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ORIGINAL ARTICLE

Transcriptional profiling in the human prefrontal cortex: evidence for two activational states associated with cocaine abuse

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ABSTRACT

CNS-focused cDNA microarrays were used to examine gene expression profiles in dorsolateral prefrontal cortex (dlPFC, Area 46) from seven individual sets of age- and post-mortem interval-matched male cocaine abusers and controls. The presence of cocaine and related metabolites was confirmed by gas chromatography-mass spectrometry. Sixty-five transcripts were differentially expressed, indicating alterations in energy metabolism, mitochondria and oligodendrocyte function, cytoskeleton and related signaling, and neuronal plasticity. There was evidence for two distinct states of transcriptional regulation, with increases in gene expression predominating in subjects testing positive for a metabolite indicative of recent 'crack' cocaine abuse and decreased expression profiles in the remaining cocaine subjects. This pattern was confirmed by quantitative polymerase chain reaction for select transcripts. These data suggest that cocaine abuse targets a distinct subset of genes in the dlPFC, resulting in either a state of acute activation in which increased gene expression predominates, or a relatively destimulated, refractory phase.
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Keywords: addiction; expression patterns; gene profiling; neuroplasticity; psychostimulants

INTRODUCTION

A hallmark feature of drugs of abuse, such as the psychostimulant cocaine, is to alter the expression of a wide array of behavioral, neurochemical, and cellular parameters.[1–4] It appears that most of the changes seen in mRNA and protein expression as well as biochemical parameters disappear rapidly when drug is discontinued,[5–9] and may even be blunted by chronic drug exposure.[10] By effecting downstream events, even short-lived changes in expression patterns may lead to more enduring modifications, and thereby provide the neurobiological substrates for sensitization, tolerance, craving, and addiction.[3, 11]

While cocaine has a distinct effect in different anatomical regions,[12–14] it appears to rely heavily on the prefrontal cortex (PFC) both for the induction and long-term sensitization to cocaine.[1, 15, 16] Imaging studies have demonstrated altered neuronal activity in the PFC of cocaine abusers responding to cocaine or cocaine-related cues.[17–22] Neuropsychological testing has indicated that executive planning and consequence evaluation, measures of the functionality of the ventromedial aspects of the PFC, are adversely affected, while explicit memory and learning, involving the dorsolateral PFC (dlPFC) circuitry, appears functional.[23–25] Consequently, the PFC appears to be critically involved in both experimental animal models and in human cocaine abuse.[1, 26–28]

Table 1 Demographic data for control- and cocaine-abusing subjects

ID	*Race/age (years)*	*PMI (h)*	*Brain pH*	*Cause of death*	*Drug abuse history*
CTR#1	CM 40	16.0	6.02	Natural	—
COC#1	AAM 36	11.5	6.95	Homicide (stabbing)	cocaine
CTR#2	AAM 24	12.5	6.59	Natural	
COC#2	AAM 23	15.0	7.05	Homicide (m gsw* torso)	cocaine
CTR#3	AAM 32	15.5	6.77	Homicide	—
COC#3	AAM 34	28.0	6.73	Cocaine toxicity	cocaine/alcohol
CTR#4	AAM 56	33.0	6.09	Pulmonary embolism	—
COC#4	AAM 51	24.5	6.58	Asphyxia (chest compression)	cocaine
CTR#5	AAM 40	31.5	6.41	Natural (asthma attack)	—
COC#5	AAM 37	37.5	6.59	Homicide (m gsw* to body)	cocaine
CTR#6	AAM 38	32.5	6.14	Pulmonary embolism	—
COC#6	AAM 29	29.0	6.45	Mixed drug intoxication	cocaine
CTR#7	AAM 42	40.0	6.63	Acute asthma attack	—
COC#7	AAM 49	36.0	6.92	Homicide (gsw* upper body)	crack/cocaine (alcohol)
CTR, all	38.9 $\pm$ 9.8	25.9 $\pm$ 10.9	6.38 $\pm$ 0.30		
COC, all	37.0 $\pm$ 10.1	25.9 $\pm$ 9.8	6.75 $\pm$ 0.22		
CTR (A)	39.5 $\pm$ 13.1	23.5 $\pm$ 10.8	6.21 $\pm$ 0.26		
COC (A)	34.8 $\pm$ 12.1	20.0 $\pm$ 8.1	6.76 $\pm$ 0.29		
CTR (B)	38.0 $\pm$ 5.3	29.0 $\pm$ 12.4	6.60 $\pm$ 0.18		
COC (B)	40.0 $\pm$ 7.9	33.8 $\pm$ 5.1	6.75 $\pm$ 0.17		

control- and cocaine-abusing male subjects were individually matched for race (C—Caucasian, AA—African-American), age, and PMI as indicated. Age, PMI, and brain pH for the control- and cocaine-abusing groups did not differ significantly. Rows indicated by 'all', 'A' or 'B' are means $\pm$SD for all subjects, and the subjects in Groups A and B, respectively. *m gsw = multiple gun shot wounds.

Profiling the expression of large sets of transcripts has been greatly facilitated by the development and miniaturization of DNA array technology. This technique enables the concurrent screening of large sets of known and novel targets of drug-induced change. Equally important, by profiling a large number of transcripts in minute amounts of human brain tissue, the microarray technology allows for a judicious use of a limited and unique resource. The present study employed complementary DNA (cDNA) microarrays to analyze differences in transcription patterns in the dlPFC of seven individual sets of gender-, age- and post-mortem interval-matched cocaine abusers and controls (Table 1).

RESULTS

Selection Criteria

Transcripts for which the expression levels differed across all or most of the seven individual cocaine–control comparisons were identified by sorting the individual transcript expression data by the mean of the *P*-values for the seven matched comparisons. Selecting transcripts with a mean *P*-value <0.05 and a minimum of three individual matched comparisons with a *P*-value ≤ 0.001 yielded 65 clones Table 2. These represent 64 different gene transcripts, and correspond to 5.6% of the total number of transcripts present on the Neuroarray (Figure 1). Two of the 65 different clones overlapped within the same region of a tubulin transcript (*TUBA2*), and those two sets of data corresponded closely (Table 2).

Main Gene Expression Pattern

Subsequent inspection of the *z*-ratios revealed two distinct expression patterns, subdividing the seven individual comparisons into two subgroups (Table 2, Figures 2–4). Pattern A included comparisons #1, #2, #4, and #6, while comparisons #3, #5, and #7 followed pattern B. As can be seen from Table 2, most of the 65 transcripts that were differentially expressed in the cocaine-control subject comparisons were changed in opposite directions in the two subgroups. Of the 65 transcripts selected, 17 (~25%) did not adhere strictly to this pattern. Re-examining the entire set of expression data, there was a similar Group A *vs* Group B subdivision for approximately 10–15% of all transcripts. These included many transcripts with weaker differences between the subject and controls that did not meet the applied criteria for differential expression. The pattern was, however, more prominent for those transcripts with the greatest differences between the cocaine and control subjects.

In other experiments (data not shown), microarray studies of pooled samples containing equal amounts of RNA from either cocaine abusers (cocaine pool) or matched controls (control pool) resulted in *z*-ratios for the transcripts selected in the present study that were intermediate in magnitude between those seen for either pattern A or pattern B, while the direction of change more closely resembled that of pattern B (data not shown).

