Published in final edited form as:

J Neurochem. 2003 May ; 85(4): 911–924.

Molecular profiling of midbrain dopamine regions in cocaine overdose victims

Wen-Xue Tang^{*}, Wendy H. Fasulo^{*}, Deborah C. Mash[†], and Scott E. Hemby^{*}

^{*}Department of Pharmacology and Psychiatry/Behavioral Sciences, Yerkes National Primate Research Center, Neuroscience Division, Emory University School of Medicine, Atlanta, Georgia, USA

[†]Department of Neurology and Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida, USA

Abstract

Chronic cocaine use in humans and animal models is known to lead to pronounced alterations in neuronal function in brain regions associated with drug reinforcement. To evaluate whether the alterations in gene expression in cocaine overdose victims are associated with specific dopamine populations in the midbrain, cDNA arrays and western blotting were used to compare gene and protein expression patterns between cocaine overdose victims and age-matched controls in the ventral tegmental area (VTA) and lateral substantia nigra (l-SN). Array analysis revealed significant up-regulation of numerous transcripts in the VTA, but not in the l-SN, of cocaine overdose victims including NMDAR1, GluR2, GluR5 and KA2 receptor mRNA ($p < 0.05$). No significant alterations between overdose victims and controls were observed for GluR1, R3 or R4 mRNA levels. Correspondingly, western blot analysis revealed VTA-selective up-regulation of CREB ($p < 0.01$), NMDAR1 ($p < 0.01$), GluR2 ($p < 0.05$), GluR5 ($p < 0.01$) and KA2 ($p < 0.05$) protein levels of cocaine overdose victims. The present results indicate that selective alterations of CREB and certain ionotropic glutamate receptor (iGluR) subtypes appear to be associated with chronic cocaine use in humans in a region-specific manner. Moreover, as subunit composition determines the functional properties of iGluRs, the observed changes may indicate alterations in the excitability of dopamine transmission underlying long-term biochemical and behavioral effects of cocaine in humans.

Keywords

cocaine; CREB; gene expression; glutamate; substantia nigra; ventral tegmental area

Neuropharmacological evidence in animal models and imaging studies in humans indicate that the mesocorticolimbic dopamine pathway is regulated by cocaine administration and that the functional integrity of this pathway is essential for the reinforcing and euphoric effects of cocaine (Ritz *et al.* 1987; Wise and Bozarth 1987; Di Chiara and Imperato 1988; Koob and Bloom 1988; Volkow *et al.* 1999). The mesocorticolimbic pathway originates in the ventral tegmental area (VTA) and projects to several forebrain regions, most notably the nucleus accumbens (NAc), a region implicated in cocaine reinforcement. Cocaine administration produces significant elevations in NAc and VTA extracellular dopamine concentrations in animal models (Pettit and Justice 1989, 1991; Iyer *et al.* 1995; Hemby *et*

al. 1997a, 1999; Czoty *et al.* 2000) and alters metabolic function and blood flow in terminal regions following chronic cocaine use in humans (Volkow *et al.* 1988; Holman *et al.* 1991, 1993; Volkow *et al.* 1991; Strickland *et al.* 1993; Levin *et al.* 1994), effects that contribute to the abuse liability of cocaine (Ritz *et al.* 1987; Bergman *et al.* 1989).

In addition to the direct interaction between cocaine and the dopamine transporter and indirect agonist effects on dopamine receptor subtypes, neuroadaptive processes are induced by cocaine that may lead to persistent or even permanent alterations in neuronal function (Nestler 1993; White *et al.* 1995b; Nestler 1997; Pierce and Kalivas 1997; White and Kalivas 1998). Alterations in the expression of genes associated with dopaminergic neurotransmission have been an area of investigation into the neuroadaptations induced by chronic cocaine administration. The intracellular cascade mediating such events include a generalized up-regulation of the cyclic adenosine 3',5'-monophosphate (cAMP) pathway (Nestler *et al.* 1990; Terwilliger *et al.* 1991; Striplin and Kalivas 1992; Miserendino and Nestler 1995; Carlezon *et al.* 1998; Self *et al.* 1998; Pliakas *et al.* 2001) and activator protein 1 family members (Hope *et al.* 1992; Couceyro *et al.* 1994; Nye *et al.* 1995; Hiroi *et al.* 1997; Pich *et al.* 1997; Haile *et al.* 2001). For example, chronic cocaine administration increased the formation of cAMP-dependent protein kinase A (PKA) and adenylate cyclase in the nucleus accumbens of rats (Terwilliger *et al.* 1991), as well as mRNA and protein levels of the catalytic subunit of PKA and other potential transcriptional regulators in the NAc of rhesus monkeys following chronic cocaine administration (Freeman *et al.* 2001). Interestingly, stimulation of PKA or over-expression of cAMP responsive element binding protein (CREB) in the NAc attenuate the rewarding effects of cocaine (Carlezon *et al.* 1998; Self *et al.* 1998; Pliakas *et al.* 2001), suggesting that adaptive changes induced by cocaine on this intracellular cascade can alter subsequent responsiveness of the drug.

In addition to dopamine, several studies indicate an involvement of glutamate transmission in the VTA and NAc in the mediation of the behavioral and neurochemical effects of cocaine as well as neuroadaptations induced by chronic cocaine administration. AMPA and kainate subunits contribute to fast neurotransmission and all three ionotropic glutamate receptor (iGluR) subtypes are thought to play roles in long-term potentiation induced by cocaine (Nestler 2001; Ungless *et al.* 2001). iGluRs are classified as NMDA (NR1, NR2A-D, NR3) (+/-)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; GluR1-4), and kainate (GluR5-7, KA1-2) receptor subunits based on their pharmacological characteristics and sequence information (Hollmann and Heinemann 1994; Borges and Dingledine 2002). Previous studies have shown cocaine administration increased extracellular glutamate concentrations in the NAc and VTA (Pierce *et al.* 1996; Reid *et al.* 1997; Kalivas and Duffy 1998), produced behavioral sensitization (Pierce *et al.* 1996) and resulted in increased responsivity of glutamate receptor stimulation in the NAc and VTA (White *et al.* 1995a; Zhang *et al.* 1997) with as little as one exposure to cocaine (Ungless *et al.* 2001). Increased AMPA and NMDA receptor expression in the VTA has been proposed as a possible mechanism for increased excitability of VTA dopamine neurons and behavioral sensitization to cocaine (White *et al.* 1995a; Zhang *et al.* 1997). Indeed, GluR1 and NR1 protein levels were increased in the VTA following chronic cocaine administration (Fitzgerald *et al.* 1996) and remained elevated following one day, but not three weeks, of withdrawal in rats that manifest behavioral sensitization to cocaine (Churchill *et al.* 1999). In contrast, others have reported elevated VTA NR1 protein levels after protracted withdrawal periods (3 and 14 days; (Loftis and Janowsky 2000) and no change in VTA GluR1 levels following 16–24 h of withdrawal (Lu *et al.* 2002). The discrepant results may be caused by several factors including dosing regimen, time since last cocaine injection, or the protein analysis procedures. In contrast to the protein data, NR1 mRNA levels were decreased in the VTA following acute, but not chronic, cocaine administration (Ghasemzadeh *et al.* 1999), whereas GluR1 and GluR2/3 subunit mRNAs in the VTA were not altered by cocaine

administration in rats (Ghasemzadeh *et al.* 1999; Lu *et al.* 2002). Alterations in iGluRs in the VTA may represent another adaptive mechanism by which chronic cocaine can exert long-term effects on mesocorticolimbic dopamine function which, in turn, may affect subsequent drug intake.

Whereas animal models have advanced our understanding of the neurobiological basis of drug addiction, the evaluation of similar questions in human tissue are few, yet essential. Parallel investigations in human post-mortem tissue are important to determine whether biochemical changes observed and characterized in animal models are relevant to human drug abuse, as well as to identify novel changes that may be indicative of the human condition. The present study was undertaken to evaluate differences in gene expression in the VTA of cocaine overdose victims and age-matched, drug-free controls. In addition, we evaluated gene expression in the l-SN to provide a measure of brain region specificity. To this end, custom-designed macroarrays were used to simultaneously assess 81 genes and test the hypothesis that functional classes of genes were differentially expressed between cocaine overdose victims and controls in the VTA. Computational analyses were used to partition the data into groups of clones with similar function in order to facilitate the interpretation of these data to the relevance of cocaine addiction. Select genes that were differentially expressed were evaluated at the protein level by western blot analysis.

Experimental procedures

Subjects and tissue

Post-mortem human brain tissue was obtained at autopsy from 11 age-matched, drug-free control individuals [eight white males, one hispanic male, one black male and one white female; aged 35.0 ± 2.3 years; post-mortem interval (PMI) = $13.2 \text{ h} \pm 0.6 \text{ h}$] and 10 cocaine overdose victims (eight white males and two black males; aged 35.5 ± 2.2 years; PMI = $12.3 \text{ h} \pm 0.9 \text{ h}$) by the University of Miami Brain Endowment Bank (Miami, FL, USA) (Table 1). For protein analysis, tissue punches were dissected from the VTA and l-SN of the contralateral hemisphere and used for RNA analysis in eight cocaine overdose victims (5WM, 3BM; aged 34.4 ± 2.7 years; PMI = $12.5 \pm 1.1 \text{ h}$) and seven age-matched, drug free controls (6WM, 1WF; aged 37 ± 2.7 years; PMI = $12.6 \pm 0.7 \text{ h}$), representing a subset of subjects from whom tissue was used in the RNA experiments. Gross and microscopic diagnostic neuropathologic examinations, which included examination of multiple cortical and subcortical regions, were performed in all cases and no neuropathological abnormalities relevant to mental status were found. All cases were retrospectively accrued based on toxicological data and circumstances surrounding the death, including review of prior arrest records and treatment admissions, as well as pathological indications (e.g. perforation of the nasal septum and needle track marks), and were reviewed carefully before classifying a cocaine intoxication case. All cases were evaluated for common drugs of abuse and alcohol, and positive urine screens were confirmed by quantitative analysis of blood. Cocaine and benzoylecgonine concentrations in brain and blood were assessed using gas chromatography/mass spectroscopy as described previously (Hernandez *et al.* 1994).

Following removal, brains were photographed and cut into 1.5-cm coronal blocks. Brain tissue was cryopreserved using a procedure adopted from previous studies (Hardy *et al.* 1983; Dodd *et al.* 1986). Punches (100 mg) were dissected from the blocks containing the VTA (posterior region of slab) and SN (anterior region containing the pars lateralis and the pars medialis): one hemisphere for RNA and the contralateral hemisphere for protein analysis. Possible neuronal loss, ischemic cell changes, and reactive gliosis were assessed using semiquantitative ratings by the neuropathologists and found to be negligible in all cases used in the present study.

