Neuropsychopharmacology (2005) 30, 1443–1454

www.neuropsychopharmacology.org

Repeated Cocaine Administration Induces Gene Expression Changes through the Dopamine D1 Receptors

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Drug addiction involves compulsive drug-seeking and drug-taking despite known adverse consequences. The enduring nature of drug addiction suggests that repeated exposure to abused drugs leads to stable alterations that likely involve changes in gene expression in the brain. The dopamine D1 receptor has been shown to mediate the long-term behavioral effects of cocaine. To examine how the persistent behavioral effects of cocaine correlate with underlying changes in gene expression, we have used D1 receptor mutant and wild-type mice to identify chronic cocaine-induced gene expression changes mediated via the D1 receptors. We focused on the caudoputamen and nucleus accumbens, two key brain regions that mediate the long-term effects of cocaine. Our analyses demonstrate that repeated cocaine administration induces changes in the expression of 109 genes, including those encoding the stromal cell-derived factor 1, insulin-like growth factor binding protein 6, sigma 1 receptor, regulators of G-protein signaling protein 4, Wnt1 responsive Cdc42 homolog, Ca^{2+}/calmodulin-dependent protein kinase II α subunit, and cyclin D2, via the D1 receptors. Moreover, the seven genes contain AP-1 binding sites in their promoter regions. These results suggest that genes encoding certain extracellular factors, membrane receptors and modulators, and intracellular signaling molecules, among others, are regulated by cocaine via the D1 receptor, and these AP-1 transcription complex-regulated genes might contribute to persistent cocaine-induced behavioral changes.
Neuropsychopharmacology (2005) **30,** 1443–1454, advance online publication, 16 March 2005; doi:10.1038/sj.npp.1300680

Keywords: chronic cocaine; dopamine D1 receptor; gene expression; AP-1 transcription complex

INTRODUCTION

Drug addiction is characterized by the compulsive seeking and taking of drugs despite adverse consequences (Leshner, 1997). A cardinal feature of this brain disease is that it is long-lasting (Koob *et al*, 1998; White and Kalivas, 1998; Berke and Hyman, 2000; Nestler, 2001; Hyman and Malenka, 2001). The neurobiological basis underlying the effects of cocaine is associated with the brain dopaminergic system that consists of dopamine (DA) neurons originating in the midbrain region and projecting to multiple brain areas including the nucleus accumbens (NAc) and caudoputamen (CPu; Koob, 1992). Abused drugs can increase synaptic levels of DA that is involved in reward and reinforcement (Ritz *et al*, 1987; Koob, 1992; White and Kalivas, 1998; Hyman and Malenka, 2001; Nestler, 2001; Kelley and Berridge, 2002; Everitt and Wolf, 2002).

The DA D1 receptor is expressed in multiple brain regions, including the CPu and NAc, areas that mediate the effects of cocaine (Civelli *et al*, 1993; Gingrich and Caron, 1993; Sibley *et al*, 1993; Missale *et al*, 1998). The D1 receptor interacts with G_s proteins, and stimulation of the receptor leads to increased intracellular levels of cAMP, resulting in the activation of a transcription factor, the cAMP-response element binding protein (CREB; Civelli *et al*, 1993; Gingrich and Caron, 1993; Sibley *et al*, 1993; Missale *et al*, 1998). Activated CREB can regulate the expression of cellular genes including the immediate-early gene (IEG) c-*fos* (Nestler, 2000, 2001). The D1 receptor plays a critical role in mediating the behavioral and cellular effects of cocaine. For example, D1 receptor agonists and antagonists can affect cocaine-induced locomotor responses, discriminative stimulus, reinforcing effects, and reinstatement in a variety of experimental animal systems (Cabib *et al*, 1991; Caine and Koob, 1994; Tella, 1994; Cervo and Samanin, 1995; Pruitt *et al*, 1995; Self *et al*, 1996; Grech *et al*, 1996; Baker *et al*, 1998; Tzschentke, 1998; Caine *et al*, 1999, 2000; Katz *et al*, 1999; Khroyan *et al*, 2000; Anderson *et al*, 2003; Nazarian *et al*, 2004). Repeated cocaine injections also lead to persistent increases in D1 receptor sensitivity within the

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Received 9 September 2004; revised 10 December 2004; accepted 15 December 2004
Online publication: 4 January 2005 at http://www.acnp.org/citations/Npp01040504011/default.pdf

NAc (Henry and White, 1995). We have used the D1 receptor mutant mice and showed that this receptor mediates the locomotor sensitization and rewarding effects of cocaine (Xu *et al*, 1994a, b, 2000; but see Miner *et al*, 1995).

The persistent nature of drug addiction suggests that repeated exposure to abused drugs leads to enduring alterations that likely involve gene expression changes in the brain (Koob *et al*, 1998; White and Kalivas, 1998; Berke and Hyman, 2000; Nestler 2000, 2001; Hyman and Malenka, 2001). Changes in gene expression through the D1 receptors may contribute to neuroadaptations to chronic exposure to cocaine (Graybiel *et al*, 1990; Koob *et al*, 1998; White and Kalivas, 1998; Berke and Hyman, 2000; Nestler, 2000, 2001; Hyman and Malenka, 2001). Much evidence suggests the involvement of the Fos family proteins and CREB in mediating the effects of cocaine. For example, acute cocaine injections induce c-Fos and CREB expression via the D1 receptors, and the expression returns to the baseline hours later (Graybiel *et al*, 1990; Moratalla *et al*, 1996; Zhang *et al*, 2002, 2004). Repeated cocaine administration induces relatively long-lasting AP-1 transcription complexes consisting of ΔFosB also via the D1 receptors (Nestler *et al*, 1999; Zhang *et al*, 2002). Persistent ΔFosB expression in D1 receptor-producing neurons in the NAc in mice can increase the locomotor-stimulating and rewarding effects of cocaine (Kelz *et al*, 1999; Colby *et al*, 2003). Increased expression of CREB is associated with a decrease in the rewarding effects of cocaine (Carlezon *et al*, 1998). These observations imply that Fos family protein- and CREB-regulated gene expression may contribute to neuroadaptations to repeated exposure to cocaine.

Drug addiction involves neuroadaptations that likely include complex changes in gene expression. The microarray method allows high-throughput analysis of gene expression patterns (Khan *et al*, 1999; Lipshutz *et al*, 1999), and it is particularly useful for studying cocaine-induced gene expression changes (Bibb *et al*, 2001; Freeman *et al*, 2001a, b; Toda *et al*, 2002; McClung and Nestler, 2003; Yuferov *et al*, 2003; Tang *et al*, 2003; Yao *et al*, 2004; Albertson *et al*, 2004). Despite these studies, the gene expression profile induced by chronic cocaine through the DA D1 receptors remains unclear. To address this issue, we have used the Affymetrix oligonucleotide genechips to identify gene expression differences in D1 receptor mutant and wild-type mice that exhibit differences in chronic cocaine-induced behavioral changes 24 h after cocaine withdrawal. We then focused on confirming the differential expression of selected candidate genes in the two groups of mice. Our analyses demonstrate that repeated cocaine administration induces changes in the expression of several groups of cellular genes that contain AP-1 binding sites in their promoter regions, suggesting that these AP-1 transcription complex-regulated genes in D1 receptor-producing neurons contribute to persistent cocaine-induced behavioral changes.

