Proteomic analysis of persistent cortico-limbic-striatal neuroadaptations following repeated cocaine exposure in Vervet monkeys.

Dilja D. Krueger[1], Peter Olausson[1], Terence Wu[2], Kenneth R. Williams[2], Jane R. Taylor[1] and Angus C. Nairn[1].

[1]Dept. of Psychiatry and [2]Molecular Biophysics and Biochemistry, Yale University, New Haven CT.

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-limbic-striatal brain circuits that normally mediate reward, motivation and inhibitory control. It has been previously shown that chronic administration of cocaine increases the density of dendritic spines in the nucleus accumbens and prefrontal cortex (e.g. Robinson and Kolb 1999, Li et al.` 2004), 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 and may thus play a role in mediating such structural rearrangements. However, the exact mechanisms underlying these 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-limbic 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 inhibitory control dependent on prefrontal cortex and facilitation of incentive aspects of motivation, supporting the notion that cocaine administration induces functionally significant deficits in cortico-limbic-striatal functions (Olausson et al. 2004). The current study sought to identify biochemical correlates of these behavioral effects in tissue taken from the same animals. Four weeks after the last cocaine injection, monkeys were sacrificed, and tissue punches were taken from a number of brain regions and subjected to two-dimensional differential fluorescence gel electrophoresis (DIGE). A number of cocaine-regulated proteins were identified that may be related to the behavioral effects observed.

METHODS

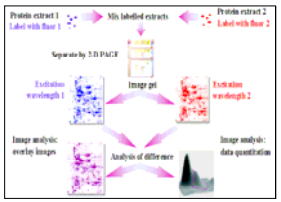
Subjects and treatment: African green monkeys (*Cercopithecus aethiops*) received daily injections of cocaine (2 mg/kg, i.m.) or saline for 14 days (n = 6 per group). Following 14 days of withdrawal, monkeys were tested on behavioral tasks measuring inhibitory control and reward-related learning as described previously (Olausson et al 2004).

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 needle. Punches were sonicated in 1% SDS containing 25 mM NaF and 1 mM Na_3VO_4, and stored at -80°C until use.

Sample preparation for DIGE analysis: DIGE analysis was performed on samples from four brain regions: Nucleus accumbens, caudate, orbitofrontal cortex and dorsolateral cortex. For each brain region, 100 µg total protein per animal from each treatment group were pooled, and proteins were precipitated using a 2D clean-up kit (Amersham Biosciences). Samples were resuspended in DIGE buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 25 mM Tris, pH 8.5 at a concentration of 5 mg/ml.

DIGE analysis and mass spectrometry: DIGE analysis and mass spectrometric identification of proteins was carried out by the Yale/NIDA Neuroproteomics Center. Briefly, saline- and cocaine-treated samples were labeled with Cy3 and Cy5, respectively, and the two samples were pooled and run on two-dimensional gel electrophoresis. Gels were scanned on an Amersham Typhoon 9400 Imager, and spots were quantified using the Amersham Biosciences DeCyder software. Spots of interest were identified and excised using the Ettan Spot Picker instrument. Following in-gel tryptic digestion, spots were analyzed using LC/MS/MS and subsequent identification of peptides using the Mascot database.

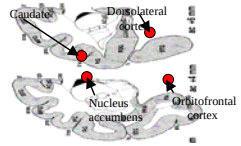
Secondary confirmation: Standard Western blotting procedures were used confirm regulation of target proteins in individual animals. The following antibodies were used: BASP1 (Chemicon), GAP43 (Chemicon), neurogranin (Upstate), calmodulin (Upstate), GFAP (Chemicon), and GAPDH (Advanced Immunochemical Co)



Amersham EttanDIGE User Manual

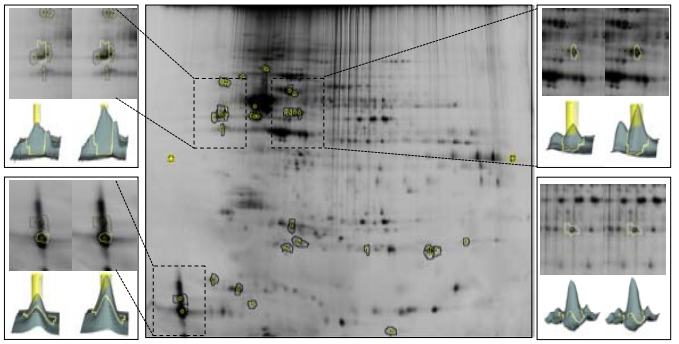
RESULTS

Figure 1: ***Location of tissue punches taken.***



(Martin and Bowden 1996)

Figure 2: ***Representative DIGE gel image from a nucleus accumbens sample.*** For each sample, 100 µg labeled protein were loaded on a gel and separated first by isoelectric focusing point, then by molecular weight. The gel was scanned, and for each spot the Cy5/Cy3 volume ratio was determined. Examples of spots showing increases in volume following cocaine administration are shown on the sides (top left = BASP1, bottom left = calmodulin, top right = GFAP, bottom right = example of a spot showing no regulation). Yellow spots indicate punches taken for mass spectrometric protein identification.



