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Discrete cell gene profiling of ventral tegmental dopamine neurons following acute and chronic cocaine self-administration

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Running Title: Cocaine-induce gene expression in VTA dopamine neurons

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Nonstandard abbreviations: aRNA—antisense RNA; CaMKII—calcium/calmodulin-dependent protein kinase II; DAT—dopamine transporter; DTT—dithiothreitol, GABA—γ-amino butyric acid; iGluR—ionotropic glutamate receptor; LCM—laser capture microdissection; LTP—long-term potentiation; NAc—nucleus accumbens; nNOS—neuronal nitric oxice synthase; PKA—cAMP-dependent protein kinase; PP2α—protein phosphatase 2A, catalytic subunit; TH—tyrosine hydroxylase; VTA—ventral tegmental area

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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 one or twenty 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 (α4, α6, β2, γ2 and δ) were downregulated at both one and twenty days of cocaine self-administration. In contrast, the catalytic subunit of protein phosphatase 2A (PP2α), GABA-A α1 and $G\alpha_{i2}$ were significantly increased at both time points. Additionally, calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) mRNA levels were increased initially followed by a slight decrease after 20 days, whereas neuronal nitric oxide synthase (nNOS) 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 appear 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.

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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 changes in 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 forebrain regions including the nucleus accumbens (NAc) (Ungerstedt 1971), considered a primary substrate for the reinforcing effects of cocaine. Acute and chronic cocaine administration produce significant elevations in NAc extracellular dopamine concentrations in animal models (Pettit and Justice 1989; Hemby et al. 1997b; Hemby et al. 1997a) - 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 increased activity of cyclic adenosine 3',5'-monophosphate (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

appears 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; Yamaguchi et al. 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 utilizing *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 utilizing 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; Hemby et al. 2003; Kamme et al. 2003).

Whereas the majority of studies investigating the molecular adaptations associated with chronic drug use have relied on non-contingent administration, several studies indicate pronounced biochemical differences between the contingent and non-contingent administration of drugs (Hemby et al. 1995; Hemby et al. 1997b; 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 following 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 following 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.

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Methods

Subjects and Surgical Procedures.

Male Sprague-Dawley rats (60-90 days; 225-275g; Charles River, 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 hour light/dark cycle (lights on: 8:00 pm) and fed *ad libitum* prior to surgery. Rats in the cocaine groups were implanted with chronic indwelling jugular catheters under isofluorane anesthesia, as described previously (Hemby et al., 1995, 1997a). Infusions of methohexital (100 µl; 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.

Following surgery, subjects were housed in standard operant conditioning chambers (24.5x23.5x21cm; Med Associates; St. Albans, VT) containing a retractable lever (0.25N 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 following surgery, rats were placed in their respective chambers and received infusions of heparinized 0.9% bacteriostatic saline (1.7 U/ml; 200 µl/30 min) for 48 h. On the following 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-1 (FR1), time-out 20 s (FR1:20 s TO) 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 following 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 CO_2 and transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde in PBS. 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 40ºC), 80% ethanol (30 min at 40ºC), twice in 95% ethanol (45 min at 40ºC), twice in 100% ethanol (45 min at 40ºC), twice in xylenes (45 min at 40ºC) and four times in paraffin (Paraplast; 30 min at 58ºC). Afterwards, 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 (58ºC). 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 (3ºC) to solidify the tissue block.

Immunocytochemistry and Laser Capture Microdissection (LCM)

Tissue blocks that included the mesencephalon were cut in 8 μm sections, then deparaffinized in ascending ethanol concentrations (1 min: 70%, 80%, and 2X 95%; 5 min: 2X 100%) followed by xylenes (2X 5 min) and RNase-free water (22ºC for 30 min). Next, sections were immersed in Tris buffer (0.1M, pH=7.4) for 5 min at 22ºC followed by immersion in 0.1M Tris buffer (pH=7.4)/3% denatured horse serum for 5 min at 22ºC. Sections were then incubated in primary monoclonal antibody against tyrosine hydroxylase (TH; 1:1000; Sigma T-1209) in 0.1M Tris buffer (pH=7.4)/3% denatured horse serum for 15 min at 22ºC. Sections were rinsed with 0.1M Tris buffer (pH=7.4) and incubated with a horse anti-mouse secondary antibody in 0.1M Tris buffer (pH=7.4) for 10 min at 22ºC, followed by a rinse with Tris buffer. Next, the conjugate was labeled using the avidin-biotin method (ABC Vectastain, Vector Laboratories; Burlingame, CA) in 0.1M Tris buffer (pH=7.4) for 10 min at 22ºC, followed by a rinse with Tris buffer. Immunolabeling was visualized with 3,5′-diaminobenzidine. Following the peroxidase reaction, sections were rinsed in RNase free water for 15 min and dehydrated in ascending ethanol concentrations (1min each in 70, 80, 95 and 100%) and xylenes for 5 min.