MATERIALS AND METHODS

Mice

The D1 receptor mutant mice were previously generated and have been back-crossed from the initial C57BL/6Jx129Sv genetic background with wild-type C57BL/6J mice for three generations (Xu *et al*, 1994a). Mutant and wild-type littermates were derived from heterozygous breeding and were genotyped by Southern blotting (Xu *et al*, 1994a). All mice were housed in a room on a 12 h light/dark cycle with food and water available *ad libitum*. The temperature and humidity of the room were controlled.

Drug

Cocaine hydrochloride and SCH23390 (Sigma, St Louis, MO) were dissolved in saline. Cocaine was used at 30 mg/kg for acute injections or at 20 mg/kg for repeated injections. SCH23390 was used at 0.5 mg/kg. Saline was injected in equal volumes as 0 h controls. All injections were administered intraperitoneally (i.p.) in 1 ml/0.1 kg body weight volumes during the light phase of the light/dark cycle (Xu *et al*, 2000).

Treatment Paradigms

Three treatment paradigms were used in the current study. The chronic injection paradigm, which was described previously (Xu *et al*, 2000; Zhang *et al*, 2002), was used to identify and verify repeated cocaine-induced and D1 receptor-mediated gene expression changes. D1 receptor mutant and wild-type mice 6–10 weeks of age (mean age was 7.8 weeks) were injected twice daily i.p. at 1100 and 1600 for 7 days with either 20 mg/kg of cocaine or saline as 0 h controls. While cocaine-treated wild-type mice exhibited increased locomotor stimulation during the injection time window, D1 receptor mutant mice failed to do so (Xu *et al*, 2000; Zhang *et al*, 2002). The use of the 20 mg/kg dose of cocaine avoided lethality of mice, which occurred when 30 mg/kg of cocaine was used. In the acute injection paradigm, D1 receptor mutant and wild-type mice were given one cocaine injection at the 30 mg/kg dose or saline as 0 h controls to compare acute cocaine-induced gene expression changes in the two groups of mice. The D1 receptor antagonist paradigm was used to compare the effects of D1 receptor gene mutation with pharmacological blockade of the D1 receptor on cocaine-induced gene expression changes. Four groups of wild-type mice were given two injections twice daily as follows: saline + saline, SCH23390 + saline, SCH23390 + cocaine, or saline + cocaine. SCH23390 or saline was administered 30 min before cocaine or saline injections. Unless indicated, identical numbers of male and female mice from each genotype were used for each condition.

RNA Isolation

To identify chronic cocaine-induced gene expression changes shortly after withdrawal, we focused on the CPu where robust cocaine-induced molecular changes occur (Berke *et al*, 1998). To avoid identifying acute cocaine-induced genes, such as the IEGs (Zhang *et al*, 2002, 2004), and to reduce noise including stress-induced genes, we isolated total RNA from the treated mice 24 h after the last injection. We used saline-treated D1 receptor mutant mice and wild-type mice to control for baseline differences.

For microarray hybridizations, chronic cocaine- and saline-treated mice ($n=12$ each) were decapitated and CPu tissues were dissected and homogenized individually in TRIZOL (Life Technology, Rockville, MD). After the extraction, total RNA was precipitated by isopropyl alcohol, washed with 70% ethanol, and dissolved in water. OD readings at 260 and 280 nm were determined and the ratio was between 1.9 and 2.1. The quality of the total RNA was further examined by gel electrophoresis and by Agilent 2001 Bioanalyzer (Agilent, Palo Alto, CA), and the two methods showed clear bands and peaks, respectively, indicating that no degradation occurred during isolation.

For quantitative real-time polymerase chain reactions (RT real-time PCR), both the chronic and acute injection paradigms were used. At 2 h after either acute cocaine or saline injections, or 24 h after the last chronic cocaine or saline injection, intact brains ($n=4$ each at each time point) were removed and the CPu tissues were isolated. Total RNA was isolated with TRIZOL reagent, precipitated, and subjected to further purification using a DNA free kit (Ambion, Austin, TX). The quality and quantity of extracted total RNA samples were examined by using a spectrophotometer and gel electrophoresis, respectively, as described previously.

Microarray Processing and Data Analysis

Sample labeling, microarray hybridization, and image scanning were carried out according to the Affymetrix Expression Analysis Technical Manual. A 10 μg portion of total RNA from individual mice was used to synthesize cDNAs that were used as templates to generate biotinylated cRNA. cRNA was fragmented and hybridized to Affymetrix mouse U74Av2 genechips that contain probe sets for more than 12 000 genes and ESTs. The genechips were washed and scanned with a laser scanner (Agilent, Palo Alto, CA). The Affymetrix GENECHIP software (MAS 5.0) was used to calculate the raw expression value of each gene from the scanned image. RNA quality was further examined by the 3′ to 5′ ratios for actin and glyceraldehyde-3-phosphate dehydrogenase. Samples were excluded if the ratio was greater than 2, or if there were visible defects on the arrays, or if the hybridization was much weaker or stronger than most other arrays. Hybridizations were performed three times for each of the four treatment conditions using RNA isolated from different 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. This procedure scaled the average intensity of all the arrays to the same level and made the comparison among different samples possible. The two-way ANOVA tool of GeneSpring 6.0 was used to identify genes that are differentially regulated among four groups of mice: saline-treated D1 receptor mutant mice, chronic cocaine-treated D1 receptor mutant mice, saline-treated wild-type mice, and chronic cocaine-treated wild-type mice, with treatment (cocaine or saline) as one parameter and genotype (D1 receptor mutant or wild type) as the second parameter. Using this test, we identified genes that are differentially expressed due to the genotype or cocaine treatment and also determined if there is any interaction between the two parameters. $p<0.05$ was considered as significant. Based on this criterion, we used a cutoff of at least a 1.2-fold difference in expression for further studies.

