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Gene Chromosomal Organization and Expression in Cultured Human Neurons Exposed to Cocaine and HIV-1 proteins gp120 and tat: Drug Abuse and NeuroAIDS

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1. ABSTRACT

As a model for Neuropsychiatric dysfunction in NeuroAIDS due to HIV-1 infection and drug abuse, we analyzed gene expression in human neurons treated with cocaine and HIV-1 proteins tat and envelope (env). One-way ANOVA showed statistically significant genes among the treatment groups ($p \leq 0.0005$). The identified genes were then subjected to a "stepwise" analysis using a repeated measures ANOVA to discover genes with parallel response group profiles across the treatment conditions. These groups were then analyzed using a repeated measures ANOVA to assess treatment main effects and gene-by-treatment interactions within groups. One-way ANOVA produced 35 genes that were significantly associated across all treatment conditions. Factorial analysis of each gene found statistically significant differences: 30 - tat, 17 - cocaine, 10 - env, 6 – tat/env, 6 – coc/env, and 4 – coc/tat. Analyses across genes found three sets of four genes, one set of three genes, and three sets of two genes with parallel profiles. Identified genes had functions included signaling, immune related, and transcription control. The genes were not stochastically arranged on the chromosomes, were in proximity to each other, and to other genes involved in neuropsychiatric diseases. We hypothesize that these genes fall in transcriptionally isolated groups and that abused drugs and HIV-1 proteins trigger transcription overload, coerced expression that may result in damage to the chromosome's control and organization of chromatin transcription machinery.

2. INTRODUCTION

An important rationale for our studies is that drug abuse is a complicating factor in the progression, diagnosis, and treatment of NeuroAIDS as well as AIDS (1). Thus, we commenced the current study of gene expression in neurons in cultures treated separately and in combination with cocaine and HIV-1 tat and env proteins. A central focal issue in drug abuse research with human populations compared to cell culture and animal studies is that humans for the most part select poly-drug use whereas specific drugs can be tested in cell culture and animal studies. Thus, a further complication in determining the mechanisms of drug addiction, drug effected pathways that lead to behavioral and organic pathology as well as HIV-1 infection is to extricate the effects of drugs including cocaine, methamphetamine, opiates, and the product of cocaine/ethanol ingestion, cocaethylene given the new technologies of Microarrays for gene expression. There are thus several factors including drug abuse and HIV-1 infection that interact to produce NeuroAIDS. The analysis of the details of these factors is vital in the development of paradigms for prevention and cure of NeuroAIDS. These are discussed briefly here.

2.1. AIDS Dementia

Cocaine abuse has been an impediment for constructive social development for many years and continues to be a growing problem in the United States. Since the last century, there has been a steady increase of cocaine abuse in the United States. From 1999 to 2002 cocaine was the most common drug reported in emergency department visits (2). Cocaine abusers were treated more frequently from ages 18 to 25. In 1999, the Office of National Drug Policy estimated that there were 3.6 million chronic cocaine users (3). According to the National Household Survey on Drug Abuse in 1997, the prevalence of cocaine use was 1.4 percent for African American, 0.8 percent for Hispanic, and 0.6 percent for Caucasian populations in the US.

HIV-1 penetrates the blood-brain barrier (BBB) early in the course of HIV-1 infection (4, 5) and causes brain damage (e.g. cerebral atrophy) (6). Further studies describe HIV-1 evolution in the brain compartment. An initial study showed HIV sequences were present in AIDS cases with dementia (7). HIV-1 infected monocyte/macrophages and microglia, and macrophage-tropic HIV-1 strains are associated with neurologic disease more than T-cell tropic isolates (8). HIV-1 may evolve divergently and separately in different regions of brain in NeuroAIDS (9, 10). Some recent studies showed that HIV sequences from deep white matter had greater similarities with bone marrow derived sequences than with other tissue or fluids. The implication is that there is frequent trafficking of monocytes from bone marrow into the brain associated with late stage dementia. A hypothesis was advanced that this is a process for late-stage HIV-1 penetration of the brain (11). During late-stage AIDS, CD16 expression increases on macrophage-monocytes that exit bone marrow due to increased M-CSF in the bone marrow. Furthermore, in late stage infection, when blood monocytes circulating in the activated state meet brain microvasculature endothelium, they are able to initiate the changes that lead to transmigration. This process then enhances additional macrophage/monocyte recruitment. HIV-1 infection of the brain further accelerates this incursion. Thus, in this model, intrusion of new virus strains occurs continually prior to death via cycles of augmented macrophage/monocyte trafficking into the brain from bone marrow (12).

Pathological studies have elucidated several components in NeuroAIDS pathogenesis. Infected macrophage and microglial cells may be the primary conduit for HIV-1 invasion of the brain. In addition, astrocytes and neurons may be infected as well (13,14). *In situ* immunohistochemical and hybridization studies on sections from autopsied AIDS brains in many laboratories detected HIV-1 at high frequency in macrophage and microglial cells (13, 15, 16, 17) and in association with multinucleated giant cells and microglial nodules, This complex set of processes and pathology is termed HIV Encephalitis (HIVE) (18, 19, 20).

2.2. Inflammation

Inflammatory cytokines as well as other inflammatory molecules are associated with brain inflammation and damage including IL-1, IL-6, TNF-α, IFN-γ, arachidonic acid, and PAF (21, 22). In addition, IFN-γ and TNF-α activate macrophages that produce excitatory compounds including quinolinic acid and reactive oxygen (e.g. nitric oxide, NO) (23, 24, 25, 26, 27, 28). Moreover, the production of chemokines (CCKs) further increases the dimension of the inflammatory condition. Several CCK receptors are on brain cells including macrophages and neurons thus furthering the impact of inflammation. MCP-1, MIP-1, and RANTES are produced that activate macrophages thru CCR5, SDF-1a binds to CXCR4, and fractalkine binds to CX3CR1 further mediating macrophage recruitment into the brain (27, 29). This is confirmed in the use of animal models; for example, the SCID mouse injected with HIV-1-infected human macrophage/monocytes demonstrated brain inflammation related to their level of virus replication and the presence of these cells (30). Of many animal models, the SIV-rhesus macaque also yields important information related to NeuroAIDS disease (31, 32). The monkey animal model using SIV recapitulates NeuroAIDS in great part (33) and infection of CNS is supported by animal model studies as well. A neurovirulent clone of SIV was isolated that causes CNS lesions. Coinfection with an immunosuppressive clone further exacerbates neural disease (34).

2.3. Neuronal damage

Neuronal injury and cell loss (neurotoxicity) are hypothesized to result from toxic mechanisms that are initiated and maintained by productive HIV-1 infection in the CNS directly involving several HIV-1 proteins and indirectly by factors produced by activated and inflamed brain cells (20, 35). This damage may vary across brain regions or compartments (36). Damage closely associated with HIVE occurs to specific subpopulations of neurons in neocortex, limbic system, and basal ganglia (18, 20, 37).

Microglial cells in specific brain regions may be more vulnerable to HIV-1 infection than in other regions (38). These processes may involve specific subpopulations of macrophage/microglial cells (39, 40) and specific regional-neurotropic mutations of HIV-1 (9, 10, 41). Alternate models have been proposed as well. For example, it was proposed that in macrophage-derived cells co-evolution of the viral LTR, tat, and Vpr are associated with HIV associated dementia (HAD) (42). In addition, elevated nitric oxide synthase levels were demonstrated in neurons in AIDS sensory ganglia and in neurons from patients who were HIV infected and cocaine users (26, 43). In summary, severity of HIVE and HAD may be associated with neuronal cell death caused by abnormal activation of the immune system induced by infection of a specific subpopulation of macrophages or microglial cells by neurovirulent subset quasispecies of HIV-1 evolving specifically in local brain regions.

2.4. Drugs, HIV-1, neural cells, and NeuroAIDS

Cocaine abuse results in clinical signs and symptoms that include immunologic, behavioral, and neurophysiologic complications and deficits. Cocaine's stimulatory and addictive effects in great part result from its well-characterized ability to inhibit neuronal reuptake of dopamine. Cocaine has pleiotrophic effects, however, and for example enhances the production of IL-6. IL-6 is known to stimulate HIV-1 replication and is produced by the stimulation of mononuclear and astrocytes that may be important to the progression of HAD (44). In addition, behavioral and neurological studies in conjunction with brain PET scans further indicate that cocaine has a detrimental effect on brain function. For example, it was established from PET scans that cocaine reduces cerebral blood flow in anterior cortical areas beyond normal vasoconstriction (45). Additionally, cocaine stimulates the CNS and acts as a strong behavioral reinforcer. Long-term cocaine abusers develop paranoia, delusions, and various disorders. Furthermore, chronic users tend to be in a higher risk for stroke, heart attacks, and pneumonia (46). Cocaine causes deficits in many neuropsychological areas including attention, concentration, learning, verbal memory, information processing speed, and calculations (46).

There are some indications of co-incidences of cocaine and amphetamine epidemiology but additional studies need to be performed more extensively to investigate that possibility (38, 47). Mechanistically, cocaine- and methamphetamine-mediated CNS damage appear to be related. There is cross-sensitization between the two drugs in mice (48). The mechanism of this effect may be in part related to NMDA and NOS (49). Further studies indicate that just as with cocaine, amphetamine administration in conjunction with HIV-1 tat results in combined toxicity to neurons. Mitochondrial alterations were found in a non-pyramidal Calbindin positive human cultured neuron cell line due to dysregulation of calcium potential in mitochondria of these neurons (50.) In terms of drug treatment paradigms, it is interesting to note that buprenorphine was effective in reducing self-administration of cocaine, heroin, and cocaine/heroin "speedballs" whereas amphetamine was effective in reducing self-administration of cocaine and cocaine/heroin "speedballs" (51).

