptor mutant mice and further decreased in D1 receptor mutant mice compared with

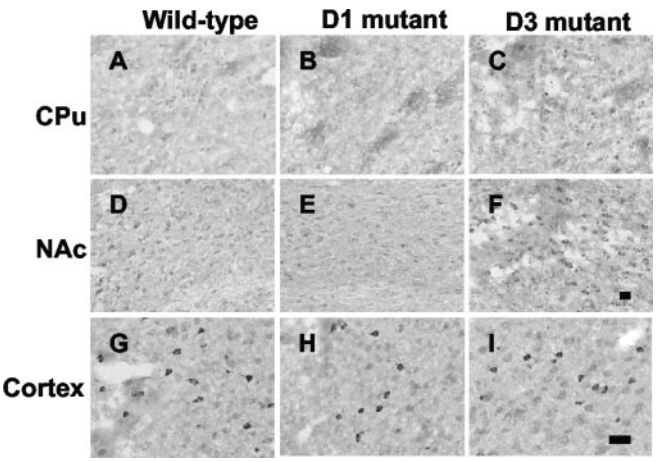

Figure 6. Dynorphin expression is oppositely regulated in the NAc and CPu of D1, D3 receptor mutant mice after repeated cocaine exposure. D1, D3 receptor mutant, and wild-type mice ($n = 4$ each) were treated with cocaine (20 mg/kg) twice daily for 7 consecutive days. Coronal sections from wild-type (*A, D, G*), D1 (*B, E, H*), and D3 receptor mutant (*C, F, I*) mice were stained with an anti-dynorphin antibody. Scale bar, 15 μm.

that in wild-type mice, respectively, after repeated cocaine treatments (Fig. 7*A*). Synaptotagmin VII was induced by repeated cocaine treatment in both the NAc and CPu in wild-type mice (Fig. 7*B*). There was no obvious induction in synaptotagmin VII expression in D1 receptor mutant mice and an increase in synaptotagmin VII expression in D3 receptor mutant mice, respectively, compared with that in wild-type mice in both the NAc and CPu after repeated cocaine administration (Fig. 7*B*). These results indicate that D1 and D3 receptors exert opposite regulation on the expression of certain target genes, including those encoding dynorphin, neogenin, and synaptotagmin VII, in the NAc and CPu after repeated cocaine administration.

The opposite regulation of gene expression by the D1 and D3 receptors after chronic cocaine injections depends on ERK activation and c-*fos* function

To determine whether neogenin and synaptotagmin VII expression is regulated by the ERK signaling pathway, we analyzed extracts prepared either from chronic cocaine-treated or from both SL327- and cocaine-treated wild-type mice. We found that, similar to those observed in the D1 receptor mutant mice, inhibition of ERK further reduced neogenin and synaptotagmin VII expression in the NAc and CPu after repeated cocaine injections (Fig. 8*A*). Interestingly, a mutation of the c-*fos* gene in D1 receptor-expressing neurons produced results that are similar to those that are caused by either the D1 receptor gene mutation or ERK inhibition after repeated cocaine injections (Fig. 8*B*). Detailed DNA sequence analyses indicate that there are AP-1 and CREB binding sites in the promoter regions of the genes encoding dynorphin, neogenin, and synaptotagmin VII (Fig. 9). Together, these results suggest that the opposite regulation of expression of these genes by the D1 and D3 receptors requires proper ERK activation and may be mediated by the AP-1 transcription complexes or CREB or both.

Discussion

Repeated cocaine administration can induce persistent changes in the brain and gene expression changes mediated by the DA receptors may contribute to the development of cocaine-induced neuroadaptations (Koob et al., 1998; White and Kalivas, 1998; Nestler, 2001; Hyman and Malenka, 2001; Laakso et al., 2002). Both the D1 and D3 receptors are expressed in the NAc and CPu,

brain areas that have been implicated in the development of drug-induced neuroadaptations (Civelli et al., 1993; Sibley et al., 1993; Missale et al., 1998; Ridray et al., 1998; Schwartz et al., 1998). Coexpression studies showed that a large percentage of D3 receptor-expressing neurons also contains the D1 receptor (Surmeier et al., 1996; Le Moine and Bloch, 1996; Schwartz et al., 1998; Ridray et al., 1998). Moreover, coexisting D1 and D3 receptors mediate both opposite and synergistic responses (Xu et al., 1994a, 1997; Ridray et al., 1998; Schwartz et al., 1998). The present study shows that the D1 and D3 receptors exert opposite regulation of target gene expression by regulating ERK activation and c-*fos* induction after acute and chronic cocaine treatment.