Quantitative Real-Time PCR

RETROscript™ (Ambion, Austin, TX) was used to reverse transcribe RNA to cDNA. A 2 μg portion of total RNA was mixed with oligo(dT) or random decamers (5 μM), and denatured. The cDNA synthesis was carried out at 42°C for 1 h using 100 U of Moloney murine leukemia reverse transcriptase, 10 U of RNase inhibitor, and 500 μM of dNTPs in a 20 μl volume (Vincent *et al*, 2002). Quantitative real-time PCR was performed using the LightCycler-DNA Master SYBR Green I kit (Roche, Mannheim, Germany). Primers were designed using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA) for genes encoding the D1 receptor, stromal cell-derived factor 1 (SDF1), insulin-like growth factor binding protein 6 (IGFBP6), sigma 1 receptor, regulators of G-protein signaling protein 4 (RGS4), Wnt1 responsive Cdc42 homolog (Wrch1), Ca^{2+}/calmodulin-dependent protein kinase II α subunit (CaMKIIα), and β-actin. Primer sequences are listed in Table 1 as forward (F) and reverse primers (R), and they were checked in the GenBank database (Bainbridge Island, WA) to avoid possible sequence homologies with other sequences. Primer concentrations were optimized before use. PCR reactions contained 2.5 ng/μl of forward and reverse primers, 4 mM $MgCl_2$, and 1 μl cDNA from the 20 μl RT reactions in a total volume of 20 μl. Cycling conditions for detecting the D1 receptor, IGFBP6, sigma 1 receptor, CaMKIIα, and Wrch1 expression were 95°C for 150 s, followed by 40 cycles at 95°C for 10 s, 58°C for 15 s, and 72°C for 20 s. Cycling conditions for SDF1 and β-actin were identical to the above except that the reaction time at 72°C was 30 s. Relative starting mRNA template concentrations were calculated using the standard curve method and normalized against β-actin mRNA levels. The PCR products were resolved on 1.5% agarose gels to confirm the expected sizes of the products. We used a two-way ANOVA 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 (Zhang *et al*, 2002).

Protein Extracts Preparation

Mice were decapitated and brains were removed after either acute or chronic cocaine or saline injections. The CPu and NAc tissues were isolated by gross dissection. For the CPu, extracts were prepared from individual mouse brains. For the NAc, tissues from 2–3 mice were pooled during extract isolation. 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 (Zhang *et al*, 2002). Homogenates were incubated on ice and centrifuged at 12 000*g* at 4°C, and protein concentrations were determined by the Bradford method as described (Zhang *et al*, 2002).

Table 1 Oligonucleotide Primers Used for Quantitative RT Real-Time PCR for the Indicated Genes

Gene	Acc. no.	Sequence 5′ to 3′	Position
SDF1	NM013655	F: GCATCAGTGACGGTAAACCAG	50
		R: GGGTCAATGCACACTTGTCTG	223
IGFBP6	NM008344	F: GCGGGGTCTACAGCCCTAAG	214
		R: CAGCAGAGGTCCGTGGATTC	413
Sigma 1 receptor	NM011014	F: ACGCCTCGCTGTCTGAGTAC	290
		R: CTCCAGAGCCGTTGCTTCTC	489
RGS4	BC003882	F: GAAATGGGCTGAATCGTTGG	170
		R: TCCGGCTTGTCTCCTCTCTG	398
Wrch1	XM125061	F: CTTGGTGTCTGGGGAGGATC	9
		R: TCTGGGTAGCTGCTCACACG	198
CaMKIIα	X14836	F: GCAGGTGTGTGAAGGTGCTG	83
		R: CCCCACCAGTAACCAGATCG	286
D1 receptor	mCT51047	F: GTGACACGAGGTTGAGCAGG	536
		R: GAGCATTCGACAGGGTTTCC	738

Acc. no. indicates GenBank accession numbers. F and R represent forward and reverse primers, respectively. The positions of the primers relative to the translational start sites of the cDNA are also indicated.

Western Blotting

Samples (20 μg) from each treatment condition were resolved on SDS–PAGE and transferred onto PVDF membranes (Zhang *et al*, 2002). The membranes were blocked with 5% nonfat dry milk in 10 mM PBS, and incubated in primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Immunoreactive signals were visualized using enhanced chemiluminescence. For each protein analyzed, Western blots were repeated at least three times. We used primary antibodies against IGFBP6 (1 : 1000), RGS4 (1 : 1000), cyclin D2 (CD2, 1 : 1000), and actin (1 : 3000) (Santa Cruz Biotechnology, Santa Cruz, CA). We also used c-Fos and ΔFosB as controls for the sensitivity of detecting protein expression (Zhang *et al*, 2002). Blocking peptides were used to verify the specificities of the antibodies.

We scanned in Western blot results for each sample from different mice. The relative band intensity is defined as densities subtracted by background for each signal and was quantified using Metamorph program as described (Zhang *et al*, 2002). Equal amounts of protein were used for expression comparisons of each individual protein at the 0 and 2 h time points. Thus, the expression level for each protein can be compared with one another at these time points. Equal amounts of protein were also used for the 7-day time point. A two-way ANOVA 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 (Zhang *et al*, 2002).

Immunohistochemistry

We processed D1 receptor mutant and wild-type mice ($n = 6$ each) 24 h after the last chronic cocaine and saline injection as described (Zhang *et al*, 2002). Mice were anesthetized with Nembutal and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS (pH 7.4). Brains were then fixed in 4% paraformaldehyde for 2 h and in 20% sucrose overnight (Zhang *et al*, 2002). Coronal sections were obtained on a cryostat at 25 μm. Free-floating sections were blocked with 0.4% Triton X-100 and 0.1% BSA in PBS for 1 h and were incubated with primary goat anti-IGFBP6 (1 : 100) and rat anti-CD2 (1 : 400) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit anti-leumorphin antibody (1 : 100, Serotec, Oxford, UK) at 4°C overnight. The brain sections were then incubated with biotin-conjugated secondary antibodies for 1 h at room temperature and were incubated in ABC reagent for 30 min (Vector Laboratories Inc., Burlingame, CA). The sections were processed in DAB and Nickel solutions. The immunostaining images were analyzed using a Metamorph program. The optical density of each cell body above the background level was measured and an average density for each mouse was calculated. A two-way ANOVA was used to compare the expression levels as before. Although the anti-RGS4 antibody detected a strong signal in Western blotting, it did not produce obvious immunostaining signals.

Immunocolocalization

For IGFBP6 and dynorphin, wild-type sections obtained for immunostaining were incubated with goat anti-IGFBP6 and rabbit anti-leumorphin antibodies either together or alone overnight. These sections were then incubated with Alexa Fluor 488-conjugated donkey anti-goat and Alexa Fluor 546-conjugated donkey anti-rabbit secondary antibodies. For CD2/dynorphin colocalization, after CD2 immunostaining with DAB using a rat anti-CD2 antibody, wild-type sections were incubated in 1% H_2O_2 in PBS for 10 min and overnight with a rabbit anti-leumorphin antibody. The sections were then incubated with biotin-conjugated anti-rat and anti-rabbit secondary antibodies for 1 h at room temperature, and incubated for 30 min in ABC reagent, and

in DAB-Nickel solution (Zhang *et al*, 2002). CPu and NAc immunostaining images of brain sections were captured by SpotCam.