HIV-1 protein and drug treatments as well as HIV-1 infection of neural cells resulted in changes in several genes. A few examples follow. Early studies pointed toward calcium and glutamate channel involvement in gp120 toxicity. Treatments of postnatal rat retinal ganglion cell neurons with gp120 concentrations of 2 to 200 pM up to10 minutes increased Ca^{+2} in these cells (52). Lipton (53) found that 2 uM memantine prevented the injury produced by 20 pM gp120. Nath et al (54) showed that env and tat HIV-1 protein damage of neurons could be reversed by memantine. In Calbindin-containing neuron cultures exposed to gp120 (at 100 pM) for 24 hr, initially neuronal survival was 19.7% and 84.6% in control cultures (55). 200 pM gp120 causes a significant accumulation of reactive oxygen species (ROS), including superoxide and accumulation of lipid peroxidation in primary hippocampal cultures (56). Rat cerebellar granule neuron cultures of 7-8 days were exposed for 15 minutes to a buffered Locke's solution containing 100 pM gp120, washed, and cultured for an additional 24 hours in their original medium. The control culture average cell death was 8% and gp120 treatment (1 pM-10 nM) caused an increase of cell death of approximately 80%. There was a lack of neurotoxic effect of media conditioned by astrocyte-enriched and by granule cell cultures, pre-exposed to gp120 or glutamate for 15 minutes (57). Cytotoxic effect induced by 10 pM gp120 in CHP100 human neuroblastoma cells was inhibited by ethanol exposure (58). Rat cortical astrocytes, human glioblastoma cells and glial restricted precursor cells, from a human embryonic terato-carcinoma cell line, were incubated with recombinant tat (100 ng/ml for 60 minutes) that induced a significant reduction of glutamate or ATP-induced intracellular Ca^{2+} increase (59). An unusual finding is that at a lethal cocaine concentration (100 ug/ml), there were no affects on neuron proliferation or cell morphology (60). In a relevant study in non-neural cells, human mesangial cells (HMC) were treated with gp120 in different doses (0-800 pM) for 16 hr. At lower concentration (800 aM) gp120 promoted and at higher concentrations (800 pM) gp120 suppressed the proliferation of HMCs (61). Consequently, the effects of HIV-1, its pure proteins, with and without abused drugs including cocaine, are complex and vary under the experimental conditions. Thus, additional work is required to further increase our understanding of these complex phenomena.

It is important to note that there may be overlapping pathways among several different types of abused drugs and this is under investigation. Morphine stimulates SIV replication in monkeys and cocaine stimulates SIV levels as well (34, 62). Kappa agonists inhibit cocaine-induced HIV-1 expression in human microglial cells by suppression of ERK (63). Human microglial cells were infected with HIV-1 SF162, an R5 isolate, and viral expression was quantified by measurement of p24 antigen in culture supernatants. KOR ligands inhibit cocaine's stimulatory effect on viral expression involves the suppression of cocaine-induced activation of extracellular signal-regulated kinase1/2

thereby blunting cocaine-enhanced up-regulation of the HIV-1 entry chemokine co-receptor CCR5. The findings of this study suggest that in addition to its neurotoxic effects, cocaine could foster development of HAD by potentiating viral expression in the brain, and that this phenomenon is inhibited by KOR ligands (63). In addition, Memantine, a possible therapeutic agent for NeuroAIDS protects neurons exposed *in vitro* to HIV-1 tat and env (54.) Cannabinoids modulate immunity, through the T cell-dendritic axis and result in a shift of TH1 to TH2 patterns (64).

3. MATERIALS AND METHODS

3.1. Neuron isolation and culture

Brain specimens were obtained from human fetuses of 12-14 weeks gestational age with consent from women undergoing elective termination of pregnancy (all IRB and NIH rules and regulations were strictly enforced). Neuronal cultures were prepared as described previously (65, 66). Briefly, the cells were mechanically disassociated, suspended in OPTI-MEM medium with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (GIBCO) and 1% antibiotic solution (penicillin G at 104 units/ml, streptomycin at 10 mg/ml, and amphotericin B at 25 ug/ml) and plated in flat bottom flasks. The cells were maintained in culture for at least one month prior to conducting the experiment.

At the time of culture treatments, the culture medium was replaced with Lock's buffer containing 154 mM NaCl, 5.6 mM KCl, 1 mM MgCl2, 3.5 mM NaHCO3, 5 mM D-glucose, 5 mM N-2-hydroxy-ethyl-piperazine-N'-ethanesulfonic acid (pH 7.2). Each of the eight culture treatments was done in triplicate: medium alone, medium with tat, env (gp120), cocaine, tat and envelope, cocaine and envelope, cocaine and tat, and tat and cocaine and env. HIV-1 tat was used at 80 nM, env at 30 pM, and cocaine at 1.6 uM and the duration of the incubations was 6 hours. All dosages were at subtoxic concentrations (65, 66, 67, 68).

Recombinant tat was prepared as described previously (67) with minor modifications. The tat gene coding for the first 72 amino acids was amplified from HIVBRU (obtained from Richard Gaynor [Galveston, TX]) through the AIDS repository at the NIH and inserted into vector PinPoint Xa-2 (Promega) and grown in *E. coli* HB101. HIV-1SF2 gp120 (originally isolated from a patient with HIV associated neurological disease) was obtained as a gift from Chiron Corporation. The tat and env proteins were shown to be 95% free of degradation products by Western Blot and free of endotoxin (< 1 pg/ml) as determined by Pyrochrome Chromogenic test kit (Associates of Cape Cod Inc, Falmouth, MA). The proteins were aliquoted and cryopreserved at -80 ^{o}C in aqueous stock solutions in endotoxin-free siliconized microfuge tubes prior to use.

3.2. Gene Expression Analysis

Cell cultures were rinsed with Locke's buffer and total RNA extracted from 3-4 million cells per culture using Trizol reagent (Invitrogen). The RNA yields ranged from 11.75 to 77.05 ug per culture. RNA was amplified using Arcturus labeling kits (Arcturus). The Affymetrix HGU95A oligonucleotide chip was used to measure the expression of over 12,565 probes. The primary data were processed using Affymetrix Microarray Suite version 4.0 (MAS 4.0) followed by further analysis. Affymetrix gene identification and description are based on NetAffx Analysis Center (69).

3.3. Statistical Analysis

We performed a one-way ANOVA between the eight treatment groups across genes (no treatment control, tat alone, envelope alone, cocaine alone, tat-envelope, tat-cocaine, envelope-cocaine, and envelope-tat-cocaine.) We then selected the genes for which the ANOVA was statistically significant at $\alpha = 0.0005$ and 35 genes fulfilled this requirement. Assuming independence across the genes, under the null hypothesis we would expect to find only six genes significant (e.g. we expect to make six Type I errors). Thus, we have identified many more genes than would be expected due to chance. Each of the identified genes was then analyzed further in an ANOVA across the 2x2x2 factorial arrangement of treatments. Primary factors are coc, env, and tat; potential interactions are coc-tat, coc-env, tat-env, and coc-tat-env.

Next, gene expression profile analysis of the 35 genes was done. Starting with the first gene in the list, a repeated measure ANOVA was performed with each remaining gene and any pair of genes that was judged to be "parallel" was noted. This pair was then analyzed with each remaining gene to find statistically parallel triples. Triples were then analyzed with each remaining gene, and similarly for quadruples. A group of genes was defined to be jointly parallel if their gene by group interaction was not significant at $p \leq 0.05$. This process yielded three sets of four genes each, one set of three genes, and three sets of two genes each, such that the response profiles within each set were parallel. There were no gene-combinations with more than four members and fourteen genes remained ungrouped.

Each set of parallel genes was then analyzed in a repeated measure ANOVA with three grouping factors and one repeated factor. The grouping factors were each at two levels in a 2x2x2 factorial arrangement: tat (with, without), envelope (with, without), and cocaine (with, without). The repeated factors were the genes within the set being analyzed. Further analysis was done as follows. The 'among-treatments' analysis included the treatment main effects and the two-factor treatment interactions, while the 'within-treatments' analysis included gene effects and the two-factor and three-factor gene interactions with treatments. The SAS statistical program PROC GLM (SAS Institute Inc, Cary, NC) was used for all analyses.

3.4. Gene-Chromosome and Pathway Analysis

The web sites used for gene identification and chromosome mapping were as follows.
http://hal.hgc.jp/cgi-bin/gene_ensembl.cgi?id=ENSG00000157349.4
http://human.genelynx.org/cgi-bin/record?glid=9366&submit=Go

Table 1. Statistical analysis of 35 genes expressed significantly across neuron culture treatments

Affymetrix Probe Set Name	p value	tat	env	coc	tat-coc	env-coc	tat-env
PPF1A1	0.000153015	0.0099					
TBL3	0.000404589	0.0244			0.09		
DTNA	0.000135239	0.011					0.021
MAT2A	0.000253903	0.02					
KIAA0582	0.000366837	0.0024		0.021			
UPP1	0.000171076	0.0008					
JUNB	0.000154163	0.0001	0.027				
DDR2	0.000448931	0.0018					
Absent [1]	1.97689E-05	0.0001		0.0171		0.0823	
KIAA0676	0.000216897	0.0002					
RNF126	0.000296945	0.0001	0.0851	0.0876			
PSMD13	4.94832E-05	0.0001				0.0496	
SOX2	0.000396344	0.0005					
CD44	0.000455041	0.0025	0.0602				
ARHB	0.000182857	0.0034	0.0759				
HS6ST1	0.000397664	0.0062		0.0242			
LOC92912	0.000242997			0.0781			
LOC51257	0.000192537	0.0008		0.011	0.0578		
ZNF75	9.17098E-05	0.0006		0.0024	0.0083		0.0003
SDC2	0.000483198	0.0001	0.0154				
AEBP1	0.000454525	0.0001		0.01		0.081	
TM4SF1	0.000201517	0.0025				0.0095	
WBSCR22	6.15542E-05	0.0003					
FUS	0.00043226	0.0003					
GK2	0.00042479			0.003			0.0334
PFKFB3	9.03837E-05	0.044		0.0578			
KIAA0478	0.000324325			0.0047			
KREMEN2	0.000101856	0.0001	0.0003	0.0336			0.0009
RAB35	1.1681E-05	0.0114	0.0014		0.0029	0.0118	0.0002
MGC10471	0.000398615	0.0003	0.0213				
ARFGEF2	0.000325642			0.0359			
DDX19	0.000407404	0.0441		0.0683			
MAP2	0.000127153	0.0006	0.0923	0.0001			0.0883
ABCC4	7.98413E-05	0.0055	0.0004	0.0014		0.0001	
IRF2	0.000236072			0.0418			

[1] Absent = no gene ID available.

http://nciarray.nci.nih.gov/cards/
http://www.gene.ucl.ac.uk/nomenclature/index.html [this is the HUGO site]
http://www.google.com/
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
http://www.ncbi.nlm.nih.gov/mapview/
Ariadne Pathways Assist Program (Stratagene Iobion Inc, Cedar Creek, TX) was used for pathway analysis.