RNA isolation and amplification

Micro-FastTrack Kits (Invitrogen, Carlsbad, CA, USA) were used to isolate polyadenylated RNA from the VTA and l-SN. mRNA yields ranged from 1 to 2 µg/100 ng of frozen tissue. Because of the quantity of mRNA, samples from each subject were amplified using a modification of the aRNA amplification procedure described previously (Ginsberg *et al.* 2000; Hemby *et al.* 2002, 2003). Oligo(dT)-T7-primer/promoter (500 ng) [AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGC(T)24] was hybridized to poly(A⁺)mRNA in the presence of 500 µM each of dNTPS- and RNase-free water for 30 min at 65°C then quick cooled on ice for 5 min. Following this, 10 mM dithiothreitol (DTT), 1X first-strand buffer and 20 U RNasin were added and samples were incubated at 42°C for 2 min (Van Gelder *et al.* 1990). Reverse transcriptase (1 µL Superscript II, RNase H⁻; Invitrogen; Carlsbad, California) was added to the solution and incubated at 42°C for 60 min. Following phenol/chloroform extraction and ethanol precipitation in the presence of 10 µg linear acrylamide, samples were re-suspended in RNase free water and heat denatured at 94°C for 5 min, then quick cooled. Next, 1X second-strand buffer, 250 mM each of dNTPs, 10 U Klenow fragment and 10 U RNase H were added to the sample and incubated at 16°C for 4–6 h (Van Gelder *et al.* 1990). At the end of the incubation, 2.5 mM -nictotanimide adenine dinucleotide and 1000 U *Escherichia coli* DNA ligase were added to the solution and incubated at room temperature (22°C) of 15 min, followed by the addition of 5 U T4 DNA polymerase at room temperature for 15 min (Sambrook and Russell 2001). At the end of the incubation, 2.5 mM ethylenediaminetetraacetic acid (EDTA) was added to stop the enzymatic reaction followed by phenol/chloroform extraction and ethanol precipitation. Following drop dialysis against RNase/DNase-free water for 15 min, purified cDNA templates were used to generate antisense RNA (aRNA). aRNA was synthesized using T7 RNA polymerase (Epicentre Technologies; Madison, WI, USA) in 1X transcription buffer (Epicentre Technologies), 10 mM DTT, 250 µM each of NTPs and 20 U RNasin at 37°C overnight. Following phenol/chloroform extraction and ethanol precipitation, aRNA was reverse transcribed using Superscript II in the presence of 1X first-strand buffer, 250 mM each of dNTPs, 100 ng of random hexamers and 20 U RNasin incubated at 37°C for 1 h. For second-strand cDNA synthesis, samples were incubated with 10 U Klenow fragment, 250 mM each of dNTPs, 500 ng of the aforementioned oligo dT-T7 primer-promoter, and 5 U T4 DNA polymerase at 16°C overnight. Samples were phenol/chloroform extracted, ethanol precipitated and drop dialyzed for 15 min. For the second round of amplification, reaction conditions were identical for the first round except for the inclusion of 30 µCi [³³P]UTP (2500 Ci/mmol; Amersham Pharmacia Biotech; Piscataway, NJ, USA), 10 µM of UTP and 250 µM each of CTP, GTP and ATP. 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. mRNA can be reliably amplified from small quantities of fixed tissue including individual neurons and neuronal processes (Van Gelder *et al.* 1990; Eberwine *et al.* 1992; Ginsberg *et al.* 1999; Hemby *et al.* 2002, 2003). Although there is a general decrease in mRNA levels in autopsied tissue, the relative abundance of gene expression remains unchanged (Castensson *et al.* 2000).

Macroarray procedures

Reverse northern blots were prepared on nylon membranes containing, but were not limited to, dopamine receptors (e.g. D1, D2, D4, D5 and DAT), G protein subunits (α_1 , α_2 , α_s , α_z , α_q , α_o , β_1 and β_2), glutamate receptor mRNAs (mGluR3, GRIA1-4, GRIK5, 7 and GRIN1), GABA_A receptor subunits (α_1 , α_2 , α_3 , α_4 , α_5 , α_6 and α_7), regulators of G protein signaling (RGS1–7, 9, 10, 12, 13 and 16) and other transcripts (cannabinoid 1 receptor, cocaine–amphetamine regulated transcript (CART), serotonin 2 and serotonin 3 receptors and tyrosine hydroxylase). Inserts were amplified in 96-well plates using PCR with GF200

primers under the following conditions: 95°C for 5 min (1 cycle); 95°C for 30 s, 52°C for 45 s and 72°C for 2 min (40 cycles); and 72°C for 7 min (1 cycle). PCR samples were purified (Multiscreen PCR filter plate; Millipore, Bedford, MA, USA) and aliquots were electrophoresed on a 1% agarose gel (1X TAE, 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, Sunnyvale, CA, USA) using a 1 : 5000 dilution of SYBR 1 Green/TE and an aliquot of the PCR product. Values were compared with concentrations of known DNA standards for quantitation. Each amplified insert (250 ng) was spotted on Nytran SuPerCharge[®] nylon transfer membrane (Schleicher and Schuell, Keene, NH, USA) using a 96-well dot blot apparatus (Minifold I; Schleicher and Schuell). DNA was crosslinked to the membrane by ultraviolet radiation.

Arrays were prehybridized with UltraHyb solution (Ambion, Austin, TX, USA) in hybridization bottles for 1 h at 42°C. Next, ³³P-labeled aRNA probes from each subject were heat denatured for 5 min at 65°C, and then immediately added to their respective bottles and allowed to hybridize for 18–24 h at 42°C in a rotisserie hybridization oven. Following hybridization, membranes were washed twice with 2X SSC/0.1% sodium dodecyl sulfate (SDS) and 0.1X SSC/0.1% SDS for 20 min each at 42°C. Labeled hybridized products were detected using phosphorimager screens, and hybridization signal intensities were analyzed using IMAGEQUANT software (Amersham Biosciences, Sunnyvale, CA, USA).

Protein preparation and western blot analysis

Tissue samples were dounce homogenized in 10 mM HEPES, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM EDTA and the following protease inhibitors (PI): 1 mM PMSF, 10 mM benzamidine, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 µg/mL pepstatin and centrifuged at 9645 *g* for 5 min. Supernatant (cytosol and membrane) was removed and the pellet (nuclei and debris) was resuspended in 20 mM Tris HCl, 1 mM EDTA (pH 8.0) with PIs and centrifuged at 9645 *g* for 5 min. This procedure was repeated twice and the pellet was resuspended in the solution and stored at –20°C (nuclear fraction). Supernatant was centrifuged at 107 170 *g* for 30 min at 4°C. Following, the supernatant containing the cytosolic fragment was removed and stored at –20°C (cytosolic fraction). The pellet was re-suspended in 10 mM Tris (pH = 7.5), 300 mM sucrose, 1 mM EDTA (pH = 8.0), 0.1% NP40 and PIs and centrifuged at 4287 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in the buffer and washed three times before re-suspension in the buffer and PIs and storing the samples at –20°C (membrane fraction).

Protein concentrations of samples were calculated using the bicinonchonic acid protein assay kit (Pierce, Rockford, IL, USA) and diluted in Laemmli sample buffer to achieve the same final protein concentration. Identical quantities of proteins were loaded into a gel electrophoresis apparatus, subjected to sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (Bio-Rad, Richmond, CA) and transferred to nitrocellulose by electroblotting (30 V, overnight at 4°C) in 1X transfer buffer (Bio-Rad). Nitrocellulose membranes were blocked in 0.5% non-fat dry milk and 0.1% Tween 20 in phosphate-buffered saline (pH 7.4, 0.12 M) for 1 h at room temperature prior to being incubated with primary antibodies in blocking buffer (Bio-Rad) overnight at 4°C followed by secondary antibody for 1 h at room temperature. Protein bands were visualized on a Kodak XAR-5 film with enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech). Primary antibodies were as follows: mouse monoclonal antibodies directed against NMDAR1 (Chemicon International, Temecula, CA, USA) and GluR5 (Upstate Biotechnologies, Waltham, MA, USA); rabbit polyclonal antibodies directed against GluR1, GluR2/3, KA2, G_s, G_{i1/2}, G_q, CREB, phospho-CREB (Upstate Biotechnologies) and fos-related antigen

2 (FRA2) (L-15; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were HRP-conjugated anti-rabbit IgG and HRP-conjugated antimouse IgG (Upstate Biotechnologies). Protein abundances were calculated by optical densitometry with a Scan Jet 2200C and imported into NIH IMAGE 1.61 software (National Institutes of Health, Bethesda, MD, USA). Film background was subtracted from the optical density values to give the optical density value for a single subject. All assays were conducted under conditions in which densitometric signal intensity was linear with protein concentration as determined in preliminary experiments.

Data analysis

Specific signal (minus background) of probe bound to each PCR product was expressed as a ratio of the total hybridization intensity of spots of the custom-designed array, thereby minimizing variations resulting from differences in the specific activity of the probe and the absolute quantity of probe present (Ginsberg *et al.* 2000; Hemby *et al.* 2002, 2003). Array data were analyzed using a two-way ANOVA with group and anatomical region as the fixed effects and normalized hybridization intensity as the dependent measure. *Post-hoc* analyses were conducted as needed using Tukey's test and the null hypothesis was rejected when $p < 0.05$. Western blot data were analyzed using *t*-tests comparing the band intensities between cocaine overdose victims and controls for each respective protein. Data were graphically depicted as the percentage of the controls for each respective protein. Null hypotheses were rejected when $p < 0.05$.

Results

Subject data

There was no significant difference in age between cocaine overdose victims and age-matched, non-drug controls ($t = -0.156$, d.f. = 19, $p = 0.877$), post-mortem interval ($t = 0.847$, d.f. = 19, $p = 0.408$), or brain pH ($t = 0.431$, d.f. = 17, $p = 0.672$) indicating that these factors did not significantly influence the observed changes in gene or protein expression. Furthermore, neuroadaptive changes in the human brain post-mortem reflect chronic cocaine abuse, as death in a naive user is a rare occurrence, and the cohort of post-mortem subjects have many surrogate measures of chronicity (Ruttenber *et al.* 1997).

Gene expression

Custom-designed macroarrays containing 81 cDNAs were used to evaluate gene expression changes in the VTA and l-SN of cocaine overdose victims and controls. Although limited in scope compared with high-density arrays, the arrays contained a variety of transcripts including iGluR subunits ($n = 8$), dopamine signaling ($n = 7$), GABA transcripts ($n = 10$), G protein subunits ($n = 14$), GTPase/RGS proteins ($n = 16$), mRNA processing transcripts ($n = 8$), cell growth/death transcripts ($n = 5$) and others ($n = 13$).

Receptors—Several groups have reported alterations in glutamate receptor subunit protein and mRNA levels following cocaine administration. Consistent with these findings, we report a significant effect of cocaine overdose on glutamate receptor subunit mRNA expression in the VTA ($F_{1,209} = 18.541$; $p < 0.001$) with a significant interaction between transcript and group ($F_{1,9} = 2.311$; $p < 0.017$). *Post-hoc* analysis revealed a significant increase in NMDAR1, GluR2, GluR5 and KA2, but not GluR1, GluR3 or GluR4 glutamate receptor subunits or the mGluR3 receptor mRNAs in the VTA of cocaine overdose victims (Table 2). mGluR3 and GluR3 were in low abundance in the VTA. Furthermore, no significant differences were observed in glutamate receptor mRNA expression in the l-SN between cocaine overdose victims and controls.

There was no significant difference in mRNA abundance for the various dopamine signaling transcripts including receptor subtypes (D1, D4 and D5), the dopamine transporter, tyrosine hydroxylase and dopa decarboxylase between cocaine overdose victims and controls in either the VTA ($F_{1,146} = 0.179$, $p = 0.679$) or the l-SN ($F_{1,139} = 0.135$, $p = 0.714$). There was a trend towards significance in GABA signaling transcripts including GABA_A receptor subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\alpha 7$) and glutamic acid decarboxylases (GAD) 65 and 67 in the VTA ($F_{1,230} = 3.843$, $p = 0.051$); however, there was no significant difference in these transcripts between the groups in the l-SN ($F_{1,219} = 1.980$, $p = 0.161$). Analysis of other neurotransmitter signaling transcripts (CART, serotonin receptor subtype 2C and 3, and cannabinoid receptor 1) revealed a significant GROUP by TRANSCRIPT interaction ($F_{1,4} = 4.440$, $p = 0.003$) that was attributable to a down-regulation of CART in the VTA of cocaine overdose victims compared with controls ($p < 0.05$).