Promoter Sequence Comparisons

DNA sequences from both Celera and GenBank were used for promoter sequence comparisons. Either TGAGTAA or TGA(C/G)TCA was used for the AP-1 binding site (Morgan and Curran, 1991; Cole *et al*, 1995; Logan *et al*, 1996), and TGACGTCA was used for the CREB binding site (Cole *et al*, 1995) to search for 10 kilobase pairs of sequences upstream of the transcription initiation sites of the genes.

RESULTS

A Total of 109 Genes are Differentially Expressed by at least 1.2-Fold in the CPu in D1 Receptor Mutant and Wild-Type Mice after Repeated Cocaine Administration

The goal of the current study is to gain insights into how the long-lasting behavioral effects of repeated cocaine administration correlate with underlying changes in gene expression through the DA D1 receptors. Repeated cocaine administration can induce persistent behavioral changes that may reflect drug-induced neuronal plasticity (Robinson and Berridge 1993; Kalivas *et al*, 1998; Vanderschuren and Kalivas, 2000), and the D1 receptor has been shown to be critical in mediating cocaine-induced long-lasting behavioral changes (Xu *et al*, 2000; Zhang *et al*, 2002).

We used the microarray method to identify chronic cocaine-induced gene expression differences in the two groups of mice 24 h after cocaine withdrawal. Statistical analysis of three independent hybridization experiments indicates that gene expression differences exist between cocaine-treated wild-type and D1 receptor mutant mice. Using a cutoff of at least a 1.2-fold difference in expression, we found that 109 genes exhibited, on the average, a significant difference in cocaine-induced expression ($p<0.05$), and at the same time no difference in saline-induced expression ($p>0.05$), between wild-type and D1 receptor mutant mice after 7 days of injections (Supplemental Table 1). These genes were either down- or upregulated in the CPu in D1 receptor mutant mice compared to that in wild-type mice by repeated cocaine exposure. Based on GeneOntology annotations and an extensive literature analysis, we found that these genes encode a wide range of molecules that can be organized into different functional groups, including extracellular factors, channels, membrane receptors and modulators, intracellular signaling molecules, gene expression modulators, structural proteins, and those with yet unidentified functions (Figure 1 and Supplemental Table 1). There were also 395 genes that exhibited at least a 1.2-fold differential expression after 7 days of saline treatment in wild-type and D1 receptor mutant mice, such as that encoding prodynorphin (Xu *et al*, 1994a; Moratalla *et al*, 1996; Supplemental Table 2). Whereas baseline expression difference of some genes between the D1 receptor mutant and wild-type mice may contribute to cocaine-induced differences, changes in many other genes may reflect compensatory changes produced by the D1 receptor gene mutation.

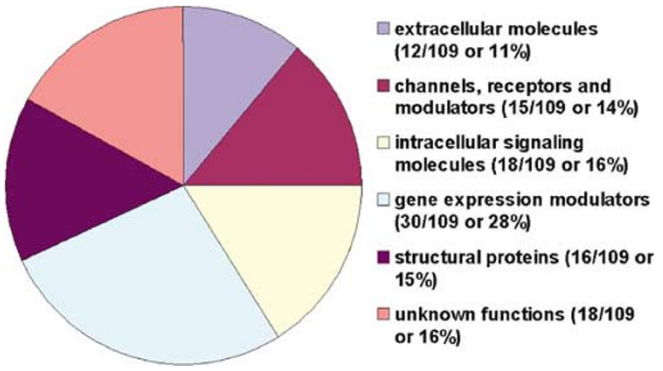

Figure 1 A total of 109 genes are differentially down- or upregulated by at least 1.2-fold in the CPu in D1 receptor mutant mice compared to wild-type mice by repeated cocaine administration. D1 receptor mutant and wild-type mice ($n=12$ each) were treated with 20 mg/kg of cocaine or saline for 7 consecutive days, and differences in gene expression in the CPu were compared using microarray genechips. Based on two-way ANOVA statistical and functional analyses, the differentially expressed genes were grouped into those encoding extracellular molecules, channels, receptors and modulators, intracellular signaling molecules, gene expression modulators, structural proteins, and proteins with unknown function.

Verifying Cocaine-Induced Gene Expression Difference by Quantitative RT Real-Time PCR

We are particularly interested in identifying chronic cocaine-induced long-lasting neuroplastic changes in the brain and selected two genes each from the extracellular factor, channel, membrane receptor and modulator as well as intracellular signaling molecule functional groups and used quantitative RT real-time PCR to verify their differential expression in the CPu in D1 receptor mutant and wild-type mice 24 h after cocaine withdrawal. These genes encode SDF1, IGFBP6, the sigma 1 receptor, RGS4, Wrch1, and CaMKIIα. SDF1 belongs to the CXC family of chemokines that are chemoattractant molecules (Baggiolini, 1998). Both SDF1 and its receptor, CXCR4, are expressed in the brain and are important for neuronal cell migration (Ma *et al*, 1998). IGFs are growth regulators of neurons and astrocytes, and IGFBPs are a family of at least six extracellular proteins that bind to IGFs with high affinities (Murphy, 1998; Zumkeller and Westphal, 2001). IGFBP6 binds IGF-II with high affinity and may inhibit IGF-II actions and both IGFBP6 and IGF-II are expressed in the central nervous system (Murphy, 1998; Zumkeller and Westphal, 2001). The sigma 1 receptor is associated with both plasma and intracellular membranes and is expressed in the CPu, NAc, and amygdala in the brain (Matsumoto *et al*, 2002; Romieu *et al*, 2003). This receptor can modulate cocaine-induced behaviors (Matsumoto *et al*, 2002; Romieu *et al*, 2003). RGS proteins are a family of diverse and multifunctional signaling proteins that modulate G-protein functions (Zhong and Neubig, 2001; Hollinger and Hepler, 2002). RGS4 is exclusively expressed in the brain (Zhong and Neubig, 2001; Hollinger and Hepler, 2002). Wrch1 is a homolog of the Rho family of GTPase that also shares 57% amino-acid sequence homology with Cdc42 (Tao *et al*, 2001). Wrch1 is expressed in the brain and its expression is increased in response to Wnt signaling, which plays important roles in cell proliferation and adhesion (Tao *et al*, 2001). CaMKII is a kinase with a broad spectrum of

substrates that coordinates a variety of neuronal functions such as gene expression, membrane excitability, and neuronal transmission (Lisman *et al*, 2002). Multiple isoforms of CaMKII exist and the α isoform is abundantly expressed in forebrain neurons (Lisman *et al*, 2002). Together, changes in the expression of these molecules can change the responsiveness of the D1 receptor-expressing neurons to further neuronal stimulation.