Slides were removed from xylene and air dried prior to microdissection. Laser dissection parameters for the Arcturus Pix Cell IIe system (Arcturus; Mountain View, CA) were as follows: diameter: 7 μm, power: 30-100 mW, duration: 400-1200 μs. 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 (Figure 1). After dissection, the cap was removed and placed on a CapSure Pad (Arcturus) to remove excess tissue.

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RNA extraction and amplification

Each cap was then secured to the top of a 0.5 ml Eppendorf microcentrifuge tube containing 200 µl Trizol reagent (Gibco BRL; Rockville, MD). The tube was inverted, vortexed and incubated at 22ºC for 15 min. RNA was then extracted with phenol/chloroform and precipitated with 100% ethanol (-80ºC) in the presence of 10 µg linear acrylamide. Following 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 then quick cooled on ice for 5 min. Complimentary DNA (cDNA) was synthesized using reverse transcriptase (AMVRT, Seikagaku; Falmouth, MA) in 1X reverse transcriptase buffer containing 7 mM dithiothreitol (DTT), 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 re-amplification procedures have been described in detail elsewhere (Van Gelder et al. 1990; Hemby et al. 2002; Hemby et al. 2003). During the second round of amplification, ^{33}P-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 10^6-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 intracellular signaling molecules. Inserts were amplified from plasmid stocks in 96 well plates using PCR with GF200 primers under the following conditions: 95ºC for five minutes (one cycle); 95ºC for twenty seconds, 55ºC for twenty seconds, and 72ºC for two minutes (40 cycles); and 72ºC for seven minutes (one cycle). Aliquots of PCR samples were electrophoresed on a 1% agarose gel (1X TAE, 0.05% ethidium bromide) at 5V/cm for PCR band size verification. Gel images were captured by digital camera and archived. PCR product concentration was determined by spectrofluorometry (Molecular Devices, Gemini; Sunnyvale, CA) using a 1:5000 dilution of SYBR 1 Green/TE and an aliquot of the PCR product. Values were compared to known concentrations of DNA standards for quantitation. Approximately 700 ng of each amplified insert was spotted on Nytran SuPerCharge® nylon transfer membrane (Schleicher and Schuell; Keene, NH) using a 96 well dot blot apparatus (Minifold I, Schleicher and Schuell). DNA was crosslinked to the membrane by ultraviolet radiation at 120,000 $\mu J/cm^2$.

Macroarrays were pre-hybridized with UltraHyb solution (Ambion; Austin, TX) in hybridization bottles for 1 hr at 42ºC. Next, ^{33}P-labelled aRNA probes from the VTA for each subject were heat denatured for 5 min at 70ºC, 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. Following hybridization, membranes were washed once briefly with 2X SSC/0.1% SDS, twice with 2X SSC/0.1% SDS for fifteen minutes each at 42°C and once with 0.1X SSC/0.1% SDS for ten minutes at 42°C. Labeled hybridized products were detected using phosphoimager cassettes, and hybridization signal intensities were analyzed using ImageQuant software (Amersham Biosciences; Sunnyvale, CA).

Data Analysis

Densitometry values (hybridization intensities) were obtained for each clone and for background (non-specific) 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; Hemby et al. 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 ANOVA (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 $p<0.05$.

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Results

Behavior

Rats in the acute cocaine group self-administered an average of 40 (± 10 S.E.M.) infusions (20 ± 5 mg cocaine) over the eight-hour self administration session (Figure 2). In the chronic cocaine group, rats self-administered an average of 31 (± 3) infusions (15 ± 3 mg cocaine) per day (Figure 2). As expected, the mean total cocaine intake for the chronic cocaine self-administration group (317.94 ± 32.19 mg) was significantly greater than the acute cocaine group (20.06 ± 5.01 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 eight-hour self-administration session. However, there was no significant difference in the number of infusions self-administered or in the gene expression pattern compared to the rest of the group.