Signaling cascade transcripts—Previous studies in rodents have indicated a role for G protein subunits in cocaine intake. Examination of a variety of G protein subunit mRNAs (G₁₁, G₁₅, G₁₂, G₁₃, G₁₆, G₂₀, G₂₁, G₂₂, G₂₃, G₂₄, G₂₅, G₂₆, G₂₇, G₂₈, G₂₉, G₃₀, G₃₁, G₃₂, G₃₃, G₃₄, G₃₅, G₃₆, G₃₇, G₃₈, G₃₉, G₄₀, G₄₁, G₄₂, G₄₃, G₄₄, G₄₅, G₄₆, G₄₇, G₄₈, G₄₉, G₅₀, G₅₁, G₅₂, G₅₃, G₅₄, G₅₅, G₅₆, G₅₇, G₅₈, G₅₉, G₆₀, G₆₁, G₆₂, G₆₃, G₆₄, G₆₅, G₆₆, G₆₇, G₆₈, G₆₉, G₇₀, G₇₁, G₇₂, G₇₃, G₇₄, G₇₅, G₇₆, G₇₇, G₇₈, G₇₉, G₈₀, G₈₁, G₈₂, G₈₃, G₈₄, G₈₅, G₈₆, G₈₇, G₈₈, G₈₉, G₉₀, G₉₁, G₉₂, G₉₃, G₉₄, G₉₅, G₉₆, G₉₇, G₉₈, G₉₉, G₁₀₀, G₁₀₁, G₁₀₂, G₁₀₃, G₁₀₄, G₁₀₅, G₁₀₆, G₁₀₇, G₁₀₈, G₁₀₉, G₁₁₀, G₁₁₁, G₁₁₂, G₁₁₃, G₁₁₄, G₁₁₅, G₁₁₆, G₁₁₇, G₁₁₈, G₁₁₉, G₁₂₀, G₁₂₁, G₁₂₂, G₁₂₃, G₁₂₄, G₁₂₅, G₁₂₆, G₁₂₇, G₁₂₈, G₁₂₉, G₁₃₀, G₁₃₁, G₁₃₂, G₁₃₃, G₁₃₄, G₁₃₅, G₁₃₆, G₁₃₇, G₁₃₈, G₁₃₉, G₁₄₀, G₁₄₁, G₁₄₂, G₁₄₃, G₁₄₄, G₁₄₅, G₁₄₆, G₁₄₇, G₁₄₈, G₁₄₉, G₁₅₀, G₁₅₁, G₁₅₂, G₁₅₃, G₁₅₄, G₁₅₅, G₁₅₆, G₁₅₇, G₁₅₈, G₁₅₉, G₁₆₀, G₁₆₁, G₁₆₂, G₁₆₃, G₁₆₄, G₁₆₅, G₁₆₆, G₁₆₇, G₁₆₈, G₁₆₉, G₁₇₀, G₁₇₁, G₁₇₂, G₁₇₃, G₁₇₄, G₁₇₅, G₁₇₆, G₁₇₇, G₁₇₈, G₁₇₉, G₁₈₀, G₁₈₁, G₁₈₂, G₁₈₃, G₁₈₄, G₁₈₅, G₁₈₆, G₁₈₇, G₁₈₈, G₁₈₉, G₁₉₀, G₁₉₁, G₁₉₂, G₁₉₃, G₁₉₄, G₁₉₅, G₁₉₆, G₁₉₇, G₁₉₈, G₁₉₉, G₂₀₀, G₂₀₁, G₂₀₂, G₂₀₃, G₂₀₄, G₂₀₅, G₂₀₆, G₂₀₇, G₂₀₈, G₂₀₉, G₂₁₀, G₂₁₁, G₂₁₂, G₂₁₃, G₂₁₄, G₂₁₅, G₂₁₆, G₂₁₇, G₂₁₈, G₂₁₉, G₂₂₀, G₂₂₁, G₂₂₂, G₂₂₃, G₂₂₄, G₂₂₅, G₂₂₆, G₂₂₇, G₂₂₈, G₂₂₉, G₂₃₀, G₂₃₁, G₂₃₂, G₂₃₃, G₂₃₄, G₂₃₅, G₂₃₆, G₂₃₇, G₂₃₈, G₂₃₉, G₂₄₀, G₂₄₁, G₂₄₂, G₂₄₃, G₂₄₄, G₂₄₅, G₂₄₆, G₂₄₇, G₂₄₈, G₂₄₉, G₂₅₀, G₂₅₁, G₂₅₂, G₂₅₃, G₂₅₄, G₂₅₅, G₂₅₆, G₂₅₇, G₂₅₈, G₂₅₉, G₂₆₀, G₂₆₁, G₂₆₂, G₂₆₃, G₂₆₄, G₂₆₅, G₂₆₆, G₂₆₇, G₂₆₈, G₂₆₉, G₂₇₀, G₂₇₁, G₂₇₂, G₂₇₃, G₂₇₄, G₂₇₅, G₂₇₆, G₂₇₇, G₂₇₈, G₂₇₉, G₂₈₀, G₂₈₁, G₂₈₂, G₂₈₃, G₂₈₄, G₂₈₅, G₂₈₆, G₂₈₇, G₂₈₈, G₂₈₉, G₂₉₀, G₂₉₁, G₂₉₂, G₂₉₃, G₂₉₄, G₂₉₅, G₂₉₆, G₂₉₇, G₂₉₈, G₂₉₉, G₃₀₀, G₃₀₁, G₃₀₂, G₃₀₃, G₃₀₄, G₃₀₅, G₃₀₆, G₃₀₇, G₃₀₈, G₃₀₉, G₃₁₀, G₃₁₁, G₃₁₂, G₃₁₃, G₃₁₄, G₃₁₅, G₃₁₆, G₃₁₇, G₃₁₈, G₃₁₉, G₃₂₀, G₃₂₁, G₃₂₂, G₃₂₃, G₃₂₄, G₃₂₅, G₃₂₆, G₃₂₇, G₃₂₈, G₃₂₉, G₃₃₀, G₃₃₁, G₃₃₂, G₃₃₃, G₃₃₄, G₃₃₅, G₃₃₆, G₃₃₇, G₃₃₈, G₃₃₉, G₃₄₀, G₃₄₁, G₃₄₂, G₃₄₃, G₃₄₄, G₃₄₅, G₃₄₆, G₃₄₇, G₃₄₈, G₃₄₉, G₃₅₀, G₃₅₁, G₃₅₂, G₃₅₃, G₃₅₄, G₃₅₅, G₃₅₆, G₃₅₇, G₃₅₈, G₃₅₉, G₃₆₀, G₃₆₁, G₃₆₂, G₃₆₃, G₃₆₄, G₃₆₅, G₃₆₆, G₃₆₇, G₃₆₈, G₃₆₉, G₃₇₀, G₃₇₁, G₃₇₂, G₃₇₃, G₃₇₄, G₃₇₅, G₃₇₆, G₃₇₇, G₃₇₈, G₃₇₉, G₃₈₀, G₃₈₁, G₃₈₂, G₃₈₃, G₃₈₄, G₃₈₅, G₃₈₆, G₃₈₇, G₃₈₈, G₃₈₉, G₃₉₀, G₃₉₁, G₃₉₂, G₃₉₃, G₃₉₄, G₃₉₅, G₃₉₆, G₃₉₇, G₃₉₈, G₃₉₉, G₄₀₀, G₄₀₁, G₄₀₂, G₄₀₃, G₄₀₄, G₄₀₅, G₄₀₆, G₄₀₇, G₄₀₈, G₄₀₉, G₄₁₀, G₄₁₁, G₄₁₂, G₄₁₃, G₄₁₄, G₄₁₅, G₄₁₆, G₄₁₇, G₄₁₈, G₄₁₉, G₄₂₀, G₄₂₁, G₄₂₂, G₄₂₃, G₄₂₄, G₄₂₅, G₄₂₆, G₄₂₇, G₄₂₈, G₄₂₉, G₄₃₀, G₄₃₁, G₄₃₂, G₄₃₃, G₄₃₄, G₄₃₅, G₄₃₆, G₄₃₇, G₄₃₈, G₄₃₉, G₄₄₀, G₄₄₁, G₄₄₂, G₄₄₃, G₄₄₄, G₄₄₅, G₄₄₆, G₄₄₇, G₄₄₈, G₄₄₉, G₄₅₀, G₄₅₁, G₄₅₂, G₄₅₃, G₄₅₄, G₄₅₅, G₄₅₆, G₄₅₇, G₄₅₈, G₄₅₉, G₄₆₀, G₄₆₁, G₄₆₂, G₄₆₃, G₄₆₄, G₄₆₅, G₄₆₆, G₄₆₇, G₄₆₈, G₄₆₉, G₄₇₀, G₄₇₁, G₄₇₂, G₄₇₃, G₄₇₄, G₄₇₅, G₄₇₆, G₄₇₇, G₄₇₈, G₄₇₉, G₄₈₀, G₄₈₁, G₄₈₂, G₄₈₃, G₄₈₄, G₄₈₅, G₄₈₆, G₄₈₇, G₄₈₈, G₄₈₉, G₄₉₀, G₄₉₁, G₄₉₂, G₄₉₃, G₄₉₄, G₄₉₅, G₄₉₆, G₄₉₇, G₄₉₈, G₄₉₉, G₅₀₀, G₅₀₁, G₅₀₂, G₅₀₃, G₅₀₄, G₅₀₅, G₅₀₆, G₅₀₇, G₅₀₈, G₅₀₉, G₅₁₀, G₅₁₁, G₅₁₂, G₅₁₃, G₅₁₄, G₅₁₅, G₅₁₆, G₅₁₇, G₅₁₈, G₅₁₉, G₅₂₀, G₅₂₁, G₅₂₂, G₅₂₃, G₅₂₄, G₅₂₅, G₅₂₆, G₅₂₇, G₅₂₈, G₅₂₉, G₅₃₀, G₅₃₁, G₅₃₂, G₅₃₃, G₅₃₄, G₅₃₅, G₅₃₆, G₅₃₇, G₅₃₈, G₅₃₉, G₅₄₀, G₅₄₁, G₅₄₂, G₅₄₃, G₅₄₄, G₅₄₅, G₅₄₆, G₅₄₇, G₅₄₈, G₅₄₉, G₅₅₀, G₅₅₁, G₅₅₂, G₅₅₃, G₅₅₄, G₅₅₅, G₅₅₆, G₅₅₇, G₅₅₈, G₅₅₉, G₅₆₀, G₅₆₁, G₅₆₂, G₅₆₃, G₅₆₄, G₅₆₅, G₅₆₆, G₅₆₇, G₅₆₈, G₅₆₉, G₅₇₀, G₅₇₁, G₅₇₂, G₅₇₃, G₅₇₄, G₅₇₅, G₅₇₆, G₅₇₇, G₅₇₈, G₅₇₉, G₅₈₀, G₅₈₁, G₅₈₂, G₅₈₃, G₅₈₄, G₅₈₅, G₅₈₆, G₅₈₇, G₅₈₈, G₅₈₉, G₅₉₀, G₅₉₁, G₅₉₂, G₅₉₃, G₅₉₄, G₅₉₅, G₅₉₆, G₅₉₇, G₅₉₈, G₅₉₉, G₆₀₀, G₆₀₁, G₆₀₂, G₆₀₃, G₆₀₄, G₆₀₅, G₆₀₆, G₆