Opposite regulation in ERK activation and c-*fos* induction in the CPu by the D1 and D3 receptors after acute cocaine treatment

MAPKs, including ERK, p38, and JNK, mediate a variety of signal transduction events in response to environmental stimulation. MAPK activation has also been implicated in mediating the rewarding effects of abused drugs. An acute cocaine treatment can activate ERK in the striatum and blockade of ERK activation abolishes the rewarding effects of cocaine (Valjent et al., 2000). Chronic cocaine treatment leads to a sustained ERK activation in the ventral tegmental area (Berhow et al., 1996). In the current study, we found that acute cocaine administration produced a time-dependent increase in ERK phosphorylation in the CPu in wild-type mice, a result in agreement with previous studies (Valjent et al., 2000). Moreover, ERK activation was enhanced in the CPu in D3 receptor mutant mice while abolished in the CPu in D1 receptor mutant mice, respectively, compared with that in wild-type mice. These data suggest that the D1 and D3 receptors exert opposite regulation in ERK activation after acute exposure to cocaine. Our results also show that cocaine-induced MAPK activation is specific for ERK because neither p38 nor JNK was activated by acute cocaine administration.

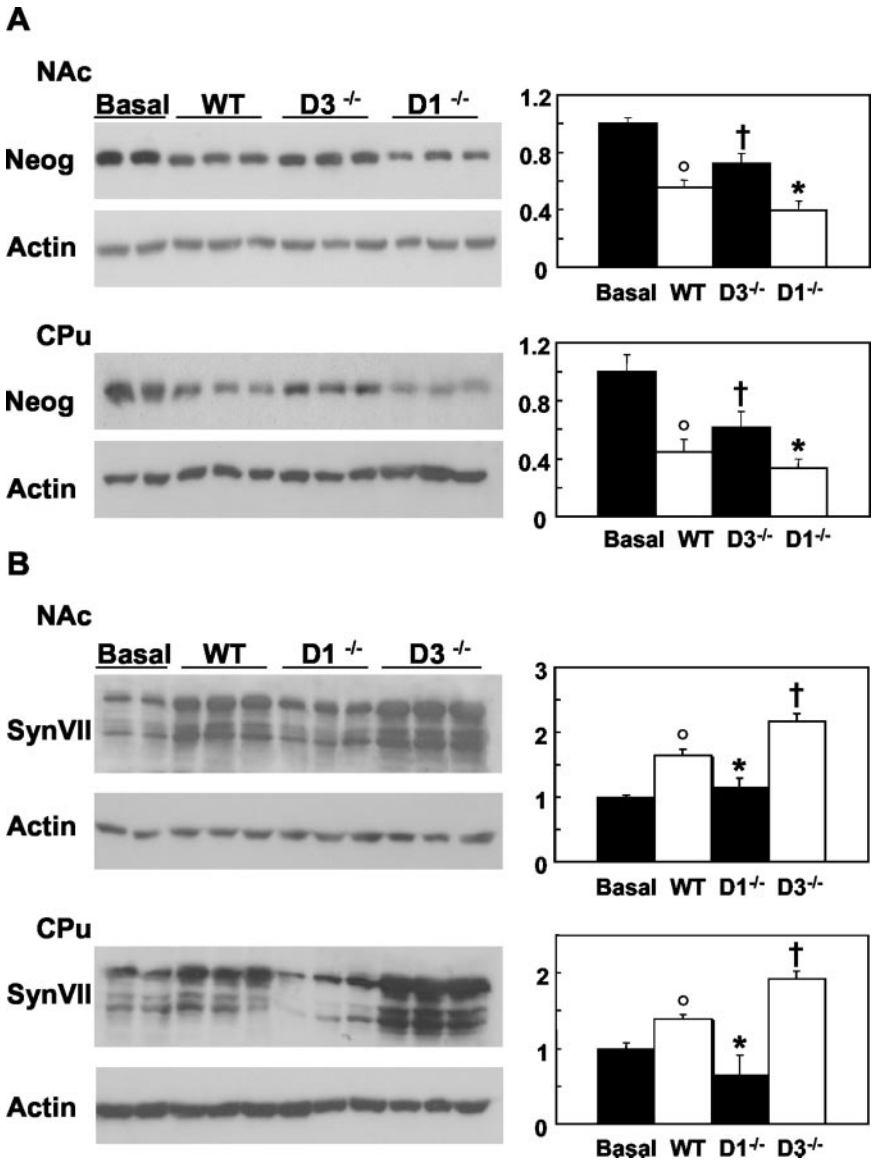

Figure 7. Neogenin and synaptotagmin VII are oppositely regulated in the NAc and CPu in D1 and D3 receptor mutant mice after repeated cocaine administration. D1 (D1−/−), D3 (D3−/−) receptor mutant, and wild-type (WT) mice were treated with 20 mg/kg of cocaine twice daily for 7 consecutive days or with saline ($n = 8$ mice each). Whole-cell extracts were isolated from both the NAc and CPu of individual mouse, and Western blotting was performed for neogenin (*A*) and synaptotagmin VII (*B*). Equal amounts of protein were loaded in each lane. Data represent mean + SEM of the