Cross-referencing Expression Profiles

While a radioactive detection format dictates the use of separate arrays for individual samples, it also provides the

Table 2 Mean *p*-value and z-ratio scores for 65 selected transcripts

Transcript		*Z-Ratio for comparison (COC#/CTR#)*							
Gene	*Accession*	*#1/#1*	*#2/#2*	*#4/#4*	*#6/#6*	*#3/#3*	*#5/#5*	*#7/#7*	*mean p-value*
Energy metabolism and mitochondrial function									
CYC1	R52654	3.33	3.50	5.40	2.45	−3.82	−3.19	−0.96	0.000025
MDH1	AA403295	3.20	4.56	6.31	4.38	−5.49	−3.85	−4.10	0.000034
SOD1	AA599127	2.23	2.73	3.50	1.24	−1.21	−2.35	0.45	0.000264
COX6C	AA456931	2.40	2.60	2.23	2.35	−0.71	−1.99	−0.51	0.000361
CASP4	H45000	2.57	2.67	1.64	0.83	−2.65	−2.89	−2.04	0.000558
ENO2	AA450189	1.70	2.03	3.48	3.06	−3.05	−3.23	−3.37	0.000617
ATP5G3	H47080	0.66	1.39	2.28	1.46	−1.64	−1.52	−1.05	0.001483
Neuronal cytoskeleton, signaling, and extracellular matrix-related									
MAP1B	AA219045	2.57	3.66	4.95	2.88	−4.07	−2.66	−3.58	0.000176
NEFL	R14230	2.39	3.70	5.37	3.42	−3.87	−2.78	−4.64	0.000108
NEF3	AA400329	2.06	2.14	1.83	2.50	−1.52	−0.89	−2.29	0.000637
TUBA1	AA180912	3.57	3.73	1.86	0.45	−3.27	−3.72	−3.78	0.000006
TUBA2	AA626698	2.89	3.45	2.58	1.82	−4.84	−4.04	−5.84	0.000010
TUBA2	AA426374	2.82	3.59	2.14	0.87	−4.13	−3.60	−4.98	0.000175
GAP43	H05445	3.32	4.06	1.33	0.74	−2.52	−4.85	−2.91	0.000087
DNCI1	H05091	0.93	1.37	2.13	2.12	−1.33	−0.21	0.05	0.006742
PTN	AA001449	1.48	2.05	−0.22	1.71	−1.95	−2.31	0.03	0.000317
CLTB	N20335	3.11	2.97	6.40	2.77	−3.54	−3.15	−1.51	0.000067
NSF	H97488	1.59	2.35	5.06	2.92	−3.05	−2.62	−1.26	0.000696
CDH17	AA088861	2.18	2.54	1.10	0.51	−2.50	−2.48	−3.50	0.000132
CDH1	H97778	1.53	1.40	0.64	0.89	−2.12	−2.64	−2.44	0.004542
CTNND2	H04985	0.74	1.35	−0.05	−0.08	−1.23	−1.39	−1.49	0.005644
PNUTL2	T64878	1.91	1.53	0.21	0.83	−0.86	−2.28	−3.72	0.000243
PFN1	AA521431	−3.61	−2.87	−3.21	−3.94	0.29	0.58	0.44	0.007903
SGCB	R55105	1.75	1.39	0.29	1.00	−1.30	−1.25	−1.48	0.000290
LAMR1	AA629897	1.61	0.63	0.78	0.32	−1.20	−1.65	−2.65	0.005599
CAPZB	AA430524	1.41	1.69	0.66	1.13	−0.63	−1.00	−0.60	0.007152
NP25	W48780	1.38	1.05	1.34	1.45	−1.63	−1.39	−3.27	0.003745
Neurotransmission and G-protein related signaling									
GNAO1	R43320	4.99	4.62	1.84	−0.06	−3.64	−5.13	−2.15	0.000041
GNB1	AA487912	−0.35	1.27	1.70	1.42	−1.17	−0.80	−3.25	0.009438
KCNJ6	H20547	2.65	3.03	3.58	1.72	−2.28	−2.65	−0.55	0.000261
GAD1	AA018457	0.40	1.07	2.04	1.53	−1.99	−1.44	−1.34	0.049829
Glycoproteins and oligodendrocyte-related transcripts									
PLP1	T75041	2.34	1.50	0.53	1.80	−4.06	−3.04	−6.69	0.000069
ST3GALVI	H19227	−1.85	−0.69	−0.78	−1.71	0.71	1.81	1.58	0.000090
GSN	H72027	0.96	0.68	1.59	1.70	−1.64	−1.53	−2.07	0.002907
GPM6A	AA448033	3.25	2.96	2.84	1.91	−1.59	−3.48	2.42	0.000311
GPM6B	AA284329	3.84	2.82	1.90	1.09	−2.23	−3.94	0.28	0.012842
Drug metabolism									
CES2	AA101996	0.86	1.66	−0.04	0.48	−2.04	−1.31	−1.30	0.001054
CYP51	AA477893	2.74	3.22	−0.63	−1.76	−0.30	−3.94	−2.47	0.001104
SLC10A1	T68568	−2.60	−1.79	−2.10	−1.75	1.00	1.20	1.35	0.006066
Transcription factors									
FOSL1	H96643	2.58	3.25	2.44	1.36	−3.02	−2.75	−3.68	0.000014
LDB2	H74106	1.23	2.22	2.53	1.98	−2.34	−1.81	−1.32	0.000463
ISGF3G	AA291577	2.01	0.78	1.79	1.39	−3.15	−1.93	−2.08	0.000810
PAX8	AA405891	−0.65	−0.65	−0.15	0.47	0.70	1.27	1.45	0.003594
TFAP4	AA284693	−2.08	−1.32	−1.03	−1.76	0.33	1.10	0.63	0.004349
HIF1A	AA598526	1.40	0.88	1.02	0.76	−1.59	−2.08	0.60	0.042555

Table 2 Mean *p*-value and *z*-ratio scores for 65 selected transcripts

Transcript		*Z-Ratio for comparison (COC#/CTR#)*							
Gene	*Accession*	*#1/#1*	*#2/#2*	*#4/#4*	*#6/#6*	*#3/#3*	*#5/#5*	*#7/#7*	*mean p-value*
Phosphatases									
PPP2CB	AA490696	2.10	2.50	2.58	1.69	−2.58	−2.32	−0.38	0.000166
PPAP2C	AA446147	1.83	2.02	2.84	2.51	−2.44	−2.00	0.15	0.000619
PTPRA	H82419	2.47	1.94	0.13	0.41	−2.45	−2.39	−1.23	0.001009
PPP1CA	AA443982	1.75	1.25	−0.71	0.38	−1.52	−1.80	−1.25	0.001148
PPP2CA	AA599092	1.32	1.10	2.08	2.34	−1.73	−1.59	−1.06	0.001362
Kinases									
PARG1	AA629603	3.28	3.91	2.52	1.15	−4.37	−3.53	−4.74	0.000007
MAPK10	T75436	2.17	2.44	1.08	0.98	−2.38	−2.41	−1.21	0.000046
PTK2	AA630298	1.52	2.18	1.92	−1.02	−0.64	−0.87	−3.39	0.000090
PRKCB1	AA479103	2.60	2.81	0.45	−2.43	−2.37	−3.09	−2.30	0.000167
MAP2K1	R19938	2.46	2.06	1.43	1.01	−1.88	−2.13	−1.91	0.000363
MAPK3	AA454819	1.12	0.09	0.07	0.78	0.32	−0.96	1.90	0.027858
Others									
UCHL1	AA670438	4.32	4.70	5.11	2.63	−2.94	−3.71	−0.48	0.000015
UBE2I	AA487197	−2.24	−1.32	−1.05	−1.40	0.99	1.29	1.33	0.014635
PCSK2	AA069517	1.92	1.90	1.53	1.34	−1.57	−1.51	−0.33	0.000079
YWHAH	N90630	3.76	3.99	7.10	2.53	−2.93	−4.90	0.37	0.000098
CGI−43	AA490124	2.68	1.99	2.21	1.31	−1.25	−1.95	2.10	0.000310
MJD	AA702422	2.05	1.79	2.09	1.41	−1.95	−1.95	−1.91	0.001318
GJB2	AA490688	1.75	1.90	1.22	0.76	−2.52	−1.95	−2.76	0.001723
FGF2	R38539	1.96	1.72	1.10	0.61	−1.01	−1.87	−0.79	0.005470
SSR4	AA486261	1.00	1.07	−0.25	−0.35	−1.41	−1.71	−1.26	0.009438

Each transcript selected for differential expression displayed a mean *p*-value (across all seven comparisons) <0.05, with a minimum of three individual comparisons displaying a mean *p*-value <0.001. A positive z-ratio indicates higher expression in the cocaine subject. Gene symbols follow the LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/) nomenclature.