		Nucleus accumbens	Caudate nucleus	Orbito-frontal ctx	Dorso-lateral ctx
Metabolic	ATP synthase, mitochondrial F1 complex	1.58	1.41	1.54	1.5
	Creatine kinase-B	1.5	1.32	1.26	1.15
	Enolase 2	1.3	1.6	1.2	1.52
	Glyceraldehyde-3-phosphate dehydrogenase	-	-	1.36	-
	Nicotinamide adenine dinucleotide	-	-	1.36	
	Phosphoglycerate mutase 1	1.54	-	-	1.48
	Superoxide dismutase	1.29	-	-	-
	Triosephosphate isomerase	1.46	1.3	1.37	1.63
Protein turnover	Chaperonin	1.13	1.16	-	-
	Heat shock protein 60 precursor	-	-	-	1.19
	Heat shock protein 70 protein 5	1.25	1.52	1.35	1.07
	Ubiquitin carboxy-terminal esterase L1	1.48	1.31	1.15	1.3
Cytoskeletal	Actin alpha	-	1.24	1.43	1.42
	Glial fibrillary acidic protein	2	-	-	-
	Internexin alpha	1.17	-	-	1.44
	Neurofilament	-	-	-	1.18
	Tubulin alpha	-	1.29	-	-
	Tubulin beta	1.29	1.41	1.64	-
PKC/CaM signaling & actin dynamics	Brain abundant signal protein 1	1.76	1.26	2.15	1.77
	Calmodulin	1.68	2.76	-	1.88
	Neurogranin	1.4	-	-	-
	Neuromodulin / GAP43	-	-	-	1.39
Other	Exportin Cse1p	-	1.42	-	-
	Guanine nucleotide binding protein	-	1.4	-	-
	Peroxiredoxin 1/2	1.29	-	-	1.42
	Synaptosomal-associated protein 25	-	-	-	1.46
	Synuclein alpha	-	-	-	1.25
	Synuclein beta	1.22	1.15	-	1.3

Figure 3: ***Table of proteins identified as regulated following chronic cocaine administration in four brain regions.*** Numbers represent the Cy5 / Cy3 ratio, i.e. the fold regulation in cocaine-administered animals. Proteins identified are classified according to function.

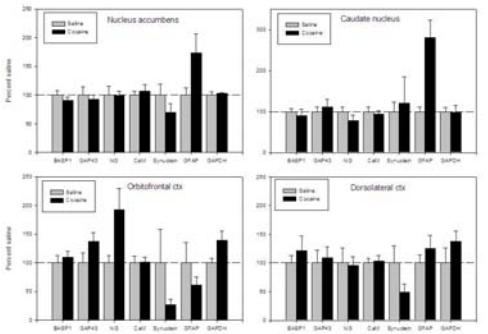


Figure 4: ***Secondary confirmation of target proteins using Western blotting.*** Antibodies were obtained for several targets of interest, and the same samples used for DIGE analysis were run on Western blots. Results are displayed for each brain region analyzed. Quantification is expressed as percentage of saline control.

CONCLUSIONS

• Chronic exposure to cocaine results in upregulation of a large number of metabolic enzymes, suggesting an increased energy demand possibly due to the increase in metabolically active spines and synapses

• In addition, a number of proteins related to protein turnover and cytoskeletal rearrangement were identified, consistent with the hypothesis that cocaine-induced changes include structural alterations and reorganization of synaptic connectivity

• Glial fibrillary acidic protein (GFAP) was found to be upregulated in the nucleus accumbens and striatum, confirming previous findings that withdrawal from cocaine induces astroglial plasticity (Bowers and Kalivas 2003)

• A number of candidate proteins, including BASP1, neurogranin and neuromodulin, were found to be targets of protein kinase C and calmodulin regulation, suggesting that PKC / Ca2+ signaling may be dysregulated in these samples

• This is of particular interest in light of the fact that the spot volume ratios identified by DIGE did not always correlate with increases in protein levels, suggesting that the DIGE results may reflect changes in post-translational modifications rather than total protein levels

SUPPORT

Supported by Yale/NIDA Neuroproteomics Research Center (1 P30 DA018343-0), NIDA grants DA11717 (JRT) and DA10044 (ACN).

REFERENCES

• Bowers MS & Kalivas PW (2003) Forebrain astroglial plasticity is induced following withdrawal from repeated cocaine administration. European Journal of Neuroscience 17 (6), 1273-1278.

• Li Y, Acerbo MJ and Robinson TE (2004) The induction of behavioural sensitization is associated with cocaine-induced structural plasticity in the core (but not shell) of the nucleus accumbens. Eur J Neurosci. 20(6):1647-54.

• Martin RF and Bowden DM (1996) A stereotaxic template atlas of the macaque brain for digital imaging and quantitative neuroanatomy. Neuroimage 4(2):119-50.

• Olausson P, Tronson NC, Kiraly DD, Krueger DD, Nairn AC and Taylor JR (2004) Selective cognitive-motivational alterations following prior cocaine exposure in monkeys. Program # 671.10, 2004 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience. Online.

• Robinson TE and Kolb B (1999) Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. European Journal of Neuroscience 11(5):1598