expression of neogenin and synaptotagmin VII in the NAc and CPu of D1 and D3 receptor mutant and wild-type mice. Basal levels in WT mice were set at 1 for quantitative comparisons. °$p < 0.05$ treated WT mice versus WT basal levels. †$p < 0.05$ treated D3 receptor mutant mice versus treated WT mice. *$p < 0.05$ treated D1 receptor mutant mice versus treated WT mice.

We also found that c-Fos induction is abolished in the CPu in D1 receptor mutant mice, and c-Fos levels are increased in the CPu in D3 receptor mutant mice, respectively, compared with that in the wild-type mice after acute cocaine injections. Moreover, the selective MEK inhibitor SL327 can inhibit acute cocaine-induced Fos family proteins, including c-Fos, FosB, and Fra2. These results suggest that the D1 and D3 receptor exert opposite regulation on cocaine-induced c-Fos induction, and this regulation depends on the ERK signaling pathway.

Because the D1 and D3 receptors are coexpressed in neurons particularly in the NAc and also CPu, these receptors may mediate cocaine-induced persistent changes by coordinately regulating gene expression via the ERK signaling pathway in the same set of neurons. Indeed, we found that acute cocaine-induced ERK activation is mostly in D1 receptor-expressing neurons. The D1 and D3 receptors are positively and negatively linked to the intracellular cAMP production and protein kinase A (PKA) activation. PKA can activate ERK through the activation of the small G-protein Rap-1 and the expression of a serine–threonine kinase B-Raf (Vossler et al., 1997; Roberson et al., 1999). Once activated, ERK translocates to the nucleus, resulting eventually in the phosphorylation and activation of a series of transcription factors, including CREB and c-Fos (Adams and Sweatt, 2002). These transcription factors may start a variety of gene expression programs that may underlie long-term neuroadaptations in the brain.