Gene Expression

Analysis of the macroarrays revealed distinct patterns of expression for the majority of transcripts investigated (Figure 3). Of the 95 mRNAs examined, 11 were found to be differentially expressed among the groups (Figure 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.

DA-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(2,129)=1.403; P=0.266] and no significant interaction [F(8,129)=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(2,363)=10.864; P<0.001] and an interaction between GROUP and TRANSCRIPT [F(26,363)=2.869; P<0.001]. Of the fourteen GABA-related transcripts examined, post-hoc analysis revealed that expression of six GABA-A receptor subunits was altered following cocaine self-administration (Table I, Figure 4). In all cases where receptor subunit mRNAs were altered after acute cocaine self-administration, expression remained altered after twenty days of self-administration. mRNA levels of four transcripts (GABA-A α4, GABA-A α6, GABA-A β2 and GABA-A δ) were significantly decreased following both acute and chronic cocaine self-administration. The GABA-A γ2 subunit was significantly decreased following 20 days, but not one day, of self-administration. In contrast, α1 subunit mRNA was upregulated with cocaine self-administration at both time points. Additionally, expression of the enzyme GAD 65 was significantly decreased after chronic cocaine self-administration when 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

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glutamate-related transcripts (Table 1). However, GRIN2C, GRIA1 and GRIA3 were decreased slightly following acute cocaine but returned to control levels following chronic cocaine self-administration . In an opposite fashion, GRIN2B, GRIA2 and GRIK4 were increased following one day and decreased towards control levels by Day 21.

Other transcripts

A significant main effect of GROUP was observed for kinases [$F(2,285)=3.611$; $P=0.043$], phosphatases [$F(2,129)=4.299$; $P=0.026$] and structural/signaling [$F(2,337)=7.840$; $P=0.003$] transcripts. Additionally, G proteins, kinases, phosphatases and structural/signaling transcripts all showed a significant interaction between GROUP and TRANSCRIPT [$F(26,363)=3.651$; $P<0.001$; $F(20,285)=4.252$; $P<0.001$; $F(8,129)=3.6$; $P=0.001$; and $F(24,337)=2.671$; $P<0.001$, respectively]. Post hoc analyses revealed a significant increase in $G\alpha_{i2}$ mRNA following acute and chronic cocaine self-administration. Though not significant, $G\alpha_{i2}$ continued increase following chronic self-administration relative to acute self-administration. Both CaMKIIα and nNOS were significantly different for all pairwise comparisons between groups. Following an initial increase in mRNA expression with acute cocaine self-administration, CaMKIIα expression declined after twenty 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. PP2α mRNA levels were upregulated over control levels following both one day and twenty days of self-administration. Although the peptide-related category showed no main effect of GROUP [$F(2,129)=0.918$; $P=0.413$] and no interaction between GROUP and TRANSCRIPT [$F(8,129)=1.399$; $P=0.208$], there was a trend towards increased levels of pCCK after both acute and chronic self-administration.

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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 towards 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 where observed for several mRNAs and likely reflect the direct pharmacological actions of cocaine on the dopamine neurons assessed in the present study. Previously, our lab and others have employed 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; Hemby et al. 2003; Kamme et al. 2003; Fasulo and Hemby in press). Future studies directed towards protein confirmation and functional relevance of the observed mRNA change are warranted. The present study provides the first molecular profile of VTA DA neurons following 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, α1 and γ2 subunits were of similarly high abundance relative to the other subunits, yet the α1 subunit was upregulated while the γ2 subunit was downregulated with cocaine self-administration. These data are supported by previous studies demonstrating significant decreases in α1, α6, β2, β3 and γ2 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 γ2 subunit could also have a potentially profound effect on normal receptor function, since γ2 subunits are essential for receptor clustering (Essrich et al. 1998). Although of lower relative abundance, α4, α6, β2, and δ subunit mRNAs were also downregulated 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.

