Synaptic Neuroadaptations following Repeated Cocaine Exposure in Vervet monkeys – a Proteomics Analysis of the Frontal Cortex

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INTRODUCTION

Cocaine addiction is known to involve long-lasting or persistent behavioral and neurochemical alterations. Of particular importance may be the drug-induced changes in synaptic connections that occur within cortico-striatal brain circuits that normally mediate reward, motivation and inhibitory control. It has been previously shown that chronic administration of cocaine alters the density of dendritic spines in the nucleus accumbens and prefrontal cortex (e.g. Robinson and Kolb 1999), structures that have been related to cocaine-induced behavioral alterations. In addition, a number of molecular substrates have been identified that are altered following cocaine administration that influences synaptic function. However, the exact mechanisms underlying these cocaine-induced structural and functional rearrangements remain to be identified.

In order to assist the identification of potential mechanisms for cocaine-induced plasticity, we attempted to provide a comprehensive analysis of protein alterations in the cortico-striatal circuitry using an unbiased proteomics approach. We previously reported that repeated cocaine exposure to Vervet monkeys (2 mg/kg/day for 14 days) was sufficient to produce concurrent and selective deficits in reversal learning which is dependent on orbitofrontal cortex (OFC) and facilitation of incentive aspects of motivation, supporting the notion that cocaine administration induces functionally significant deficits in cortico-striatal functions (Olausson et al. 2007). The current study sought to identify biochemical correlates of these behavioral effects and to perform an unbiased proteomic analysis of cocaine-induced alterations in additional regions of the frontal cortex in tissue taken from the behaviorally characterized animals. Four weeks after the last cocaine injection, monkeys were sacrificed, and tissue punches were taken from a number of regions of the frontal cortex and synaptoneurosomes generated. We determined several biochemical changes in synaptic composition that may be related to the behavioral effects observed.

METHODS

Subjects and treatment: African green monkeys (*Cercopithecus aethiops sabaeus*) were trained to perform food-rewarded object discriminations, and subsequently received daily injections of cocaine (2 mg/kg, i.m.) or saline for 14 days (n = 8 per group). Following 14 days of withdrawal, monkeys were tested on behavioral tasks, including attentional set-shifting, and sacrificed 4 weeks after the last injection.

Tissue preparation: Four weeks after the last cocaine injection, monkeys were anesthetized with ketamine, and brains were removed for biochemical analysis. During this process, monkeys were intracardially perfused with ice-cold saline containing 25 mM sodium fluoride and 1 mM sodium orthovanadate to minimize protein degradation and loss of post-translational modifications. Brains were then cut into 5 mm thick slices using a primate brain matrix, and tissue punches were taken from 20 brain regions of interest using a large gauge tissue punch. Immediately, synaptoneurosomes were isolated from the brain tissue using a modified version of Hollingsworth protocol (Hollingsworth, 1985) in a HEPES buffer and frozen in liquid nitrogen until use.

Sample preparation for iTRAQ analysis: iTRAQ analysis and mass spectrometric identification of proteins was carried out by the Yale/NIDA Neuroproteomics Center on samples from four brain regions: Nucleus accumbens, caudate, orbitofrontal cortex and medial prefrontal cortex. For each brain region, 100 µg total protein per animal from each treatment group were pooled and resuspended in iTRAQ buffer. Following Amino Acid Analysis, samples from saline- and cocaine-treated monkeys were digested using trypsin and labeled with iTRAQ reagents 114 or 116 respectively. Pairs of differentially labeled samples were pooled, subjected to cation exchange fractionation and on average 20 fractions analyzed using reverse-phase LC/MS/MS. Subsequent identification of peptides and quantification of protein expression was conducted and searched using the Celera Primate and UniSwiss databases with Protein Pilot 2.0. Secondary confirmations were made using Western blot.

Protein Expression of Sp1, Cofactors of Sp1, and Downstream Targets: Immunoblotting was done on nuclear and cytosolic fractions, in addition to total protein homogenates from the dorsal striatum of each subject. Standard Western blotting procedures were used. Primary antibodies used to quantify protein level included: Sp1 (Santa Cruz), Dopamine D1 Receptor (Abcam), Dopamine D2 Receptor (Abcam), ERK (Cell Signaling), phospho-ERK (Cell Signaling), Histone H3 (Cell Signaling), GFAP and S100-beta (Abcam), and GAPDH (Advanced Immunochemical). Fluorescent secondary antibodies for rabbit and mouse primary antibody detection were used (Rockland Immunochemicals), and relative fluorescence was detected using a LiCor Odyssey imaging system.

mRNA Expression of Sp1, Cofactors of Sp1, and Downstream Targets: Total RNA was extracted from one bilateral punch from each subject using the mirVana miRNA Isolation Kit (Applied Biosystems). Equal amounts of RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The following TaqMan (Applied Biosystems) gene expression assays were used to quantify mRNA: Sp1 (Rn00561953_m1), Dopamine Drd1a (Rn03062203_s1), Dopamine Drd2 (Rn00561126_m1), Rat actin, beta. Equal amounts of cDNA from each subject was run in triplicate for each assay according to the manufacturer's suggestions. mRNA levels were quantified using the 7500 Fast Real-Time PCR System and Sequence Detection Software (SDS). The ddCt method of analysis was used to determine relative quantification of mRNA.

