Published in final edited form as:

J Pharmacol Exp Ther. 2003 November ; 307(2): 450–459. doi:10.1124/jpet.103.054965.

Discrete Cell Gene Profiling of Ventral Tegmental Dopamine Neurons after Acute and Chronic Cocaine Self-Administration

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Abstract

Chronic cocaine administration induces a number of biochemical alterations within the mesolimbic dopamine system that may mediate various aspects of the addictive process such as sensitization, craving, withdrawal, and relapse. In the present study, rats were allowed to self-administer cocaine (0.5 mg/infusion) for 1 or 20 days. Tyrosine hydroxylase immunopositive cells were microdissected from the ventral tegmental area (VTA) using laser capture microdissection, and changes in the abundances of 95 mRNAs were assessed using cDNA macroarrays. Five GABA-A receptor subunit mRNAs ($\alpha 4$, $\alpha 6$, $\beta 2$, $\gamma 2$, and δ) were down-regulated at both 1 and 20 days of cocaine self-administration. In contrast, the catalytic subunit of protein phosphatase 2A (PP2A), GABA-A $\alpha 1$, and $G\alpha_{i2}$ were significantly increased at both time points. Additionally, calcium/calmodulin-dependent protein kinase II α mRNA levels were increased initially followed by a slight decrease after 20 days, whereas neuronal nitric-oxide synthase mRNA levels were initially decreased but returned to near control levels by day 20. These results indicate that alterations of specific GABA-A receptor subtypes and other signal transduction transcripts seem to be specific neuroadaptations associated with cocaine self-administration. Moreover, as subunit composition determines the functional properties of GABA-A receptors, the observed changes may indicate alterations in the excitability of dopamine transmission underlying long-term biochemical and behavioral effects of cocaine.

Repeated cocaine use induces biochemical adaptations in reinforcement-relevant brain regions (White and Kalivas, 1998; Koob and Le Moal, 2001; Nestler, 2001). These neuroadaptations are important in that they may underlie processes of sensitization, craving, withdrawal, and relapse (Nestler and Aghajanian, 1997). Numerous studies have examined biochemical changes that occur within the mesolimbic dopamine system (Nestler and Aghajanian, 1997), a pathway considered to be a primary substrate for various types of reinforcing stimuli, including cocaine (Koob, 1996; Wise, 1998). The mesolimbic dopamine pathway originates in the ventral tegmental area (VTA) and projects to several fore-brain regions, including the nucleus accumbens (NAc) (Ungerstedt, 1971), considered a primary substrate for the reinforcing effects of cocaine. Acute and chronic cocaine administration produces significant elevations in NAc extra-cellular dopamine concentrations in animal

models (Pettit and Justice, 1989; Hemby et al., 1997a,b), effects that contribute to the abuse liability of cocaine (Ritz et al., 1987).

In addition to the effects of acute administration, chronic cocaine administration induces neuroadaptations that may represent persistent or even permanent alterations in neuronal function (Nestler and Aghajanian, 1997; White and Kalivas, 1998). For example, cocaine induces a generalized up-regulation of the cAMP pathway (Nestler and Aghajanian, 1997) as well as activator protein 1 family members (Hope et al., 1992; Nye et al., 1995; Haile et al., 2001). Specifically, chronic cocaine administration increases activity of cAMP-dependent protein kinase (PKA) and adenylate cyclase in the NAc of rats (Terwilliger et al., 1991), as well as mRNA and protein levels of the α -catalytic subunit of PKA in the NAc of cynomolgus monkeys (Freeman et al., 2001). Furthermore, manipulation of specific components of these biochemical pathways has been shown to alter subsequent responsivity to the drug (Carlezon et al., 1998; Self et al., 1998). Numerous studies have also indicated that chronic cocaine induces hyperglutamatergic function in the VTA that seems to mediate the behavioral and neurochemical effects of cocaine (Fitzgerald et al., 1996; Ghasemzadeh et al., 1999; Loftis and Janowsky, 2000; Ungless et al., 2001; Tang et al., 2003). Given that chronic cocaine administration also alters the abundance of GABA-A receptor subunit mRNAs (Suzuki et al., 2000; Yamaguchi et al., 2000, 2002) and proteins (Lilly and Tietz, 2000; Jung and Peris, 2001) in the NAc and elsewhere, decreased GABA neurotransmission may complement the hyperglutamatergia in the VTA to increase cell excitability.

At present, there is a paucity of literature demonstrating the cellular specificity of cocaine-induced changes in gene expression in the mesolimbic dopamine system or other areas. Studies using in situ hybridization allow the analysis of mRNAs in defined cell populations, yet the sensitivity may not allow analysis of low-abundance mRNAs, the means to evaluate numerous transcripts, or the ability to reliably quantify such changes. Studies using regional assessment of gene expression emphasize transcripts contained in the majority of neuronal and glial populations and/or transcripts in highest abundance in the region but may not adequately reflect alterations in gene expression in target neuronal populations. Such limitations can be overcome by the combination of laser capture microdissection and array technology that enables precise localization of coordinate changes in gene expression within defined cell types (Eberwine et al., 1992; Hemby et al., 2002, 2003; Kamme et al., 2003).

Whereas the majority of studies investigating the molecular adaptations associated with chronic drug use have relied on noncontingent administration, several studies indicate pronounced biochemical differences between the contingent and noncontingent administration of drugs (Hemby et al., 1995, 1997a; Mark et al., 1999; McFarland et al., 2003). The concept of contingency is critical to interpreting changes associated with cocaine administration as they relate to the reinforcing effects of the drug. The present study was undertaken to evaluate differences in gene expression in tyrosine hydroxylase immunopositive neurons in the VTA after acute and chronic cocaine self-administration in rats. To this end, custom-designed macroarrays were used to simultaneously assess 95 genes and determine whether functional classes of genes were differentially expressed as a function of cocaine self-administration. The present study sought to expand upon previous findings in three ways: 1) examine gene expression changes in the VTA after self-

administered rather than experimenter-administered cocaine, 2) monitor gene expression changes occurring specifically in VTA dopaminergic neurons, and 3) evaluate glutamate and GABA-A receptor subunits to delineate cocaine's effects on excitatory as well as inhibitory neurotransmission in the VTA.