4. RESULTS

4.1. Gene Identification

Thirty-five genes were identified in the neuron treatment experiments using one-way ANOVA (70). Table 1 shows the Affymetrix identifications, abbreviations, and functions of each of the 35 genes. This table also contains the observed significance values (i.e. p-values) of the overall test of model usefulness for each gene, as well as those for the main effects and interactions (association of each gene with each treatment condition). A blank cell indicates no significant association. There are significant effects with primary factors as follows: 30 genes with tat, 17 genes with cocaine, and 10 genes with env. Several genes show main effects with more than one primary factor. Thus, 10 genes are significant with tat and env, four with env and coc, and 12 with tat and coc. Of these, four genes show significant effects due to all three primary factors. Of four genes that showed no effects due to tat, one had effects due to coc and tat-env, and three due to cocaine alone.

Significant interactions are as follows: 6 with tat-env, 6 with coc-env, 4 with coc-tat, and none with tat-env-coc. One gene shows effects with 2 interactive factors tat-coc and tat-env. One gene shows effects with all three interactive factors coc-env, tat-coc, and tat-env.

Several genes showed effects due to primary and interactive factors as follows: 6 with tat and env-coc, 3 with tat and tat-coc, 5 with tat and env-tat, 3 with tat and coc and env-coc, 4 with coc and env-tat (of these one included tat and tat-coc, and two with tat and env), 2 with tat, coc, and tat-coc, 2 with tat, env, and env-coc, 1 with tat, env, and tat-coc, 3 with tat, env, and env-tat. One gene showed effects due to tat, coc, tat-coc, and tat-env, one due to tat, env, coc, and tat-env, and one due to tat, env, tat-coc, env-coc, and tat-env.

The search for gene-sets with similar response profiles across treatment groups yielded three sets of four genes each, one set of three genes, and three sets of two genes each (Figures 1-7). The remaining fourteen genes each reflected unique 'hermit' profiles. In these figures, the y-axis label represents gene expression intensity. The x-axis labels are T (tat), E (env), C (cocaine), their combinations, and None (no treatment). The genes are