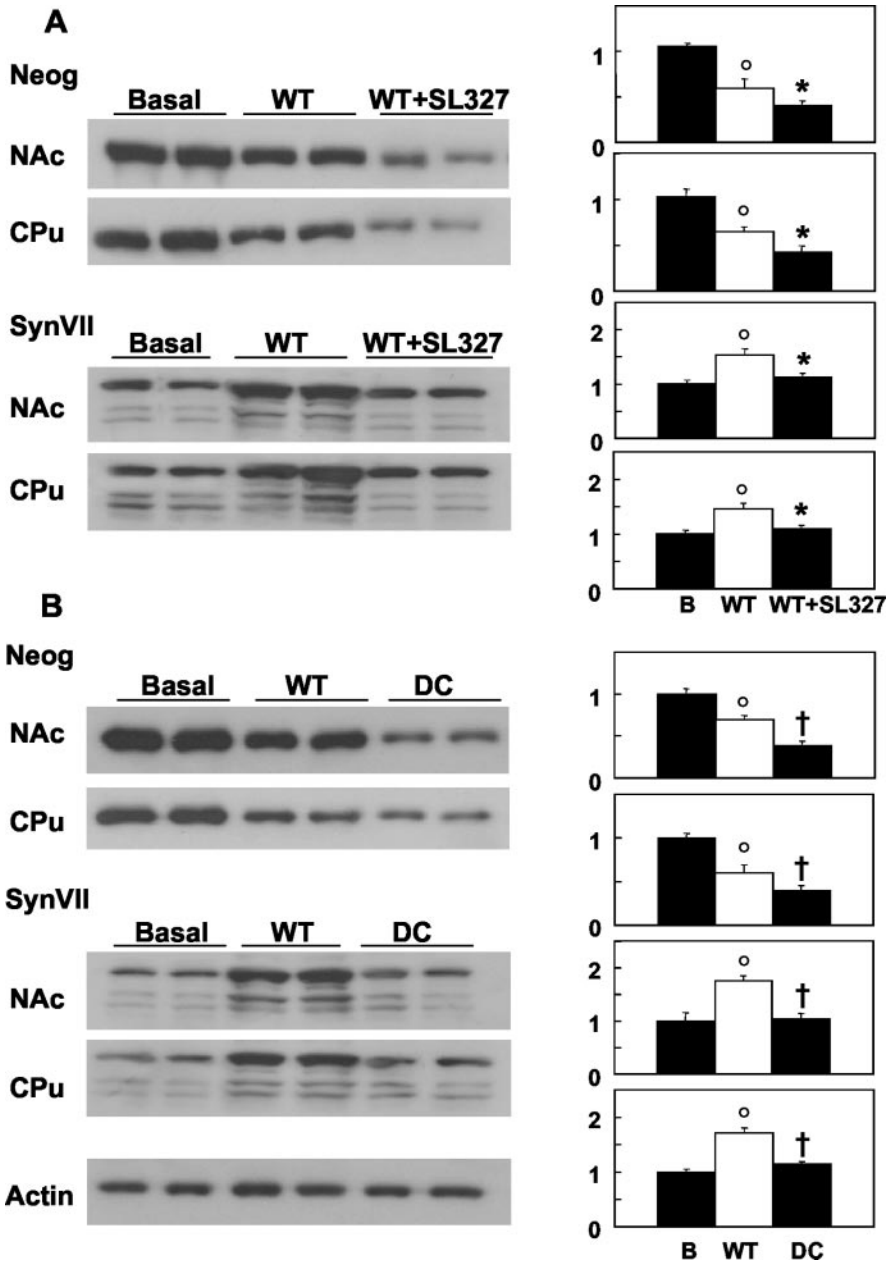

Figure 8. Changes in the expression of neogenin and synaptotagmin VII in the NAc and CPu of wild-type mice induced by repeated cocaine exposure depend on ERK activation and c-Fos function. *A,* Wild-type (WT) mice were treated with cocaine twice daily for 7 consecutive days in the absence or presence of SL327 or with saline ($n = 4$ mice each). SL327 were injected 15 min before cocaine treatment. Whole-cell extracts from the NAc and CPu were isolated, and Western blotting was performed for neogenin and synaptotagmin VII. *B,* Wild-type and D1 receptor-expressing neuron-specific c-*fos* mutant (DC) mice ($n = 4$ mice each) were treated with cocaine at the 20 mg/kg dose twice daily for 7 consecutive days or with saline ($n = 4$ mice each). Whole-cell extracts were isolated from both the NAc and CPu, and Western blotting was performed for neogenin and synaptotagmin VII. Equal amounts of protein were loaded in each lane. Results using extracts from two different mice are shown. Data represent the mean + SEM percent change over the basal levels in wild-type mice. B indicates basal levels. $^{\circ}p < 0.05$ cocaine-treated WT versus WT basal. $^{*}p < 0.05$ cocaine and SL327-treated WT versus cocaine-treated WT. $^{\dagger}p < 0.05$, cocaine-treated DC mice versus cocaine-treated WT mice.

cocaine exposure. To understand the molecular mechanisms of cocaine-induced persistent neuroadaptations, we investigated the role of D1 and D3 receptors in regulating gene expression after chronic exposure to cocaine. We found that three genes that encode dynorphin, neogenin, and synaptotagmin VII show opposite expression patterns in the NAc and CPu in D1 and D3 receptor mutant mice after repeated cocaine administration.