Materials and Methods

Subjects and Surgical Procedures

Male Sprague-Dawley rats (60–90 days; 225–275 g; Charles River Laboratories, Inc., Wilmington, MA) were randomly assigned to one of three groups: acute cocaine ($n = 9$), chronic cocaine ($n = 8$), and control ($n = 9$). Animals were housed in pairs under a reverse 12-h light/dark cycle (lights on 8:00 PM) and fed ad libitum before surgery. Rats in the cocaine groups were implanted with chronic indwelling jugular catheters under isoflurane anesthesia, as described previously (Hemby et al., 1995, 1997b). Infusions of methohexital (100 μ l; 10 mg/kg i.v.) were administered as needed to assess catheter patency. Health of the rats was monitored twice daily by the experimenter and biweekly by institutional veterinarians according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Self-Administration

After surgery, subjects were housed in standard operant conditioning chambers (24.5 $\times$ 23.5 $\times$ 21 cm; MED Associates; St. Albans, VT) containing a retractable lever (0.25 N to operate) and a stimulus light mounted directly above the lever. The chambers were enclosed in sound-attenuating boxes containing an exhaust fan, a house light, a tone source, and a water bottle. A motor-driven syringe pump was located on the side of the external chamber. Extraneous noise was masked by the exhaust fan. Immediately after surgery, rats were placed in their respective chambers and received infusions of heparinized 0.9% bacteriostatic saline (1.7 U/ml; 200 μ l/30 min) for 48 h. The next day, the self-administration procedure began. Rats assigned to the acute cocaine self-administration group were allowed to self-administer cocaine (0.5 mg/infusion) during one 8-h self-administration session under a fixed ratio (FR) 1, time-out 20-s schedule of reinforcement. Upon completion of the response requirement, a cocaine infusion was delivered and a 20-s time-out was in effect. During the time-out, the lever light was extinguished, the house light illuminated, and a tone was generated. The end of the time-out was signaled by illumination of the lever light and extinguishing of the house light and tone. During the time-out period, responses on levers were recorded but had no scheduled consequence. For rats in the chronic cocaine self-administration group, the ratio was gradually increased to FR5 (the terminal ratio) over 7 days. Rats self-administered cocaine under 8 h limited access conditions for 20 days after attainment of the terminal ratio. Rats assigned to the control group were drug naïve and did not receive jugular catheter implants. IBM-compatible computers were used for session programming and data collection.

After completion of the experimental procedures, rats were sacrificed using CO₂ and transcardially perfused with 0.1 M phosphate-buffered saline followed by 4% paraformaldehyde in phosphate-buffered saline. After removal, brains were placed in a brain

matrix, and 3-mm coronal blocks were taken from the rostral to the caudal portion of the brain. The blocks were placed in cassettes, post-fixed in 4% paraformaldehyde for 1 h and then transferred to 70% ethanol/150 mM NaCl for 2 h. All blocks were then placed in a Tissue Tek VIP5 vacuum infiltration processor (Torrance, CA) and processed in 70% ethanol (30 min at 40°C), 80% ethanol (30 min at 40°C), twice in 95% ethanol (45 min at 40°C), twice in 100% ethanol (45 min at 40°C), twice in xylenes (45 min at 40°C), and four times in paraffin (Paraplast; 30 min at 58°C). Afterward, tissue was transferred from the tissue processor to a Tissue Tek TEC tissue embedding console where each cassette containing processed tissue was transferred to an orientation platform (58°C). The tissue was removed and placed in a base mold before paraffin was dispensed into the mold. The top of the cassette was placed on top of the base mold, and the cassette and tissue were moved onto the chiller (3°C) to solidify the tissue block.

Immunocytochemistry and Laser Capture Microdissection (LCM)

Tissue blocks that included the mesencephalon were cut into 8- μ m sections and then deparaffinized in xylenes (2 $\times$ 5 min) followed by descending concentrations of ethanol (1 min, 70, 80, and 2 $\times$ 95%; 5 min, 2 $\times$ 100%) and RNase-free water (22°C for 30 min). Next, sections were immersed in Tris buffer (0.1 M, pH 7.4) for 5 min at 22°C followed by immersion in 0.1 M Tris buffer (pH 7.4)/3% denatured horse serum for 5 min at 22°C. Sections were then incubated in primary monoclonal antibody against tyrosine hydroxylase (TH; 1:1000; T-1209; Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris buffer (pH 7.4)/3% denatured horse serum for 15 min at 22°C. Sections were rinsed with 0.1 M Tris buffer (pH 7.4) and incubated with a horse anti-mouse secondary antibody in 0.1 M Tris buffer (pH 7.4) for 10 min at 22°C, followed by a rinse with Tris buffer. Next, the conjugate was labeled using the avidin-biotin method (ABC Vectastain; Vector Laboratories; Burlingame, CA) in 0.1 M Tris buffer (pH 7.4) for 10 min at 22°C, followed by a rinse with Tris buffer. Immunolabeling was visualized with 3,5'-diaminobenzidine. After the peroxidase reaction, sections were rinsed in RNase-free water for 15 min and dehydrated in ascending ethanol concentrations (1 min each in 70, 80, 95, and 100%) and xylenes for 5 min.

Slides were removed from xylene and air-dried before microdissection. Laser dissection parameters for the Arcturus Pix Cell Ite system (Arcturus, Mountain View, CA) were as follows: diameter, 7 μ m; power, 30 to 100 mW; and duration, 400 to 1200 μ s. Approximately 200 TH-immunopositive cells were dissected from the VTA (−5.8 to −6.3 mm caudal to bregma, ventral to the red nucleus, and dorsomedial to the medial lemniscus (Paxinos and Watson, 1998). Cells close to the boundary between the VTA and the substantia nigra were not dissected to ensure distinction between the regions (Fig. 1). After dissection, the cap was removed and placed on a CapSure Pad (Arcturus) to remove excess tissue.