As identified by the Affymetrix genechips, SDF1, IGFBP6, the sigma 1 receptor, RGS4, and CaMKIIα were all induced in the CPu in wild-type mice, and Wrch1 was induced in the CPu in D1 receptor mutant mice after repeated cocaine injections (Figure 2). Moreover, these genes were differentially expressed in the CPu of the two groups of mice after repeated cocaine injections (Figure 2). Importantly, the expression of these genes in the CPu was not different in saline- or acute cocaine-treated D1 receptor mutant and wild-type mice (Figure 2), suggesting the involvement of these molecules in chronic cocaine-induced neuroadaptations. As a control, we found that the D1 receptor is differentially expressed in the wild-type and D1 receptor mutant mice (Figure 2).

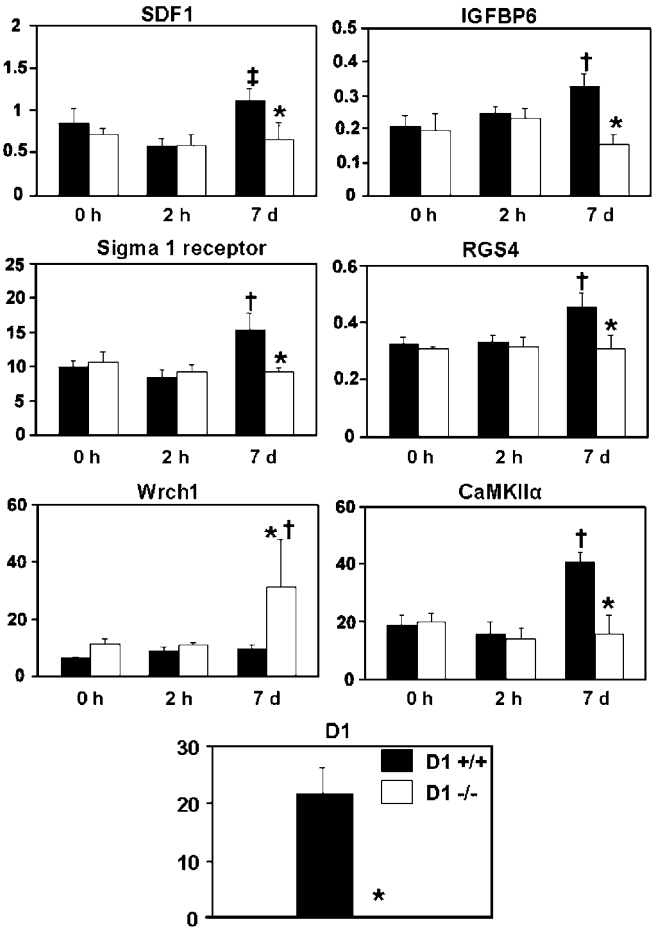

Figure 2 Differential gene expression in the CPu in D1 receptor mutant and wild-type mice after repeated exposure to cocaine. D1 receptor mutant and wild-type mice ($n = 4$ mice at each time point) were treated either with an acute 30 mg/kg dose of cocaine or saline, or with 20 mg/kg of cocaine or saline (0 h control) for 7 consecutive days. RNA was isolated from individual brains and quantitative RT real-time PCR was performed. *Y*-axes represent relative expression levels. $^{*}p < 0.05$ and $^{\dagger}p < 0.05$ between and within groups, respectively. $0.05 < {}^{\ddagger}p < 0.1$ within group. The D1 receptor gene was used as a control.

Verifying Cocaine-Induced Gene Expression Difference by Western Blotting and Immunostaining

We next selected IGFBP6 in the extracellular factor group, RGS4 in the channel, membrane receptor and modulator group, and CD2 in the intracellular signaling molecule group and examined their differential expression in the CPu and NAc in D1 receptor mutant and wild-type mice at the protein level. Our microarray studies indicate that, while it is not induced in the CPu in wild-type mice (data not shown), CD2 expression is reduced in the CPu in D1 receptor mutant mice after repeated exposure to cocaine (Supplemental Table 1). CD2 is an essential mediator for the progression through the G1 phase of the cell cycle (Coqueret, 2002). CD2 may also have a role in maintaining neural cell proliferation and differentiation (Huard *et al*, 1999).

Western blot analyses indicated that IGFBP6 and RGS4 expression is increased in the CPu in wild-type mice after repeated cocaine injections (Figure 3a). Moreover, all three genes showed attenuated expression in the CPu in D1 receptor mutant mice upon repeated exposure to cocaine compared to that in saline-treated mutant mice and that in wild-type mice (Figure 3a). Repeated cocaine injections induced RGS4 expression in the NAc in wild-type mice but not in D1 receptor mutant mice (Figure 3b). Chronic cocaine injections also attenuated CD2 expression in the D1 receptor mutant mice compared to that in wild-type mice (Figure 3b). There was no difference in the expression of these genes in the CPu or NAc in the two groups of mice either after saline or acute cocaine injections (Figure 3a and b). These results suggest that repeated cocaine administration influences the expression of these genes via the D1 receptor.

To further confirm the above findings, we performed immunostaining experiments. Quantitative analysis indicates that, consistent with the Western blot results, the expression of IGFBP6 and CD2 is lower in the CPu in D1 receptor mutant mice than in wild-type mice after repeated cocaine injections (Figure 4a–d and g). Moreover, whereas dynorphin expression is similar to previous reports (Xu *et al*, 1994a; Moratalla *et al*, 1996; Drago *et al*, 1996), cocaine-induced dynorphin expression is reduced in the CPu in D1 receptor mutant mice (Figure 4e–g).

Pretreatment of the D1 Receptor Antagonist SCH23390 Attenuates the Induction of IGFBP6, RGS4, and CD2 in the CPu by Repeated Cocaine Administration

Since both basal and acute cocaine-induced expressions of IGFBP6, RGS4, and CD2 are not different in the CPu and NAc of D1 receptor mutant and wild-type mice, our results suggest that the induction of these genes by repeated cocaine administration depends on a functional D1 receptor, and that these findings are unlikely to be related to developmental compensations of the D1 receptor gene mutation. To further confirm this notion, we pretreated wild-type mice with the D1 receptor antagonist SCH23390 and then compared IGFBP6, RGS4, and CD2 induction in