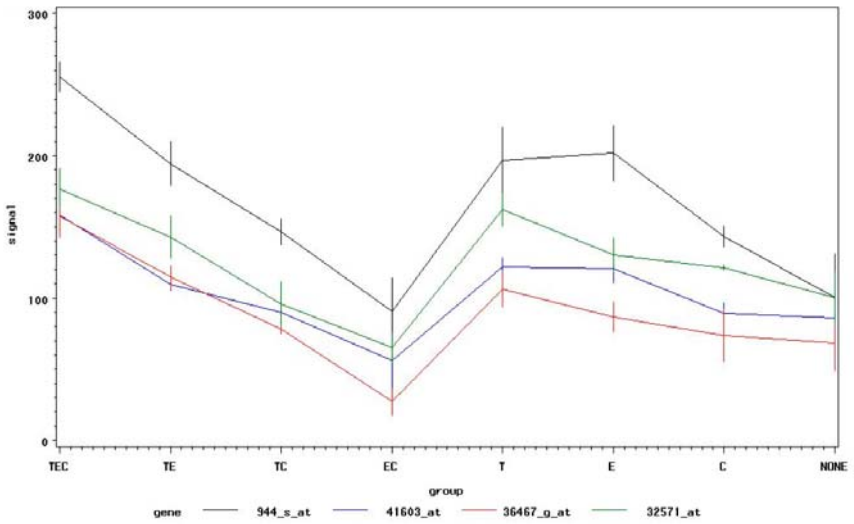

Figure 1. Four gene-set. See Table 2. T = tat, E = env, C = cocaine

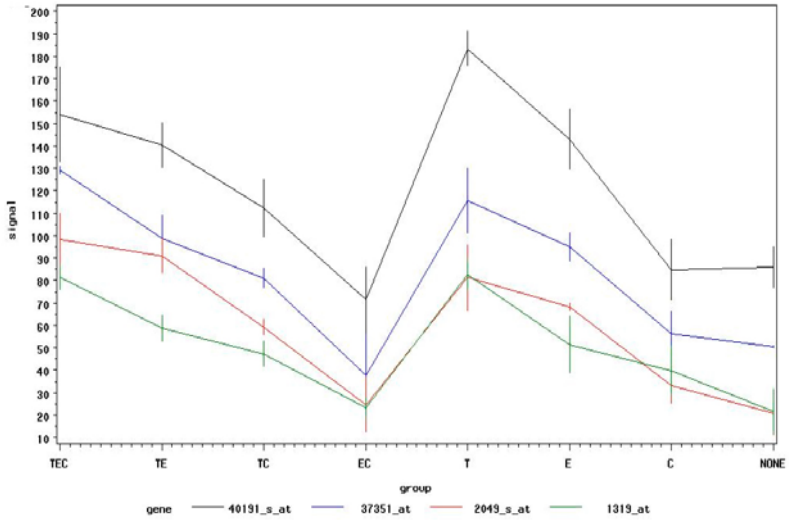

Figure 2. Four gene-set. See Table 2. T = tat, E = env, C = cocaine

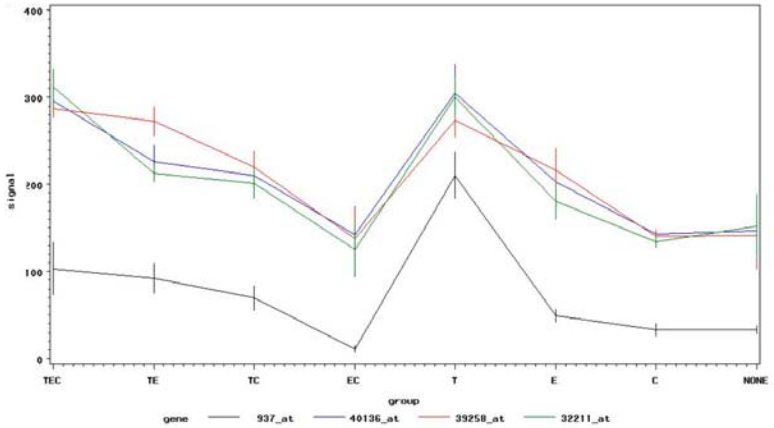

Figure 3. Four gene-set. See Table 2. T = tat, E = env, C = cocaine

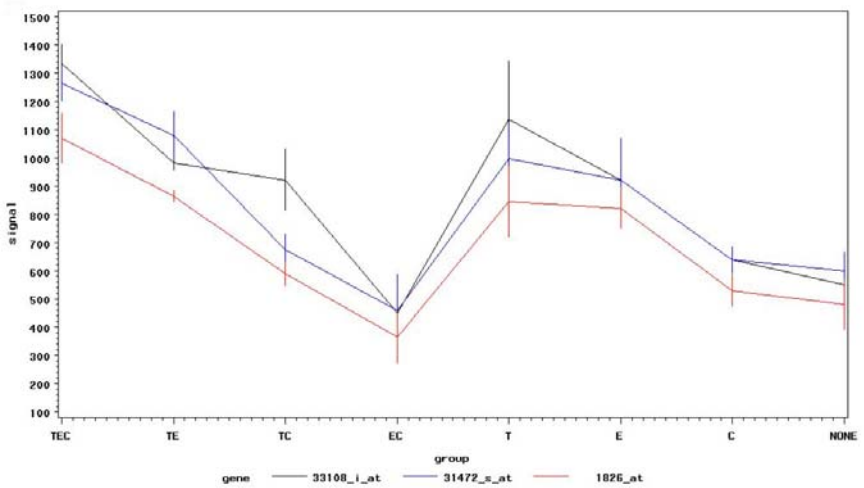

Figure 4. Three gene-set. See Table 2. T = tat, E = env, C = cocaine

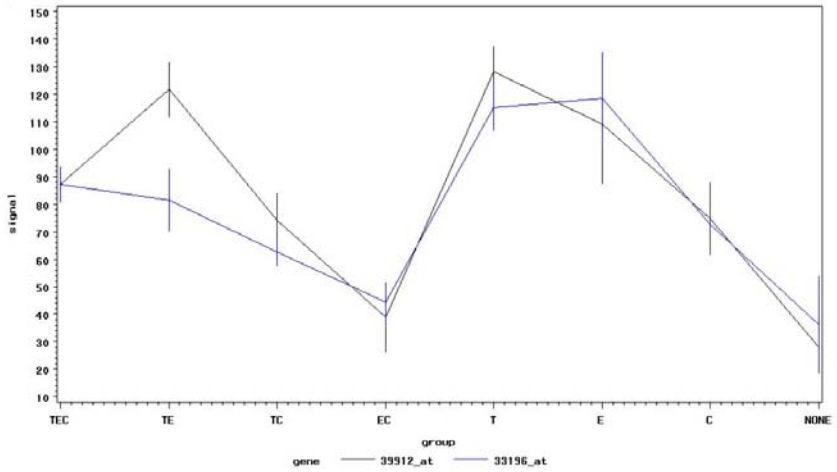

Figure 5. Two gene-set. See Table 2. T = tat, E = env, C = cocaine

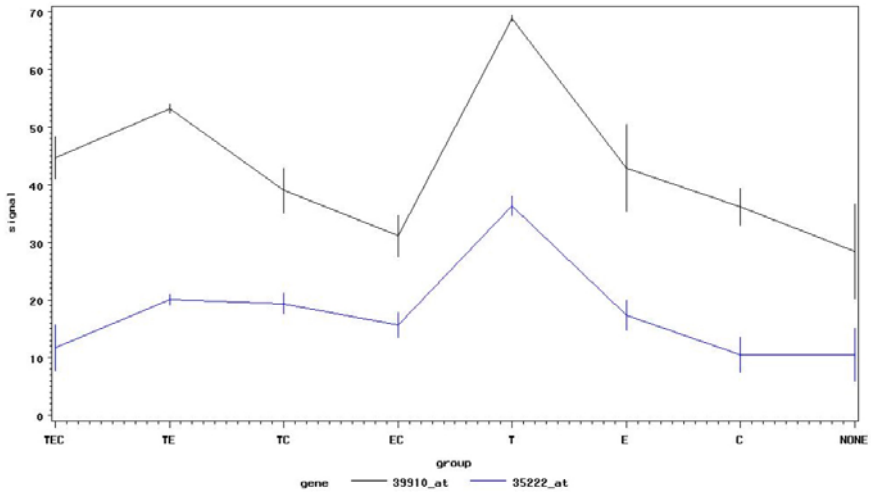

Figure 6. Two gene-set. See Table 2. T = tat, E = env, C = cocaine

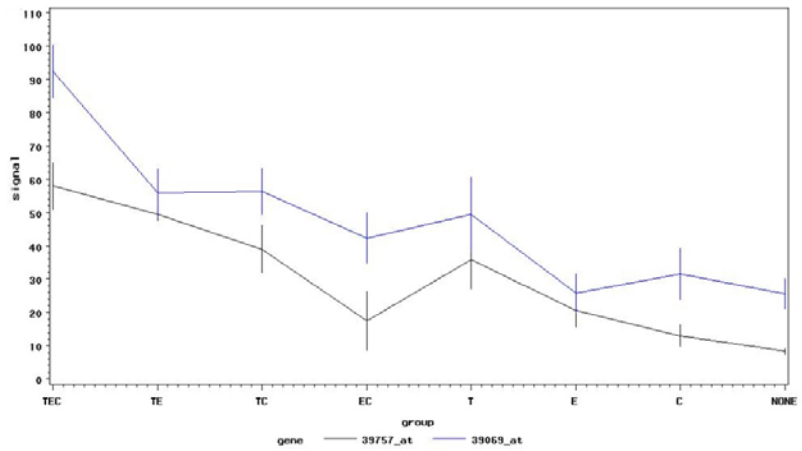

Figure 7. Two gene-set. See Table 2. T = tat, E = env, C = cocaine

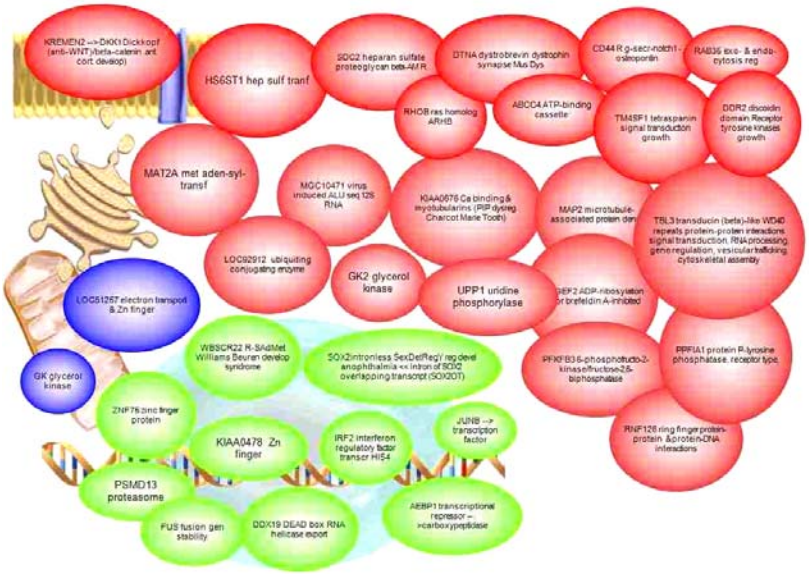

Figure 8. Locations in cell for 34 study genes. Ariadne Pathways Assist Program was used for pathway analysis.

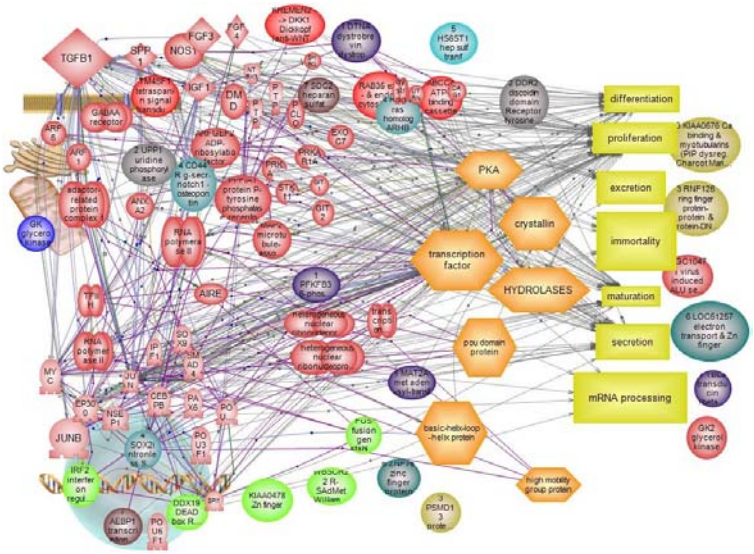

Figure 9. Interaction among 34 study genes. Ariadne Pathways Assist Program was used for pathway analysis.

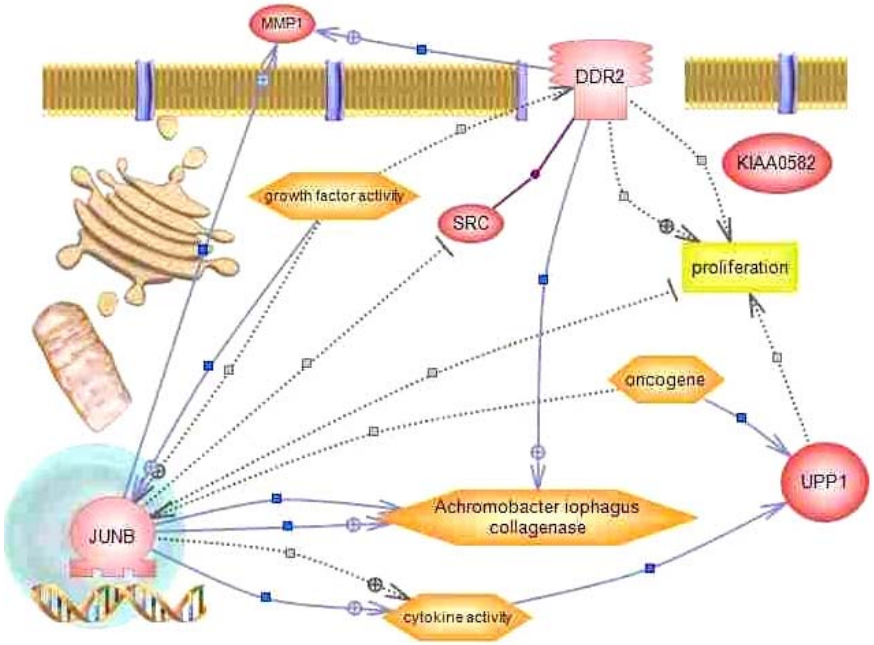

Figure 10. Interactions among DDR2, JUNB, and UPP1. Ariadne Pathways Assist Program was used for pathway analysis.

Table 2. Statistical analysis of gene groups with parallel responses within group

Affymetrix Probe Set Name	Group number [1]	tat	Env	coc	x gene	tat x gene	env x gene	coc x gene
		***p* value**	**significant at**	**alpha**	**=.05**	**Blank = no**	**significance**	
PPF1A1								
TBL3	1	0.0042			< .0001	0.0883		
DTNA								
MAT2A								
KIAA0582								
UPP1	2	0.0002		0.0676	< .0001			0.0228
JUNB								
DDR2								
Absent [2]								
KIAA0676	3	< .0001			< .0001		0.0035	
RNF126								
PSMD13								
SOX2								
CD44	4	0.0010			< .0001	0.0142		
ARHB								
HS6ST1	5	0.0348		0.0365		0.0084		
LOC92912								
LOC51257	6	0.0001		0.0018	< .0001	0.0505		
ZNF75								
SDC2	7	< .0001	0.0125	0.0465	< .0001			0.0117
AEBP1								

p values as shown, blank = ns, [1] Group number corresponds to Figures with the same numbers, respectively [2] Absent = no identity provided, ns = not significant

identified by the probe set names from the Affymetrix HGU95A oligonucleotide chip.