RNA Extraction and Amplification

Each cap was then secured to the top of a 0.5-ml Eppendorf microcentrifuge tube containing 200 μ l of TRIzol reagent (Invitrogen, Carlsbad, CA). The tube was inverted, vortexed, and incubated at 22°C for 15 min. RNA was then extracted with phenol/chloroform and precipitated with 100% ethanol (−80°C) in the presence of 10 μ g of linear acrylamide. After

extraction, a 66-base pair oligo(dT)-T7-primer/promoter (Tecott et al., 1988) was hybridized to poly(A+) mRNA for 7 min at 70°C and then quickly cooled on ice for 5 min. cDNA was synthesized using reverse transcriptase (AMVRT, Seikagaku; Falmouth, MA) in 1× reverse transcriptase buffer containing 7 mM dithiothreitol; 250 μM each of dATP, dCTP, dGTP, and dTTP; and 0.12 U/μl of RNasin and incubated at 42°C for 90 min.

The amplification and reamplification procedures have been described in detail elsewhere (Van Gelder et al., 1990; Hemby et al., 2002, 2003). During the second round of amplification, [³³P]UTP was incorporated into the aRNA probes for hybridization with reverse Northern blots. Under optimal conditions, the first round of aRNA amplification results in approximately 1000-fold yield, and two rounds of amplification result in approximately 10⁶-fold yield versus the original amount of poly(A+) mRNA. The aRNA procedure is a linear amplification process with minimal change in the relative abundance of the mRNA population in the native state of the neuron.

cDNA Macroarray Construction

Macroarrays were prepared on nylon membranes and consisted of 95 cDNAs selected for likely involvement in the effects of cocaine, including dopamine receptors, glutamate receptors, GABA receptors, synaptic proteins, and intra-cellular signaling molecules. Inserts were amplified from plasmid stocks in 96-well plates using PCR with GF200 primers under the following conditions: 95°C for 5 min (1 cycle); 95°C for 20 s, 55°C for 20 s, and 72°C for 2 min (40 cycles); and 72°C for 7 min (1 cycle). Aliquots of PCR samples were electrophoresed on a 1% agarose gel (1× Tris-acetate-EDTA, 0.05% ethidium bromide) at 5 V/cm for PCR band size verification. Gel images were captured by digital camera and archived. PCR product concentration was determined by spectrofluorometry (Gemini; Molecular Devices Corp., Sunnyvale, CA) using a 1:5000 dilution of SYBR 1 Green/Tris-EDTA and an aliquot of the PCR product. Values were compared with known concentrations of DNA standards for quantitation. Approximately 700 ng of each amplified insert was spotted on Nytran SuPerCharge nylon transfer membrane (Schleicher & Schuell, Keene, NH) using a 96-well dot blot apparatus (Minifold I; Schleicher & Schuell). DNA was crosslinked to the membrane by ultraviolet radiation at 120,000 μJ/cm².

Macroarrays were prehybridized with UltraHyb solution (Ambion, Austin, TX) in hybridization bottles for 1 h at 42°C. Next, ³³P-labeled antisense RNA probes from the VTA for each subject were heat denatured for 5 min at 70°C, placed on ice for 5 min, and hybridized to their respective arrays overnight at 42°C in a rotisserie hybridization oven. Samples from the VTA of each rat were hybridized to separate macroarrays and were not pooled within groups. After hybridization, membranes were washed once briefly with 2× SSC/0.1% SDS, twice with 2× SSC/0.1% SDS for 15 min each at 42°C, and once with 0.1× SSC/0.1% SDS for 10 min at 42°C. Labeled hybridized products were detected using PhosphorImager cassettes, and hybridization signal intensities were analyzed using ImageQuant software (Amersham Biosciences Inc., Sunnyvale, CA).

Data Analysis

Densitometry values (hybridization intensities) were obtained for each clone and for background (nonspecific) hybridization on the array. Background hybridization values were obtained from spots on the macroarrays in which no clone was loaded as well as regions between spotted clones. Only clones with spot symmetry and lack of significant artifactual signal intensity as assessed by visual identification were accepted for analysis. Values below background were not included in the analysis. The background value for each macroarray was subtracted from the densitometry value for each clone on that array. This “signal – background” value was then divided by the summed values for all of the clones on the array (global normalization) to yield a normalized value for each clone, thereby minimizing variations due to differences in the specific activity of the probe and the absolute quantity of probe present (Ginsberg et al., 2000; Hemby et al., 2002, 2003; Tang et al., 2003). Genes were grouped into nine classes (dopamine-related, glutamate-related, GABA-related, G proteins, kinases, peptides and lipids, phosphatases, structural and signaling, and transcription factors), and a two-factor analysis of variance (Transcript and Group) with repeated measures on one factor (Transcript) was then used for each group of genes. Post hoc comparisons were then conducted as needed using Tukey’s test. The null hypothesis was rejected when $p < 0.05$.

Results

Behavior

Rats in the acute cocaine group self-administered an average of $40 (\pm 10 \text{ S.E.M.})$ infusions ($20 \pm 5 \text{ mg}$ of cocaine) over the 8-h self-administration session (Fig. 2). In the chronic cocaine group, rats self-administered an average of $31 (\pm 3)$ infusions ($15 \pm 3 \text{ mg}$ of cocaine) per day (Fig. 2). As expected, the mean total cocaine intake for the chronic cocaine self-administration group ($317.94 \pm 32.19 \text{ mg}$) was significantly greater than the acute cocaine group ($20.06 \pm 5.01 \text{ mg}$; $t = -9.707$, $df = 15$, $p < 0.001$). Four rats in the chronic cocaine group received sham injections in the NAc on day 15 as part of another study. Animals were then returned to their operant chambers and allowed to self-administer cocaine for a normal 8-h self-administration session. However, there was no significant difference in the number of infusions self-administered or in the gene expression pattern compared with the rest of the group.