₀₇, G₆₀₈, G₆₀₉, G₆₁₀, G₆₁₁, G₆₁₂, G₆₁₃, G₆₁₄, G₆₁₅, G₆₁₆, G₆₁₇, G₆₁₈, G₆₁₉, G₆₂₀, G₆₂₁, G₆₂₂, G₆₂₃, G₆₂₄, G₆₂₅, G₆₂₆, G₆₂₇, G₆₂₈, G₆₂₉, G₆₃₀, G₆₃₁, G₆₃₂, G₆₃₃, G₆₃₄, G₆₃₅, G₆₃₆, G₆₃₇, G₆₃₈, G₆₃₉, G₆₄₀, G₆₄₁, G₆₄₂, G₆₄₃, G₆₄₄, G₆₄₅, G₆₄₆, G₆₄₇, G₆₄₈, G₆₄₉, G₆₅₀, G₆₅₁, G₆₅₂, G₆₅₃, G₆₅₄, G₆₅₅, G₆₅₆, G₆₅₇, G₆₅₈, G₆₅₉, G₆₆₀, G₆₆₁, G₆₆₂, G₆₆₃, G₆₆₄, G₆₆₅, G₆₆₆, G₆₆₇, G₆₆₈, G₆₆₉, G₆₇₀, G₆₇₁, G₆₇₂, G₆₇₃, G₆₇₄, G₆₇₅, G₆₇₆, G₆₇₇, G₆₇₈, G₆₇₉, G₆₈₀, G₆₈₁, G₆₈₂, G₆₈₃, G₆₈₄, G₆₈₅, G₆₈₆, G₆₈₇, G₆₈₈, G₆₈₉, G₆₉₀, G₆₉₁, G₆₉₂, G₆₉₃, G₆₉₄, G₆₉₅, G₆₉₆, G₆₉₇, G₆₉₈, G₆₉₉, G₇₀₀, G₇₀₁, G₇₀₂, G₇₀₃, G₇₀₄, G₇₀₅, G₇₀₆, G₇₀₇, G₇₀₈, G₇₀₉, G₇₁₀, G₇₁₁, G₇₁₂, G₇₁₃, G₇₁₄, G₇₁₅, G₇₁₆, G₇₁₇, G₇₁₈, G₇₁₉, G₇₂₀, G₇₂₁, G₇₂₂, G₇₂₃, G₇₂₄, G₇₂₅, G₇₂₆, G₇₂₇, G₇₂₈, G₇₂₉, G₇₃₀, G₇₃₁, G₇₃₂, G₇₃₃, G₇₃₄, G₇₃₅, G₇₃₆, G₇₃₇, G₇₃₈, G₇₃₉, G₇₄₀, G₇₄₁, G₇₄₂, G₇₄₃, G₇₄₄, G₇₄₅, G₇₄₆, G₇₄₇, G₇₄₈, G₇₄₉, G₇₅₀, G₇₅₁, G₇₅₂, G₇₅₃, G₇₅₄, G₇₅₅, G₇₅₆, G₇₅₇, G₇₅₈, G₇₅₉, G₇₆₀, G₇₆₁, G₇₆₂, G₇₆₃, G₇₆₄, G₇₆₅, G₇₆₆, G₇₆₇, G₇₆₈, G₇₆₉, G₇₇₀, G₇₇₁, G₇₇₂, G₇₇₃, G₇₇₄, G₇₇₅, G₇₇₆, G₇₇₇, G₇₇₈, G₇₇₉, G₇₈₀, G₇₈₁, G₇₈₂, G₇₈₃, G₇₈₄, G₇₈₅, G₇₈₆, G₇₈₇, G₇₈₈, G₇₈₉, G₇₉₀, G₇₉₁, G₇₉₂, G₇₉₃, G₇₉₄, G₇₉₅, G₇₉₆, G₇₉₇, G₇₉₈, G₇₉₉, G₈₀₀, G₈₀₁, G₈₀₂, G₈₀₃, G₈₀₄, G₈₀₅, G₈₀₆, G₈₀₇, G₈₀₈, G₈₀₉, G₈₁₀, G₈₁₁, G₈₁₂, G₈₁₃, G₈₁₄, G₈₁₅, G₈₁₆, G₈₁₇, G₈₁₈, G₈₁₉, G₈₂₀, G₈₂₁, G₈₂₂, G₈₂₃, G₈₂₄, G₈₂₅, G₈₂₆, G₈₂₇, G₈₂₈, G₈₂₉, G₈₃₀, G₈₃₁, G₈₃₂, G₈₃₃, G₈₃₄, G₈₃₅, G₈₃₆, G₈₃₇, G₈₃₈, G₈₃₉, G₈₄₀, G₈₄₁, G₈₄₂, G₈₄₃, G₈₄₄, G₈₄₅, G₈₄₆, G₈₄₇, G₈₄₈, G₈₄₉, G₈₅₀, G₈₅₁, G₈₅₂, G₈₅₃, G₈₅₄, G₈₅₅, G₈₅₆, G₈₅₇, G₈₅₈, G₈₅₉, G₈₆₀, G₈₆₁, G₈₆₂, G₈₆₃, G₈₆₄, G₈₆₅, G₈₆₆, G₈₆₇, G₈₆₈, G₈₆₉, G₈₇₀, G₈₇₁, G₈₇₂, G₈₇₃, G₈₇₄, G₈₇₅, G₈₇₆, G₈₇₇, G₈₇₈, G₈₇₉, G₈₈₀, G₈₈₁, G₈₈₂, G₈₈₃, G₈₈₄, G₈₈₅, G₈₈₆, G₈₈₇, G₈₈₈, G₈₈₉, G₈₉₀, G₈₉₁, G₈₉₂, G₈₉₃, G₈₉₄, G₈₉₅, G₈₉₆, G₈₉₇, G₈₉₈, G₈₉₉, G₉₀₀, G₉₀₁, G₉₀₂, G₉₀₃, G₉₀₄, G₉₀₅, G₉₀₆, G₉₀₇, G₉₀₈, G₉₀₉, G₉₁₀, G₉₁₁, G₉₁₂, G₉₁₃, G₉₁₄, G₉₁₅, G₉₁₆, G₉₁₇, G₉₁₈, G₉₁₉, G₉₂₀, G₉₂₁, G₉₂₂, G₉₂₃, G₉₂₄, G₉₂₅, G₉₂₆, G₉₂₇, G₉₂₈, G₉₂₉, G₉₃₀, G₉₃₁, G₉₃₂, G₉₃₃, G₉₃₄, G₉₃₅, G₉₃₆, G₉₃₇, G₉₃₈, G₉₃₉, G₉₄₀, G₉₄₁, G₉₄₂, G₉₄₃, G₉₄₄, G₉₄₅, G₉₄₆, G₉₄₇, G₉₄₈, G₉₄₉, G₉₅₀, G₉₅₁, G₉₅₂, G₉₅₃, G₉₅₄, G₉₅₅, G₉₅₆, G₉₅₇, G₉₅₈, G₉₅₉, G₉₆₀, G₉₆₁, G₉₆₂, G₉₆₃, G₉₆₄, G₉₆₅, G₉₆₆, G₉₆₇, G₉₆₈, G₉₆₉, G₉₇₀, G₉₇₁, G₉₇₂, G₉₇₃, G₉₇₄, G₉₇₅, G₉₇₆, G₉₇₇, G₉₇₈, G₉₇₉, G₉₈₀, G₉₈₁, G₉₈₂, G₉₈₃, G₉₈₄, G₉₈₅, G₉₈₆, G₉₈₇, G₉₈₈, G₉₈₉, G₉₉₀, G₉₉₁, G₉₉₂, G₉₉₃, G₉₉₄, G₉₉₅, G₉₉₆, G₉₉₇, G₉₉₈, G₉₉₉, G₁₀₀₀, G₁₀₀₁, G₁₀₀₂, G₁₀₀₃, G₁₀₀₄, G₁₀₀₅, G₁₀₀₆, G₁₀₀₇, G₁₀₀₈, G₁₀₀₉, G₁₀₁₀, G₁₀₁₁, G₁₀₁₂, G₁₀₁₃, G₁₀₁₄, G₁₀₁₅, G₁₀₁₆, G₁₀₁₇, G₁₀₁₈, G₁₀₁₉, G₁₀₂₀, G₁₀₂₁, G₁₀₂₂, G₁₀₂₃, G₁₀₂₄, G₁₀₂₅, G₁₀₂₆, G₁₀₂₇, G₁₀₂₈, G₁₀₂₉, G₁₀₃₀, G₁₀₃₁, G₁₀₃₂, G₁₀₃₃, G₁₀₃₄, G₁₀₃₅, G₁₀₃₆, G₁₀₃₇, G₁₀₃₈, G₁₀₃₉, G₁₀₄₀, G₁₀₄₁, G₁₀₄₂, G₁₀₄₃, G₁₀₄₄, G₁₀₄₅, G₁₀₄₆, G₁₀₄₇, G₁₀₄₈, G₁₀₄₉, G₁₀₅₀, G₁₀₅₁, G₁₀₅₂, G₁₀₅₃, G₁₀₅₄, G₁₀₅₅, G₁₀₅₆, G₁₀₅₇, G₁₀₅₈, G₁₀₅₉, G₁₀₆₀, G₁₀₆₁, G₁₀₆₂, G₁₀₆₃, G₁₀₆₄, G₁₀₆₅, G₁₀₆₆, G₁₀₆₇, G₁₀₆₈, G₁₀₆₉, G₁₀₇₀, G₁₀₇₁, G₁₀₇₂, G₁₀₇₃, G₁₀₇₄, G₁₀₇₅, G₁₀₇₆, G₁₀₇₇, G₁₀₇₈, G₁₀₇₉, G₁₀₈₀, G₁₀₈₁, G₁₀₈₂, G₁₀₈₃, G₁₀₈₄, G₁₀₈₅, G₁₀₈₆, G₁₀₈₇, G₁₀₈₈, G₁₀₈₉, G₁₀₉₀, G₁₀₉₁, G₁₀₉₂, G₁₀₉₃, G₁₀₉₄, G₁₀₉₅, G₁₀₉₆, G₁₀₉₇, G₁₀₉₈, G₁₀₉₉, G₁₁₀₀, G₁₁₀₁, G₁₁₀₂, G₁₁₀₃, G₁₁₀₄, G₁₁₀₅, G₁₁₀₆, G₁₁₀₇, G₁₁₀₈, G₁₁₀₉, G₁₁₁₀, G₁₁₁₁, G₁₁₁₂, G₁₁₁₃, G₁₁₁₄, G₁₁₁₅, G₁₁₁₆, G₁₁₁₇, G₁₁₁₈, G₁₁₁₉, G₁₁₂₀, G₁₁₂₁, G₁₁₂₂, G₁₁₂₃, G₁₁₂₄, G₁₁₂₅, G₁₁₂₆, G₁₁₂₇, G₁₁₂₈, G₁₁₂₉, G₁₁₃₀, G₁₁₃₁, G₁₁₃₂, G₁₁₃₃, G₁₁₃₄, G₁₁₃₅, G₁₁₃₆, G₁₁₃₇, G₁₁₃₈, G₁₁₃₉, G₁₁₄₀, G₁₁₄₁, G₁₁₄₂, G₁₁₄₃, G₁₁₄₄, G₁₁₄₅, G₁₁₄₆, G₁₁₄₇, G₁₁₄₈, G₁₁₄₉, G₁₁₅₀, G₁₁₅₁, G₁₁₅₂, G₁₁₅₃, G₁₁₅₄, G₁₁₅₅, G₁₁₅₆, G₁₁₅₇, G₁₁₅₈, G₁₁₅₉, G₁₁₆₀, G₁₁₆₁, G₁₁₆₂, G₁₁₆₃, G₁₁₆₄, G₁₁₆₅, G₁₁₆₆, G₁₁₆₇, G₁₁₆₈, G₁₁₆₉, G₁₁₇₀, G₁₁₇₁, G₁₁₇₂, G₁₁₇₃, G₁₁₇₄, G₁₁₇₅, G₁₁₇₆, G₁₁₇₇, G₁₁₇₈, G₁₁₇₉, G₁₁₈₀, G₁₁₈₁, G₁₁₈₂, G₁₁₈