While 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; Ikemoto et al. 1997b). 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 upregulated 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 NR1 and GluR1 protein levels in the VTA following chronic cocaine administration (Fitzgerald et al. 1996; Churchill et al. 1999; Loftis and Janowsky 2000) as well as NR1, GluR2, GluR5 and KA2 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.

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Often activated in response to glutamatergic transmission, neuronal nitric oxide synthase (nNOS) was also differentially regulated in response to cocaine self-administration. nNOS produces nitric oxide (NO), 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 following 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 NO deficiency in other brain regions or in non-dopaminergic 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 amongst 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 IIα (CaMKIIα), 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 LTP 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 following 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 PP2α 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 following 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 subpopulations 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 in order to refine/generate more appropriate biological models of the cocaine addictive process.

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Footnotes:

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FIGURE LEGENDS

Figure 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 20x magnification. Note the specificity of the dissections and the minimal disruption of surrounding neuropil. Abbreviations: MM=mammillary nucleus; PAG=periaqueductal grey area; SNc= substantia nigra – pars compacta; SNr=substantia nigra – pars reticulata; RN= red nucleus, ml=medial lemniscus; cp=cerebral peduncle; VTA=ventral tegmental area.

Figure 2: The mean (± S.E.M.) number of infusions for cocaine self-administration for one (open circle) or twenty days (filled circles). There was no significant difference in the number of infusions on day one between the two groups.

Figure 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. Abbreviations: TH=tyrosine hydroxylase; 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 65kD; GAD67=glutamate decarboxylase 65kD; Gαi1=G protein alpha inhibiting activity polypeptide 1; Gαi2=G protein alpha inhibiting activity polypeptide 2; Gαi3=G protein alpha inhibiting activity polypeptide 3; Gαs= G protein alpha stimulating activity polypeptide; Gαo= G

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protein alpha activating activity polypeptide O; Gαz= G protein alpha z polypeptide; Gα15= G protein alpha 15 (Gq class); Gβ1= G protein beta polypeptide 1; Gβ2=G protein beta polypeptide 2; Gβ3= G protein beta polypeptide 3; Gαq=G protein Q polypeptide; Gγ5= G protein gamma polypeptide 5; PP1α= protein phosphatase 1, catalytic subunit, alpha isoform; PP1β= protein phosphatase 1, catalytic subunit, beta isoform; PP2α= protein phosphatase 2, catalytic subunit, alpha isoform; PP1γ= protein phosphatase 1, catalytic subunit, gamma isoform; PP2β= protein phosphatase 2, catalytic subunit, beta isoform; ABP=AMPA 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, NMDA 1; GRIN2A=glutamate subunit receptor, ionotropic, NMDA 2A; GRIN2B=glutamate subunit receptor, ionotropic, NMDA 2B; GRIN2C=glutamate subunit receptor, ionotropic, NMDA 2C; GRIN2D=glutamate subunit receptor, ionotropic, NMDA 2D; GRIA1=glutamate receptor subunit, ionotropic, AMPA 1; GRIA2= glutamate receptor subunit, ionotropic, AMPA 2; GRIA3=glutamate receptor subunit, ionotropic, AMPA 3; GRIA4=glutamate receptor subunit, ionotropic, AMPA 4; GRIK1=glutamate receptor subunit, ionotropic, AMPA 5; GRIK2=glutamate receptor subunit, ionotropic, AMPA 6; GRIK3= glutamate receptor subunit, ionotropic, AMPA 7; GRIK4=glutamate receptor subunit, ionotropic, kainate 4; GRIK5=glutamate receptor subunit, ionotropic, kainate 5; SPCT=spectrin; nNOS= nitric oxide synthase 1; citron; cript= cysteine-rich interactor of PDZ3; hom1c=homer 1c; PSD95=post-synaptic 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; CaMKIIα= calcium/calmodulin-dependent protein kinase 2 alpha; CaMKIIβ3= calcium/calmodulin-dependent protein kinase 2 beta 3 subunit; CaMKIIδ=calcium/calmodulin-dependent protein kinase 2 delta