Gene Expression

Analysis of the macroarrays revealed distinct patterns of expression for the majority of transcripts investigated (Fig. 3). Of the 95 mRNAs examined, 11 were found to be differentially expressed among the groups (Fig. 4; Table 1), and at least one transcript was differentially expressed in five of the nine categories examined: GABA-related, G proteins, kinases, phosphatases, and structural/signaling molecules.

Dopamine-Related Transcripts and Transcription Factors

There was no significant difference for seven dopamine-related transcripts (Table 1) in either Group [$F(2,181) = 0.00958$; $P = 0.990$] or Group and Transcript interaction [$F(12,181)$]

= 0.712; $P = 0.738$]. Likewise, there was no significant effect of cocaine on the levels of transcription factor mRNAs [$F(2129) = 1.403$; $P = 0.266$] and no significant interaction [$F(8129) = 0.615$; $P = 0.763$] (Table 1).

GABA-Related Transcripts

The most extensively affected by cocaine self-administration, the GABA-related transcripts exhibited both a significant effect of Group [$F(2363) = 10.864$; $p < 0.001$] and an interaction between Group and Transcript [$F(26,363) = 2.869$; $p < 0.001$]. Of the 14 GABA-related transcripts examined, post hoc analysis revealed that expression of six GABA-A receptor subunits was altered after cocaine self-administration (Table 1; Fig. 4). In all cases where receptor subunit mRNAs were altered after acute cocaine self-administration, expression remained altered after 20 days of self-administration. mRNA levels of four transcripts (GABA-A $\alpha 4$, GABA-A $\alpha 6$, GABA-A $\beta 2$, and GABA-A δ) were significantly decreased after both acute and chronic cocaine self-administration. The GABA-A $\gamma 2$ subunit was significantly decreased after 20 days, but not 1 day, of self-administration. In contrast, $\alpha 1$ subunit mRNA was up-regulated with cocaine self-administration at both time points. Additionally, expression of the enzyme GAD 65 was significantly decreased after chronic cocaine self-administration compared with acute self-administration.

Glutamate-Related Transcripts

Previous studies have indicated significant involvement of VTA glutamate receptors in the behavioral effects of cocaine (Pierce et al., 1996; White and Kalivas, 1998). However, in the present study, there was no significant difference in glutamate receptor subunit mRNA expression between acute and chronic cocaine self-administration and controls [$F(2,545) = 0.171$; $P = 0.844$]. Furthermore, there was no significant Group and Transcript interaction [$F(40,545) = 1.154$; $P = 0.245$] for the glutamate-related transcripts (Table 1). However, GRIN2C, GRIA1, and GRIA3 were decreased slightly after acute cocaine but returned to control levels after chronic cocaine self-administration. In an opposite manner, GRIN2B, GRIA2, and GRIK4 were increased after 1 day and decreased toward control levels by day 21.

Other Transcripts

A significant main effect of Group was observed for kinases [$F(2285) = 3.611$; $P = 0.043$], phosphatases [$F(2129) = 4.299$; $P = 0.026$], and structural/signaling [$F(2337) = 7.840$; $P = 0.003$] transcripts. Additionally, G proteins, kinases, phosphatases, and structural/signaling transcripts all showed a significant interaction between Group and Transcript [$F(26,363) = 3.651$; $P < 0.001$; $F(20,285) = 4.252$; $P < 0.001$; $F(8129) = 3.6$; $P = 0.001$; and $F(24,337) = 2.671$; $P < 0.001$, respectively]. Post hoc analyses revealed a significant increase in $G\alpha_{i2}$ mRNA after acute and chronic cocaine self-administration. Although not significant, $G\alpha_{i2}$ continued increase after chronic self-administration relative to acute self-administration. Both CaMKII α and neuronal nitric-oxide synthase (nNOS) were significantly different for all pairwise comparisons between groups. After an initial increase in mRNA expression with acute cocaine self-administration, CaMKII α expression declined after 20 days of self-administration, although levels did not return to control levels. nNOS followed the opposite

pattern of expression with an initial decrease after acute cocaine self-administration followed by an increase that did not return to control levels. PP2 α mRNA levels were up-regulated over control levels after both 1 day and 20 days of self-administration. Although the peptide-related category showed no main effect of Group [$F(2129) = 0.918$; $P = 0.413$] and no interaction between Group and Transcript [$F(8129) = 1.399$; $P = 0.208$], there was a trend toward increased levels of precursor to cholecystokinin after both acute and chronic self-administration.

Discussion

The present study was undertaken to identify coordinate changes in the expression of multiple genes in VTA dopamine neurons as a function of cocaine self-administration. A membrane-based macroarray platform with radioactive hybridization was used to query 95 cDNAs of interest. Significant alterations were observed for a variety of protein families, including GABA-A receptor subunits, G protein subunits, kinases, phosphatases, other miscellaneous transcripts, and a trend toward significance in iGluR receptor subunits. A variety of effects on gene expression were observed between acute and chronic cocaine administration and are likely due to the chronicity of drug administration. However, similar effects between acute and chronic cocaine self-administration were observed for several mRNAs and likely reflect the direct pharmacological actions of cocaine on the dopamine neurons assessed in the present study. Previously, our laboratory and others have used similar approaches to identify molecular profiles of cocaine in rats (Ang et al., 2001; Freeman et al., 2002; Toda et al., 2002), monkeys (Freeman et al., 2001), and humans (Tang et al., 2003). Regional assessments of gene expression create an informative mosaic of expression level changes; however, determining the cellular origins of the gene expression has been complicated by cellular heterogeneity of subcortical brain regions and the ability to assess multiple genes in discrete neuronal populations. Single cell gene expression methodology combined with array technology can overcome some of these limitations by assessing multiple transcripts in specific target neuronal populations, providing a level of assessment heretofore unattainable (Ginsberg et al., 2000; Hasenkamp and Hemby 2002; Hemby et al., 2002, 2003; Fasulo and Hemby, 2003; Kamme et al., 2003). Future studies directed toward protein confirmation and functional relevance of the observed mRNA change are warranted. The present study provides the first molecular profile of VTA dopamine neurons after acute and chronic cocaine self-administration in rats.