The neuropeptide dynorphin is believed to be involved in drug-induced neuroadaptations, and previous studies demonstrated that the level of dynorphin increased after either acute or repeated exposure to cocaine (Moratalla et al., 1996a; Willuhn et al., 2003; Fagergren et al., 2003). We and others previously also showed that dynorphin induction in the striatum depends on a functional D1 receptor (Xu et al., 1994a; Drago et al., 1996; Moratalla et al., 1996a). Similar to the previous studies, we found that dynorphin levels were decreased in the NAc and CPu in D1 receptor mutant mice compared with that in wild-type mice after repeated cocaine treatment. Interestingly, dynorphin levels were increased in the NAc and CPu in D3 receptor mutant mice compared with those in wild-type mice after repeated cocaine administration. CREB has been shown to bind to the prodynorphin promoter and regulate its expression (Fig. 9) (Konradi et al., 1994; Collins-Hicok et al., 1994; Cole et al., 1995). The D1 and D3 receptors might exert their opposite regulatory effects on dynorphin expression through opposite regulation of CREB after repeated exposure to cocaine.

Neogenin belongs to a distinct subfamily of NCAM-like cell surface receptors that is expressed during terminal neuronal differentiation and is also involved in neurite outgrowth (Vielmetter et al., 1994; Gad et al., 1997). Neogenin, together with its ligands, the netrins, is a major guidance system in neural cell migration (Keino-Masu et al., 1996; Keynes and Cook, 1996; Tessier-Lavigne and Goodman, 1996). We observed that neogenin expression was reduced by repeated cocaine treatment in both the NAc and CPu in wild-type mice and was further decreased in D1 receptor mutant mice and less reduced in D3 receptor mutant mice, respectively, compared with that in wild-type mice. These results indicate that the D1 and D3 receptors also oppositely regulate neogenin expression. Repeated administration of abused drugs, including cocaine, amphetamine, and morphine can cause alterations in dendritic morphology in specific brain regions, including the NAc (Robinson and Kolb, 1999; Robinson et al., 2002). These observations suggest that fundamental changes in the neu-

Opposite regulation in target gene expression by the D1 and D3 receptors after repeated cocaine administration depends on ERK activation and c-*fos* function

Repeated cocaine administration can induce persistent neuroadaptations that may contribute to an addicted state that is fundamentally different from the state after the acute or an occasional

ronal circuits including synaptic strength and connections occur after repeated exposure to abused drugs. Because of the guidance role of neogenin in neural cell migrations and neurite outgrowth, neogenin might participate in the alterations of dendritic morphology after repeated exposure to cocaine.

Synaptotagmins represent a family of 13 membrane-trafficking proteins with a common domain structure characterized by an N-terminal transmembrane region, a connecting sequence and two C-terminal C2 domains (Chapman, 2002; Sudhof, 2002). Synaptotagmins I and II are vesicular calcium sensors that are involved in synaptic vesicle exocytosis (Lin and Scheller, 2000; Chapman, 2002; Sudhof, 2002). Synaptotagmin VII is mainly located on the synaptic plasma membrane, and it functions as an active zone plasma membrane calcium sensor in exocytosis (Sudhof, 2002; Sugita et al., 2002). In agreement with previous studies, we found that the synaptotagmin VII antibody recognizes a range of proteins in the NAc and CPu, likely because synaptotagmin VII is expressed in multitude alternatively spliced forms (Sugita et al., 2001; Fukuda et al., 2002). After repeated cocaine treatment, synaptotagmin VII was induced in both the NAc and CPu in wild-type mice. Moreover, there was no obvious induction in synaptotagmin VII expression in both the NAc and CPu in D1 receptor mutant mice and an increase in D3 receptor mutant mice, respectively, compared with that in wild-type mice. These results suggest that the expression of synaptotagmin VII is oppositely regulated by the D1 and D3 receptors after repeated cocaine injections. Synaptotagmin VII is involved in regulating neurotransmitter release (Lin and Scheller, 2000; Sudhof, 2002; Chapman, 2002) and changes in its expression can change synaptic transmission and synaptic plasticity in the NAc and CPu after chronic exposure to cocaine.