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subunit; CaMKIIγ=calcium/calmodulin-dependent protein kinase 2 gamma subunit; CaMKIV=calcium/calmodulin-dependent protein kinase; CaMKKα=calcium/calmodulin-dependent protein kinase kinase alpha subunit; PKR1α=cAMP-dependent protein kinase type I-alpha regulatory chain; PKR1β=cAMP-dependent protein kinase type I-beta regulatory chain; PKR2β=cAMP-dependent protein kinase type II-beta regulatory chain; GABAγ1=GABA-A receptor subunit gamma 1; GABAγ2= GABA-A receptor subunit gamma 2; GABAγ3= GABA-A receptor subunit gamma 3; GABAα1= GABA-A receptor subunit alpha 1; GABAα3= GABA-A receptor subunit alpha 3; GABAα4= GABA-A receptor subunit alpha 4; GABAα6= GABA-A receptor subunit alpha 6; GABAβ1= GABA-A receptor subunit beta 1; GABAβ2= GABA-A receptor subunit beta 2; GABAβ3= GABA-A receptor subunit beta 3; GABAδ=GABA-A receptor subunit delta; GABAε=GABA-A receptor subunit epsilon; 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= cAMP responsive element binding protein; blank.

Figure 4: Comparisons of gene expression changes in VTA tyrosine-hydroxylase immunopositive neurons following one or twenty days of cocaine self-administration. mRNA expression values correspond to hybridization intensity for individual transcripts and represent the relative abundance of mRNAs normalized to the summated signal intensities for all spots on the blot (minus background; see Methods). * $p < 0.05$, ** $p<0.01$ compared with controls; # $p < 0.05$, ## $p < 0.01$ compared with one day cocaine.

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Table 1. Summary of results for differences in relative gene expression following cocaine self-administration.

Transcript	Main Effect	Interaction	*Post Hoc* Analysis		
			Control vs. 1 day	Control vs. 20 days	1 da vs. 20 day
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 (TFstat5b)			-	-	-
Rat lung zinc finger protein Y1 (RLZF-Y)			-	-	-
cAMP response element binding protein (CREB)			-	-	-
GABA-Related	<0.001	<0.001			
Glutamic acid decarboxylase 65 (GAD 65)			-	-	0.01
Glutamic acid decarboxylase 67 (GAD 67)			-	-	-
GABA receptor type A, γ1 subunit (GABA-A γ1)			-	-	-
GABA receptor type A, γ2 subunit (GABA-A γ2)			-	0.017	-
GABA receptor type A, γ3 subunit (GABA-A γ3)			-	-	-
GABA receptor type A, α1 subunit (GABA-A α1)			<0.001	<0.001	-
GABA receptor type A, α3 subunit (GABA-A α3)			-	-	-
GABA receptor type A, α4 subunit (GABA-A α4)			0.033	0.030	-
GABA receptor type A, α6 subunit (GABA-A α6)			0.025	0.017	-
GABA receptor type A, β1 subunit (GABA-A β1)			-	-	-
GABA receptor type A, β2 subunit (GABA-A β2)			0.030	0.023	-
GABA receptor type A, β3 subunit (GABA-A β3)			-	-	-
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 δ1 (GluRδ1)			-	-	-
Glutamate receptor δ2 (GluRδ2)			-	-	-
NMDA receptor 1 (NR1)			-	-	-
NMDA receptor 2A (NR2A)			-	-	-
NMDA receptor 2B (NR2B)			-	-	-
NMDA receptor 2C (NR2C)			-	-	-
NMDA receptor 2D (NR2D)			-	-	-