The major finding of the present study was a significant alteration in the expression of GABA-A receptor subunit mRNAs. GABA-A receptors are composed of α , β , γ , δ , ϵ , θ , π , and ρ subunits that form a pentameric structure with an integrated chloride channel. The expression and assembly of different subunits confer different physiological and pharmacological properties to the receptor complex. In the present study, $\alpha 1$ and $\gamma 2$ subunits were of similarly high abundance relative to the other subunits, yet the $\alpha 1$ subunit was up-regulated and the $\gamma 2$ subunit was down-regulated with cocaine self-administration. These data are supported by previous studies demonstrating significant decreases in $\alpha 1$, $\alpha 6$, $\beta 2$, $\beta 3$, and $\gamma 2$ mRNAs in the striatum and NAc (Suzuki et al., 2000; Yamaguchi et al., 2000), along with decreased receptor number and function in this region (Peris, 1996; Yamaguchi et al., 2002). Cocaine-induced alterations in these subunits alter subsequent responsivity to the

drug (Peris et al., 1998). Decreased expression of the $\gamma 2$ subunit could also have a potentially profound effect on normal receptor function, because $\gamma 2$ subunits are essential for receptor clustering (Essrich et al., 1998). Although of lower relative abundance, $\alpha 4$, $\alpha 6$, $\beta 2$, and δ -subunit mRNAs were also down-regulated in response to cocaine self-administration. The regulation of α and γ subunits in the present study and their involvement in benzodiazepine modulation of the GABA-A receptors provides a possible explanation of the utility of benzodiazepine therapy for cocaine-related anxiety.

Although several scenarios have been proposed, growing evidence suggests that GABA-A receptors contribute to the tonic inhibition of dopamine neurons in the VTA, especially projections to the NAc. For example, intra-VTA infusions of GABA decrease VTA dopaminergic cell activity as well as dopamine release in the NAc (Suaud-Chagny et al., 1992). Other studies have demonstrated that induction of hyperpolarization in VTA neurons by focal stimulation was reversed by the GABA-A antagonists picrotoxin and bicuculline (Johnson and North, 1992b). Additionally, bicuculline administration stimulated VTA dopaminergic neurons (Johnson and North, 1992a) and supported intra-VTA self-administration (Ikemoto et al., 1997a,b). Together, these data suggest that decreased function or abundance of GABA-A receptors may result in increased firing of mesoaccumbal dopamine neurons, an effect that would likely contribute to cocaine reinforcement.

Previous studies have indicated that chronic cocaine administration significantly alters the abundance of iGluR subunit protein, but not mRNA, levels within the mesolimbic dopamine system in rats, which may lead to increased excitability of dopamine neurons (White et al., 1995; Fitzgerald et al., 1996; Ghasemzadeh et al., 1999; Loftis and Janowsky 2000). Chronic cocaine administration in rats failed to alter mRNA levels of GRIA1-4, GRIN1 or mGluR5 in the VTA (Ghasemzadeh et al., 1999; Lu et al., 2002), although specific subunits were significantly up-regulated in the VTA of human cocaine overdose victims (Tang et al., 2003). The present study supported and extended the findings in rats by demonstrating no significant alteration in iGluR subunits in VTA dopamine neurons in rats with acute and chronic self-administration histories. However, significant elevations have been demonstrated in NMDAR 1 and GluR1 protein levels in the VTA after chronic cocaine administration (Fitzgerald et al., 1996; Churchill et al., 1999; Loftis and Janowsky, 2000) as well as NMDAR 1, GluR2, GluR5, and kainate receptor 2 protein levels in cocaine overdose victims (Tang et al., 2003). The possibility of cocaine-induced alterations in iGluR protein but not mRNA levels in rats should be evaluated further with particular attention to quantification and localization of the subunits with respect to VTA dopamine neurons. The inability to recapitulate the changes observed in cocaine overdose victims in rats self-administering cocaine is likely due to several factors, including differences in anatomical complexity between the rat and human and the chronicity and regimen of cocaine administration. Further studies are warranted to investigate the changes in the levels of these and other transcripts in rats with histories of binge self-administration and abstinence from cocaine as well as similar studies in rhesus monkeys.

Often activated in response to glutamatergic transmission, nNOS was also differentially regulated in response to cocaine self-administration. nNOS produces nitric oxide, which can

be either neurotoxic or neuroprotective, depending upon experimental conditions (Khaldi et al., 2002). nNOS inhibitors have been shown to protect against methamphetamine-induced dopamine neurotoxicity, but prolonged exposure to nNOS inhibitors may also lead to apoptosis. In the current experiment, nNOS mRNA decreased with acute cocaine and returned to control levels after chronic cocaine self-administration. For example, previous studies have found that nNOS knockout mice were resistant to locomotor sensitization to cocaine (Itzhak et al., 1998); however, the nNOS knockout was not restricted to the VTA and the results could reflect a nitric oxide deficiency in other brain regions or in nondopaminergic cells of the VTA. Recent studies suggest nNOS is increased by NMDA activation (Khaldi et al., 2002), and nNOS may increase CREB activity (Ciani et al., 2002), proving a mechanism by which nNOS may contribute to cocaine-induced plasticity in the VTA. However, as the activity of nNOS varies among different brain regions, a specific role(s) for nNOS in the VTA as a function of cocaine self-administration remains to be elucidated.