4.2. Gene groups

The gene sets shown in Table 2 resulted from the analyses designed to discover whether the response profile across treatment for each individual gene was essentially parallel to the response profiles of one or more of the other selected genes. The p-values shown in the table are with respect to the factorial arrangement of treatments in a repeated measures ANOVA to test whether the responses to tat, env, and coc were significant when pooled across the gene set and whether the responses to tat, env, and coc were similar across the genes within the set. The particular interest in this analysis was whether the effect of the individual treatments is different across the genes as measured by the tat-gene, env-gene, and coc-gene interactions. Gene groups 1-7, (three quartets, one trio, and three duos) correspond to figures 1-7, respectively. Tat has a significant effect for groups 1-7. In addition, env and coc

are each significant factors for group 7, coc is a significant factor for groups 5 and 6, and coc shows a trend for group 2. There is at least one significant gene by treatment interaction within all parallel gene groups except for group 5. These interactions indicate that the individual genes within the parallel group are responding differently to the specific treatment. Most of the interactions are with tat. There is a trend for tat-gene interaction for group 1 and groups 4-6 show significant tat-gene interactions. There is only one significant interaction for env-gene (group 3) and two for coc-gene in groups 2 and 7.

For the identified 35 genes, Table 3 shows the Affymetrix IDs, the standard abbreviations, the HUGO site IDs, and a few of the salient functions of these genes. One gene identified by Affymetrix location (937_at) did not have any other abbreviation (although it was stated to be a G1-phase specific protein) and was not used in subsequent analyses.

4.3. Pathways

The Ariadne program was used to examine possible gene interactions based on transcription, translation, and signaling as well as location in the cell including nucleus, cytoplasm, mitochondrion, Golgi, and cell membrane (Figure 8). Genes within each statistical groups 1-7 were distributed among the various protein locations in the cell. Colors and cell location representations are respectively red -cell membrane, pink - cytoplasm, blue - mitochondrion, and green - nucleus. All 34 genes were used for pathway analysis as were each of the 7 grouped and the ungrouped 'hermit" genes. Many genes showed direct pathway connections and those that did not may have 2nd order or higher pathway connections (analysis not shown). Many genes in statistical groups showed pathways connections (groups 1, 2, 4, and 7) and some genes did not (groups 3, 5, and 6). Examples of pathways produced by Ariadne are shown in Figures 9 and 10. Figure 9 shows that 11 of the 34 genes did not have any direct interconnections among these genes. This figure also shows that the genes in the pathways and interconnections include functions such as differentiation, growth, secretion, maturation, signaling, and mRNA processing. The genes that are in statistical groups 1-7 also have various degrees of pathway interaction. The genes that are in pathways in each group are respectively 3 of 4 genes in Group 1, 3 of 4 genes in Group 2, 0 of 4 genes in Group 3, 3 of 3 in Group 4, 0 of 2 in Group 5, 0 of 2 in Group 6, and 2 out of 2 in Group 7. Of the 15 ungrouped 'hermit' genes, 9 showed direct pathway interconnections. Figure 10 is an example of genes that are in direct interactive pathways and shows interactions among discoidin domain receptor tyrosine kinase signaling (DDR2), immediate early transcription factor (JUNB), and uridine phosphorylase 1 (UPP1). DDR2 and JunB also interact with matrix metalloproteinase 1 (MMP1) involved in the breakdown of the extracellular matrix and cell growth. They also interact with the SRC gene that is involved in cell growth.

4.4. Gene Chromosome maps

Table 4 shows the results of a calculation of the possible stochasticity of the occurrence of the 34 Affymetrix-identified genes per human chromosome. A Chi-square statistic was calculated comparing the expected and observed numbers of genes per chromosome. The expected number of genes/chromosome was taken as 34 genes/24 chromosomes = 1.416. The Chi-square was 38.11, with 23 degrees of freedom, $p = 0.0248$. To verify the adequacy of the Chi-square statistic a Monte Carlo simulation was carried out. It was assumed that the elements (genes) are distinct and that there was no exclusion, i.e. that any number of the 34 genes could be located on any chromosome (Maxwell-Boltzmann statistics), with the probability that any chromosome would be selected at each trial equal to 1/24= 0.04167. The calculations were reiterated 5,000 times using the SAS function RNTBL. A quantile-quantile plot of the cumulative frequency distribution against the theoretical 23-df Chi-square showed virtual identity between the simulated and theoretical distributions. Thus it is concluded that the distribution of the identified genes across chromosomes is not random at $p \leq 0.0248$.

Table 5 shows the 35 genes, their chromosome locations in the human genome, absolute nucleotide number locations on each respective chromosome, their lengths, the chromosome lengths on which they fall, and their % length compared to the respective chromosome length. Table 6 tabulates the genes that appear on each of the human chromosomes (22 autosomal and two gender chromosomes), and other relevant disease genes on both p and q arms of each chromosome. Analysis of the number of genes (identified in the current study) that occur on each human chromosome showed that six chromosomes had none, nine chromosomes had one gene, three chromosomes had two genes, two chromosomes had three genes, three chromosomes had four genes, and one chromosomes had five genes. This is highly divergent from a stochastic distribution of the genes across the human chromosomes. When more refined structure maps are examined then several genes are found not only on the same chromosome arm but also located in proximity. For example, MAT2A is at 2p11.2, KIAA0582 at 2p14, ARHB at 2p12, HS6ST1 at 2q21, and MAP2 at 2q34-q35. JUNB, RNF126, LOC51257, and MGC10471 are located at 19p13.2, 19p13.3, 19p13.3, and 1913.3-p13.1 respectively. Table 7 shows a brief synopsis of genes that are associated with neuropsychiatric diseases on the human genome. Each of the 34 identified genes in our study is on a chromosome with neuropsychiatric diseases as shown in Tables 6 and 7. Table 8 shows the distribution of the identified genes among several categories. The neuropsychiatric category contains 17.1% of the genes, transcription 28.6%, signaling 11.4%, neuronal synapse 8.6%, proteasome 5.7%, cell surface function 5.7%, metabolism 14.3%, and genome maintenance, ion channel, cell cycle, electron transport, RNA & viral RNA induced, and nuclear pore each 2.9%.

5. DISCUSSION

5.1. Gene expression in drug abuse and NeuroAIDS

In a classical study comparing frontal cortex from patients with HIVE vs. controls, Masliah et al (71) showed that 74 genes were down-regulated and 59 genes were upregulated out of 12,625 genes analyzed. Down-regulated genes included cytoskeletal components, transcription

Table 3. Descriptions of 35 genes

Gene number	Affymetrix Probe Set Name	gene ID	OMIM number	Descriptions
1	944_s_at	PPFIA1		Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF),, interacting protein (liprin), alpha-1, ATP binding, magnesium ion binding
2	41603_at	TBL3	605915	Transducin (beta)-like 3 Retinal Light Energy Transfer, GTPase-Binding
3	36467_g_at	DTNA	601239	Dystrobrevin, alpha synapse synaptic junction
4	32571_at	MAT2A	601468	Methionine adenosyl-transferase ii, alpha, ATP binding, ATP binding activity, magnesium ion binding, one-carbon compound metabolism, transferase activity
5	40191_s_at	KIAA0582		Brain, K/Na hyperpolarizing activated gated channel, A kinase anchor protein.
6	37351_at	UPP1	191730	Uridine phosphorylase1 Transferase
7	2049_s_at	JUNB	165161	Proto-oncogene Transcription factor
8	1319_at	DDR2	191311	Discoidin Domain Receptor Family, Member 2 Membrane Signaling
9	937_at	Absent		G1 phase specific gene
10	40136_at	KIAA0676		Ca binding and myotubularins (endocytic traffic, Charcot-Marie-Tooth Syndrome, PIP dysregulation)
11	39258_at	RNF126		Ring finger protein #126
12	32211_at	PSMD13	603481	Proteasome cytosol
13	33108_i_at	SOX2	184429	Sry-related hmg box (sox) transcription family factors, Sox1 (602148), Sox2, and Sox3 (313340) were co-expressed Further studies showed that the ability of the proneural transcription factor neurogenin-2 (NEUROG2; 606624) to promote neuronal differentiation was based on its ability to suppress Sox gene expression, thus showing that neurogenesis is regulated by an interplay between proneural proteins and inhibitory proteins
14	31472_s_at	CD44	107269	Homing, CD44 is an integral cell membrane glycoprotein with a postulated role in matrix adhesion lymphocyte activation and lymph node homing. The nucleotide sequence of CD44 cDNAs predicts a 37-kD polypeptide with homology to cartilage link protein (115435) in a phylogenetically conserved amino-terminal domain. Aruffo et al. (1990) demonstrated that CD44 is the main cell surface receptor for hyaluronate. Mature lymphocytes in the circulation migrate selectively from the bloodstream to different lymphatic tissues through specialized high endothelial venules (HEV). Molecules on the surface of (homing function and Indian blood group system)
15	1826_at	ARHB	165370	Ras homolog gene family, member b, RhoB
16	39912_at	HS6ST1	604846	Heparan sulfate 6-0-sulfotransferase 1, ATP- or GTP-binding site
17	33196_at	LOC92912		Hypothetical protein, Ubiquitin conjugating enzyme
18	39910_at	LOC51257		Hypothetical protein, Electron transport, Zinc finger,
19	35222_at	ZNF75	314997	Zn finger pr 75, Xq24-qter
20	39757_at	SDC2	142460	Syndecan 2 (heparan sulfate proteoglycan 1), 4 members of the syndecan family show a remarkable physical relationship with 4 members of the MYC gene family.
21	39069_at	AEBP1	602981	Adiposite enhancer binding protein 1, basement membrane of lung airways, and in the dermis
22	41531_at	TM4SF1	191155	Transmembrane 4 superfamily member 1 tetraspanin family, cell surface signaling structural---Raft-related
23	40089_at	WBSCR22		Methyltransferase, Williams Beuren syndrome, contiguous genes deleted at 7q11.23 at region 22, Mental retardation when deleted on Chromosome 7
24	39180_at	FUS		Fusion (involved in t(12;16) in malignant liposarcoma)
25	38130_s_at	GK2		Glycerol kinase 2 Pseudogene
26	37111_g_at	PFKFB3	605319	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
27	35990_at	KIAA0478		May contain zinc finger
28	35977_at	KREMEN2		Kringle containing transmembrane protein 2. High-affinity dickkopf homolog 1 (dkk1) trans-membrane receptor functionally cooperates with dkk1 to block wingless (wnt)/□-catenin signaling, Kremen2 interacts in a complex with Dickkopf and DKK1 to inhibit WNT/Beta-Catenin. Arrests cortical development.
29	35508_at	RAB35	604199	RAS oncogene family, GTP binding, exocytotic and endocytotic pathways, 3.6-kb RAB35 transcript
30	35135_at	MGC10471		Hypothetical protein, Human 12S RNA induced by poly(rI), poly(rC) and Newcastle disease virus, Alu repetitive sequence; induced 12S RNA; induced RNA.
31	34484_at	ARFGEF2	605371	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited) Autosomal recessive periventricular heterotopia with microcephaly (ARPHM; 608297) is a severe malformation of the cerebral cortex, characterized by severe developmental delay and recurrent infections
32	33393_at	DDX19	606181	RNA helicase. Deadbox (Asp-Glu-Ala-As) box polypeptide 19, DDX19 , Subunit: Interacts with NUP214, Subcellular location: Cytoplasmic and nuclear pore complex Cytoplasmic fibrils
33	220_r_at	MAP2	157130	Microtubule-associated protein 2 Assembly, coassemble with tubulin into microtubules in vitro. MAP2, a 280-kD protein, is highly concentrated in neuronal somata and dendrites, early brain development, and largely disappears from the mature brain except for the retina, olfactory bulb, and cerebellum. MAP2A and MAP2B are encoded by 9-kb mRNAs, and MAP2C by a 6-kb transcript.
34	1931_at	ABCC4	605250	ATP-binding cassette, sub-family C (CFTR/MRP), member 4, member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MRP subfamily which is involved in multi-drug resistance. The specific function of this protein has not yet been determined; however, this protein may play a role in cellular detoxification as a pump for its substrate, organic anions
35	1219_at	IRF2	147576	Interferon regulatory localizations of the human IRF1 and IRF2 genes and further characterized their promoters. TRANSCRIPTION factor 2. Activate IFNs □ and □, Histone h4