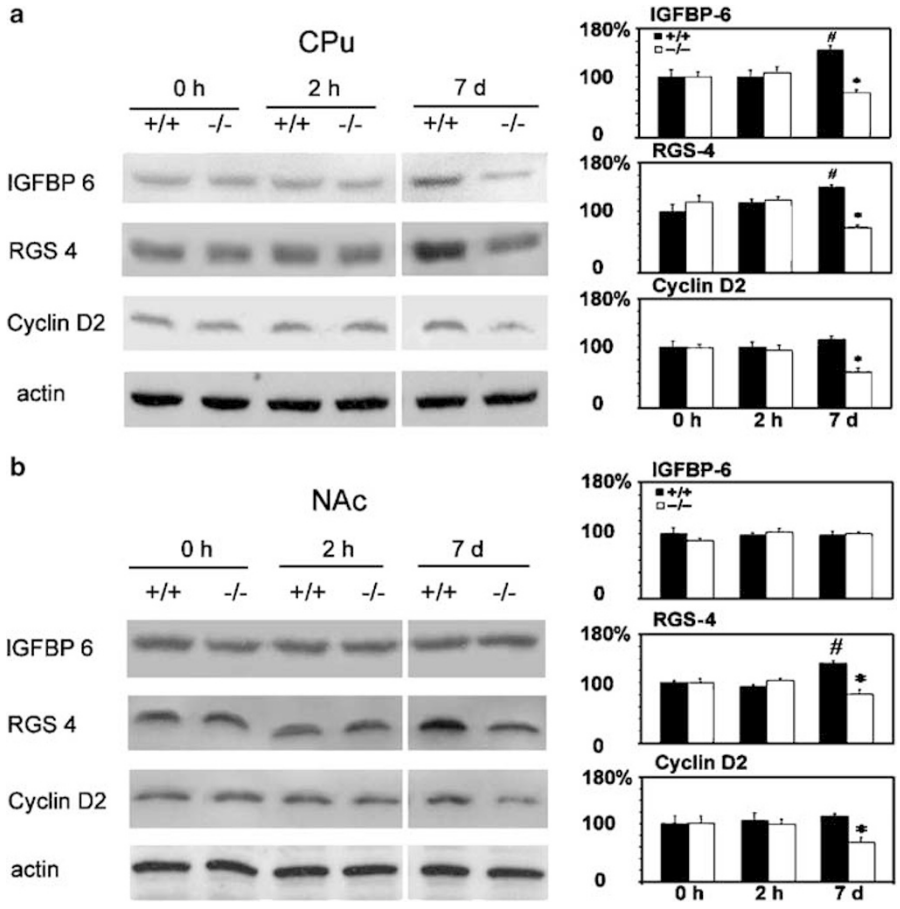

Figure 3 Differential gene expression in D1 receptor mutant mice and wild-type mice after repeated cocaine administration. Western blotting for IGFBP6, RGS4, and CD2 using extracts prepared from the CPu ((a) $n=4$ mice at each time point) and NAc ((b) $n=8$–12 mice with 4–7 males and 4–5 females at each time point) of individual wild-type (+/+) and D1 receptor mutant (−/−) mice after an acute cocaine injection at the 30 mg/kg dose (2 h) or 7 days (7 d) of cocaine or saline (0 h) treatment. Equal amounts of protein were loaded in each lane. We repeated each Western blot experiment for at least three times, and individual mouse gave parallel gene expression changes within each group. Mean + SEM of expression levels with saline-treated wild type as 100% controls. $^{*}p<0.05$ and $^{\#}p<0.05$ between and within groups, respectively.

the CPu by chronic cocaine administration. As shown in Figure 5, SCH23390 treatment before cocaine injections blocked cocaine-induced IGFPB6 and RGS4 expression and attenuated CD2 expression, while SCH23390 itself had no effects on the expression of these genes. These results are parallel to those obtained using the D1 receptor mutant mice.

IGFBP6 and CD2 are Produced in Dynorphin-Positive Neurons

Repeated cocaine injections can induce target gene expression via the D1 receptors either directly in D1 receptor-expressing neurons, or indirectly in adjacent neurons via interactions with the D1 receptor-expressing neurons. To understand whether the attenuated IGFBP6 and CD2 expression can be at least partially attributed to a reduced expression in D1 receptor-expressing neurons following repeated cocaine injections, we performed colocalization experiments. We previously found that dynorphin expression is dependent on the D1 receptor (Xu *et al*, 1994a; Moratalla *et al*, 1996; Drago *et al*, 1996) and thus used dynorphin as a marker for D1 receptor-expressing neurons. As shown in Figure 6, most of IGFBP6- and CD2-positive neurons also expressed dynorphin in the CPu, suggesting that a lack of D1 receptor led to a reduced expression of these two proteins in D1 receptor-expressing neurons after repeated cocaine injections.

Many of the Differentially Expressed Genes Contain AP-1 Binding Sites in Their Promoter Regions

Activation of the D1 receptor by cocaine leads to activation of the transcription factors c-Fos, ΔFosB, and CREB (Graybiel *et al*, 1990; Moratalla *et al*, 1996; Carlezon *et al*, 1998; Kelz *et al*, 1999; Nestler *et al*, 1999; Nestler, 2000; Zhang *et al*, 2002; Colby *et al*, 2003). These observations suggest that AP-1 transcription complexes and CREB may participate in the regulation of target genes upon repeated exposure to cocaine. To gain insights into how the differentially expressed genes are regulated in the D1 receptor-expressing neurons following repeated cocaine administration, we analyzed the promoter regions of the genes whose differential expression through the D1 receptors has been verified by the above methods. We found that all of these genes contain AP-1 binding sites in their promoter regions (Figure 7). Two genes also contain the CREB binding sites (Figure 7). These results suggest that

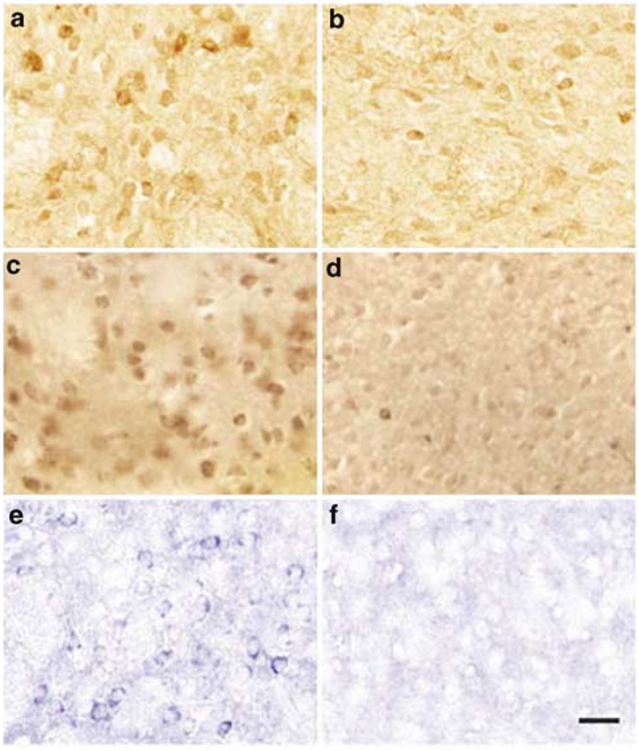

Figure 4 Differential IGFBP6 and CD2 expression in the CPu of D1 receptor mutant mice and wild-type mice after repeated cocaine administration. D1 receptor mutant and wild-type mice ($n = 6$ each) were treated with cocaine or saline twice daily for 7 consecutive days. Coronal sections ($n = 4$ for each mouse) through the CPu of wild-type (a, c, e) and D1 receptor mutant (b, d, f) mice were stained with antibodies that recognize IGFBP6 (a, b), CD2 (c, d), and dynorphin (e, f). The scale bar is 50 μm. (g) Quantification of IGFBP6, CD2, and dynorphin expression under different conditions. $^{*}p < 0.05$ between groups.