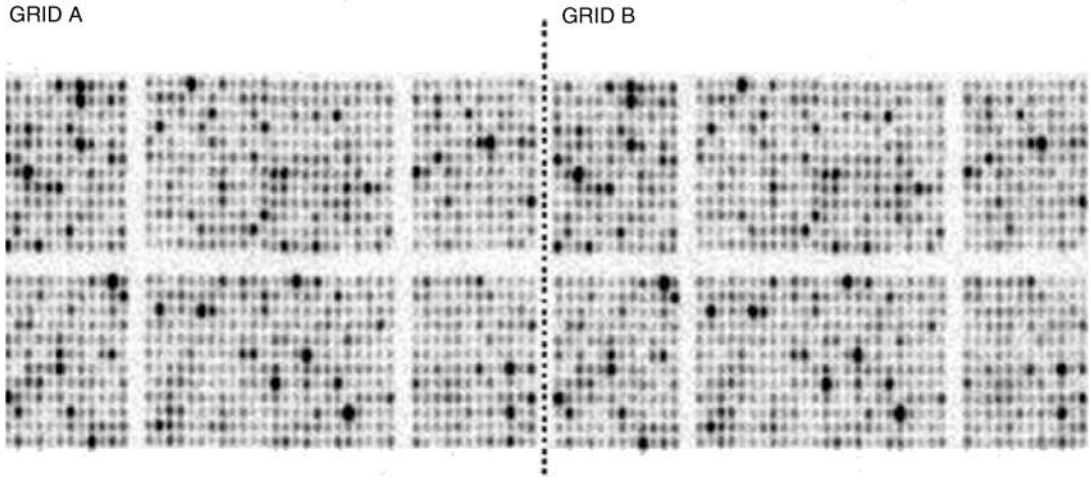

Figure 1 Example of a neuroarray used in the present study. Note that each clone is represented twice, once in Grid A, and once in Grid B.

opportunity to compare each individual array to any other individual array processed similarly. The normalized ratio provides an estimate of the relative changes in transcript expression for the matched comparisons, and concurred with the *z*-ratio data in both the general ranking of transcripts as well as the direction of change (data not shown). Normalized ratio comparisons among the cocaine-abusing individuals in pattern B showed that overall expression levels for COC#3 and COC#7 were similar, while those of COC#5 appeared generally depressed compared to either. Only a few differences in transcript expression were present between the cocaine-abusing individuals in pattern A, and none were transcripts selected by the *z*-ratio criteria.

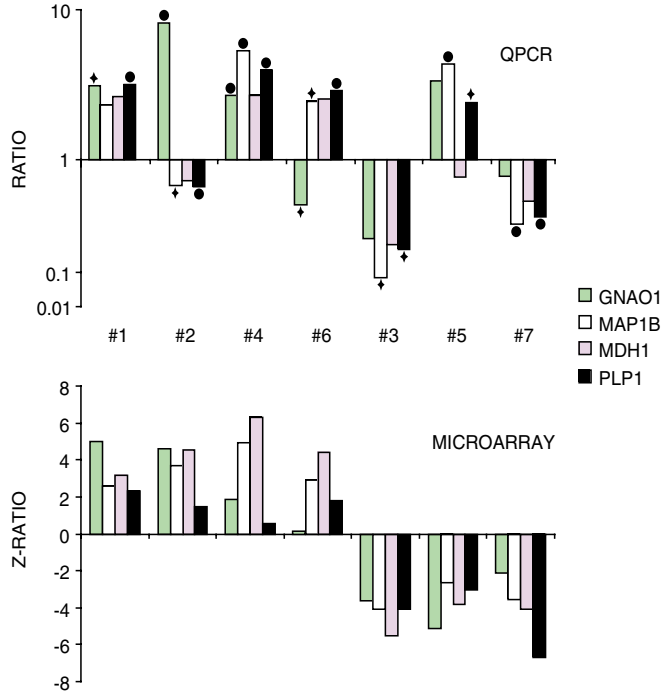

Figure 2 Comparison of individual QPCR ratios and microarray *z*-ratios for *GNAO1*, *MDH1*, *MAP1B*, and *PLP1*. For each transcript, the upper graph represents the QPCR results, expressed as numerical ratios between the cocaine subject and the respective matched control. Ordinate values above 1 indicate higher expression, while values below 1 indicate lower expression, with respect to the cocaine subject. The lower graph illustrates the corresponding microarray *z*-ratios. Positive values on the ordinate indicate higher, and negative values indicate lower, levels of expression with respect to the cocaine subject. For both types of experiments, the seven matched cocaine–control pairs are indicated on the abscissa. For each cocaine subject and matched control, the QPCR assay was run for eight paired replicated samples. For each transcript, the significance of the difference between each cocaine subject and the corresponding control was tested using a two-tailed Wilcoxon matched-pairs signed-ranks test.[73] The significance of the difference in MDH1 expression could not be tested because of a smaller number of replicates. (♦ = $P<0.05$, ● = $P<0.01$).

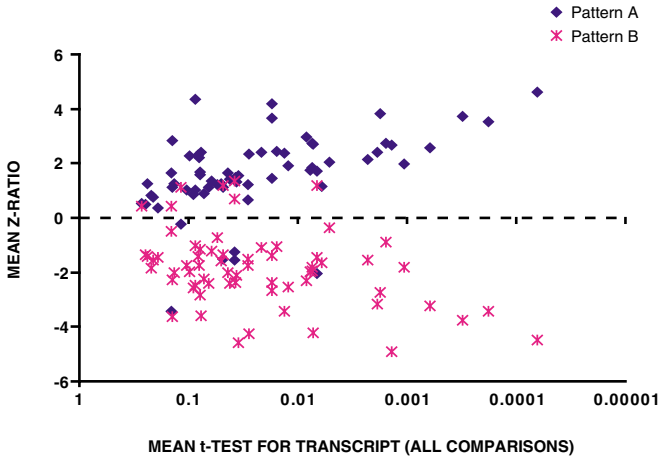

Figure 3 Divergence of transcript expression levels in cocaine–control subject comparisons. A divergent pattern emerged when the overall mean *t*-test was used to select the genes. This is illustrated by plotting the average *t*-test value against the average *z*-ratio for the pattern. For clarity, only the selected transcripts are included. Blue squares indicate average *z*-ratios for the four pattern A subjects, while pink asterisks indicate average *z*-ratios for the three pattern B subjects. This illustrates that increased differential expression between controls and cocaine subjects, as indicated by *t*-test *P*-value, is also reflected in a greater divergence between the transcriptional profile of the patterns.

GC-MS of cocaine, opiates, and related analytes (Table 3 and below), revealed the presence of low levels of cocaine and benzoylecgonine (BE) in the dlPFC of one control subject, CTR#5. By comparing the normalized ratio of two pattern B subjects, COC#5 and COC#7, to the A pattern CTRs that best approximated their age and PMI, CTR#4 and CTR#6, we found that the normalizing individual had some effect on both magnitude and direction of transcript expression for COC#7. In contrast, when comparing COC#5 to CTR#4 and to CTR#6, the magnitudes and directions of changes were nearly identical to those obtained using CTR#5, indicating that the presence of cocaine in this control had not affected the outcome of the comparison. Therefore, the results obtained for selected transcripts in the A and B groups were retained across a number of different analysis.