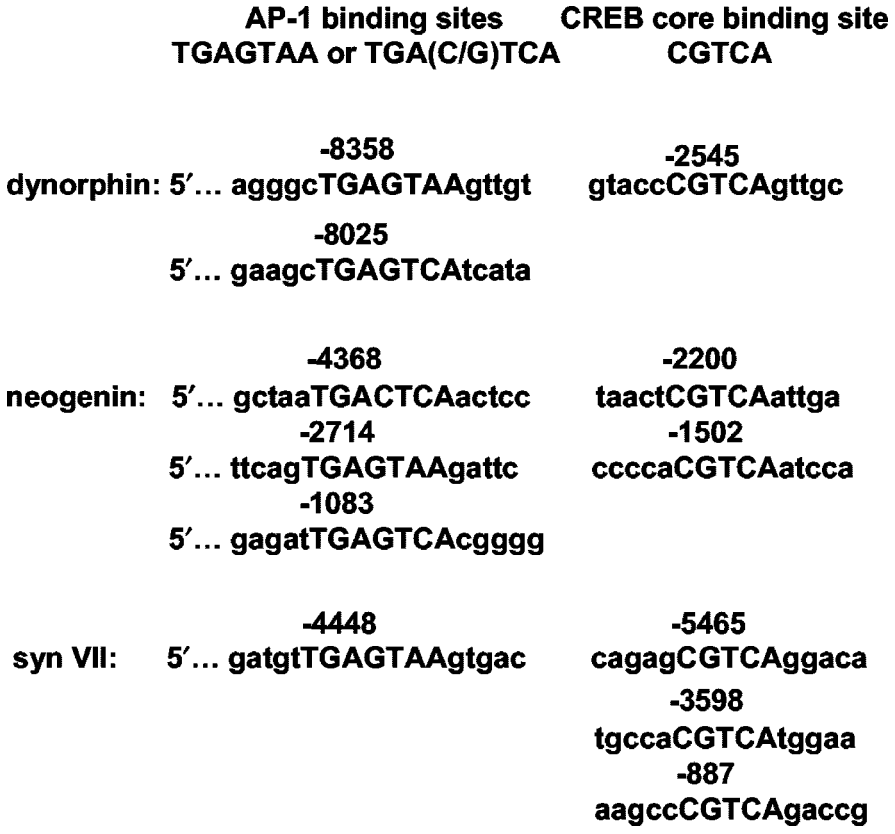

Figure 9. Promoter sequence comparisons of genes encoding dynorphin, neogenin, and synaptotagmin VII. Promoter sequences were obtained from GenBank. TGAGTAA and TGA(C/G)TCA were used for the AP-1 binding site, and CGTCA was used for the core CRE binding site, respectively, to search for sequences upstream from the transcription initiation sites of the three genes.

We found that the expression of neogenin and synaptotagmin VII in the NAc and CPu after repeated cocaine administration was influenced by proper ERK activation and a mutation of the c-*fos* gene in D1 receptor-expressing neurons. Interestingly, the requirement for ERK activation and c-*fos* function is similar to that of the D1 receptor. The promoter regions of these two genes contain AP-1 binding sites and CREB binding sites. These findings suggest that the opposite regulation of the two genes by the D1 and D3 receptors is mediated sequentially by ERK activation and then by the AP-1 transcription complexes or CREB or both.

The D3 receptor can modulate D1 and D2 class receptor interactions and gene expression at the level of ERK activation in the CPu

To determine the molecular mechanism of how D3 receptors modulate D1 and D2 class receptor interactions, we investigated how the D3 receptor modulates ERK activation in response to D1 and D2 class receptor costimulation. We found that D3 receptor mutant mice exhibited a higher ERK activation in the CPu than the wild-type mice when both D1 and D2 class receptors were costimulated either by cocaine or by a combination of selective D1 and D2 class receptor agonists but not when either class of receptors was activated alone. Moreover, SKF81297 produced a marked increase in ERK phosphorylation in D3 receptor mutant and wild-type mice, but was totally ineffective in D1 receptor mutant mice, suggesting the importance of the D1 receptor in ERK activation. In contrast, the D2 class receptor agonist PD128907 activated ERK equally well in wild-type and D3 receptor mutant mice, indicating that the D2 or D4 receptors, rather than the D3 receptors are directly involved in ERK activation. ERK activation by the D2 receptor agonist PD128907 may occur in D2 receptor-containing neurons or in both D1 and D2 receptor-containing neurons. Our current studies do no distinguish these possibilities.

Together, these results support a model in which the D3 receptors inhibit ERK activation and gene expression induced by combined stimulation of D1 and D2 class receptors. Cocaine can induce ERK activation by stimulating the D1 and D2 class receptors. Activated ERK, in turn, induces CREB phosphorylation and c-Fos expression that further regulate gene expression, including that of the dynorphin, synaptotagmin VII, and neogenin. These gene expression changes may contribute to cocaine-induced persistent neuroadaptations. Because a large percentage of the D1 and D3 receptors are coexpressed particularly in the NAc and also CPu, the inhibitory actions of the D3 receptors may occur in D1 receptor expression neurons.

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