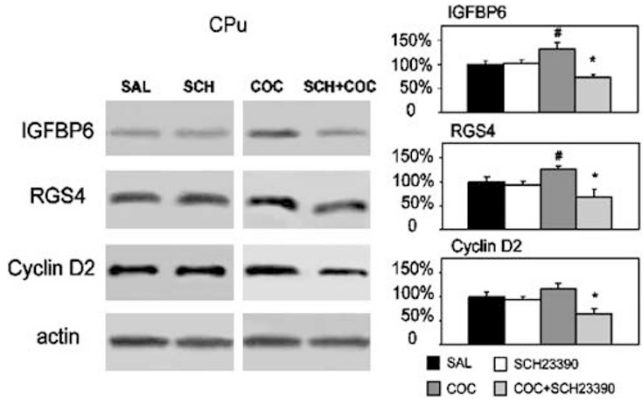

Figure 5 The D1 receptor antagonist SCH23390 can block IGFBP6, RGS4, and CD2 expression in the CPu induced by repeated cocaine administration. Wild-type mice ($n = 4$ for each condition) were treated with SCH23390 or saline 30 min before cocaine or saline injections twice daily for 7 consecutive days. Left: Equal amounts of protein were loaded in each lane for Western blotting. Right: Mean + SEM of expression levels with saline-treated wild type as 100% controls. We repeated the experiment for at least six times using different mice, and all mice in each group gave similar results. SCH, SCH23390; COC, cocaine; SAL, saline. $^{*}p < 0.05$ compared with the saline + cocaine group. $^{\#}p < 0.05$ compared with the saline + saline and saline + SCH23390 groups.

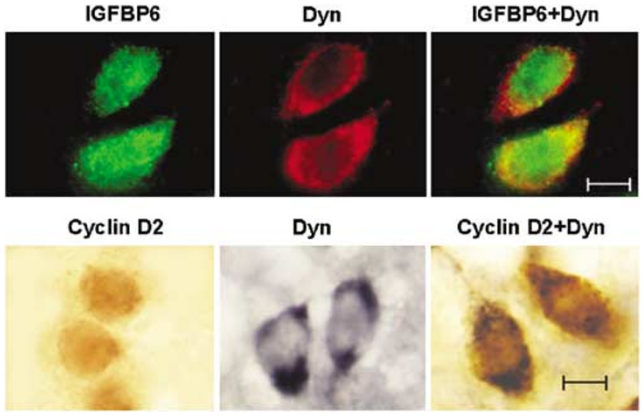

Figure 6 IGFBP6 and CD2 are coexpressed in dynorphin-positive neurons in the CPu. Wild-type mice ($n = 6$ each) were treated with cocaine or saline twice daily for 7 consecutive days. Coronal sections ($n = 4$ for each mouse) through the CPu of the mice were stained with antibodies that recognize IGFBP6, CD2, or dynorphin or in combination. The scale bar is 5 μm.

these genes are potential candidates that are regulated by either AP-1 or CREB or both in D1 receptor-expressing neurons following repeated exposure to cocaine.

DISCUSSION

We previously investigated acute cocaine-induced IEG expression and MAPK activation via the D1 receptors (Zhang *et al*, 2002, 2004). We have now used D1 receptor mutant mice that exhibit attenuated cocaine-induced behaviors compared to wild-type mice and microarrays to identify chronic cocaine-induced genes whose expression depends on a functional D1 receptor 24 h after cocaine withdrawal. We found that, whereas their expression is not different after saline injections, 109 genes are either up- or downregulated by at least 1.2-fold in the CPu D1 receptor mutant mice compared to wild-type mice by repeated cocaine injections. These genes can be classified into several functional groups. We selected six candidate genes from three functional groups and verified their differential expression in the CPu of cocaine- and saline-treated D1

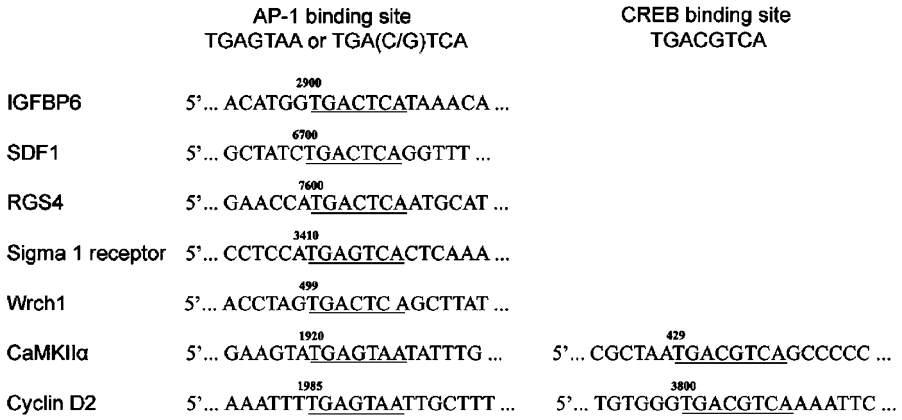

Figure 7 Promoter sequence comparisons of the differentially expressed genes. Promoter sequences were obtained from either Celera or GenBank. TGAGTAA and TGA(C/G)TCA were used for the AP-1 site, and TGACGTCA was used for the CRE site to search for sequences upstream of the transcription initiation sites of the candidate genes.

receptor mutant and wild-type mice by performing quantitative RT real-time PCR. Because the expression level and cellular location of the proteins of the candidate genes may be different from those of the mRNA, we selected one candidate molecule each from the same three functional groups and performed Western blotting and immunostaining using brains from cocaine- and saline-treated D1 receptor mutant and wild-type mice. We also verified the dependency of their expression on the D1 receptor after cocaine treatment using a D1 receptor antagonist. We found that genes encoding extracellular factors, receptors and modulators, and intracellular signaling molecules can be regulated by chronic cocaine treatment via the D1 receptors. Moreover, these genes all contain AP-1 binding sites in their promoter regions, making them potential targets for AP-1 regulation following repeated cocaine administration.

Extracellular Molecules

We found that the expression of IGFBP6 and SDF1 is induced in the CPu in wild-type mice and it is attenuated in the CPu in D1 receptor mutant mice following repeated cocaine injections. These results suggest that IGFBP6 and SDF1 can be induced by repeated cocaine administration in the CPu in mice, and that the induction is at least partially dependent on a functional D1 receptor. IGFs promote the proliferation of neurons and astrocytes (Murphy, 1998; Zumkeller and Westphal, 2001). IGFBP6 binds to IGF-II with high affinity and may inhibit IGF-II actions (Murphy, 1998; Zumkeller and Westphal, 2001). SDF1 affects neuronal cell migration through its receptor CXCR4 (Ma *et al*, 1998). Chronic cocaine administration leads to increased dendritic branching and dendritic spine density on the medium spine neurons and the prefrontal cortex pyramidal neurons (Robinson and Kolb, 1999). These dendritic morphological changes may contribute to cocaine-induced neuroadaptations. Although definitive evidence is still lacking, it is possible that IGFBP6 and SDF1 may influence chronic cocaine-induced increases in dendritic reorganization in medium spiny neurons by regulating local neuronal proliferation and migration. The significance of differential regulation of IGFBP6 in the CPu and NAc by chronic cocaine administration is not yet known.