Cluster Analysis

Clustering of the data using the *z*-ratio comparisons for matched comparisons (Figure 5a), or a *z*-ratio based on comparing individual cocaine abusers *vs* pooled control values (Figure 5b), both produced two discrete clusters of cocaine abusers, corresponding to the Group A/Group B patterning (Table 2, Figures 2 and 3).

Quantitative polymerase chain reaction (PCR) (QPCR)

The unexpected finding of a divergent pattern of expression between individual cocaine–control comparisons was probed using an alternate RNA-based quantitative methodology. Real-time QPCR for select transcripts (guanine nucleotide binding protein (G protein) alpha activating activity polypeptide O (*GNAO1),* soluble malate dehydrogenase *(MDH1)*, microtubule-associated protein-1B (*MAP1B)*, and proteolipid protein 1 (*PLP1)* confirmed the observation that individual cocaine–control comparisons exhibited opposite changes in expression level (Figure 2). QPCR for comparison #2 and, in particular, comparison #5 did not confirm the *z*-ratio data from the microarray; nevertheless, the divergent pattern was confirmed. The Wilcoxon matched-pairs signed-rank test confirmed the statistical significance of most of the changes demonstrated by quantitative polymerase chain reaction (QPCR) (Figure 2). Multiple rounds of RNA extraction and subsequent cDNA transcription were performed on aliquots of the brain samples to guard against accidental loss or contamination of the sample, and to allow for replication. In the last round

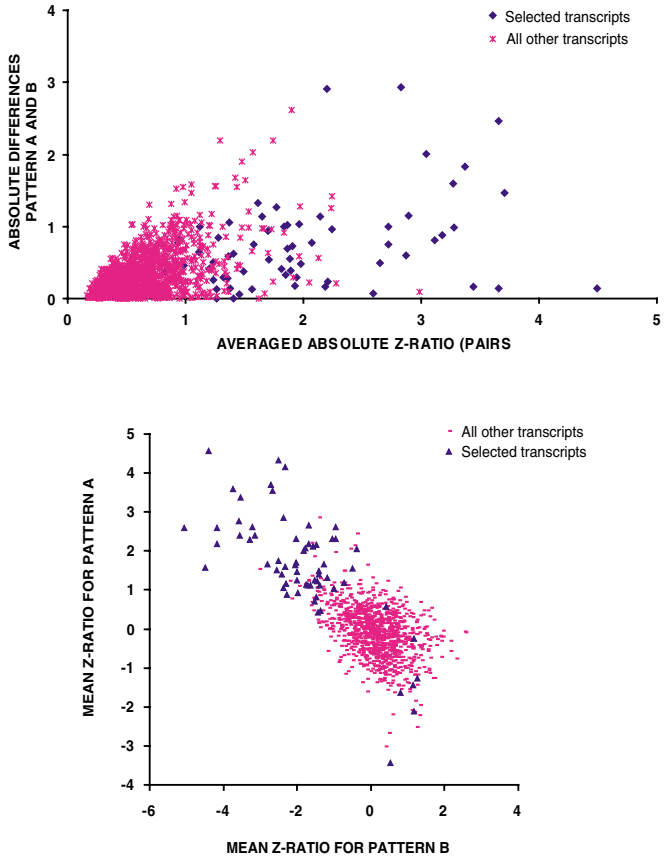

Figure 4 Relation between overall differential gene expression (cocaine *vs* controls) and the divergence between patterns A and B expression profiles for all 1152 transcripts on the neuroarray. (a) The absolute *z*-ratio differences for patterns A and B expression levels as a function of the mean absolute *z*-ratio difference between patterns A and B. This illustrates that differentially expressed transcripts tend to show larger differences in expression between the two patterns. The selected 65 differentially expressed transcripts are indicated by blue squares, while all other transcripts are indicated by pink asterisks. (b) The relation between expression profiles for the two patterns. The *z*-ratios for all 1152 transcripts for pattern A are indicated on the ordinate, while the corresponding *z*-ratios for each transcript in pattern B comparisons are indicated on the abscissa. There is a tendency for transcripts with decreased expression for pattern B cocaine subjects to show increased expression in pattern A cocaine subjects and *vice versa.*

of extractions, each individual dlPFC tissue aliquot was subdivided prior to RNA extraction, yielding both QPCR data and GC-MS information on the presence of cocaine, opiates, and related analytes from the same dlPFC aliquot (Table 3). These results conclusively demonstrated the existence of two different patterns of regulation of the same set of transcripts in the cocaine-abusing cohort.

Toxicology

An individual aliquot of dlPFC brain powder was subdivided for use in GC-MS and QPCR. GC-MS demonstrated the presence of cocaine and two major cocaine metabolites, BE and ecgonine methyl ester (EME), in the dlPFC for all individuals in the cocaine-abusing group (Table 3). The presence of anhydroecgonine methyl ester (AEME) for three pattern A individuals (COC#1–2, COC#4) suggested that cocaine in those individuals had been smoked ('crack' cocaine). The only other drugs of abuse detected were alcohol for COC#2, indicated by metabolites cocaethylene (CE), ecgonine ethyl ester (EEE), and norcocaethylene (NCE), while morphine was detected for COC#6. One control subject, CTR#5, tested positive for cocaine and BE in the dlPFC, even though no drugs were detected at the post-mortem toxicology screen.

All samples were negative for methamphetamine, amphetamine, PCP, norbenzoylecgonine, m- and *p*-hydroxycocaine, *m*- and *p*-hydroxybenzoylecgonine, codeine, norcodeine, normorphine, and 6-acetylmorphine. As the GC-MS data were obtained from a tissue aliquot that was used for QPCR, the presence of two different transcriptional patterns could not have been caused by the inadvertent misidentification of samples at some point during dissection and other preparative routines.

Genotyping

There have been reports that polymorphisms in the catechol-*O*-methyltransferase (COMT) gene influences prefrontal functioning[29] and that dopamine transporter (DAT) function is altered in cocaine abusers.[30, 31] Genotyping was performed to determine if DAT or COMT polymorphisms could account for the observed divergence in transcriptional patterns. There were no obvious differences in DAT or COMT genotypes between the cocaine abusers and their respective controls, or between Group A and Group B cocaine subjects (Table 4).

Functional Groupings

The 65 selected transcripts fell into several distinct functional categories. Seven transcripts (*CYC1, MDH1, SOD1, COX6C, CASP4, ENO2, ATP5G3)* were associated with mitochondrial function and energy metabolism. Of the selected transcripts 24 related to the neuronal cytoskeleton (*NEFL, NEF3, MAP1B, TUBA1, TUBA2, CAPZB, NP25*), extracellular matrix (*CDH1, CDH17, CTNND2, PNUTL2*), growth cones (*GAP43, DNCl1, PTN*), and G-protein-related signaling and neurotransmission (*GNAO1*, *GNB1*, *KCNJ6/GIRK2*, *GAD1*, *NSF*, *CLTB*). Five transcripts were related to oligodendrocyte function and glycoproteins (*PLP1*, *GPM6A*, *GPM6B*, *GSN*, *ST3GALV1*). Six transcription factors were selected, including the immediate early gene fra-1 (FOSL1). Nine transcripts related to protein processing (*UCHL1*, *UBE2I*), growth factors (*FGF2*), and various other functions did not fall clearly into larger categories. Three transcripts specifically related to drug metabolism were altered (*CES2*, *CYP51*, *SLC10A1*). Phosphatases and kinases were highly represented with 11 different transcripts. The mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) signaling cascade was represented by MAPK10