Both acute and chronic cocaine self-administration increased mRNA levels of Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α), a predominant protein in the post synaptic density which is necessary for LTP and is hypothesized to be a molecular correlate of memory (Lisman et al., 2002). The present data support previous studies demonstrating that both cocaine-induced behavioral sensitization and increased dopamine levels in the NAc are dependent upon activation of CaMKII-dependent mechanisms (Pierce and Kalivas, 1997; Pierce et al., 1998). Although the present study found no alterations in the expression levels of iGluR subunits, CaMKII is an important modulator of glutamate transmission, acting as a sensor of Ca^{2+} current when bound to NMDA receptors (Lisman et al., 2002). In addition, CaMKII phosphorylates AMPA receptor subunits, thereby increasing calcium conductance through the AMPA channels, stimulating CREB and possibly contributing to long-term potentiation in the VTA observed in response to cocaine (Liu and Anand, 2001; Ungless et al., 2001).

Investigators have demonstrated cocaine-induced neuroadaptations in the cAMP pathway in mesolimbic brain regions (Nestler and Aghajanian, 1997). Assessment of various α , β , and γ G protein subunits, CREB, and various protein kinase A subunits revealed only a significant increase in $G_{\alpha i2}$ subunit mRNA after acute and chronic cocaine self-administration. The present data contrast previous studies showing decreased ADP ribosylation and immunoreactivity $G_{\alpha i}$ and $G_{\alpha o}$ in the VTA of cocaine-treated rats (Nestler et al., 1990; Striplin and Kalivas, 1993). Increased $G_{\alpha i2}$ mRNA levels in the present study may serve to decrease adenylate cyclase activity and thereby decrease PKA phosphorylation of CREB. In addition, increased expression of PP2 α which dephosphorylates a number of different kinases and their substrates, including CaMKII (Millward et al., 1999), may further serve to regulate the activity of CREB and other constituents of the cAMP pathway in response to cocaine.

In summary, the present study provides direct evidence of cocaine-induced adaptations in VTA dopamine neurons after cocaine self-administration. Understanding alterations in the functional integrity of neurotransmission within the mesocorticolimbic dopamine system is a critical step in the development and/or refinement of pharmacotherapies for cocaine

addiction. Future characterization of altered gene and protein expression will provide a panoramic view of the potential molecular underpinnings of cocaine addiction. Future studies should include a comparison of genomic and proteomic alterations, examination of gene expression in sub-populations of VTA dopaminergic neurons based on axonal targets, assessment of binge cocaine access on gene and protein expression in these populations. Efforts should also include attempts to recapitulate biochemical alterations identified in human cocaine overdose victims in animal models to refine/generate more appropriate biological models of the cocaine addictive process.

Acknowledgments

This research was supported by the National Institute on Drug Abuse Grants DA13234 and DA13772 (to S.E.H.).

ABBREVIATIONS

VTA	ventral tegmental area
NAc	nucleus accumbens
PKA	cAMP-dependent protein kinase
FR	fixed ratio
LCM	laser capture microdissection
TH	tyrosine hydroxylase
PCR	polymerase chain reaction
SSC	sodium chloride-sodium citrate
nNOS	neuronal nitric-oxide synthase
PP2α	protein phosphatase 2A, catalytic subunit α
iGluR	ionotropic glutamate receptor
mGluR	metabotropic glutamate receptor
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR	NMDA receptor
CREB	cAMP response element-binding protein
CaMKII	calcium/calmodulin-dependent protein kinase II

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Fig. 1.

A, schematic of region for laser capture microdissection. Rat coronal midbrain section at -5.30 mm (relative to bregma) showing boundaries for VTA (shaded in black) from which tyrosine hydroxylase immunopositive cells were dissected. Midbrain section immunolabeled with anti-TH antibody (B) and the same section after microdissection of the indicated neurons (C) under $20\times$ magnification. Note the specificity of the dissections and the minimal disruption of surrounding neuropil. MM, mammillary nucleus; PAG, periaqueductal gray area; SNc, substantia nigra-pars compacta; SNr, substantia nigra-pars reticulata; RN, red nucleus; ml, medial lemniscus; cp, cerebral peduncle.

Fig. 2.

The mean ($\pm$ S.E.M.) number of infusions for cocaine self-administration for 1 (open circle) or 20 days (filled circles). There was no significant difference in the number of infusions on day one between the two groups.

Fig. 3.

Representative cDNA macroarray (95 clones and one blank spot) demonstrating the expression profile of VTA dopamine neurons. The signal intensity of each spot is proportional to the abundance of the particular mRNA in the sample. CCK, cholecystokinin; D1, dopamine receptor 1; D2, dopamine receptor 2; D3, dopamine receptor 3; D4, dopamine receptor 4; D1b, dopamine receptor 1b; DAT, dopamine transporter; BDNF, brain-derived neurotrophic factor; CART, cocaine and amphetamine regulated transcript; GAD65, glutamate decarboxylase 65 kDa; GAD67, glutamate decarboxylase 65 kDa; *Gai1*, G protein α inhibiting activity polypeptide 1; *Gai2*, G protein α inhibiting activity polypeptide 2; *Gai3*, G protein α inhibiting activity polypeptide 3; *Gas*, G protein α stimulating activity polypeptide; *Gao*, G protein α activating activity polypeptide O; *Gaz*, G protein α z polypeptide; *Gai15*, G protein α 15 (Gq class); *Gβ1*, G protein β polypeptide 1; *Gβ2*, G protein β polypeptide 2; *Gβ3*, G protein β polypeptide 3; *Gaq*, G protein Q polypeptide; *Gγ5*, = G protein γ polypeptide 5; *PP1α*, protein phosphatase 1, catalytic subunit, α isoform; *PP1β*, protein phosphatase 1, catalytic subunit, β isoform; *PP2α*, protein phosphatase 2, catalytic subunit, α isoform; *PP1γ*, protein phosphatase 1, catalytic subunit, γ isoform; *PP2β*, protein phosphatase 2, catalytic subunit, β isoform; *ABP*, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor binding protein; *GRM1a*, metabotropic glutamate receptor 1a; *GRM3*, metabotropic glutamate receptor 3; *GRM4*, metabotropic glutamate receptor 4; *GRM5*, metabotropic glutamate receptor 5; *GluRδ1*, glutamate receptor subunit delta1; *GluRδ2*, glutamate receptor subunit; *GRIN1*, glutamate receptor subunit, ionotropic, *N*-methyl-D-aspartate 1; *GRIN2A*, glutamate subunit receptor, ionotropic, *N*-methyl-D-aspartate 2A; *GRIN2B*, glutamate subunit receptor, ionotropic, *N*-methyl-D-aspartate 2B; *GRIN2C*, glutamate subunit receptor, ionotropic, *N*-methyl-D-aspartate 2C; *GRIN2D*, glutamate subunit receptor, ionotropic, *N*-methyl-D-aspartate 2D; *GRIA1*, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 1; *GRIA2*, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 2; *GRIA3*, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 3; *GRIA4*, glutamate receptor subunit,

ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 4; GRIK1, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 5; GRIK2, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 6; GRIK3, glutamate receptor subunit, ionotropic, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 7; GRIK4, glutamate receptor subunit, ionotropic, kainate 4; GRIK5, glutamate receptor subunit, ionotropic, kainate 5; SPCT, spectrin; citron; cript, cysteine-rich interactor of PDZ3; hom1c, homer 1c; PSD95, postsynaptic density protein SAPAP =/PSD-95-associated protein; GRIP1, glutamate receptor interacting protein 1; FRA1, fos-related antigen 1; FRA2, fos-related antigen 2; pDYN, prodynorphin; CaMKII α , calcium/calmodulin-dependent protein kinase 2 α ; CaMKII β , calcium/calmodulin-dependent protein kinase 2 β 3 subunit; CaMKII δ , calcium/calmodulin-dependent protein kinase 2 δ subunit; CaMKII γ , calcium/calmodulin-dependent protein kinase 2 γ subunit; CaMKIV, calcium/calmodulin-dependent protein kinase; CaMKK α , calcium/calmodulin-dependent protein kinase kinase α subunit; PKR1 α , cAMP-dependent protein kinase type I- α regulatory chain; PKR1 β , cAMP-dependent protein kinase type I- β regulatory chain; PKR2 β , cAMP-dependent protein kinase type II- β regulatory chain; GABA γ 1, GABA-A receptor subunit γ 1; GABA γ 2, GABA-A receptor subunit γ 2; GABA γ 3, GABA-A receptor subunit γ 3; GABA α 1, GABA-A receptor subunit α 1; GABA α 3, GABA-A receptor subunit α 3; GABA α 4, GABA-A receptor subunit α 4; GABA α 6, GABA-A receptor subunit α 6; GABA β 1, GABA-A receptor subunit β 1; GABA β 2, GABA-A receptor subunit β 2; GABA β 3, GABA-A receptor subunit β 3; GABA δ , GABA-A receptor subunit δ ; GABA ϵ , GABA-A receptor subunit ϵ ; PLD, phospholipase D; AKAP, protein kinase A anchor protein; synj2, synaptojanin 2; syntx5, syntaxin 5; synbv2, synaptobrevin 2; stat5b, signal transducer and activator of transcription 5B; RLZF-Y, rat lung zinc finger-Y; CB1, cannabinoid receptor 1; GralA, GTP-binding protein ral A; GralB, GTP-binding protein ral B; GRK4, G protein-dependent receptor kinase 4; grk5, G protein-dependent receptor kinase 5; CREB, blank.

Fig. 4.

Comparisons of gene expression changes in VTA tyrosine-hydroxylase immunopositive neurons after 1 or 20 days of cocaine self-administration. mRNA expression values correspond to hybridization intensity for individual transcripts and represent the relative abundance of mR-NAs normalized to the summated signal intensities for all spots on the blot (minus background; see *Materials and Methods*). *, $p < 0.05$; **, $p < 0.01$, compared with controls; #, $p < 0.05$; ##, $p < 0.01$ compared with 1-day cocaine.

TABLE 1

Summary of results for differences in relative gene expression following cocaine self-administration

Normalized expression values for designated transcript classes were analyzed using two way ANOVA (Group $\times$ Transcript) with repeated measures (Transcript). Post hoc analyses were conducted as needed using Tukey's test, and the null hypothesis was rejected when $P < 0.05$. P values are indicated for the main effect, interaction, and post hoc analyses.

Transcript	Main Effect	Interaction	Post Hoc Analysis	
			Control vs. 1 Day	Control vs. 20 Days
Dopamine-related	0.990	0.738		
Tyrosine hydroxylase (TH)				
Dopamine receptor 1 (D1)				
Dopamine receptor 2 (D2)				
Dopamine receptor 3 (D3)				
Dopamine receptor 4 (D4)				
Dopamine receptor 5 (D5)				
Dopamine transporter (DAT)				
Transcription factors	0.266	0.763		
Fos-related antigen 1 (FRA-1)				
Fos-related antigen 2 (FRA-2)				
Transcription factor stat 5b (TF-stat5b)				
Rat lung zinc finger protein Y1 (RLZF-Y)				
cAMP response element binding protein (CREB)				
GABA-related	<0.001	<0.001		0.014
Glutamic acid decarboxylase 65 (GAD 65)				
Glutamic acid decarboxylase 67 (GAD 67)				
GABA receptor type A, $\gamma 1$ subunit (GABA-A $\gamma 1$)				
GABA receptor type A, $\gamma 2$ subunit (GABA-A $\gamma 2$)			0.017	
GABA receptor type A, $\gamma 3$ subunit (GABA-A $\gamma 3$)				
GABA receptor type A, $\alpha 1$ subunit (GABA-A $\alpha 1$)			<0.001	
GABA receptor type A, $\alpha 3$ subunit (GABA-A $\alpha 3$)				
GABA receptor type A, $\alpha 4$ subunit (GABA-A $\alpha 4$)			0.033	0.030
GABA receptor type A, $\alpha 6$ subunit (GABA-A $\alpha 6$)			0.025	0.017