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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 ($G_{\alpha i}1$)			-	-	-
GTP-binding protein, α inhibiting polypeptide 2 ($G_{\alpha i}2$)			<0.001	<0.001	-
GTP-binding protein, α inhibiting polypeptide 3 ($G_{\alpha i}3$)			-	-	-
GTP-binding protein, α stimulating polypeptide 1 ($G_{\alpha s}1$)			-	-	-
GTP-binding protein, αo polypeptide ($G_{\alpha o}$)			-	-	-
GTP-binding protein, αz polypeptide ($G_{\alpha z}$)			-	-	-
GTP-binding protein, α15 plypeptide ($G_{\alpha 15}$)			-	-	-
GTP-binding protein, β1 polypeptide (Gβ1)			-	-	-
GTP-binding protein, β2 polypeptide (Gβ2)			-	-	-
GTP-binding protein, β3 polypeptide (Gβ3)			-	-	-
GTP-binding protein, q polypeptide ($G_{\alpha q}$)			-	-	-
GTP-binding protein, γ polypeptide (Gγ5)			-	-	-
GTP-binding protein ral A (GralA)			-	-	-
GTP-binding protein ral B (GralB)			-	-	-
Kinases	0.043	<0.001			
Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα)			<0.001	<0.001	0.04
Ca^{2+}/calmodulin-dependent protein kinase IIβ3 (CaMKIIβ3)			-	-	-
Ca^{2+}/calmodulin-dependent protein kinase IIδ (CaMKIIδ)			-	-	-
Ca^{2+}/calmodulin-dependent protein kinase IIγ (CaMKIIγ)			-	-	-
Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV)			-	-	-
CaMKII kinase α (CaMKKα)			-	-	-
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	0.026	0.001			
Protein phosphatase 1A, catalytic subunit (PP1CA)			-	-	-
Protein phosphatase 1B, catalytic subunit (PPP1CB)			-	-	-
Protein phosphatase 2A, catalytic subunit (PP2α)			<0.001	<0.001	-
Protein phosphatase 1, γ subunit (PP1γ)			-	-	-
Protein phosphatase 2A, β subunit (PP2Aβ)			-	-	-
Structural/Signaling	0.003	<0.001			
Spectrin α subunit (spectrin)			-	-	-
Neuronal nitric oxide synthase (nNOS)			0.001	0.019	0.00
Rho-interacting, serine/threonine kinase 21 (citron)			-	-	-
Cysteine-rich interactor of PDZ3 (CRIPT)			-	-	-
Homer, neural immediate early, gene 1C (homer1c)			-	-	-
Post-synaptic density protein 95kD (PSD-95)			-	-	-
PSD-95/SAP90-associated protein-1 (SAPAP)			-	-	-
Glutamate receptor interacting protein 1 (GRIP1)			-	-	-
Phospholipase D (PLD)					

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Phospholipase D (PLD)			-	-	-
A-kinase anchoring protein (AKAP)			-	-	-
Synaptojanin II (SYNJN II)			-	-	-
Syntaxin 5 (SYNTX 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)			-	-	-

Normalized expression values for designated transcript classes were analyzed using two way ANOVA (Group x 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.