Table 3 GC-MS of dorsolateral prefrontal cortex (dlPFC) tissue

Substance (ng/mg tissue)	*AEME*	*EME*	*EEE*	*COC*	*CE*	*NCOC*	*NCE*	*BE*	*MOR*
COC#1	0.271	0.060	0.0	0.224	0.0	0.0	0.0	0.061	0.0
COC#2	0.277	0.677	0.096	1.570	0.237	*	0.303	0.326	0.0
COC#3	0.0	0.298	0.0	0.838	0.0	0.0	0.0	0.344	0.0
COC#4	0.136	0.459	0.0	0.171	0.0	0.0	0.0	0.607	0.0
COC#5	0.0	0.655	0.0	0.623	0.0	0.0	0.0	1.380	0.0
COC#6	0.0	0.339	0.0	0.148	0.0	0.0	0.0	0.932	0.361
COC#7	0.0	0.398	0.0	0.140	0.0	0.0	0.0	0.381	0.0
CTR#1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTR#2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTR#3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTR#4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTR#5	0.0	0.0	0.0	0.122	0.0	0.0	0.0	0.376	0.0
CTR#6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CTR#7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Anhydroecgonine methyl ester (AEME), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), cocaine (COC), norcocaine (NCOC), norcocaethylene (NCE), benzoylecgonine (BE) and morphine (MOR) were quantified in dlPFC brain tissue as described. Samples were negative (limit of quantitation (LOQ) <125 pg/ng) for methamphetamine, amphetamine, PCP, norbenzoylecgonine, *m*- and *p*-hydroxycocaine, *m*- and *p*-hydroxybenzoylecgonine, codeine, norcodeine, normorphine, and 6-acetylmorphine (*sample positive for analyte but below LOQ).

(JNK3A/SAPK), MAPK3 (ERK1), and MAP2K1 (MEK1), while other selected transcripts interacting with the MAPK/ERK pathway are exemplified by *PTK2* (FAK), *PARG1*, and *PRKCB1*.

Association With 'crack' cocaine Use

GC-MS detected the cocaine metabolite AEME, indicative of smoked or 'crack' cocaine, in the dlPFC of cocaine subjects COC#1, COC#2, and COC#4. Consequently, we examined the individual transcripts (Table 2) *post hoc* for changes potentially associated with recent crack cocaine use. Each of these three cocaine–control comparisons were in the Group A pattern. Approximately 45% (29 of 64) of the selected transcripts showed a similar pattern of consistently larger increases in COC#1, COC#2, and COC#4, and a smaller increase in COC#6 (Table 2). These transcripts included *CYC1, SOD1, CASP4, TUBA1, TUBA2, GAP43, GPM6A, GPM6B, Goα, KCNJ6, FOSL1, PARG1, PTK2, PRKCB1, MAP2K1, UCHL1*, and *YWHAH*.

DISCUSSION

Main Findings

A consistent subset of 65 transcripts met our selection criteria for altered expression across the seven cocaine–control comparisons. The cocaine-abusing cohort was, however, divided into two subgroups, distinguished by opposite directions of change. Pattern A was associated with comparisons #1, #2, #4, and #6, while pattern B was associated with comparisons #3, #5, and #7. The pattern of bidirectional change was identifiable for 10–15% of all transcripts, including some transcripts that failed the selection criteria, but was most pronounced for transcripts exhibiting low mean *P*-values and large *z*-ratios. The pattern was replicated by QPCR for select transcripts (*GNAO1, MDH1*, *MAP1B*, *PLP1)*. GC-MS confirmed the presence of cocaine and cocaine metabolites in samples originating from cocaine abusers. Differences in COMT and DAT genotypes did not account for differences between pattern A and pattern B. There was, however, an association of 'crack' cocaine use with the pattern A change in gene expression.

Divergent Expression Pattern

There is precedence for divergent expression of mRNA, protein or receptor function within subgroups of substance abusers. Decreased DAT mRNA was demonstrated in the substantia nigra in a subgroup of cocaine abusers exhibiting preterminal cocaine-induced excited delirium,[32] while cocaine, ethanol, or co-abuse differentially affected serotonin transporter binding sites in the dorsal raphe.[33] In addition, three sets of pooled samples of frontal or motor cortex from chronic alcoholics differed in the direction of change in select transcripts,[34] among which were two oligodendrocyte-related genes selected presently (*PLP1, GPM6B*). In schizophrenia, it was noted that individual subjects differ, such that group means are not representative of the pathophysiology of the disorder.[35] Individual differences in schizophrenia were observed in an *in situ* hybridization study in support of microarray data.[36] The dynamic regulation of the soluble *MDH1* transcript was illustrated by a downregulation in the dlPFC of schizophrenics in contrast to an upregulation in long-term haldol-treated monkeys.[37]

Implications of the Two Patterns

Pattern A could represent a state of general activation, as exemplified by increases in transcripts associated with metabolic or mitochondrial activity (*MDH1, CYC1*), neurite growth and cytoskeletal remodeling (*NEFL, NEF3, MAP1B, GAP43*), protein metabolism (*UCHL1, UBE2I*), and the transcription factor *fra-1* (*FOSL1*). The recent crack use

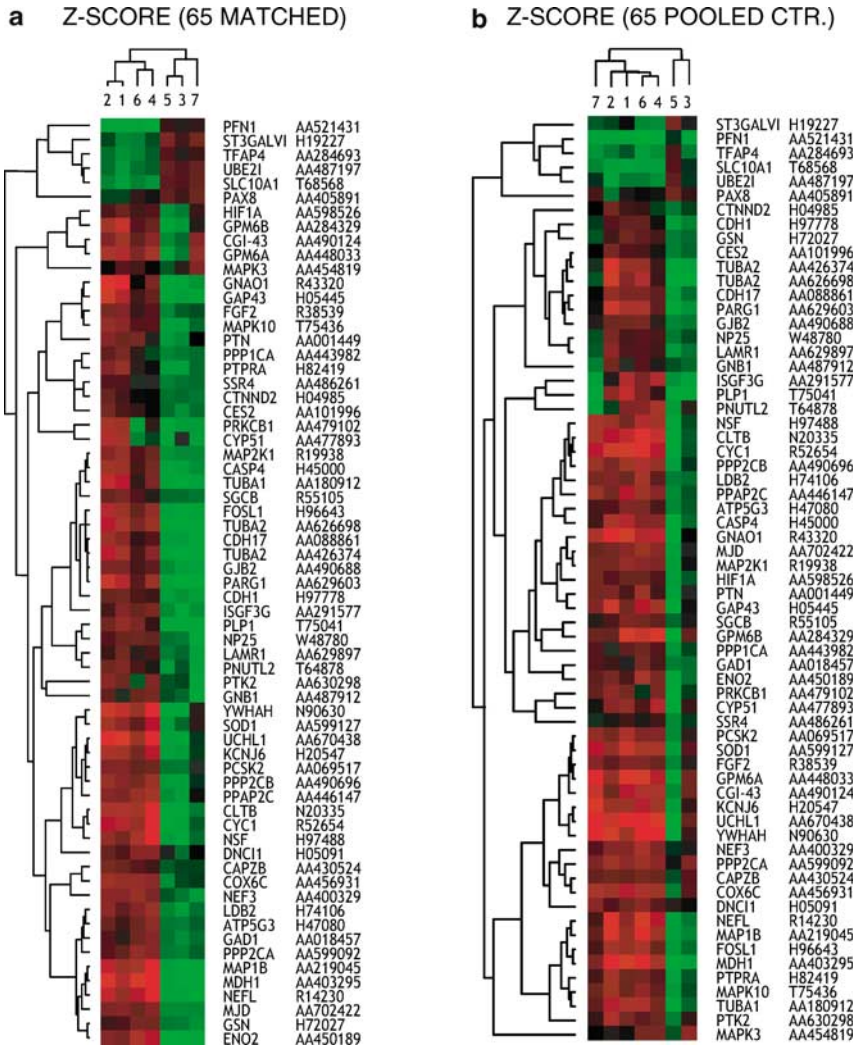

Figure 5 Cluster analysis. Cluster analysis was performed using average-linkage hierarchical cluster analysis, described by Eisen *et al*,[72] of the selected set of 65 transcripts or the entire set of 1152 transcripts (not shown). This analysis demonstrated the clustering of the A pattern cocaine-abusing individuals, differing from the B pattern cocaine-abusing individuals. This is illustrated by the clustering of the *z*-ratio for the individual cocaine–control comparisons (a) and for the comparison of individual cocaine abusers to a control pool, formed by combining all *z*-score data from all seven controls (b). The color and intensity indicate direction and level of change with respect to the cocaine-abusing individual: green spectrum colors indicate decreased expression, while red spectrum colors indicate upregulated transcript expression.