In membrane fractions, NR1 immunoreactive protein was increased 111.2% ($p < 0.05$), GluR2/3 by 69.1% ($p < 0.05$), GluR5 by 507.9% ($p < 0.01$) and KA2 by 72.1% ($p < 0.05$) in the VTA of cocaine overdose victims, whereas GluR1 showed a non-significant 50.3% increase in this region (Fig. 1). Comparatively, these proteins were slightly increased in the l-SN, albeit in a non-significant manner (NR1, 12.9%; GluR1, 56.1%; GluR2/3, 30.1%; GluR5, 13.4%; KA2, 50.4%). A previous study indicates that the aforementioned iGluR subunits are stable up to 18 h PMI (Wang *et al.* 2000), suggesting that the present results are not compromised by proteolysis.

Protein levels were increased 23.3% for $G_{i1/2}$ and 23.6% for G_s whereas G_o levels were decreased 21.7% in the VTA of cocaine overdose victims compared with controls in cytosolic fragments. In the l-SN, $G_{i1/2}$ was increased by 10%, whereas G_s was decreased by 15.1% and G_o by 10.1% in cocaine overdose victims compared with controls, all in a statistically non-significant manner (Fig. 2).

Previous studies have hypothesized that adaptive changes induced by cocaine on the cAMP intracellular cascade can alter subsequent responsiveness of the drug (Carlezon *et al.* 1998; Self *et al.* 1998; Pliakas *et al.* 2001). Evaluation of CREB immunoreactive protein revealed a significant up-regulation (69.6%; $p < 0.05$) of CREB in the cytosolic fraction from the VTA, but not l-SN, of cocaine overdose victims (Fig. 3). There was a slight but statistically nonsignificant increase in CREB immunoreactivity in the nuclear protein fraction from the VTA (34.3%), whereas levels in the l-SN were similar between cocaine overdose victims and controls. We were unable to reliably detect phospho-CREB immunoreactivity in either the cytosolic or the nuclear fractions in either brain regions of cocaine overdose victims or controls (data not shown). There was no significant difference in FRA2 protein levels between cocaine overdose victims and controls in either the VTA or the l-SN (data not shown).

Discussion

In the present study, macroarrays and western blot analyses were used to examine the expression of select genes and proteins in the VTA and l-SN of cocaine overdose victims and controls. A membrane-based macroarray platform with radioactive hybridization was used to query 81 human cDNAs of interest. Significant alterations were observed for a variety of protein families including iGluR subunits, G protein subunits, RGS proteins, miscellaneous transcripts including CART, and a trend towards significance in GABA_A receptor subunits. Previously, we have employed similar approaches to identify molecular profiles of other diseases in human brain tissue including Alzheimer's disease, schizophrenia, drug abuse, as well as normal aging (Ginsberg *et al.* 2000; Hasenkamp and Hemby 2002; Hemby *et al.* 2002, 2003). Western blot analyses were used to confirm changes in protein levels for some of the differentially expressed transcripts and revealed significant up-regulation of iGluR subunit and CREB protein levels in the VTA of cocaine overdose victims. The present results provide a composite assessment of cocaine-induced alterations in gene expression in a brain region commonly associated with the drug reinforcement.

The major finding of the present study was the up-regulation of NR1, GluR2, GluR5 and KA2 glutamate receptor subunit mRNA and protein levels specific to the VTA in human cocaine overdose victims. Previous studies in rodents indicated that up-regulation of iGluR subunits was associated with augmented dopamine–glutamate interactions in the mesolimbic pathway (White *et al.* 1995a,b; Zhang *et al.* 1997; White and Kalivas 1998; Wolf 1998; Vanderschuren and Kalivas 2000; Giorgetti *et al.* 2001). Results of the majority of cocaine administration studies in rodents contrast with the present findings. For example, NR1

mRNA levels were decreased in the VTA following acute cocaine administration whereas chronic administration failed to alter mRNA levels of GluR1-4, NR1 or mGluR5 in this region (Ghasemzadeh *et al.* 1999; Lu *et al.* 2002). Likewise, GluR1 protein levels are elevated in the VTA following chronic cocaine administration (Fitzgerald *et al.* 1996) but were no different than controls 16–24 h after the last cocaine injection (Lu *et al.* 2002). In agreement with the present results, NR1 protein levels were elevated in the VTA following chronic cocaine administration (Fitzgerald *et al.* 1996) and remain elevated 1, 3 and 14 days following the last administration of cocaine (Churchill *et al.* 1999; Loftis and Janowsky 2000). These data suggest that increased NR1 protein levels in cocaine overdose victims may be induced by acute or subacute cocaine exposure or possibly reflect the last acute cocaine binge prior to death. Up-regulation of mRNA and protein levels of GluR2, GluR5 and KA2 may be indicative of chronic cocaine use and/or cocaine overdose in humans.

In the VTA and SN, GluR1-4 and NR1 immunoreactivity was found to be localized almost exclusively on dopaminergic neurons (Paquet *et al.* 1997); however, less is known about the localization of kainate subunits in these regions. Glutamatergic afferents in the midbrain originate from the medial frontal cortex, subthalamic and pedunculopontine tegmental nuclei (Christie *et al.* 1985; Sesack *et al.* 1989; Sesack and Pickel 1992; Lu *et al.* 1997). A recent study suggested prefrontal glutamatergic projections selectively synapse on mesoprefrontal dopamine neurons and mesoaccumbens GABA neurons in the VTA in rats (Carr and Sesack 2000), although the topography in non-human primates and humans remains unknown. The presence of these subunits on dopamine neurons and the up-regulation reported in the present study may provide a means by which to influence dopamine excitability in this region. Up-regulation of the kainite-preferring subunits GluR5 and KA2 in cocaine overdose victims and the probable localization of these subunits on dopamine neurons represent additional means by which increased Ca^{2+} influx may lead to hyperexcitability of VTA dopamine neurons. Interestingly, GluR2 subunit protein levels were increased in the hippocampus of alcoholics (Breese *et al.* 1995) and GluR5 mRNA and protein levels were increased in the dorsal prefrontal cortex of cocaine-treated rats (Toda *et al.* 2002). Because extensive editing of GluR2 in the adult brain renders ionophores less permeable to Ca^{2+} , the up-regulation observed in the present study may represent a molecular compensation for the effects of increased Ca^{2+} influx generated by the combined up-regulation of NR1, GluR5 and KA2 (Paschen *et al.* 1994). As a result of the influence of RNA editing for each of these glutamate receptor subunits, which confer different desensitization kinetics and other pharmacological properties (Borges and Dingledine 2002), the possibility remains that cocaine administration may differentially affect the expression of specific variants (Lomeli *et al.* 1994; Mosbacher *et al.* 1994). Inasmuch as splice variants were not discriminated using either cDNAs or antibodies in the present study, future studies are warranted to assess the regulation of splice variants as a result of cocaine exposure. In summary, up-regulation of the iGluR subunit mRNA and protein levels in the present study are likely attributable to changes in VTA dopaminergic neurons, suggesting a neuroadaptive response to cocaine in these subjects.

Dopamine receptor mRNAs were not differentially regulated as a function of cocaine overdose, in agreement with previous literature (Peris *et al.* 1990; Meador-Woodruff *et al.* 1993; Moore *et al.* 1998), opening the possibility that intracellular signaling mechanisms may also contribute to the altered function of these neurons. Neuroadaptations in the cAMP pathway have been implicated in mesolimbic brain regions as a function of cocaine exposure (Nestler *et al.* 1990; Terwilliger *et al.* 1991; Striplin and Kalivas 1992; Miserendino and Nestler 1995; Carlezon *et al.* 1998; Self *et al.* 1998; Pliakas *et al.* 2001). Assessment of various α and β G protein subunits revealed only a significant decrease in G_i subunit mRNA, but not in protein, in the VTA of cocaine overdose victims. The discrepancy between G_i mRNA and protein levels may be reflective of post-transcriptional,

translational or post-translational processing/degradation and/or trafficking of the protein outside of the regions studied. In addition, decreased G mRNA levels may manifest as down-regulated protein levels in axonal targets of the mesoaccumbens dopamine neurons, as shown previously in cocaine-treated rats (Wang *et al.* 1997). The present data contrast previous studies showing decreased ADP ribosylation and immunoreactivity G_i and G_o in the VTA of cocaine-treated rats (Nestler *et al.* 1990; Striplin and Kalivas 1993). However, a significant increase in CREB mRNA and protein levels in the cytosolic fraction and a trend towards significance in the nuclear fraction in the VTA of cocaine overdose victims was observed – the first demonstration of increased CREB protein levels in the VTA of either humans or animal models as a function of cocaine exposure. Unfortunately, the length of the post-mortem intervals for these subjects are likely to have contributed to the inability to detect phosphorylated CREB immunoreactivity; precluding speculation on the role of elevated CREB levels in the present study. Although CREB is almost exclusively expressed in the nucleus, CREB protein has been identified in the cytoplasm by light and electron microscopy (Ferrer *et al.* 1996; Hermanson *et al.* 1996; Suzuki *et al.* 1998; Shaywitz and Greenberg 1999) as well as in postsynaptic density fractions (Suzuki *et al.* 1998) and dendrites (Crino *et al.* 1998) leading to the hypothesis of nuclear translocation as a means of site-specific plasticity (Crino *et al.* 1998; Suzuki *et al.* 1998).

In relation to the present data, differential regulation of cytosolic vs. nuclear CREB should be addressed. In order to avoid overspeculation regarding this difference, statistical analysis indicated a trend towards significance in nuclear CREB ($p < 0.07$) in the VTA of cocaine overdose victims. Secondly, the ability of the antibody to detect the antigen in the nucleus may be impaired by conformational changes in the protein caused by dimerization, binding of the dimer to DNA, and/or masking of the antigen by CREB binding proteins or other transcriptional regulators. A more precise role of cytoplasmic CREB and the mechanism underlying elevated CREB levels remains to be determined in animal models of the disease. The relevance of altered CREB levels in the present study supports several studies in rodents that indicated CREB levels were inversely proportional to the rewarding effects of the drug (Carlezon *et al.* 1998; Walters and Blendy 2001). One manner in which CREB activity may be regulated is by Ca²⁺ influx via NMDA receptors, demonstrating a requirement for CREB–NMDA receptor interactions in dopamine-regulated gene expression (Konradi *et al.* 1996).

One of the transcripts regulated by CREB is CART (Dominguez *et al.* 2002). Discovered as a novel transcript in the striatum, the regulation of which was induced by acute cocaine (Douglass *et al.* 1995), CART peptides were shown to be abundant in the NAc, VTA and SN as well as other brain regions (Smith *et al.* 1997; Smith *et al.* 1999; Hurd and Fagergren 2000; Dallvechia-Adams *et al.* 2002). The present study is the first to demonstrate CART mRNA regulation by chronic cocaine use in humans. Future studies are warranted to explore the location, abundance and regulation of these CART peptides by cocaine administration.

The up-regulation of RGS3 and RGS12 mRNAs in the VTA of cocaine overdose victims represents another potential mechanism for cocaine-induced alterations in cell signaling. Previous studies have shown elevated RGS2, 3 and 5 mRNA levels in the striatum caused by acute amphetamine administration (Burchett *et al.* 1999) and RGS2 mRNA levels by cocaine in the hippocampus, cortex and striatum (Ingi *et al.* 1998). RGS proteins modulate/integrate G protein and other intracellular signaling proteins, some of which reduce the duration of G protein activation by increasing the rate of GTP hydrolysis (Berman *et al.* 1996; Hunt *et al.* 1996; Chatterjee *et al.* 1997; Hepler *et al.* 1997; Berman and Gilman 1998). *In vivo*, RGS3 interacts with the G protein complex to accelerate the rate of recovery of N-type Ca²⁺ inhibition from G_i thereby leading to increased Ca²⁺ conductance (Jeong and Ikeda 1998; Melliti *et al.* 1999; Hollinger and Hepler 2002) and potentially to

increased excitability of VTA dopamine neurons. There is limited information regarding the functionality of RGS12 and how cocaine may regulate its function. The manner in which cocaine and other drugs of abuse may regulate RGS expression and, in turn, how this regulation affects subsequent drug responsiveness and intake remain unanswered. The lack of effective antisera for RGS3 and 12 proteins and their potentially low protein expression (Hollinger and Hepler 2002) preclude a more complete understanding of the regulation of the proteins at the present time; however, it is reasonable to speculate that RGS proteins appear to be located in dopamine pathways and may be involved in neuroadaptations in signal transduction mechanisms associated with chronic cocaine exposure.