RESULTS

Persistent Synaptic Neuroadaptations at 1 Month Following Prior Chronic Cocaine Exposure

OFC (Area 11/12)

Protein ID	Fold regulation
Rho guanine nucleotide exchange factor (GEF) 17	-1.51
DJ179M20.1 (Adenosine deaminase)	-1.68
Putative lipid kinase	-1.65
Thymosin-like 4	-1.60
GDAP1-like1	-1.58
Claudin 11	-1.56
Seven transmembrane helix receptor	-1.56
DJ-1	-1.56
Endosepine (Fragment)	-1.55
Tropomyosin	-1.55
Mitochondrial NADP(+)-dependent malic enzyme 3	-1.54
Aquaporin 4	-1.51
Hypothetical protein DKFZp459K2229	-1.51
Neuronal pentraxin I precursor (NP-I)	-1.51
S100 calcium binding protein A1	-1.51
S100 protein, beta chain	-1.47
Fumarate hydratase	-1.47
GTP-binding protein G(i)/G(S)/G(O) gamma-3 subunit	-1.44
Carbonic Anhydrase II (Carbonate Dehydratase)	-1.44
Myelin Basic Protein (MBP)	-1.44
ACTA2 protein	-1.43
ELKS protein	-1.43
Hypothetical protein DKFZp468H1910	-1.43
Actin related protein 2/3 complex, subunit 4	-1.43
2",3"-cyclic nucleotide 3" phosphodiesterase	-1.42
Alpha-internexin (Neurofilament-66)	-1.41
Phosphoserine aminotransferase (PSAT)	-1.41
RAN protein	-1.40
Carbohydrate sulfotransferase 11	-1.40
Vacuolar protein sorting-associated protein 35	1.43
WD-repeat protein 37	1.43
Protein PM1	1.43
Cytochrome c oxidase subunit Vb isoform 1 (COX Vb-1)	1.43
NADH-ubiquinone oxidoreductase 30 kDa, mitochondrial	1.43
Kinesin-like protein KIF22	1.49
Hypothetical protein DKFZp459C1015	1.51
Succinyl-CoA ligase beta-chain, mitochondrial	1.52
Dipeptidylpeptidase VI isoform 1	1.53
MLL/SEPTIN6 fusion protein	1.55
Monoacyl glycerol lipase	1.57
Tubulin beta Class II	1.60
Regulator of G-protein signaling 6 (RGS6)	1.62
Antigen MLAA-34	1.64
Flotillin 1	1.67
KIAA0851 protein (Fragment)	1.74
Macrophage migration inhibitory factor	1.74
Ras homolog gene family, member T1	1.74
Rap1 GTPase-GDP dissociation stimulator 1	1.76
Protein phosphatase 2A, regulatory subunit B'	1.77
Transketolase	1.86
Rho-associated protein kinase 1	1.95
PKA catalytic subunit beta, isoform 2	2.06
Microsomal glutathione S-transferase 3	2.17
Ubiquinol-cytochrome c reductase complex 7.2kDa	2.28

Dorsolateral PFC

Protein ID	Fold regulation
Hypothetical protein DKFZp459A0327	-1.59
WD-repeat protein 1 (Actin interacting protein 1)	-1.45
Hypothetical protein DKFZp459G219	-1.44
Crystallin, alpha B	1.41
Carbonyl reductase 1	1.41
Sirtuin 2 (NAD-dependent deacetylase)	1.48
Myelin-oligodendrocyte glycoprotein precursor	1.48
Versican V2 core protein precursor	1.49
ATP-binding cassette, sub-family C, member 1 isoform 7	1.56
Dorsal neural-tube nuclear protein	1.61
2",3"-cyclic nucleotide 3" phosphodiesterase	1.88
Myelin basic protein (MBP)	1.93
Myelin proteolipid protein (PLP)	1.93
Cytochrome c oxidase subunit Va (Fragment)	2.00
Myelin-associated glycoprotein precursor (Siglec-4a)	2.14
Rho/rac-interacting citron kinase	2.35
CD9 antigen	2.60