evidenced in three of four pattern A subjects, and in none of the pattern B subjects, potentially indicates a different, or more acute, state of cocaine stimulation in the pattern A subjects. It is tempting, therefore, to speculate that cocaine abusers are either in an acute state of hyperstimulation with increased demand on gene expression, or in a refractory, destimulated state with decreased gene expression. Such surges or peaks in transcriptional activity might occur during the experiences of COC-related craving or euphoria reported by COC abusers.[17, 22]

Cytoskeleton and Signal Transduction

Virtually every aspect of cellular proliferation, differentiation, and cytoskeletal-linked signal transduction is regulated by changes in tyrosine phosphorylation. Tyrosine phosphorylation, in turn, is controlled by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs, *PTPRA*). The mitogen-activated protein kinase (MAPK) family is thought to mediate pivotal intracellular signaling cascades implicated in neuronal plasticity, and includes the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK, *JNK3A1*), the focal adhesion kinase (FAK/*PTK2*), and the extracellular signal-regulated protein kinases (ERKs, *MEK1*, *ERK1*). A different FAK transcript (PYK2) was reported among highly cocaine-responsive transcripts in the rat hippocampus,[38] while PYK2 and *MEK1* expression were increased in the nucleus accumbens of chronic cocaine-treated macacaques.[39] Acti-

Table 4 Individual DAT and COMT genotypes

COMPARISON	*DAT*	*COMT*
COC#1	9/10	Met/Met
CTR#1	9/10	Val/Met
COC#2	10/10	Val/Val
CTR#2	9/10	Val/Val
COC#3	9/10	Val/Met
CTR#3	10/10	Val/Val
COC#4	10/10	Val/Val
CTR#4	8/10	Val/Met
COC#5	10/10	Val/Val
CTR#5	10/10	Val/Met
COC#6	10/10	Met/Met
CTR#6	10/10	Val/Val
COC#7	9/10	Val/Val
CTR#7	10/10	Val/Met

The DAT genotypes differ by 40 bp (**8**: 400 bp, **9**: 440 bp, **10**: 480 bp allele). The enzyme COMT is critical in the metabolic degradation of dopamine, a neurotransmitter hypothesized to influence human cognitive function, and contains a functional polymorphism, Val158Met, that exerts a four-fold effect on enzyme activity. No difference in these genotypes could account for the divergent transcriptional pattern observed.

vation of the MAPK/MEK/ERK signaling pathway occurs via soluble growth factors like basic fibroblast growth factor (bFGF/*FGF2*) or cell-adhesion molecules (CAMs), such as catenin (*CTNND2*) and cadherins (*CDH1, CDH17*). Downstream genes activated include the ERK-dependent Fos-related antigen-1 (Fra-1, *FOSL1*), which is central to the mitogenic activation by activator protein-1 (AP-1).[40] The rewarding effects of cocaine were demonstrated to be abolished by selective systemic inhibition of MEK,[41] further underscoring the central role of the MAPK signal transduction cascade in mediating cocaine-associated cellular communication.

Similar to tyrosine phosphorylation, phosphoprotein phosphorylation appears tightly regulated by the opposing action of kinases and phosphatases. Cocaine, morphine, opioids, and nicotine have all been demonstrated to regulate both the levels and phosphorylation state of neurofilaments.[42–46] The major dephosphorylating agents, protein phosphatase 1 (*PP1CA*) and protein phosphatases 2A and 2B (*PP2CA, PP2CB*),[47, 48] were prominently regulated along with their target proteins (*NEFL, NEF3, MAP1B, TUBA1, TUBA2*).

Synaptic Transmission and Neuroplasticity

Like memory and learning processes that activate specific pathways directing cytoskeletal organization and axonal growth, so has cocaine been demonstrated to produce distinct regional alterations in dendritic arborizations and spine densities.[4, 49] Neuronal plasticity is a dynamic process requiring a coordinated restructuring of neuronal connectivity, and transcripts encoding growth cone-associated proteins, such as the 43 kDa growth-associated protein GAP43, dynein and pleiotropin (*GAP43*, *DNCl1*, *PTN*), were significantly altered.

G-protein-mediated signaling features prominently in the pathways thought to mediate cocaine-induced alterations.[50–52] Protein kinase C (PKC)-β_1 (*PRKCB1*), a target of both G-protein-mediated signaling and diacylglycerol, has been associated with the PFC and sensitization in rats.[8] The heterotrimeric G_0 protein is the major noncytoskeletal protein in the growth cone membrane, where it is colocalized with and appears to be regulated by GAP43.[51, 53] Disrupting the $G_0\alpha$ gene (*GNA01*) abolishes G_0 function and uncouples dopamine D2 receptors from their effectors,[54, 55] while $G_0\beta_1$ (*GNB1*) has been associated with the establishment of cocaine sensitization[52]. The activation/deactivation of G-proteins is mediated via the α subunit by activating guanine nucleotide exchange factors (GEFs, *GAP43*) and inactivating GTPase-activating proteins (GAPs). PTPL1-associated RhoGAP 1 (*PARG1*)[56] is a strong negative regulator of the active form of the small Ras-related monomeric GTPase Rho, relaying signals from cell-surface receptors to, among others, the actin cytoskeleton. PARG1 shifts the balance towards an inactive Rho-GDP form, which increases axonal branching and dendritic stability. The active modulation of actin-cytoskeletal modulators was further noted by the strong regulation of transcripts encoding the actin-severing protein gelsolin (*GSN*) and actin-capping proteins beta (*CAPZB*) and profilin 1 (*PFN1*).

None of the nine named transcripts associated with dopamine neurotransmission were selected presently. Changes in parameters associated with an altered inhibitory modulation in response to cocaine[57, 58] were, however, suggested by the differential expression of transcripts encoding the 67 kDa isoform of the GABA biosynthetic enzyme glutamate decarboxylase (*GAD1*), and a G-protein activated inwardly rectifying potassium channel (GIRK) protein (*GIRK2/KCNJ6)* thought to regulate inhibitory responses by decreasing neuronal excitability.[59, 60]

Energy Metabolism and Mitochondrial Function

A number of differentially regulated transcripts were indicative of changes in energy metabolism and mitochondrial function. The mitochondrion is the converging point of all cellular catabolic processes and the locus of the tricarboxylic acid (TCA) cycle, fueled by factors such as γ-enolase (*ENO2*) and *MDH1*. Oxidative phosphorylation and ATP generation were represented by the inner mitochondrial membrane cytochrome b–c_1 complex (*cytochrome c-1, CYC1*), the cytochrome oxidase complex (*COX6C*), and a mitochondrial ATP synthase (*ATP5G3*). The cytosolic antioxidant superoxide dismutase 1 (*SOD1*) additionally guards against mitochondrial oxidative damage and may mitigate activation of procaspases, such as caspase 4 (*CASP4*).