Understanding the consequences of long-term cocaine abuse on post-mortem human brain requires rigorous investigation. The present study demonstrated that some of the adaptations that occur in animal models of drug abuse are applicable to the human brain. Discrepancies between the present data and the existing rodent literature may reflect differences in the (i) comparative complexities of chronic cocaine use, (ii) anatomical connectivity of the VTA (Alheid *et al.* 1990; Heimer *et al.* 1995), (iii) as well as differences in the response contingency of drug administration (Hemby *et al.* 1997b). With regard to the present data, whether the observed changes could be attributed to acute or chronic cocaine use or cocaine overdose remains to be addressed. Future studies should include comparison of gene and protein profiles with individuals diagnosed with cocaine addiction histories but who do not die of cocaine overdose as well as individuals who die during excited cocaine delirium (Ruttenber *et al.* 1997). More importantly, additional efforts should be directed towards the recapitulation of these findings in rodent and non-human primate models (e.g. self-administration) in order to develop a better understanding of the variables that may contribute to these alterations. Although there are many difficulties with post-mortem brain studies, it is one of the most promising ways to view biochemical changes that are relevant to human drug abusers and to educate the public about the consequences of cocaine abuse. Whereas animal studies have advanced our understanding of the neurobiological basis of drug addiction, the evaluation of similar questions in human tissue are so far few and yet are essential. By assessing changes in defined biochemical pathways in human post-mortem tissue, we can begin to ascertain the fundamental molecular and biochemical processes that are associated with long-term cocaine use.

Acknowledgments

This work was supported in part by grants from the National Institute on Drug Abuse (DA13772, SEH; F31 DA15941, WHF). The Emory University Health Sciences Center Microarray Facility provided the cDNA clones. The authors thank the staff of the Miami Brain Endowment Bank for their assistance in case accrual and evaluation.

Abbreviations used

AMPA	(+/-)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aRNA	antisense ribonucleic acid
BE	benzylecognine
cAMP	cyclic adenosine 3',5'-monophosphate
CART	cocaine-amphetamine regulated transcript
CE	cocethylene
COC	cocaine
COD	cocaine overdose

CREB	cAMP response element binding protein
CTR	control
DHPA	diphenhydramine
EtOH	ethanol
FRA2	fos-related antigen 2
GAD	glutamic acid decarboxylases
GluR	glutamate receptor
iGluR	ionotropic glutamate receptor
LC	lidocaine
l-SN	lateral substantia nigra
mGluR	metabotropic glutamate receptor
NAc	nucleus accumbens
ND	not detectable
PI	protease inhibitors
PKA	protein kinase A
PMI	postmortem interval
RGS	regulators of G protein signaling
SDS	sodium dodecyl sulfate
VTa	ventral tegmental area

References

- Alheid, GF.; Heimer, L.; Switzer, RC. Basal Ganglia, in *The Human Nervous System*. Paxinos, G., editor. Academic Press; San Diego: 1990. p. 483-582.
- Bergman J, Madras BK, Johnson SE, Spealman RD. Effects of cocaine and related drugs in nonhuman primates. III. Self-administration by squirrel monkeys. *J. Pharmacol. Exp Therapeutics*. 1989; 251:150–155.
- Berman DM, Gilman AG. Mammalian RGS proteins: barbarians at the gate. *J. Biol. Chem*. 1998; 273:1269–1272. [PubMed: 9430654]
- Berman DM, Kozasa T, Gilman AG. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem*. 1996; 271:27209–27212. [PubMed: 8910288]
- Borges, K.; Dingledine, R. Molecular pharmacology and physiology of glutamate receptors, in *Glutamate and Addiction*. Zukin, SR., editor. Humana Press; Totawa, NJ: 2002. p. 3-22.
- Breese CR, Freedman R, Leonard SS. Glutamate receptor subtype expression in human postmortem brain tissue from schizophrenics and alcohol abusers. *Brain Res*. 1995; 674:82–90. [PubMed: 7773698]
- Burchett SA, Bannon MJ, Granneman JG. RGS mRNA expression in rat striatum: modulation by dopamine receptors and effects of repeated amphetamine administration. *J. Neurochem*. 1999; 72:1529–1533. [PubMed: 10098858]
- Carlezon WA Jr, Thome J, Olson VG, Lane-Ladd SB, Brodtkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ. Regulation of cocaine reward by CREB. *Science*. 1998; 282:2272–2275. [PubMed: 9856954]

- Carr DB, Sesack SR. Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J. Neurosci.* 2000; 20:3864–3873. [PubMed: 10804226]
- Castensson A, Emilsson L, Preece P, Jazin EE. High-resolution quantification of specific mRNA levels in human brain autopsies and biopsies. *Genome Res.* 2000; 10:1219–1229. [PubMed: 10958640]
- Chatterjee TK, Eapen AK, Fisher RA. A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylyl cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.* 1997; 272:15481–15487. [PubMed: 9182581]
- Christie MJ, Bridge S, James LB, Beart PM. Excitotoxin lesions suggest an aspartatergic projection from rat medial prefrontal cortex to ventral tegmental area. *Brain Res.* 1985; 333:169–172. [PubMed: 2859910]
- Churchill L, Swanson CJ, Urbina M, Kalivas PW. Repeated cocaine alters glutamate receptor subunit levels in the nucleus accumbens and ventral tegmental area of rats that develop behavioral sensitization. *J. Neurochem.* 1999; 72:2397–2403. [PubMed: 10349849]
- Couceyro P, Pollock KM, Drews K, Douglass J. Cocaine differentially regulates activator protein-1 mRNA levels and DNA-binding complexes in the rat striatum and cerebellum. *Mol. Pharmacol.* 1994; 46:667–676. [PubMed: 7969045]
- Crino P, Khodakhah K, Becker K, Ginsberg S, Hemby S, Eberwine J. Presence and phosphorylation of transcription factors in developing dendrites. *Proc. Natl Acad. Sci. USA.* 1998; 95:2313–2318. [PubMed: 9482882]
- Czoty PW, Justice JB Jr, Howell LL. Cocaine-induced changes in extracellular dopamine determined by microdialysis in awake squirrel monkeys. *Psychopharmacology.* 2000; 148:299–306. [PubMed: 10755743]
- Dallvechia-Adams S, Kuhar MJ, Smith Y. Cocaine- and amphetamine-regulated transcript peptide projections in the ventral midbrain: colocalization with gamma-aminobutyric acid, melanin-concentrating hormone, dynorphin, and synaptic interactions with dopamine neurons. *J. Comp. Neurol.* 2002; 448:360–372. [PubMed: 12115699]
- Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl Acad. Sci. USA.* 1988; 85:5274–5278. [PubMed: 2899326]
- Dodd PR, Hardy JA, Baig EB, Kidd AM, Bird ED, Watson WE, Johnston GA. Optimization of freezing, storage, and thawing conditions for the preparation of metabolically active synaptosomes from frozen rat and human brain. *Neurochem. Pathol.* 1986; 4:177–198. [PubMed: 3561893]
- Dominguez G, Lakatos A, Kuhar MJ. Characterization of the cocaine- and amphetamine-regulated transcript (CART) peptide gene promoter and its activation by a cyclic AMP-dependent signaling pathway in GH3 cells. *J. Neurochem.* 2002; 80:885–893. [PubMed: 11948252]
- Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J. Neurosci.* 1995; 15:2471–2481. [PubMed: 7891182]
- Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M, Coleman P. Analysis of gene expression in single live neurons. *Proc. Natl Acad. Sci. USA.* 1992; 89:3010–3014. [PubMed: 1557406]
- Ferrer I, Blanco R, Rivera R, Carmona M, Ballabriga J, Olive M, Planas AM. CREB-1 and CREB-2 immunoreactivity in the rat brain. *Brain Res.* 1996; 712:159–164. [PubMed: 8705300]
- Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ. Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J. Neurosci.* 1996; 16:274–282. [PubMed: 8613793]
- Freeman WM, Nader MA, Nader SH, Robertson DJ, Gioia L, Mitchell SM, Daunais JB, Porrino LJ, Friedman DP, Vrana KE. Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. *J. Neurochem.* 2001; 77:542–549. [PubMed: 11299316]
- Ghasemzadeh MB, Nelson LC, Lu XY, Kalivas PW. Neuroadaptations in ionotropic and metabotropic glutamate receptor mRNA produced by cocaine treatment. *J. Neurochem.* 1999; 72:157–165. [PubMed: 9886066]

- Ginsberg SD, Crino PB, Hemby SE, Weingarten JA, Lee VM, Eberwine JH, Trojanowski JQ. Predominance of neuronal mRNAs in individual Alzheimer's disease senile plaques. *Ann. Neurol.* 1999; 45:174–181. [PubMed: 9989619]
- Ginsberg SD, Hemby SE, Lee VM, Eberwine JH, Trojanowski JQ. Expression profile of transcripts in Alzheimer's disease tangle-bearing CA1 neurons. *Ann. Neurol.* 2000; 48:77–87. [PubMed: 10894219]
- Giorgetti M, Hotsenpiller G, Ward P, Teppen T, Wolf ME. Amphetamine-induced plasticity of AMPA receptors in the ventral tegmental area: effects on extracellular levels of dopamine and glutamate in freely moving rats. *J. Neuroscience.* 2001; 21:6362–6369.
- Haile CN, Hiroi N, Nestler EJ, Kosten TA. Differential behavioral responses to cocaine are associated with dynamics of mesolimbic dopamine proteins in Lewis and Fischer 344 rats. *Synapse.* 2001; 41:179–190. [PubMed: 11391778]
- Hardy JA, Dodd PR, Oakley AE, Perry RH, Edwardson JA, Kidd AM. Metabolically active synaptosomes can be prepared from frozen rat and human brain. *J. Neurochem.* 1983; 40:608–614. [PubMed: 6827264]
- Hasenkamp, W.; Hemby, SE. Functional genomics and psychiatric illness. In: Swaab, DF., editor. *Progress in Brain Research: Plasticity in the Adult Brain.* Vol. 138. Elsevier; Amsterdam: 2002. p. 277–305.
- Heimer, L.; Zahm, DS.; Alheid, GF. Basal Ganglia, in *The Rat Nervous System.* 2nd edn. Paxinos, G., editor. Academic Press; San Diego: 1995.
- Hemby SE, Co C, Koves TR, Smith JE, Dworkin SI. Differences in extracellular dopamine concentrations in the nucleus accumbens during response-dependent and response-independent cocaine administration in the rat. *Psychopharmacology.* 1997a; 133:7–16. [PubMed: 9335075]
- Hemby, SE.; Johnson, BA.; Dworkin, SI. Neurobiological basis of drug reinforcement. In: Roache, JD., editor. *Drug Addiction and its Treatment: Nexus of Neuroscience and Behavior.* Lippincott-Raven Publishers; Philadelphia: 1997b. p. 137–169.
- Hemby SE, Co C, Dworkin SI, Smith JE. Synergistic elevations in nucleus accumbens extracellular dopamine concentrations during self-administration of cocaine/heroin combinations (Speedball) in rats. *J. Pharmacol. Exp. Ther.* 1999; 288:274–280. [PubMed: 9862781]
- Hemby SE, Ginsberg SD, Brunk B, Trojanowski JQ, Eberwine JH. Gene expression profile for schizophrenia: discrete neuron transcription patterns in the entorhinal cortex. *Arch. Gen. Psych.* 2002; 59:631–640.
- Hemby SE, Trojanowski JQ, Ginsberg SD. Neuron specific age related decreases in dopamine receptor subtype mRNAs. *J. Comp. Neurol.* 2003; 456:176–183. [PubMed: 12509874]
- Hepler JR, Berman DM, Gilman AG, Kozasa T. RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. *Proc. Natl Acad. Sci. USA.* 1997; 94:428–432. [PubMed: 9012799]
- Hermanson O, Gustavsson J, Stralfors P, Blomqvist A. Cytoplasmic CREB alpha-like antigens in specific regions of the rat brain. *Biochem. Biophys. Res. Commun.* 1996; 225:256–262. [PubMed: 8769127]
- Hernandez A, Andollo W, Hearn WL. Analysis of cocaine and metabolites in brain using solid phase extraction and full-scanning gas chromatography/ion trap mass spectrometry. *Forensic Sci. Int.* 1994; 65:149–156. [PubMed: 8039772]
- Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ. FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc. Natl Acad. Sci. USA.* 1997; 94:10397–10402. [PubMed: 9294222]
- Hollinger S, Hepler JR. Cellular Regulation of RGS Proteins: Modulators and Integrators of G Protein Signaling. *Pharmacol. Rev.* 2002; 54:527–559. [PubMed: 12223533]
- Hollmann M, Heinemann S. Cloned glutamate receptors. *Ann. Rev. Neurosci.* 1994; 17:31–108. [PubMed: 8210177]
- Holman BL, Carvalho PA, Mendelson J, Teoh SK, Nardin R, Hallgring E, Hebben N, Johnson KA. Brain perfusion is abnormal in cocaine-dependent polydrug users: a study using technetium-99m-HMPAO and ASPECT. *J. Nucl. Med.* 1991; 32:1206–1210. [PubMed: 2045934]