Receptors and Modulators

We found that the expression of sigma 1 receptor and RGS4 in the D1 receptor mutant mice, unlike that in the wild-type mice, is not upregulated following repeated cocaine administration, suggesting the importance of the D1 receptor in the induction of these genes. The sigma 1 receptor is involved in modulating the rewarding effects of cocaine (Romieu *et al*, 2003). Antagonists for this receptor block the acute locomotor stimulating effect of cocaine and attenuate cocaine-induced behavioral sensitization (Ujike *et al*, 1996; Matsumoto *et al*, 2002). The sigma 1 receptor may also modulate dopaminergic neurotransmission by modulating DA release (Ault and Werling, 1999). Changes in the expression of the sigma 1 receptor can thus change the behavioral responses to cocaine by exerting both pre- and postsynaptic influences. RGS4 proteins can modulate G-protein-coupled receptor function in the brain (Zhong and Neubig, 2001; Hollinger and Hepler, 2002). Selective pharmacological stimulation of the DA D1 and D2 receptors has been shown to decrease and increase RGS4 expression in the striatum, respectively (Geurts *et al*, 2002; Taymans *et al*, 2003). Changes in RGS4 expression might thus be involved in alterations of signal transduction occurring after activation of D1 receptors by repeated cocaine stimulation.

Intracellular Signaling Molecules

We also found that Wrch1 is upregulated by repeated cocaine injections in the CPu in D1 receptor mutant mice but not wild-type mice. We speculate that Wrch1 may inhibit cocaine-induced and D1 receptor-mediated behavioral changes. We previously found that β-catenin, which is a key member in the Wnt signaling pathway, was induced by acute cocaine administration in the NAc and by repeated cocaine treatment in both the NAc and CPu in wild-type mice (Zhang *et al*, 2002). On the contrary, acute cocaine administration reduced β-catenin expression in the CPu and chronic cocaine injections decreased β-catenin

expression in the NAc in D1 receptor mutant mice (Zhang *et al*, 2002). Changes in the Wnt signaling pathway can influence Wrch1 function and neuronal cell proliferation and cell adhesion and thus contribute to chronic cocaine-induced neuroadaptations.

We found that, unlike that in the CPu in wild-type mice, CaMKIIα is not upregulated in D1 receptor mutant mice after chronic cocaine treatment. CaMKII is activated in response to increases in intracellular calcium levels and it can modulate synaptic plasticity both pre- and postsynaptically (Lisman *et al*, 2002). In the postsynaptic density, CaMKII can directly interact with and phosphorylate many proteins, including the *N*-methyl-D-aspartate receptor 2B subunit and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor 1, both of which mediate excitatory neurotransmission (Yoshimura *et al*, 2000; Lisman *et al*, 2002). The D1 receptor-dependent change in CaMKIIα expression following repeated cocaine injections can thus change neurotransmission in the striatum.

Finally, we found that CD2 expression is downregulated in the CPu in D1 receptor mutant mice but not in wild-type mice after repeated cocaine injections. CD2 is a mediator for the progression through the G1 phase of the cell cycle (Coqueret, 2002). In the central nervous system, CD2 has also been shown to be required for both the proliferation of the granule cell precursors and the proper differentiation of granule and stellate interneurons in the cerebellum (Huard *et al*, 1999). It is possible that continuous CD2 expression during repeated cocaine injections is necessary for the maintenance of dendritic morphology or for the local proliferation of neuronal cells in the NAc and CPu.

AP-1-Regulated Genes in D1 Receptor-Expressing Neurons may Contribute to Cocaine-Induced Neuroadaptations

We found that the D1 receptor antagonist SCH23390 blocked cocaine-induced changes in IGFBP6, RGS4, and CD2 expression in the CPu while it did not affect the expression of these genes. These results are completely parallel to those obtained using the D1 receptor mutant mice, and they argue that these gene expression changes are indeed induced by chronic cocaine administration via the D1 receptor and are not due to compensatory effects produced from the D1 receptor gene mutation.

Analyses of the promoter sequences of the above candidate genes showed that they all contain AP-1 binding sites. Two genes also contain the CREB binding sites in the promoters. These results and the fact that IGFBP6 and CD2 can be induced in dynorphin-positive neurons in the CPu by cocaine support a molecular model in which different classes of genes can serve as potential targets for regulation by either AP-1 transcription complexes or CREB or both in D1 receptor-expressing neurons following repeated cocaine injections. These changes in gene expression may contribute to cocaine-induced persistent neurochemical and behavioral changes (Zhang *et al*, 2004).

Our current study focused on confirming genes whose expression changes correlate with chronic cocaine-induced behavioral changes, and whose expression in the CPu and NAc depends on a functional D1 receptor. A more detailed bioinformatics analysis of the results in the future may uncover more functionally related changes in gene expression following repeated cocaine injections. Moreover, the gene expression changes identified in our study involve a relatively short 24 h cocaine withdrawal time window, and how these changes relate to the persistent nature of drug addiction remains to be determined. To truly identify genes that are physiologically relevant to chronic cocaine-induced neuroadaptations, we will need to functionally perturb the expression of the candidate genes *in vivo* and investigate whether and how they affect behavioral changes in response to chronic cocaine administration. Whether these candidate genes are regulated by AP-1 transcription complexes in response to repeated cocaine administration also needs further investigation. We used both male and female mice in the current study, and potential sex-related gene expression differences following cocaine administration need to be addressed. It should also be noted that post-translational modifications, such as protein phosphorylation, are an integral part of molecular responses to repeated exposure to cocaine (Greengard *et al*, 1999). The proteomics method will address this possibility (Husi and Grant, 2001). The combined use of gene-targeted mice that exhibit altered responses to cocaine, with microarrays and proteomics, and proper physiological testing of the putative target genes may provide novel insights into the molecular basis of the persistence of drug dependence.

ACKNOWLEDGEMENTS

We thank Bruce Aronow and Sarah Williams at the Bioinformatics Core for advice on the analysis of the microarray results, and the Microarray Core at the Children's Hospital Research Foundation at Cincinnati for microarray hybridization and data scanning. JZ is supported by NIDA (DA11284). MX is supported by NIDA (DA11005 and DA13786).

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Supplementary information accompanies the paper on Neuropsychopharmacology website (http://www.nature.com/npp)