Oligodendrocyte-associated Transcripts

A number of oligodendrocyte-associated transcripts implicated changes relating to the function of this cell type. The major myelin constituent, *PLP1*, was regulated, as also found in human brain tissue from chronic alcohol abu-

sers.[34, 61] Notably, the two-fold downregulation of *PLP1* in the frontal cortex of chronic alcohol abusers[61] is reminiscent of the downregulation present for the two individuals with an alcohol coabuse history (COC#3, COC#7). Phosphatidate phosphohydrolase (PAPase, *PPAP2c*) converts phosphatidic acid to diacylglycerol, regulating the *de novo* synthesis of glycerolipids and phospholipase-mediated signal transduction.[62]

Conclusion

The existence of at least two different, but consistent, gene expression patterns associated with cocaine abuse in the present study suggests that cocaine targets a distinct subset of genes. While we cannot conclusively attribute these differences to the mode, duration or recency of abuse, the two patterns may represent acute stimulation *vs* a refractory, destimulated state. Three of the four pattern A cocaine subjects, all of whom showed generally increased gene expression, had recently abused 'crack' cocaine, as indicated by the presence of AEME in the dlPFC. The increases in gene expression in the pattern A subjects may therefore represent a general state of activation, in contrast to the generally decreased expression of the same genes in pattern B. Experimental models that induce similar differential transcriptional changes may aid our understanding of the human condition of drug abuse. Emerging from this study is the cocaine-induced transcriptional regulation of cytoskeletal elements and signal transduction, synaptic transmission and neuroplasticity, energy metabolism and mitochondrial function, and oligodendrocytic function/myelination. Changes converging on the MEK/ERK pathway were especially noted, and it is conceivable that a host of up- and downstream modulatory inputs onto this pathway produce both acute and enduring changes in response to cocaine. By combining post-mortem studies with regional and temporal maps of gene expression from experimental studies, it may be feasible to associate cellular transcriptional signatures of activation, and deactivation, with different abuse patterns.

MATERIALS AND METHODS

Experimental Brain Samples

DlPFC from cocaine abusers ($N=7$) and controls with no history of drug abuse or mental illness ($N=7$) was obtained from the brain repository of the Clinical Brain Disorders Branch, NIMH. Anatomically, the dlPFC was defined as Area 46 rostral to the most anterior aspect of the corpus callosum. In an attempt to circumvent a number of ante- and post-mortem factors affecting brain RNA quantity and quality, we matched as closely as possible for age, gender, and post-mortem interval (PMI) (Table 1). A short agonal state was suggested by the cause of death and brain pH,[63] indicating that the samples were appropriate for use in microarray experiments.[64] Determination of brain pH was performed as described previously.[65] All control subjects tested negative for drugs of abuse and alcohol by post-mortem toxicology screens.

Microarray experiments

RNA extraction

As previously described,[66] aliquots of dlPFC brain powder were kept at -80°C until extraction of total RNA by a modified guanidinium thiocyanate method using the TRIzol extraction protocol (Life Technologies, Inc., Gaithersburg, MD, USA). Individual RNA extractions utilized in the study had a minimum $OD_{260/280}$ ratio >1.6. The RNA was further checked by formaldehyde agarose gel electrophoresis for evidence of degraded RNA and for the integrity of the 28S and 18S rRNA bands. No evidence of high molecular weight bands, representing potential DNA contamination, was found.

Neuroarray

The neuroarray (Figure 1) was produced by sorting a set of 15 000 human cDNA clones (http://image.llnl.gov/, IMAGE Consortium clones, Research Genetics, Huntsville, AL, USA) for CNS relevant genes, and including additional candidate genes identified in mRNA or protein screenings of patients with neuropsychiatric disorders.[67] The resulting gene list contained 1152 clones, representing functional families such as synaptic, neuronal, neuroglial and cell adhesion molecules, transcription factors, kinases, phosphatases, proteases, oncogenes, and structural genes.

RNA labeling and hybridization

Total RNA (5 μg) from each individual was reverse transcribed in the presence of ^{33}P-dCTP to produce ^{33}P-labeled cDNA following a previously published protocol.[66] The reaction product was purified by size separation through a spin column (BioSpin, BioRad, CA, USA). Heat-denatured probe ($\sim 5\times10^{6}$ c.p.m.) was diluted in 4 ml Microhyb solution (Research Genetics), and hybridized to the arrays for 16–18 h at 50°C with rotation. Two posthybridization washes were performed with $2\times$SSC/0.1% SDS at room temperature to remove nonspecific hybridization, and the array was placed under Saran wrap and exposed to a low-energy phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA). After a 7-day exposure, the phosphor screen was scanned in a Phosphoimager 860 (Molecular Dynamics) at 50 μm resolution, and the pixel intensities quantified using ImageQuant (Molecular Dynamics).

QPCR analysis of gene expression

Reverse transcription of sample RNA for PCR

All cDNA and PCR-related reagents were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA) if not otherwise indicated. Reverse transcription (RT) of the sample RNA into cDNA was performed by incubating 24 μl RNA sample with 32 μl AMV buffer, 1 mm dNTP, 1 μg random hexamers (Promega, Madison, WI, USA), 160 U RNAse inhibitor, 20 μM DTT, and 100 U AMV RT for 1 h at 42°C. The reaction mix was subsequently purified using the HighPure PCR product purification kit.

Primer design

MacVector software (Oxford Molecular Group, Madison, WI, USA) was used to design custom primers targeting the region on genes-of-interest corresponding to that of the microarray clone. The primers were purchased from Gene Probe Technology (Gaithersburg, MD, USA). As the regulation of most, if not all, genes appear context dependent,[68] no gene can be universally ascribed the role of a standardizing, 'housekeeping' gene. Literature searches suggested 18S and cyclophilin as the best candidates for standardization.[32, 69] Both transcripts concurred in experimental/control cDNA ratios across a number of samples and conditions in pilot experiments, verifying them as suitable internal standards for standardization of cDNA and, by inference, of starting RNA concentration. For this study, we used an 18S primer set (489 nt amplicon, QuantumRNA 18S Classic; Ambion, Austin, TX, USA) and cyclophilin (206 nt amplicon; atg gtc aac ccc acc gtg ttc ttc g (F); cgt gtg aag tca cca ccc tga cac (R)). The following primers ($5' \rightarrow 3'$) were used for selected genes-of-interest: *GNAO1* (244 nt): aca gaa ata cac agc cgt cag tca c (F), ccc cag cag tag tca aaa tgt tcc (R); *MDH1* (339 nt): tcc atc cat ccc caa gga gaa c (F), gag ttg cca tca gag ata aca ccc (R); *MAP1B* (151 nt): cag tca aga gca gca agg aaa tg (F), gga tgc cac aca atc aaa gat gag (R); *PLP1* (263 nt): tgg att gtg ttt ctt tgg ggt g (F); cag atg gtg gtc ttg taa gtc gc (R).

Real-time quantitative PCR (QPCR)

QPCR was performed using the Roche LightCycler (software version 3.5) and the FastStart DNA Master SYBR Green I kit. Correct primer and $MgCl_2$-concentrations were determined in pilot experiments, and primer concentrations ranged from 0.5 to 1.0 μM with a standard Mg^{2+}-concentration of 4 μM. Preparation of a general PCR master mix (MM) for all samples ensured testing under near-identical conditions. This consisted of 11.6 μl DEPC-treated water, 2.4 μl $MgCl_2$, and 2.0 μl FastStart DNA master SYBR Green I per sample. To further ensure that comparable cDNA content was probed for both the 18S standard and the gene-of-interest, cDNA-specific MM were prepared for each individual cDNA sampled. Aliquots of 18 μl cDNA MM were mixed with 2 μl of the appropriate primer to a final reaction volume of 20 μl per test, and a final 1 : 100 cDNA dilution. All PCR products were checked by melting curve analysis and by agarose gel electrophoresis to exclude the possibility of multiple products or incorrect product size.