- Holman BL, Mendelson J, Garada B, Teoh SK, Hallgring E, Johnson KA, Mello NK. Regional cerebral blood flow improves with treatment in chronic cocaine polydrug users. *J. Nucl. Med.* 1993; 34:723–727. [PubMed: 8478703]
- Hope B, Kosofsky B, Hyman SE, Nestler EJ. Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. *Proc. Natl Acad. Sci. USA.* 1992; 89:5764–5768. [PubMed: 1631058]
- Hunt TW, Fields TA, Casey PJ, Peralta EG. RGS10 is a selective activator of G alpha i GTPase activity. *Nature.* 1996; 383:175–177. [PubMed: 8774883]
- Hurd YL, Fagergren P. Human cocaine- and amphetamine-regulated transcript (CART) mRNA is highly expressed in limbic- and sensory-related brain regions. *J. Comp. Neurol.* 2000; 425:583–598. [PubMed: 10975881]
- Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, Barnes CA, Lanahan AA, Siderovski DP, Ross EM, Gilman AG, Worley PF. Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J. Neurosci.* 1998; 18:7178–7188. [PubMed: 9736641]
- Iyer RN, Nobiletti JB, Jatlow PI, Bradberry CW. Cocaine and cocaethylene: effects on extracellular dopamine in the primate. *Psychopharmacology.* 1995; 120:150–155. [PubMed: 7480546]
- Jeong SW, Ikeda SR. G protein alpha subunit G alpha z couples neurotransmitter receptors to ion channels in sympathetic neurons. *Neuron.* 1998; 21:1201–1212. [PubMed: 9856474]
- Kalivas PW, Duffy P. Repeated cocaine administration alters extracellular glutamate in the ventral tegmental area. *J. Neurochem.* 1998; 70:1497–1502. [PubMed: 9523566]
- Konradi C, Leveque JC, Hyman SE. Amphetamine and dopamine-induced immediate early gene expression in striatal neurons depends on postsynaptic NMDA receptors and calcium. *J. Neurosci.* 1996; 16:4231–4239. [PubMed: 8753884]
- Koob GF, Bloom FE. Cellular and molecular mechanisms of drug dependence. *Science.* 1988; 242:715–723. [PubMed: 2903550]
- Levin JM, Holman BL, Mendelson JH, Teoh SK, Garada B, Johnson KA, Springer S. Gender differences in cerebral perfusion in cocaine abuse: technetium-99m-HMPAO SPECT study of drug-abusing women. *J. Nucl. Med.* 1994; 35:1902–1909. [PubMed: 7989967]
- Loftis JM, Janowsky A. Regulation of NMDA receptor subunits and nitric oxide synthase expression during cocaine withdrawal. *J. Neurochem.* 2000; 75:2040–2050. [PubMed: 11032893]
- Lomeli H, Mosbacher J, Melcher T, Hoger T, Geiger JR, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH. Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science.* 1994; 266:1709–1713. [PubMed: 7992055]
- Lu XY, Churchill L, Kalivas PW. Expression of D1 receptor mRNA in projections from the forebrain to the ventral tegmental area. *Synapse.* 1997; 25:205–214. [PubMed: 9021901]
- Lu W, Monteggia LM, Wolf ME. Repeated administration of amphetamine or cocaine does not alter AMPA receptor subunit expression in the rat midbrain. *Neuropsychopharmacology.* 2002; 26:1–13. [PubMed: 11751027]
- Meador-Woodruff JH, Little KY, Damask SP, Mansour A, Watson SJ. Effects of cocaine on dopamine receptor gene expression: a study in the postmortem human brain. *Biol. Psychiatry.* 1993; 34:348–355. [PubMed: 8218601]
- Melliti K, Meza U, Fisher R, Adams B. Regulators of G protein signaling attenuate the G protein-mediated inhibition of N-type Ca channels. *J. Gen. Physiol.* 1999; 113:97–110. [PubMed: 9874691]
- Miserendino MJ, Nestler EJ. Behavioral sensitization to cocaine: modulation by the cyclic AMP system in the nucleus accumbens. *Brain Res.* 1995; 674:299–306. [PubMed: 7796110]
- Moore RJ, Vinsant SL, Nader MA, Porrino LJ, Friedman DP. Effect of cocaine self-administration on dopamine D2 receptors in rhesus monkeys. *Synapse.* 1998; 30:88–96. [PubMed: 9704885]
- Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP. A molecular determinant for sub-millisecond desensitization in glutamate receptors. *Science.* 1994; 266:1059–1062. [PubMed: 7973663]
- Nestler EJ. Cellular responses to chronic treatment with drugs of abuse. *Crit. Rev. Neurobiol.* 1993; 7:23–39. [PubMed: 8385579]

- Nestler EJ. Molecular mechanisms of opiate and cocaine addiction. *Curr. Opin. Neurobiol.* 1997; 7:713–719. [PubMed: 9384550]
- Nestler EJ. Neurobiology. Total recall-the memory of addiction. *Science.* 2001; 292:2266–2267. [PubMed: 11423644]
- Nestler EJ, Terwilliger RZ, Walker JR, Sevarino KA, Duman RS. Chronic cocaine treatment decreases levels of the G protein subunits Gi alpha and Go alpha in discrete regions of rat brain. *J. Neurochem.* 1990; 55:1079–1082. [PubMed: 2117048]
- Nye HE, Hope BT, Kelz MB, Iadarola M, Nestler EJ. Pharmacological studies of the regulation of chronic FOS-related antigen induction by cocaine in the striatum and nucleus accumbens. *J. Pharmacol. Exp. Ther.* 1995; 275:1671–1680. [PubMed: 8531143]
- Paquet M, Tremblay M, Soghomonian JJ, Smith Y. AMPA and NMDA glutamate receptor subunits in midbrain dopaminergic neurons in the squirrel monkey: an immunohistochemical and in situ hybridization study. *J. Neurosci.* 1997; 17:1377–1396. [PubMed: 9006980]
- Paschen W, Hedreen JC, Ross CA. RNA editing of the glutamate receptor subunits GluR2 and GluR6 in human brain tissue. *J. Neurochem.* 1994; 63:1596–1602. [PubMed: 7523595]
- Peris J, Boyson SJ, Cass WA, Curella P, Dwoskin LP, Larson G, Lin LH, Yasuda RP, Zahniser NR. Persistence of neurochemical changes in dopamine systems after repeated cocaine administration. *J. Pharmacol. Exp. Ther.* 1990; 253:38–44. [PubMed: 2329520]
- Pettit HO, Justice JB Jr. Dopamine in the nucleus accumbens during cocaine self-administration as studied by in vivo microdialysis. *Pharmacol. Biochem. Behav.* 1989; 34:899–904. [PubMed: 2623043]
- Pettit HO, Justice JB Jr. Effect of dose on cocaine self-administration behavior and dopamine levels in the nucleus accumbens. *Brain Res.* 1991; 539:94–102. [PubMed: 2015506]
- Pich EM, Pagliusi SR, Tessari M, Talabot-Ayer D, Hooft van Huijsduijnen R, Chiamulera C. Common neural substrates for the addictive properties of nicotine and cocaine. *Science.* 1997; 275:83–86. [PubMed: 8974398]
- Pierce RC, Kalivas PW. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. *Brain Res. Brain Res. Rev.* 1997; 25:192–216. [PubMed: 9403138]
- Pierce RC, Bell K, Duffy P, Kalivas PW. Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. *J. Neurosci.* 1996; 16:1550–1560. [PubMed: 8778304]
- Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr. Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J. Neurosci.* 2001; 21:7397–7403. [PubMed: 11549750]
- Reid MS, Hsu K Jr, Berger SP. Cocaine and amphetamine preferentially stimulate glutamate release in the limbic system: studies on the involvement of dopamine. *Synapse.* 1997; 27:95–105. [PubMed: 9266771]
- Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science.* 1987; 237:1219–1223. [PubMed: 2820058]
- Ruttenber AJ, Lawler-Heavner J, Yin M, Wetli CV, Hearn WL, Mash DC. Fatal excited delirium following cocaine use: epidemiologic findings provide new evidence for mechanisms of cocaine toxicity. *J. Forensic Sci.* 1997; 42:25–31. [PubMed: 8988571]
- Sambrook, J.; Russell, DW. *Molecular Cloning: A Laboratory Manual.* 3rd edn. Vol. 2. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2001. p. 11.43–11.47.
- Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ. Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J. Neurosci.* 1998; 18:1848–1859. [PubMed: 9465009]
- Sesack SR, Pickel VM. Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. *J. Comp. Neurol.* 1992; 320:145–160. [PubMed: 1377716]

- Sesack SR, Deutch AY, Roth RH, Bunney BS. Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J. Comp. Neurol.* 1989; 290:213–242. [PubMed: 2592611]
- Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Ann. Rev. Biochem.* 1999; 68:821–861. [PubMed: 10872467]
- Smith Y, Kieval J, Couceyro PR, Kuhar MJ. CART peptide-immunoreactive neurones in the nucleus accumbens in monkeys: ultrastructural analysis, colocalization studies, and synaptic interactions with dopaminergic afferents. *J. Comp. Neurol.* 1999; 407:491–511. [PubMed: 10235641]
- Smith Y, Koylu EO, Couceyro P, Kuhar MJ. Ultra-structural localization of CART (cocaine- and amphetamine-regulated transcript) peptides in the nucleus accumbens of monkeys. *Synapse.* 1997; 27:90–94. [PubMed: 9268069]
- Strickland TL, Mena I, Villanueva-Meyer J, Miller BL, Cummings J, Mehringer CM, Satz P, Myers H. Cerebral perfusion and neuropsychological consequences of chronic cocaine use. *J. Neuropsych. Clin. Neurosci.* 1993; 5:419–427.
- Striplin CD, Kalivas PW. Correlation between behavioral sensitization to cocaine and G protein ADP-ribosylation in the ventral tegmental area. *Brain Res.* 1992; 579:181–186. [PubMed: 1628210]
- Striplin CD, Kalivas PW. Robustness of G protein changes in cocaine sensitization shown with immunoblotting. *Synapse.* 1993; 14:10–15. [PubMed: 8511715]
- Suzuki T, Usuda N, Ishiguro H, Mitake S, Nagatsu T, Okumura-Noji K. Occurrence of a transcription factor, cAMP response element-binding protein (CREB), in the postsynaptic sites of the brain. *Brain Res. Mol. Brain Res.* 1998; 61:69–77. [PubMed: 9795144]
- Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.* 1991; 548:100–110. [PubMed: 1651140]
- Toda S, McGinty JF, Kalivas PW. Repeated cocaine administration alters the expression of genes in corticolimbic circuitry after a 3-week withdrawal: a DNA macroarray study. *J. Neurochem.* 2002; 82:1290–1299. [PubMed: 12358776]
- Ungless MA, Whistler JL, Malenka RC, Bonci A. Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. *Nature.* 2001; 411:583–587. [PubMed: 11385572]
- Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl Acad. Sci. USA.* 1990; 87:1663–1667. [PubMed: 1689846]
- Vanderschuren LJ, Kalivas PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacologia.* 2000; 151:99–120.
- Volkow ND, Mullani N, Gould KL, Adler S, Krajewski K. Cerebral blood flow in chronic cocaine users: a study with positron emission tomography. *Br. J. Psych.* 1988; 152:641–648.
- Volkow ND, Fowler JS, Wolf AP, Hitzemann R, Dewey S, Bendriem B, Alpert R, Hoff A. Changes in brain glucose metabolism in cocaine dependence and withdrawal. *Am. J. Psych.* 1991; 148:621–626.
- Volkow ND, Fowler JS, Wang GJ. Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans. *J. Psychopharmacol.* 1999; 13:337–345. [PubMed: 10667609]
- Walters CL, Blendy JA. Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. *J. Neurosci.* 2001; 21:9438–9444. [PubMed: 11717377]
- Wang XB, Funada M, Imai Y, Revay RS, Ujike H, Vandenbergh DJ, Uhl GR. rGbeta1: a psychostimulant-regulated gene essential for establishing cocaine sensitization. *J. Neurosci.* 1997; 17:5993–6000. [PubMed: 9221795]
- Wang Y, Tesfaye E, Yasuda RP, Mash DC, Armstrong DM, Wolfe BB. Effects of post-mortem delay on subunits of ionotropic glutamate receptors in human brain. *Brain Res. Mol. Brain Res.* 2000; 80:123–131. [PubMed: 11038245]
- White FJ, Kalivas PW. Neuroadaptations involved in amphetamine and cocaine addiction. *Drug Alcohol Depend.* 1998; 51:141–153. [PubMed: 9716936]