Transcript	Main Effect	Interaction	Post Hoc Analysis		
			Control vs. 1 Day	Control vs. 20 Days	1 Day vs. 20 Days
GABA receptor type A, β 1 subunit (GABA-A β 1)					
GABA receptor type A, β 2 subunit (GABA-A β 2)			0.030	0.023	
GABA receptor type A, β 3 subunit (GABA-A β 3)					
GABA receptor type A, δ subunit (GABA-A δ)			0.042	0.020	
GABA receptor type A, ε subunit (GABA-A ε)					
Glutamate-related	0.844	0.245			
AMPA receptor binding protein (AMPAbp)					
Metabotropic glutamate receptor 1a (mGluR1a)					
Metabotropic glutamate receptor 3 (mGluR3)					
Metabotropic glutamate receptor 4 (mGluR4)					
Metabotropic glutamate receptor 5 (mGluR5)					
Glutamate receptor δ 1 (GluR δ 1)					
Glutamate receptor δ 2 (GluR δ 2)					
NMDA receptor 1 (NR1)					
NMDA receptor 2A (NR2A)					
NMDA receptor 2B (NR2B)					
NMDA receptor 2C (NR2C)					
NMDA receptor 2D (NR2D)					
Glutamate receptor 1 (GluR1)					
Glutamate receptor 2 (GluR2)					
Glutamate receptor 3 (GluR3)					
Glutamate receptor 4 (GluR4)					
Glutamate receptor 5 (GluR5)					
Glutamate receptor 6 (GluR6)					
Glutamate receptor 7 (GluR7)					
Kainate receptor 1 (KA1)					
Kainate receptor 2 (KA2)					
G proteins	0.136	<0.001			
GTP-binding protein, α inhibiting polypeptide 1 (G α 1)					
GTP-binding protein, α inhibiting polypeptide 2 (G α 2)			<0.001	<0.001	

Transcript	Main Effect	Interaction	Post Hoc Analysis		
			Control vs. 1 Day	Control vs. 20 Days	1 Day vs. 20 Days
GTP-binding protein, α inhibiting polypeptide 3 ($G_{\alpha i3}$)					
GTP-binding protein, α stimulating polypeptide 1 ($G_{\alpha s1}$)					
GTP-binding protein, αo polypeptide ($G_{\alpha o}$)					
GTP-binding protein, αz polypeptide ($G_{\alpha z}$)					
GTP-binding protein, $\alpha 15$ polypeptide ($G_{\alpha 15}$)					
GTP-binding protein, $\beta 1$ polypeptide ($G_{\beta 1}$)					
GTP-binding protein, $\beta 2$ polypeptide ($G_{\beta 2}$)					
GTP-binding protein, $\beta 3$ polypeptide ($G_{\beta 3}$)					
GTP-binding protein, q polypeptide ($G_{\alpha q}$)					
GTP-binding protein, γ polypeptide ($G_{\gamma 5}$)					
GTP-binding protein α 1 ($G_{\alpha 1A}$)					
GTP-binding protein α 2 ($G_{\alpha 2B}$)	0.043	<0.001	<0.001	<0.001	0.042
Kinases					
Ca ²⁺ /calmodulin-dependent protein kinase II α (CaMKII α)					
Ca ²⁺ /calmodulin-dependent protein kinase II β (CaMKII β)					
Ca ²⁺ /calmodulin-dependent protein kinase II δ (CaMKII δ)					
Ca ²⁺ /calmodulin-dependent protein kinase II γ (CaMKII γ)					
Ca ²⁺ /calmodulin-dependent protein kinase IV (CaMKIV)					
CaMKII kinase α (CaMKK α)					
Protein kinase A, regulatory subunit 1A (PRKAR1A)					
Protein kinase A, regulatory subunit 1B (PRKAR1B)					
Protein kinase A, regulatory subunit 2B (PRKAR2B)					
G protein-coupled receptor kinase 4 (GRK4)					
G protein-coupled receptor kinase 5 (GRK5)					
Phosphatases					
Protein phosphatase 1A, catalytic subunit (PP1CA)	0.026	0.001			
Protein phosphatase 1B, catalytic subunit (PP1CB)					
Protein phosphatase 2A, catalytic subunit (PP2A)			<0.001	<0.001	
Protein phosphatase 1, γ subunit (PP1 γ)					

Transcript	Main Effect	Interaction	Post Hoc Analysis		
			Control vs. 1 Day	Control vs. 20 Days	1 Day vs. 20 Days
Protein phosphatase 2A, β subunit (PP2A β)					
Structural/signaling					
Spectrin α subunit (spectrin)	0.003	<0.001			
nNOS			0.001	0.019	0.001
Rho-interacting, serine/threonine kinase 21 (citron)					
Cysteine-rich interactor of PDZ3 (CRIPT)					
Homer, neural immediate early, gene 1C (homer1c)					
Post-synaptic density protein 95 kDa (PSD-95)					
PSD-95/SAP90-associated protein-1 (SAPAP)					
Glutamate receptor interacting protein 1 (GRIP1)					
Phospholipase D (PLD)					
A-kinase anchoring protein (AKAP)					
Synaptojanin II (SYNJN II)					
Syntaxin 5 (SYNTAX 5)					
Synaptobrevin 2 (SYNBREV 2)					
Peptide-related	0.413	0.208			
Precursor to cholecystokinin (pCCK)					
Brain-derived neurotrophic factor (BDNF)					
Cocaine/amphetamine-related transcript (CART)					
Prodynorphin (PDYN)					
Cannabinoid receptor 1 (CB1)					