Medial PFC

Protein ID	Fold regulation
Kinase insert domain rec (type III receptor tyr kinase)	-2.03
SNAP gamma	-1.81
CD81 antigen	-1.70
Mitotic checkpoint protein	-1.62
Sirtuin type 2 (NAD-dependent deacetylase)	-1.62
Tropomyosin	-1.61
Dynamin-1	-1.59
NAD-dependent aldehyde dehydrogenases	-1.58
Neuronal calcium sensor 1 (NCS-1)	-1.41
NCK-associated protein 1	-1.41
Neurocan core protein precursor	1.40
Programmed cell death 8 isoform 2	1.41
Peroxiredoxin 6	1.43
Hypothetical protein DKFZp459G2127	1.44
BASP 1	1.48
TPA: Ras-related small GTPase	1.55
Flotillin 1	2.09

Figure 1: Tables of synaptic proteins identified as regulated following chronic cocaine based on the ratio between iTRAQ ligand 116 (cocaine) and 114 (saline).

Immunoblot Analysis Confirmed Regulation of Several Targets in Whole Tissue Punches

	mRNA	COC lOFC	COC mOFC	COC ACg	COC Cau
D1		=	=	Down	=
D2	Down	Down	Down	Down	Down
Sp1	=	=	=	=	=
GFAP		Down	=	=	Up
TrkB		=	=		Up
ERK		=	=	=	=
pERK/ERK		=	Down	=	=
DARPP-32					Up
pAKT/AKT		Up	AKT down		Up
CaM		=	Down	=	Up
Neurogranin		=	Down		=
Cytochrome C			Down	Down (trend)	Down
a-synuclein					=
PSD-95				Up	Up

Figure 3: Western blot analysis of Sp1-regulated proteins and selected cocaine-regulated targets from the behaviorally characterized monkeys.

Transcriptional Analyses of Cocaine-Induced Changes in Synaptic Protein Expression

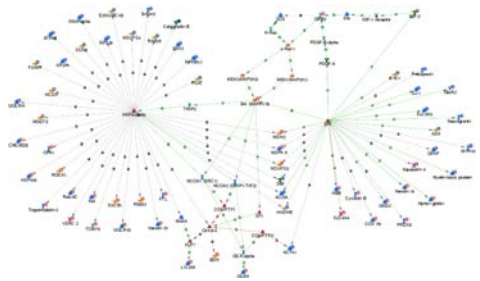

Figure 2: Bioinformatic transcriptional analysis of alterations in protein expression within the OFC and the striatal regions using GeneGo MetaCore (see tables) suggest that the transcription factors HNF4alpha and SP1 are highly integrated in the proteomic map. Red circles identify up-regulated proteins, blue circles down-regulated proteins.

Alterations in Sp1 Activity may Result in Decreased Expression of D2 Receptors in the OFC and ACg

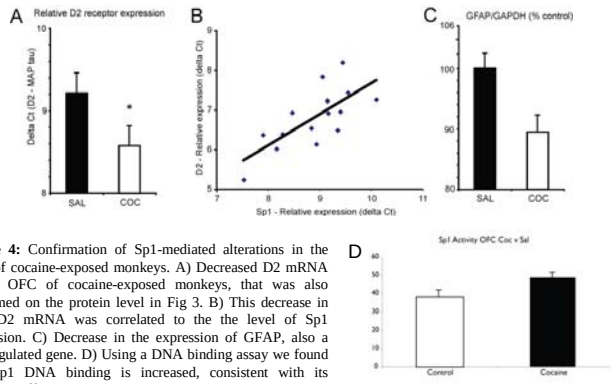

Figure 4: Confirmation of Sp1-mediated alterations in the OFC of cocaine-exposed monkeys. A) Decreased D2 mRNA in the OFC of cocaine-exposed monkeys, that was also confirmed on the protein level in Fig 3. B) This decrease in OFC D2 mRNA was correlated to the the level of Sp1 expression. C) Decrease in the expression of GFAP, also a Sp1-regulated gene. D) Using a DNA binding assay we found that Sp1 DNA binding is increased, consistent with its inhibitory effect on D2 receptor expression.

CONCLUSIONS

• A number of proteins related to intracellular signaling (including small GTPases, kinases, phosphatases and calcium-binding proteins), protein turnover and cytoskeletal rearrangement were identified, consistent with the hypothesis that cocaine-induced changes include both structural and functional alterations of synaptic function.

• Chronic cocaine exposure also regulates a large number of metabolic enzymes, suggesting an changes in energy demand possibly due to alterations in metabolically active spines and synapses.

• Transcriptional analysis of cocaine-induced regulation of synaptic proteins using bioinformatics suggest the involvement of the transcription factor SP1 and its regulation of dopamine D2 receptor expression in the orbitofrontal and anterior cingulate cortices that may be causally related to cocaine-induced cognitive-motivational impairments

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