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Figure 1

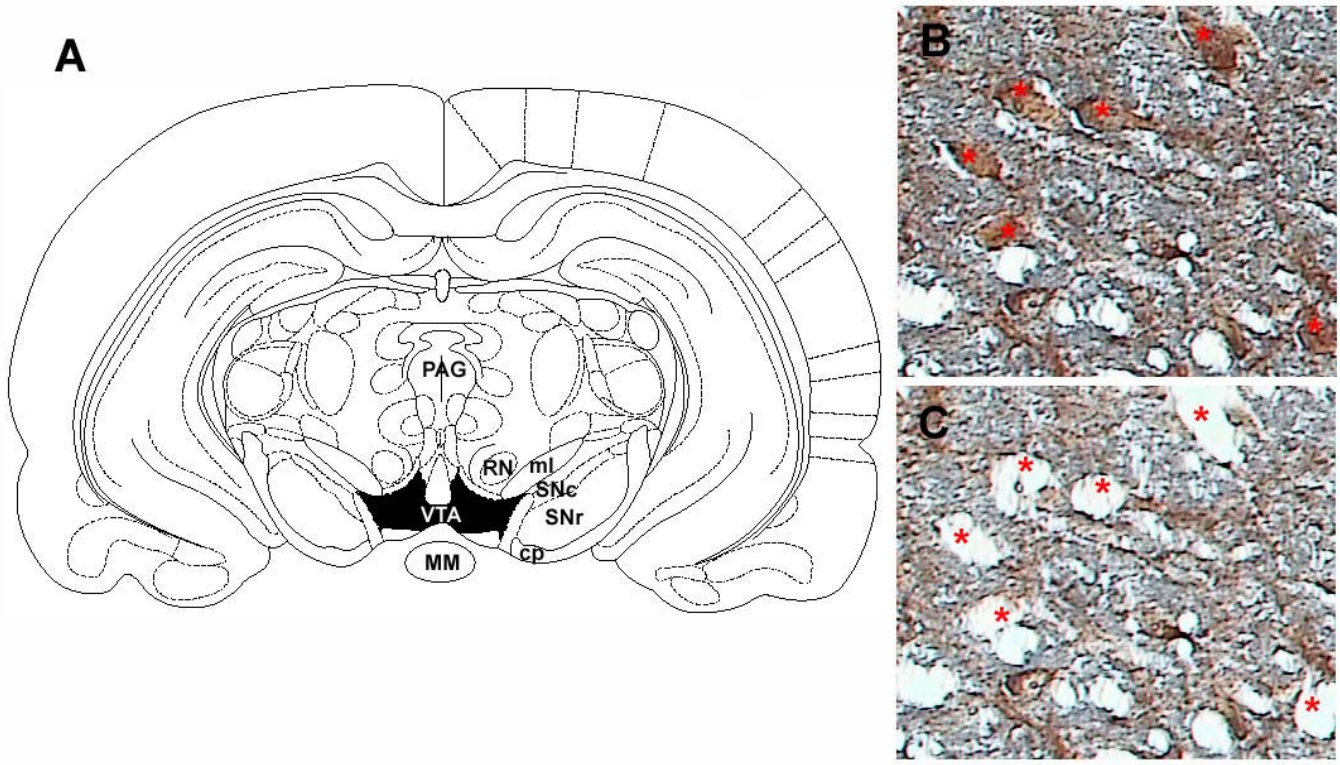

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Figure 2

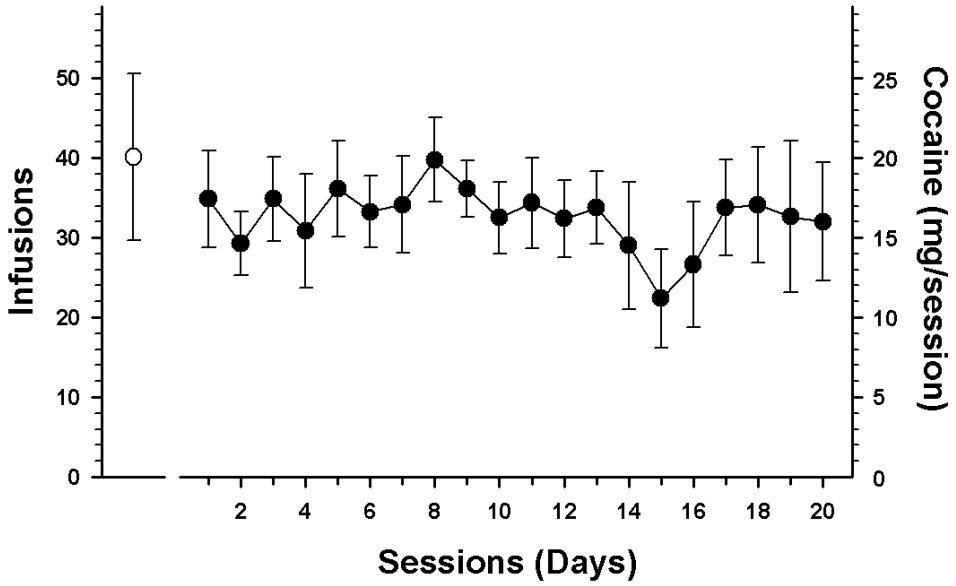

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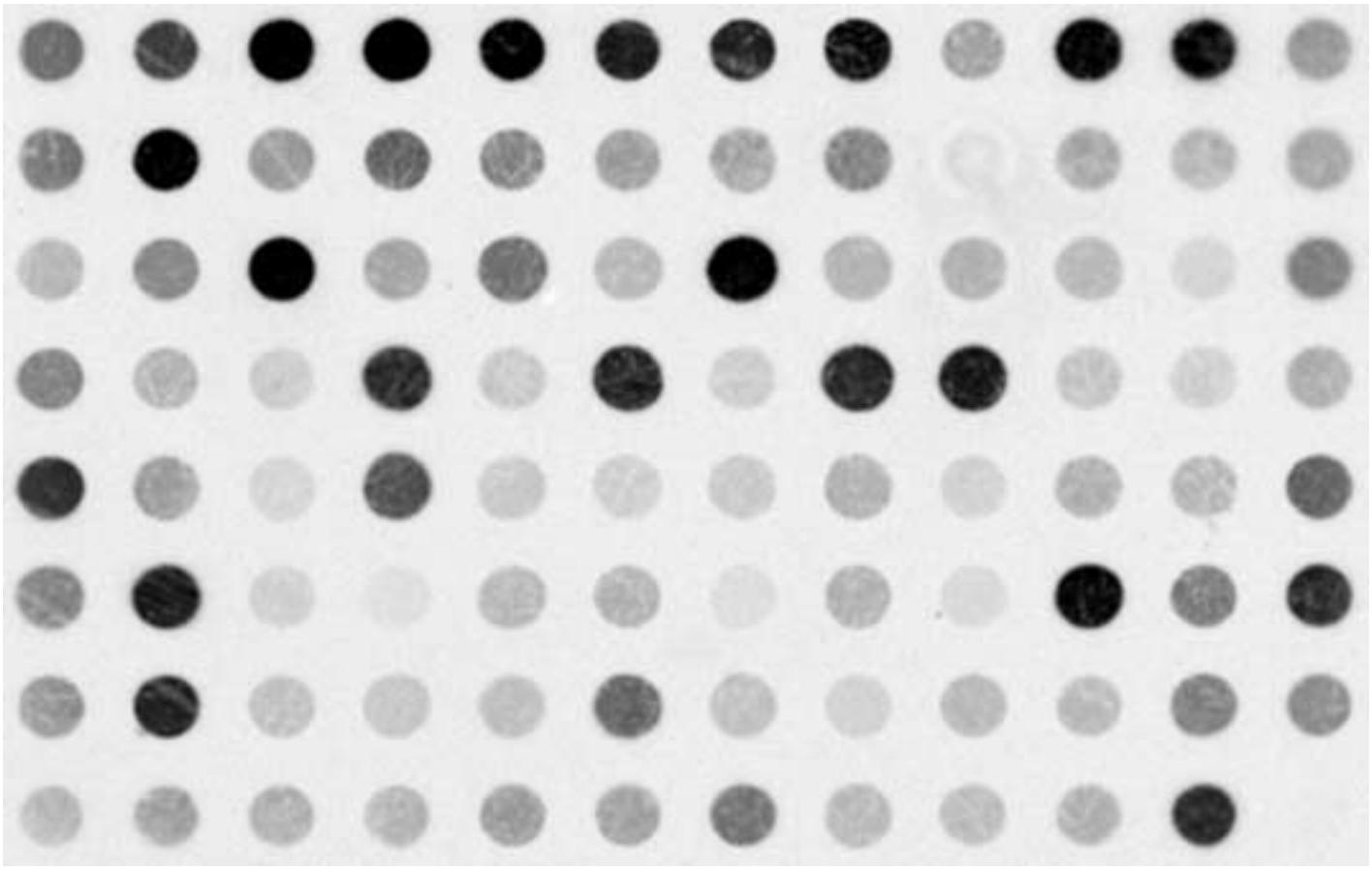
Figure 3

A



B

TH	pCCK	D1	D2	D3	D4	D1b	DAT	BDNF	CART	GAD65	GAD67
Gαi1	Gαi2	Gαi3	Gαs	Gαo	Gαz	Gα15	Gβ1	Gβ2	Gβ3	Gαq	Gγ5
PP1α	PP1β	PP2α	PP1γ	PP2β	ABP	GRM1a	GRM3	GRM4	GRM5	GRID1	GRID2
GRIN1	GRIN2A	GRIN2B	GRIN2C	GRIN2D	GRIA1	GRIA2	GRIA3	GRIA4	GRIK1	GRIK2	GRIK3
GRIK4	GRIK5	SPCT	nNOS	citron	CRIPT	homer1c	PSD95	SAPAP	GRIP1	FRA1	FRA2
pDYN	CaMKIIα	CaMKIIβ3	CaMKIIδ	CaMKIIγ	CaMKIV	CaMKKα	PKAR1α	PKAR1β	PKAR2β	GABAγ1	GABAγ2
GABAγ3	GABAα1	GABAα3	GABAα4	GABAα6	GABAβ1	GABAβ2	GABAβ3	GABAδ	GABAε	PLD	AKAP
synjnII	syntx5	synbrev2	stat5b	RLZFY	CB1	GralA	GralB	GRK4	GRK5	CREB	blank

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Figure 4