Absent = no gene ID available.

Table 4. Thirty four Selected Gene chromosome location Chi square calculation

number of genes /chromosome	Data	Maxwell-Boltzman Statistics	Maxwell-Boltzman Statistics
	number of chromosomes with that number of genes	%	number of chromosomes with that number of genes
0	6	23.43	5.62
1	9	35.36	8.49
2	3	24.43	5.86
3	2	11.46	2.75
4	3	3.90	0.94
5	1	1.12	0.27
6	0	0.26	0.06
7	0	0.04	0.01
8	0	~0	0

Chi square statistic = 38.11, 23df, p = 0.0248, Maxwell-Boltzman Statistics was calculated assuming distinction among elements and with no exclusion, Calculations were reiterated 1,000 times. Stochastic hypothetical mean based on 34 genes/24 chromosomes = 1.416 genes/chromosome.

Table 5. Cocaine/tat/env neuron gene locations, distances, and dimensions

#	Gene ID	Chromosome site	nucleotide number	Gene length (bp)	Chromosome Length (millions bp)	Gene % of Chromosome Length
1	PPFIA1	11q13.2	69,843,130 - 69,956,808	113,678	144	.08
2	TBL3	16p13.3	1,962,064 – 1,968,752	6,688	98	.007
3	DTNA	18q12	30,325,267 – 30,661,279	336,012	85	.40
4	MAT2A	2p11.2	85,677,946 – 85,684,061	6,115	255	.0024
5	KIAA0582	2p14	65,195,146 – 65,225,788	30,642	255	.012
6	UUP1	7p12.3	47,901,481 – 47,921,570	20,089	171	.012
7	JUNB	19p13.2	12,763,310 – 12,765,125	1,815	67	.0027
8	DDR2	1q12-q23	159,333,913 – 159,481,893	147,980	263	.056
9	Absent					
10	KIAA0676	5q35.3	179,221,677 – 179,267,464	45,787	194	.024
11	RNF126	19p13.3	598,529 – 614,227	15,698	67	.023
12	PSMD13	11p15.5	226,977 – 242,981	16,004	144	.011
13	SOX2	3q26-q27	189,912,424 – 182,914,926	2,502	214	.0012
14	CD44	11pter-p13	35,125,036 – 35,215,882	90,846	144	.063
15	ARHB	2pter-p12	20,631,738 – 20,632,329	531	255	.0002
16	HS6ST1	2q21	128,781,262 – 128,792,482	11,220	255	.0044
17	LOC92912	15q23	73,922,855 – 73,980,440	57,585	106	.054
18	LOC51257	19p13.3	8,384,276-8,409,901	25,625	67	.038
19	ZNF	Xq26	134,108,412 – 134,203,532	95,120	164	.058
20	SDC2	8q22	97,575,072 – 97,693,176	118,104	155	.076
21	AEBP1	7p13	43,917,200 – 43,927,399	10,199	171	.0060
22	TM4SF1	3q21-q25	150,569,507-150,578,244	8,737	214	.0041
23	WBSCR22	7q11.23	72,542,549-72,557,193	14,644	171	.012
24	FUS	16p11.2	31,098,960-31,110,424	11,464	98	.012
25	GK2	4q27	47,235,268-47,409,294	174,026	203	.086
26	PFKFB3	10p15-p14	6,226,887-6,317,501	90,614	144	.063
27	KIAA0478	1p36	22,523,656-22,600,286	76,630	263	.029
28	KREMEN2	16p13.3	2,954,218-2,958,382	4,164	98	.0042
29	RAB35	12q24.31	118,995,626-119,017,319	21,693	143	.015
30	MGC10471	19p13.13	13,719,720-13,735,106	15,386	67	.023
31	ARFGEF2	20q13.13	46,971,834-47,083,645	111,811	72	.16
32	DDX19	16q22.1	70,109,307-70,143,922	34,615	98	.035
33	MAP2	2q34-q35	210,114,288-210,424,340	310,052	255	.12
34	ABCC4	13q32	94,470,091-94,751,684	281,593	114	.25
35	IRF2	4q34.1-q35.1	185,684,064-185,770,852	86,788	203	.043

bp = base pairs

factors, signaling, cell cycle, synaptic transmission, and synaptic plasticity. Upregulated genes included cytoskeleton transcription, signaling modulation, anti-viral response, and neuroimmune response. These results were directly translated into a model of Neurotoxicity subsequent to HIV-1 infection of the brain with neuronal effects due to the categories as listed. And indeed many studies have been performed using the two-way approach comparing cases and controls (72, 73.) We have recently performed gene expression studies related to AIDS dementia using other statistical methods (22, 26, 40, 70, 74, 75). Overall, similar classes of genes were implicated in these studies although not precisely identical genes across all studies. This is an important issue since it seems unlikely that the current state of the art of statistical analysis will implicate precisely identical genes across studies with different patients, tissues, and methods.

In the current preliminary analysis of human neuron gene expression in culture, results using the one-way ANOVA produced a list of 35 genes that were significant across treatments. Subsequent application of a

Table 6. Genes, chromosome locations, and disease genes

Chromosome #	Study Gene ID-Location	Study Gene ID-Location	Study Gene ID-Location	Study Gene ID-Location	Study Gene ID-Location	Diseases on chromosome "p" arm	Diseases on chromosome "q" arm
1	DDR-2 1q12-q23	KIAA0478 1p36				HTR1D 1p36.3-34.3 (Serotonin receptor) PARK7 (DJ-1 protein, chaperone and anti-oxidant) 1p36.33-p36.12 (early onset Parkinson)	MPZ 1q22 (Neuropathy), DISC1 1q42.1 (Schizophrenia, if disrupted), CHRNB2 1q21.3 (nicotine addiction susceptibility) PS2 (AD4) 1q42.13 (Alz dis), IL10 1q31-q32 (GVH), TAR 1q42.3 (HIV) binding protein
2	MAT2A 2p11.2	KIAA0582 2p14	ARHB 2pter-p12	HS6ST1 2q21	MAP2 2q34-q35	ETM2 2p25-p22 (Essential Tremor 2) DYSF 2p13 (Muscular Dystrophy 2B) PARK3 2p13 (also known as SPR gene and is implicated in dopamine synthesis) (early onset Parkinson)	CSEN 2q21.1 calsenilin, presenilin binding protein (Alz dis) HRB 2q36.3 HIV-1 Rev binding protein SLC25A12 2q24 (Autism)
3	SOX2 3q26.3-q27	TM4SF1 3q21-q25				CCR5 3p21 (HIV infection susceptibility/resistance to) DMT1 3p11.1-q11.2 (Dementia, familial)	PSARL 3q27.3 presenilin associated, rhomboid like (Alz dis) ETM1 3q13 (Essential Tremor 1)
4	GK2 4q13	IRF2 4q34.1-q35.1				PARK4 4p15 (Parkinson) HD 4p16.3 (Huntington) DRD5 4p16.1 Dopamine receptor)	SNCA 4q21.3-q22 (Parkinson, PD1)
5	KIAA0676 5q35.3						HTR4 5q31-q33 (Serotonin receptor) SCZD1 5q11-q13 (Schizophrenia)
6						NOTCH4 6p21.3 (Schizophrenia)	PARK2 6q25.2-q27 (Parkinson) EPM2A 6q24 (Epilepsy)
7	UPP1 7p12.3	AEBP1 7p13	WBSCR2 2 7q11.23			AUT2 7p13 (Autism susceptibility 2) GBAS 7p12 (Glioblastoma)	HRBL 7q22.1 HIV-1 Rev binding protein like, CFTR 7q31-q32 (Cystic Fibrosis) AUT1 7q (Autism susceptibility 1)
8	SDC2 8q22-q23					CLN8 8p23 (Epilepsy)	TARBP2P 8q22-q24 , TAR(HIV)RNA binding protein 2 pseudogene
9							PCPH3 9q34 (Microcephaly)
10	PFKFB3 10p15-p14					PHYH 10p15.3-p12.2 (Refsum desease, leukodystrophy)	EPT 10q24 (Epilepsy)
11	PPFIA1 11q13.3	PSMD13 11p15.5	CD44 11p13			BDNF 11p14.1 (Huntington)	TCIRG1 11q13.4-q13.5 (T-cell immune regulator), DRD 11q23 (Schizophrenia)
12	RAB35 12q.24.31					PXR1 12p13.3 (Zellweger syndrome)	HRB2 12q21.2 HIV-1 Rev binding protein 2, HIVE1 12q11-q13.3 HIV-1 expression elevated in TARBP2 12q12 TAR (HIV) RNA binding protein
13	ABCC4 13q31						HTR2A 13q14-q21 (Schizophrenia)
14							PS1(AD3) 14q24.3 (Alz dis) LOC145414 14q22.3 similar to ribosomal protein L3, 60S ribosomal protein, HIV-1 TAR RNA-binding protein GCH1 14q22.1-q22.2 (DOPA Dystonia)
15	LOC92912 15q23						UBE3A 15q11-q13 (Angelman syndrome) SNRPN 15q11-q13 (Prader-Willi syndrome)
16	TBL3 16p13.3	FUS 16p11.2	KREMEN2 16p13.3	DDX19 16q22.3		MEFV 16p13.3 (Familial Mediterranian Fever)	GAN 16q24.1 (Giant axonal neuropathy)
17						CMT1A 17p13.1-q12 (Charcot-Marie-Tooth Neuropathy)	FALZ 17q24 (Alz D, fetal)
18	DTNA 18q12					SCZD 18p (Schizophrenia)	LYZ 18q12.1(Ameloidosis)
19	JUNB 19p13.2	RNF126 19p13.3	LOC51257 19p13.3	MGC10471 19p13.3-p13.1		FEB2 19p13.3 (Convulsions)	MCPH2 19q13 (Microcephaly)
20	ARFGEF2 20q13.13					PRNP 20pter-p12 (Creutzfeldt-Jakob Dis, fatal insomnia), CST3 20p11.2 (ameloid angiopathy, cerebral hemorrhage)	KCNQ2 20q13.3 (Epilepsy, neonatal)
21							APP 21q21.3 (Alz dis) DSCR1 21q22.12 (Down's) SOD1 21q22.1 (ALS)
22							NF2 22q12.2 (Neurofibromatosis)
X	ZNF75 q26					DMD Xp21.2 (Duchenne muscular dystrophy)	HTATSP1 Xq26.1-q27.2 HIV-TAT specific factor 1, GJB1 Xq13.1 (Charcot-Marie-Tooth, neuropathy)
Y							