- White FJ, Hu XT, Zhang XF, Wolf ME. Repeated administration of cocaine or amphetamine alters neuronal responses to glutamate in the mesoaccumbens dopamine system. *J. Pharmacol. Exp. Ther.* 1995a; 273:445–454. [PubMed: 7714800]
- White, FJ.; Xiu, Y-H.; Henry, DJ.; Zhang, X-F. Neurophysiological alterations in the mesocorticolimbic dopamine system during cocaine administration. In: Hammer, RP., Jr, editor. *The Neurobiology of Cocaine Addiction*. CRC Press; Boca Raton, FL: 1995b. p. 99-120.
- Wise RA, Bozarth MA. A psychomotor stimulant theory of addiction. *Psychol. Rev.* 1987; 94:469–492. [PubMed: 3317472]
- Wolf ME. The role of excitatory amino acids in behavioral sensitization to psychomotor stimulants. *Prog. Neurobiol.* 1998; 54:679–720. [PubMed: 9560846]
- Zhang XF, Hu XT, White FJ, Wolf ME. Increased responsiveness of ventral tegmental area dopamine neurons to glutamate after repeated administration of cocaine or amphetamine is transient and selectively involves AMPA receptors. *J. Pharmacol. Exp Ther.* 1997; 281:699–706. [PubMed: 9152375]

Fig. 1.

(a) Ionotropic glutamate receptor subunit protein levels in VTA and I-SN of cocaine overdose victims. Membrane fractions were isolated as described in Experimental procedures and 10 μ g was separated on 10% SDS-PAGE to assess glutamate receptor subunit immunoreactivity. Data are expressed as mean $\pm$ SEM of the percentage of control values per quantity of protein loaded; * p < 0.05; ** p < 0.01. (b) Representative bands from two cocaine overdose victims (+) and two control subjects (-) for each subunit.

Fig. 2.

(a) Levels of G protein subunits in VTA and I-SN of cocaine overdose victims. Cytoplasmic fractions were isolated as described in the Experimental procedures and 10 μ g was separated on 10% SDS-PAGE to assess G protein subunit immunoreactivity. Data are expressed as mean $\pm$ SEM of the percentage of control values per quantity of protein loaded. (b) Representative bands from two cocaine overdose victims (+) and two control subjects (-).

Fig. 3.

(a) CREB protein levels in the cytosolic and nuclear fractions of VTA and I-SN in cocaine overdose victims. Cytoplasmic and nuclear fractions were isolated as described in Experimental procedures and 15 μ g was separated on 10% SDS-PAGE to assess CREB immunoreactivity. Phospho-CREB immunoreactivity was not detectable in either the cytoplasmic or the nuclear fractions. Data are expressed as mean $\pm$ SEM of the percentage of control values; * p < 0.05. (b) Representative bands from two cocaine overdose victims (+) and two control subjects (-).

Table 1

Clinical information of study subjects

Subject	Race/ Gender	Age (yrs)	PMI (h)	Cause of death	Toxicology	Blood		Brain		Cocethylene (blood/brain)
						Coc (mg/L)	BE (mg/L)	Coc (mg/kg)	BE (mg/kg)	
COD1	W/M	39	12.5	Cocaine intoxication	COC, CE	0.05	0.16	0.056	1.224	NA
COD2	W/M	36	13	Cocaine intoxication Atherosclerotic heart disease	COC, CE, EtOH, LC	0.05	0.11	0.136	0.276	0.204
COD3	W/M	41	12	Cocaine intoxication; Cardiac arrest	COC, EtOH	0.07	0.22	NA	NA	ND
COD4	B/M	32	18	Cocaine intoxication	COC, NIC	0.2	0.31	1.11	0.89	–
COD5	W/M	40	11.5	Cocaine intoxication	COC, DPHA	0.05	0.22	0.119	<0.05	ND/<0.05
COD6	B/M	34	14	Cocaine intoxication	COC, ME	0.049	2.4	–	–	0.09/ND
COD7	W/M	41	12	Cocaine intoxication	COC, ME	6.7	10	22.8	2.8	ND
COD8	W/M	23	8	Cocaine intoxication	COC, ME	0.05	0.28	0.11	0.15	0.1/0.14
COD9	W/M	27	12	Cocaine intoxication	COC	–	–	2.241	1.801	–
COD10	W/M	42	10.5	Cocaine intoxication	COC, CE	0.05	1.3	0.1	0.73	0.05/0.20
CTR1	W/M	35	16	Calcific aortic stenosis	ND					
CTR2	W/F	39	11	Sharp/blunt force injuries	ND					
CTR3	W/M	44	11.5	Acute myocardial infarction	ND					
CTR4	W/H/M	26	14	Idiopathic cardiac conduction system disease	ND					
CTR5	W/M	24	15.5	Occlusive coronary artery disease	ND					
CTR6	W/M	46	11	Aortic aneurysm	ND					
CTR7	W/M	34	14	Coronary arteriosclerosis	ND					
CTR8	W/M	41	12	Atherosclerotic heart disease	ND					
CTR9	W/M	34	11.5	Asthmatic bronchitis	ND					
CTR10	W/M	37	14.5	NA	ND					
CTR11	B/M	33	14	Surgical complications	ND					

Abbreviations: BE, benzylecognine; CE, cocethylene; COC, cocaine; COD, cocaine overdose; CTR, control; DPHA, diphenhydramine; EtOH, ethanol; LC, lidocaine; NA, not available; ND, not detectable; PMI, post-mortem interval.

Table 2

Statistical analysis of gene expression profiles for VTA and I-SN of cocaine overdose victims and controls

Gene class	<u>Main effect</u>		<u>Interaction</u>		<u>Post-hoc analysis</u>	
	VTA	I-SN	VTA	I-SN	VTA	I-SN
Glutamate Receptor	0.001 ^a	0.070	0.017	0.972	–	–
GRIN (encoding NMDAR1)	–	–	–	–	0.033 ^a	–
GRIA1 (encoding GluR1)	–	–	–	–	–	–
GRIA2 (encoding GluR2)	–	–	–	–	0.002 ^a	–
GRIA3 (encoding GluR3)	–	–	–	–	–	–
GRIA4 (encoding GluR4)	–	–	–	–	–	–
GRIK1 (encoding GluR5)	–	–	–	–	0.003 ^a	–
GRIK5 (encoding KA2)	–	–	–	–	0.004 ^a	–
GRM3 (encoding mGluR3)	–	–	–	–	–	–
GABA transcripts	0.051	0.161	0.239	0.812	–	–
GABA subunit 1	–	–	–	–	–	–
GABA subunit 2	–	–	–	–	–	–
GABA subunit 1	–	–	–	–	–	–
GABA subunit 3	–	–	–	–	–	–
GABA subunit 2	–	–	–	–	–	–
GABA subunit	–	–	–	–	–	–
GABA subunit	–	–	–	–	–	–
GABA subunit	–	–	–	–	–	–
GAD65	–	–	–	–	–	–
GAD67	–	–	–	–	–	–
G protein subunits	0.590	0.033 ^b	0.023	1.000	–	–
G i1	–	–	–	–	–	–
G i2	–	–	–	–	–	–
G o	–	–	–	–	–	–
G l	–	–	–	–	–	–
G s	–	–	–	–	–	–
G t	–	–	–	–	–	–
G q	–	–	–	–	–	–
G z	–	–	–	–	–	–
G 11	–	–	–	–	–	–
G 15	–	–	–	–	–	–
G 1	–	–	–	–	0.001 ^b	–
G 1	–	–	–	–	–	–
G 2	–	–	–	–	–	–
G 4	–	–	–	–	–	–
Dopamine transcripts	0.673	0.714	0.531	0.760	–	–
D1 receptor	–	–	–	–	–	–

Gene class	Main effect		Interaction		Post-hoc analysis	
	VTA	I-SN	VTA	I-SN	VTA	I-SN
D2 receptor	–	–	–	–	–	–
D4 receptor	–	–	–	–	–	–
D5 receptor	–	–	–	–	–	–
Dopamine transporter	–	–	–	–	–	–
Tyrosine hydroxylase	–	–	–	–	–	–
Dopa decarboxylase	–	–	–	–	–	–
Regulators of G protein signaling (RGS) & GTPases	0.175	0.027 ^b	0.002	1.000	–	–
RGS1	–	–	–	–	–	–
RGS2	–	–	–	–	–	–
RGS3	–	–	–	–	0.001 ^a	–
RGS4	–	–	–	–	–	–
RGS5	–	–	–	–	–	–
RGS6	–	–	–	–	–	–
RGS7	–	–	–	–	–	–
RGS9	–	–	–	–	–	–
RGS10	–	–	–	–	–	–
RGS11	–	–	–	–	–	–
RGS12	–	–	–	–	0.002 ^b	–
RGS13	–	–	–	–	–	–
RGS16	–	–	–	–	–	–
Rho guanine nucleotide exchange factor (GEF) 5	–	–	–	–	–	–
RAB7, member RAS oncogene family like 1	–	–	–	–	–	–
Ras homolog enriched in brain 2	–	–	–	–	–	–
mRNA processing	0.204	0.210	0.442	0.998	–	–
Fos related antigen 2	–	–	–	–	–	–
Early growth response 2	–	–	–	–	–	–
Early growth response 3	–	–	–	–	–	–
protein kinase, interferon-inducible RNA dependent	–	–	–	–	–	–
CUG triplet repeat, RNA binding protein 2	–	–	–	–	–	–
RNApII	–	–	–	–	–	–
TATA box binding protein-associated factor, 30 kDa	–	–	–	–	–	–
RNA polymerase II polypeptide C, 33 kDa	–	–	–	–	–	–
Cell growth/death	0.759	0.515	0.118	0.943	–	–
activity-regulated cytoskeleton-associated protein (arc)	–	–	–	–	–	–
Brain derived neurotrophic factor (BDNF)	–	–	–	–	–	–
growth associated protein 43 (GAP43); neuromodulin	–	–	–	–	–	–
fodrin	–	–	–	–	–	–
BCL2-associated X protein (bax)	–	–	–	–	–	–
Miscellaneous	0.481	0.278	0.003	0.205	–	–
Cocaine-amphetamine related transcript	–	–	–	–	0.001 ^b	–

Gene class	<u>Main effect</u>		<u>Interaction</u>		<u>Post-hoc analysis</u>	
	VTA	I-SN	VTA	I-SN	VTA	I-SN
5-hydroxytryptamine (serotonin) receptor 2 A	–	–	–	–	–	–
5-hydroxytryptamine (serotonin) receptor 2C	–	–	–	–	–	–
5-hydroxytryptamine (serotonin) receptor 3	–	–	–	–	–	–
Cannabinoid receptor 1	–	–	–	–	–	–

Normalized expression values for designated transcript classes were analyzed using a two-way ANOVA (Group $\times$ 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 Main effect, Interaction and *post-hoc* analysis.

^a Indicates significant up-regulation in cocaine overdose victims

^b Indicates significant down-regulation in cocaine overdose victims.