GC-MS Analysis of Cocaine, Opiates, and Related Analytes in dlPFC Brain Tissue

Lyophilized dlPFC specimens were weighed (30–52 mg), suspended in sodium acetate buffer (2.0 M, pH 4.0), and internal standards (100 ng of $[^2H_{11}]$-methamphetamine, $[^3H_{10}]$-amphetamine, $[^2H_3]$-COC, $[^2H_3]$-EME, $[^2H_3]$-CE, $[^2H_3]$-BE, $[^2H_3]$-norcocaine, $[^2H_3]$-codeine, $[^2H_3]$-morphine, and $[^2H_3]$-6 acetyl morphine in 100 μl of H_2O) added. Samples were vortex mixed, allowed to stand at room temperature for 30 min, and centrifuged. Analytes were isolated by solid phase extraction (Clean Screen DAU solid phase extraction columns, United Chemical Technologies, Bristol, PA, USA) and eluted with methylene chloride, 2-propanol, and ammonium hydroxide (80 : 20 : 2, v : v : v). A 20 μl aliquot of *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide + 1% *tert*-butyldimethyl-chlorosilane (MTBSTFA + TBDMCS, Pierce Chemical, Rockford, IL, USA) was added, followed by evaporation under nitrogen at 40°C until dry. Residues were reconstituted in acetonitrile, transferred to autosampler vials, 20 μl MTBSTFA+TBDMCS was added and extracts were heated at 80°C for 15–20 min. Then, 20 μl of *N*,*O*-bis(trimethyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+TMCS, Pierce Chemical, Rockford, IL, USA) was added, and samples were heated at 80°C for 45 min. Analyzes were performed on a Hewlett-Packard 6890 gas chromatograph with a Hewlett-Packard 5973 mass selective detector (GC-MS). A capillary inlet system, operated in splitless mode, and an HP-1 fused-silica capillary column (15×0.2 mm^2 ID, 0.10 μm film thickness) were employed for chromatographic separation. The mass selective detector was operated in a selected ion monitoring mode with positive chemical ionization. A 1 μl aliquot was injected into the GC-MS. Analytes were quantified by comparing respective analyte-internal standard target ion response ratios to calibration curves prepared in drug-free brain homogenate. Limits of quantitation (LOQ) were $\leqslant$125 pg/mg. At the LOQ, signal-to-noise ratios for all ions were $\geqslant$3 and analytes quantified within $\pm$20% of target. Correlation coefficients were $\geqslant$0.985 from the LOQ (31–125 pg/mg) to 3125 pg/mg. Duplicate controls at 312.5 and 3125 pg/mg were required to quantify within $\pm$20% of target values.

Individual DAT and COMT Genotypes

Genotyping for a DAT VNTR polymorphism was performed according to a previously published protocol.[70] The Val158-Met COMT polymorphism was determined by a 5′ exonuclease allelic discrimination Taqman assay.[71] The following primers and probes were custom designed using Primer Express (Applied Biosystems) software: forward primer: tcg aga tca acc ccg act gt; reverse primer: aac ggg tca ggc atg ca; Fam-labeled probe (Val allele): cct tgt cct tca cgc cag cga, VIC-labeled probe (Met allele): acc ttg tcc ttc atg cca gcg aaa t.

Data Analysis

Ratio-based comparisons

Z-ratio. The *z*-ratio was in essence obtained as described previously.[66] Pixel intensities were $\log_{10}$-transformed to reduce the variance introduced by extreme values. All 1152 transcripts were included in the distribution. The normalized *z*-score for each individual membrane was calculated by subtracting the average array log intensity from the gene log intensity, and dividing with the standard deviation (SD) of the array grid. This yielded an *z*-score distribution with a mean value of 0 and an SD of 1. The *z*-difference, with respect to the cocaine-abusing individual, was obtained for each gene by subtracting the mean matched control gene *z*-score from the mean gene *z*-score

for the cocaine-abusing individual. The *z*-ratio, expressed with respect to the cocaine-abusing individual, was then calculated by dividing the *z*-difference for each gene by the SD of the *z*-differences distribution. The z-ratio represents a normalized, log-transformed fold-like change for each gene.

Normalized ratios. Normalized ratios were used to evaluate the absolute change in expression level of individual genes between any two individuals (control and/or cocaine). This enabled additional information on specific individual aberrations and the effect of the normalizing individual. Normalized ratios were calculated as a ratio of the scaled mean raw intensity values.

Selection criteria for transcripts associated with differential expression in cocaine abusers. A two-tailed Student's *t*-test assuming equal variance of the two samples was performed for each cocaine–control comparison to identify statistically significant gene expression changes. Our null hypothesis was that, for each transcript, the *z*-scores for the cocaine subject and the respective matched control would not differ. Two criteria determined whether the null hypothesis was accepted (transcript not differentially expressed) or rejected (transcript differentially expressed) for each individual transcript. The null hypothesis was rejected for transcripts where the average *P*-value was less than 0.05. The second criteria demanded that minimally three of the seven cocaine–control comparisons displayed individual *P*-values <0.001. No correction was made for the use of multiple *t*-tests. It is argued, that for a rejection criteria of $P>0.001$, we would have expected significant differences in 1–2 genes (0.001×1152) to occur at random per comparison. The probability that a $P<0.001$ or less would occur for the same gene for three or more subject comparisons would consequently be small ($P \approx 0.01$ to find one gene with $P<0.001$ for any three comparisons). The *t*-tests examined the four repeated measurements (*z*-scores) from each individual as representative of a larger population of measurements of the same individuals. The results of the *t*-tests therefore apply to the generality of the current arrays as extrapolated to a larger population of arrays, rather than to the more usual circumstance wherein a *t*-test examines the generality of a set of results if extrapolated to a larger population of subjects.

Cluster analysis. Average-linkage hierarchical clustering (Cluster software;[72]) was used to organize the relative expression patterns of the transcripts into dendrograms, which were visualized in Treeview (Cluster/Treeview software: http://rana.lbl.gov/EisenSoftware.htm). These indicate both the degree of similarity (clustering) of transcriptional expression patterns in the individual cocaine–control comparisons as well as the clustering of individual transcripts.

Analysis of the QPCR Data

A standard curve for each primer was used to calculate the respective relative concentrations. Using the same cDNA mastermix for both the primers for the gene-of-interest and the standardizing gene, the median value of minimally three replicate samples for each primer was obtained. To correct for potentially different amounts of cDNA subject to amplification, the ratio of the mean concentrations of standardizing gene for the cocaine and the matched controls was calculated. This ratio was applied to correct the concentration of the gene-of-interest for the COC individual. The QPCR ratio was then calculated between the standardized values for the cocaine and matched control individual. A ratio below 1 reflects a downregulation, a ratio of 1 indicates no change, while a ratio above 1 indicates upregulation with respect to the cocaine-abusing individual.

A statistical approach was included to evaluate the statistical reliability of the standardized (cDNA-content corrected) raw expression values. The QPCR analyses were performed multiple times (usually eight replicates). It was therefore possible to test the statistical reliability of each cocaine–control matched comparison, with the exception of MDH1 for which there were only four replicates. A nonparametric test had to be employed, because absolute values differed between runs. Differences between each cocaine subject and the corresponding matched control were tested for each transcript using Wilcoxon matched-pairs signed-ranks tests.[73]

NOTE ADDED IN PROOF:

Levels of expression of selected genes, as measured by z-scores for the individual subjects, were examined for a possible correlation with brain pH. No relationship was found, confirming that brain pH differences were not a factor in the present study.

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DUALITY OF INTEREST

None declared.

ABBREVIATIONS

PFC	***prefrontal cortex***
DlPFC	***dorsolateral prefrontal cortex***
CDNA	***complementary DNA***
DNA	***deoxyribonucleic acid***
RNA	***ribonucleic acid***
PCR	***polymerase chain reaction***
QPCR	***quantitative polymerase chain reaction***

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