GLM analysis to individual genes for significant interactions indicated most genes were significant due to tat, some due to tat-env, tat-cocaine, and env-cocaine interactions and none due to tat-env-cocaine interactions. The analysis of gene expression data in the literature has predominately taken the approach of conceptually transposing the observed data so that the genes being probed become experimental units (i.e. "subjects") and the samples, cultures, or human subjects become the "variables" in the analysis. This has been done mainly because the number of gene probes (e.g. Affymetrix chips with 12,565 to 40,000 probes and CodeLink chips with 55,000 gene sequences) is so much larger than the number of samples that the conventional methods of Multivariate Analysis of Variance (MANOVA) or its analogues cannot be applied (76). However, an alternative statistical approach, the repeated measures ANOVA can be applied which takes into account the correlated nature of gene responses within subject (77). This approach has been recommended by Emptage et al (78) in their treatment of microarray experiments as split-plot designs.

In this neuron study we tested eight different neuron cell culture conditions that were composed of three

Table 7. Chromosomes and diseases

Chromosome #	Diseases
1	Alzheimer's, Schizophrenia, Neuroblastoma, Migraine, Hypomyelination, Recurrent viral infections, Lupus, Serotonin receptor, Brain cancer
2	Epilepsy, Serotonin receptor, Myastena Gravis, ALS-juvenile, Parkinson's , Muscular Dystrophy, Spastic Paraplegia, Neurogenic differentiation
3	Encephalopathy, Dementia, Apnea, Neuropathy, Tremor, Muscular Dystrophy, HIV susceptibility
4	Parkinson's, Huntington's, Alcoholism susceptibility , Hepatitis B virus integration site, Dopamine receptor
5	Schizophrenia, Dopamine transporter, Attention deficit disorder, Convulsions, Immunodeficiency, Cortisol resistance
6	Parkinson's, Epilepsy, Schizophrenia, Complex neurologic disorder, Nystagmus, Spino-cerebellar ataxia, Immuno-interferon, Serotonin receptor
7	Autism, Serotonin receptor, Turcot-glioblastoma
8	Epilepsy, Schizophrenia, Neuropathy, Opiate receptor, Convulsions
9	ALS-juvenile, Prostaglandin D2 synthase(brain), Dopamine-beta-hydroxylase deficiency, Interferon-□-deficiency
10	Immunodeficiency disorder, Malignant brain tumors, Serotonin receptor, Schizencephaly, Interleukin receptor deficiency, Neuropathy
11	Amyloidosis, Serotonin receptor, Dopamine receptor, Immunodeficiency, T-cell immune regulator, APO-A & APO-C-III deficiency,
12	Alzheimer's, HIV-1 expression, Interferon, Immunodeficiency, Human papilloma virus integration site
13	Dementia, Schizophrenia, Serotonin receptor
14	Alzheimer's, Cerebrovascular disorder, Immunodeficiency, DOPA-Dystonia, Basal ganglia calcification, Chorea
15	Epilepsy, Schizophrenia, Mental retardation
16	Cocaine & antidepressant sensitivity, Giant axonal neuropathy, Cerebral degeneration, GABA-transaminase deficiency,□-thalassemia (mental retardation)
17	Alzheimer's, Huntington's, Medulloblastoma, HIV-disease delayed progress, Neurotransmitter transporter, Serotonin (anxiety related), Dementia
18	Parkinson's, Amyloid neuropathy, Schizophrenia, Hepatitis B virus integration site
19	Nicotine addiction protection, Epilepsy, Alzheimer's, Convulsions, Glioblastoma, Cerebral arteriopathy, Microcephaly, Cancers
20	Creuzfeldt-Jakob disease, Insomnia, Cerebral amyloid angiopathy, Epilepsy, Immunodeficiency, Huntington-like neurodegenerative disorder
21	Alzheimer's, Amyloidosis, Down's, Schizophrenia, Epilepsy, Coxsackie, adenovirus, & myxovirus (influenza) receptors,
22	Autism, Epilepsy, Schizophrenia, Neuroepithelioma, Meningioma, Ubiquitin fusion degradation
X	Mental retardation, Immunodeficiency, Migraine, Autoimmunity, Hemophilia B
Y	Gender-determining region

Table 8. Gene categories and Neuropsychiatric disease

Gene Category	Study Gene number	Gene ID	Neuropsychiatric disease
Neuropsychiatric Disease 6/35 = 17.1%	10	KIAA0676	Charcot-Marie-Tooth (Endocytic Traffic)
	16	HS6ST1	Alz D Beta-Amyloid Receptor (Alz Dis)
	20	SDC2	Alz D Beta-Amyloid Receptor (Alz Dis)
	23	WBSCR22	William-Buren Mental Retardation (When Deleted)
	28	KREMEN2	Dickhof Syndrome [Catenin Wnt Signaling]
	31	ARFGEF2	Micro-Encephaly
Signaling 4/35 = 11.4%	1	PPFIA1	
	2	TBL3	
	8	DDR2	
	28	KREMEN2	
Transcription 10/35 = 28.6%	7	JUNB	
	11	RNF126	
	13	SOX2	
	15	ARHB	
	19	ZNF	
	21	AEBP1	
	27	KIAA0478	
	29	RAB35	
	31	ARFGEF2	
	35	IRF2	
Genome Maintenance 1/35 = 2.9%	24	FUS	
Ion Channel 1/35 = 2.9%	5	KIAA0582	
Endocytic Traffic	10	KIAA0676	Charcot-marie-tooth
Neuronal, Synapse 3/35 = 8.6%	2	TBL3	
	3	DTNA	
	33	MAP2	
Cell Cycle 1/35 = 2.9%	9	-	
Proteasome 2/35 = 5.7%	12	PSMD13	
	17	LOC92912	
Cell Surface Cell Function 2/35 = 5.7%	14	CD44	
	22	TM4SF1	
Electron Transport 1/35 = 2.9%	18	LOC51257	
Metabolism 5/35 = 14.3%	4	MAT2A	
	6	UUP1	
	25	GK2	
	26	PFKFB3	
	34	ABCC4	
Rna & Viral Rna Induced 1/35 = 2.9%	30	MGC10471	
Nuclear Pore 1/35 = 2.9%	32	DDX	

treatments (tat, envelope, cocaine), each at two levels (present, absent) in a factorial arrangement. This yields eight ($2x2x2 = 2^3$) different conditions. The first analysis was to discover individual genes for which the response was significantly different across the eight treatment conditions. We used an ANOVA and selected a low probability ($p < 0.0005$) in order to obtain a feasible number of genes to analyze further. This is similar to examinations including cluster analysis and principal components analysis (PCA) to produce a manageable number of genes indicative of treatment differences. In this manner we found 35 genes that show a significant response across the 8 treatment conditions. In order to further characterize the significant differences among conditions, the response of each selected gene was analyzed in a factorial ANOVA, testing the primary factors tat, (as previously defined) envelope (env), and cocaine (coc) and their two-factor interactions tat-env, tat-coc, and env-coc. Thus, the results in Table 1 support the strength of effects on gene expression by tat, cocaine, and env (in decreasing order.) These three treatments show interactive effects. However, genes such as CD44 (cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration), SDC2 (syndecan 2, heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan, also a receptor for both Fibroblast Growth Factor-2 (FGF-2) and β-amyloid), and ARHB (Aplysia ras-related homolog, small guanosine triphosphatase Rho regulates actin-filament remodeling), although they have tat and env primary effects do not show any interactive effects with tat-env. This phenomenon is true for a few additional genes including tat and coc primacy effects for genes HS6ST1 (a heparan sulfate biosynthetic enzyme, thus related to SDC2 mentioned above) and tat, coc, and env primary effects for MAP2 (microtubule-associated protein related to neurofilaments and dendrite remodeling and thus related to ARHB mentioned above) but only an interactive effect with tat-env for this gene (MAP2). The possible explanation for this phenomenon is that for genes where the primary effects and interactive effects do not match may be due to a cancellation mechanism in the over-all networks of the neuronal homeostatic machinery. This is an important phenomenon because when we extrapolate to actual mechanisms in brain where the panoply of reactions is orders of magnitude more complex, cancellation and stimulation interactions among the pathways are to be expected. In reference to SDC2 mentioned above, it should be noted that mechanistically that Heparan Sulfate serves in a neuroprotective role as a binding site for FGF-2 and in a neurodamaging role as a binding site for β-amyloid fibrils (79).

Laboratory findings support the notion of cancellation treatment effects. In work focusing on cocaine related effects on HIV-1 replication, *in vitro*, we have previously shown that two primary conditions that effect HIV-1 gene expression do not result in an additive interactive effects, although additive interactive effects were expected. Thus, cocaine and cocaethylene each stimulate HIV-1 reverse transcriptase gene expression in HIV-1-infected normal donor PBMNC cultures but their interactive effect actually results in a 3-fold suppression of HIV-1 reverse transcriptase gene expression. In the same study, ethanol treatments restored stimulation of HIV-1 gene expression in a mixture of cocaine, cocaethylene, and ethanol (80, 81).

Even prior to the current widespread use of Microarray studies, approximately 103 genes were identified with altered expression after drug administration as of 2001 (82). One of the goals in this type of work related to the pathobiology of drug abuse is to establish a causal relationship between drug abuse and genes beyond genetics. An example is a protein termed CART - cocaine and amphetamine regulated transcript. This protein responds to psychostimulant drug administration and is remarkable. When CART peptides are injected into the rodent ventral tegmental area, the resulting behavioral changes include pharmacologic psychostimulant-like effects (82).

Thus, in the current study, a few examples of genes, their drug and HIV-1 protein treatment interactions, and their pathways for pathogenesis follow. The expression of two ungrouped 'hermit' genes, MAP2 and KREMEN2, are each associated with tat, env, coc, and tat-env treatments. These combinations result in effects on these genes even though they are not in any of the 'grouped' genes. They are however interrelated functionally as follows, though. The gene MAP2 is the microtubule associate protein-2 and is involved in neuronal intracellular filament assembly. KREMEN2 is Kringle containing transmembrane protein-2. This protein is a high-affinity dickkopf homolog 1 (dkk1) trans-membrane receptor that functionally cooperates with dkk1 to block wingless (wnt)/β-catenin signaling. Furthermore, β-catenin and its regulatory kinase, GSK-3 beta, (also known as MAP-tau protein kinase-1) are associated with activity of presenilin-1 (PS-1) (83.) Perturbation of β-catenin integrity may lead to hyperphosphorylation of MAP-tau and neurofibrillary tangle formation (NTF) and to neuronal apoptosis via different signaling pathways (83, 84, 85, 86, 87, 88, 89). It should also be noted that Disrupted-in-Schizophrenia-1 (DISC1) was identified as a potential susceptibility gene for Schizophrenia and was shown to co-localize with MAP2 in neurons of the olfactory bulb, cortex, hippocampus, hypothalamus, cerebellum and brain stem (90.) Thus, perturbation of MAP2 gene expression due to the drug and HIV-1 protein treatments may perturb the activity of DISC1 and further exacerbate neuronal dysfunction.

Another example is the interactions among genes mentioned above, DDR2, discoidin receptor that is a tyrosine receptor kinase, JUNB an immediate early transcription factor crucial in gene expression control in neurons (as well as in other cells), and UPP1, uridine phosphorylase 1. These genes show pathway interactions related to growth cytokine (inflammation), and matrixmetaloproteinase (MMP) activities and thus may be indicative of repair or restorative responses of the neuron related to the cocaine, HIV-1 tat, and envelope treatment effects.

An additional example of a gene identified in our study that shows novel regulatory mechanisms is TM4SF1. Expression of this gene is associated with tat and env-coc treatments. TM4SF1 (Transmembrane 4 superfamily member 1) is a tetraspanin family cell surface signaling structural raft-related protein. The mechanism of tetraspanin family proteins in signaling and their association with HIV-1 proteins in the immune synapse is under recent study (91, 92). This protein may be related to presentation of HIV-1 proteins; thus, the perturbation in its expression due to tat and env-coc treatments may play an important role in the ability of immune surveillance to screen cells expressing HIV-1 proteins.

5.2. Hypothesis 1. Genes identified related to drug abuse and HIV-1 protein treatments are associated with transcription isolation

The genes identified in our studies are related among several different levels of analysis, dimension, or category. There is some molecular basis for the statistical analytic results for several of the genes through Ariadne analysis as well as identification of their chromosome grouped locations. This is remarkable in that the statistical analyses are done by computer programs with no abrogation of objectivity during the analysis, since the programs run and analyze the data in a 'black box' mode. Furthermore, the associations of several genes in groups occur on the same chromosome, chromosome arm, and in proximity on the chromosome arm. This supports functional, pathobiological, and pharmacologic associations for the genes. For example, the gene we identified as KIAA0478 is in the same karyotype band 1p36 as HTR1D, a serotonin receptor. These are furthermore near PARK7 or DJ-1, an early onset Parkinson's disease gene. DJ-1 is a chaperone protein with anti-oxidant function. An additional example is that a few identified genes on chromosome 2 (MAT2A at 2p11.2, KIAA0582, and ARHB, p12) cluster near PARK3 at 2p13 (also known as SPR gene and is implicated in dopamine synthesis and early onset Parkinson's disease.) MAT2A is methionine adenosyltransferase II alpha, KIAA0582 is brain K/Na hyper-polarization activated gated channel, a kinase anchor protein, and ARHB as mentioned above is a small guanosine triphosphatase Rho protein that regulates actin-filament remodeling. Prior to the use of Microarray studies, 103 tabulated genes were in the following categories: Receptors, Transcription factors, Enzymes, Neuropeptides, Apoptotic genes, Cytokines, G proteins, Transporters, Growth factors, HIV-related genes, and Heat shock proteins (82). This overlaps categories identified through Microarray analysis, however, a wider range of types of genes were identified more recently including signaling, genome maintenance, RNA and viral RNA induced, nuclear pore, electron transport, cell cycle, endocytic traffic, proteasome, and most importantly gene directly involved in neuropsychiatric disease.

Transcription insulation is a current concept in molecular biology. The hypothesis is that there are transcription insulators between genes on chromosomes so that gene expression activation does not spill over indiscriminately across the chromosome. Thus, as chromatin conditions or states change there are barriers at various junctions between various gene groups. It remains to be determined how widespread this phenomenon is across all the human chromosomes (93). This may be related to a mechanics of imprinting, the regulation of expression on paternal vs. maternal autosomal alleles. This exists for example on chromosome 11, at region 11p15.5 for genes H19DMR and intron 10 of gene KCNQ1. Gene insulators and silencers appear are involved and a protein common to each site is CTCF protein that may mediate this control (94).

That implies that when we find groups of genes that are coordinately expressed that they may be located on the same chromosome and may be even near each other. Thus, the molecular biology term, transcription insulator boundary, could also be termed a gene expression fire wall. This is an alternative to metabolic or signaling networks, genes that are also controlled in concert.

5.3. Hypothesis 2. Drug abuse and HIV-1 proteins coerce transcription overload

We hypothesize that the deleterious effects on neurons (in culture and brain) caused by abused drugs and HIV-1 proteins include mechanisms that trigger imbalanced transcription or what we term transcription overload. This overload is due to the coerced expression caused by the treatments that may result in damage to the chromosome's control and organization of the chromatin machinery for transcription. The normal homeostatic checks and balances of the neuron are disrupted to an extent that damages the cell's transcription machinery. Cocaine causes DNA strand breaks and this has been supposed due to its induction of apoptosis (95.) However, we suggest that DNA strand scissions may also result from weakening of chromosome integrity due to coerced and dysregulated transcription. Further investigation is required to dissect and confirm mechanisms of drug and HIV-1 protein effects on human neurons as a model for NeuroAIDS in the context of drug abuse.

6. CONCLUSIONS AND FUTURE DIRECTIONS

This preliminary work adds a larger dimension and scope to our prior studies that supported the notion that multiple types of analyses are needed for Microarray work (70, 73, 74, 75). We find that gene expression differs significantly across different treatment conditions (preparations), that treatments show significant interactive effects on gene expression, that expression of genes varies in groups in parallel across different treatment conditions, and there are specific genes that show gene by treatment effects within these groups. We also find that the genes identified in our study are arranged on the human chromosomes in a non-stochastic fashion, and possibly linked to genes known to be associated with human neuropsychiatric illness. Furthermore, this work lead to hypotheses relating to both transcription isolation and overload in damage due to drug abuse and HIV infection. This emboldens our approach and supports its productive scope.

Currently, we are performing laser capture dissection (LCM) to analyze gene expression in neurons from AIDS brain tissue with and without HIV encephalitis and with and without dementia using Microarray analysis. In addition, we are applying representations of data, processes, and queries to develop detailed tools to assist in the development of automated tools for simulating complex biological processes. Furthermore, we are applying intuitive and programmatic network approaches, cluster methodologies, and to identify key genes and gene-combinations in the pathogenesis of dementia due to HIV-1 infection and drug abuse. The application of gene expression, pathway and gene-chromosome architecture analysis are thus productive in this regard.

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