Proteomic analysis of synaptic cortico-striatal neuroadaptations following repeated PCP exposure in Vervet monkeys

Alexia T. Kedves[1], Peter Olausson[1], Dilja D. Krueger[1], Christopher Colangelo[2], Kenneth R. Williams[2], Angus C. Nairn[1], and Jane R. Taylor[1].

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

INTRODUCTION

Repeated exposure to phencyclidine (PCP) is suggested to be a useful pharmacologic animal model of psychosis (Jentsch and Roth 1999). Indeed, chronic PCP produces long-lasting or persistent behavioral and neurochemical alterations that recapitulate multiple aspects of schizophrenia, in particular the cognitive impairments associated with this disorder. The biochemical consequences of chronic PCP, in particular those that occur within cortico-striatal brain circuits that mediate and coordinate cognitive functions, largely remain unknown. We, and others, have previously reported that repeated PCP exposure to Vervet monkeys (0.3 mg/kg/day for 14 days) induces persistent cognitive abnormalities in monkeys. The current study was designed to identify alterations in synaptic function associated with these cognitive deficits using an unbiased proteomics approach. Four weeks after the last PCP injection, monkeys were sacrificed and synaptoneurosomes were generated from tissue punches taken from orbitofrontal and dorsolateral prefrontal cortex, as well as the dorsal (caudate and putamen) and ventral (nucleus accumbens) striatum. Differential expression of proteins in the synaptic compartment of saline vs. PCP-exposed animals was subsequently analyzed using multiplexed isobaric tagging technology (iTRAQ) with mass spectrometric methods (LC-MS-MS). A number of PCP-regulated proteins involved in intracellular signaling, cytoskeletal and metabolic functions were identified in cortical and striatal regions, confirming the profound effects of this exposure on synaptic function. Ongoing analyses are exploring the relationships between PCP-induced alterations in protein expression and cognitive processes, and in order to identify potential target proteins and cellular functions potentially involved in the neurobiology of schizophrenia

METHODS

Subjects and PCP exposure: Male and female African green monkeys (*Cercopithecus aethiops sabaeus*) received daily injections of PCP (0.3 mg/kg, i.m.) or saline for 14 days. Following 14 days of withdrawal, monkeys were tested on behavioral tasks and sacrificed 4 weeks after the last PCP injection.

Tissue preparation: Monkeys were anesthetized with ketamine, and brains removed for biochemical analyses. 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 25 brain regions of interest. 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.

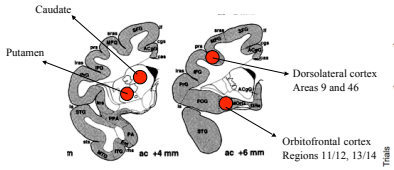
Sample preparation for iTRAQ analysis: iTRAQ analysis and mass spectrometric identification of proteins was carried out by the Yale/NIDA Neuroproteomics Center on samples from four cortical and two striatal brain regions. For each brain region, 100 µg total protein per animal from each exposure group were pooled and resuspended in iTRAQ buffer. Following Amino Acid Analysis, samples from saline- and PCP-treated monkeys were digested using trypsin and labeled with iTRAQ reagents 114 (saline) or 115 (PCP) 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. Identification and quantification of protein expression was conducted and searched using the Celera Primate database.

Secondary confirmation: Standard Western blotting procedures were used to confirm differential regulation of target proteins identified by iTraq. The following antibodies were used: Akt/pAkt (Cell Signaling), Cytochrome C (Mitosciences), ERK/pERK (Cell Signaling), Dopamine D1 Receptor (Abcam), Dopamine D2 Receptor (Abcam), GAPDH (Advanced Immunochemical Inc), GFAP (Millipore), Neurogranin (Cell Signaling), Sp1 (Santa Cruz), TrkB (BD Transduction Laboratories).

Sp1 Immunoprecipitation: In the absence of phospho-specific Sp1 antibodies, PCP-induced changes in Sp1 activity was assayed with kinase substrate antibodies following Sp1 immunoprecipitation with the Sp1 antibody from Santa Cruz and Protein A sepharose beads (Sigma). Approximately 100ug of total protein was IP'd per sample for each kinase assay. Standard Western blotting methods were used, and substrate antibodies for MAPK, PKA, CDKs, and Akt (PKB) (all Cell Signaling) were used to identify modification sites contained within the Sp1 protein sequence.

RESULTS

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



(Martin and Bowden, 1996)

Figure 2: ***Prior Chronic PCP Exposure Produces Persistent Cognitive Deficits.*** Prior PCP Exposure elicits deficits in Reversal Learning and Extra-Dimensional Shifting as compared to saline treated controls two weeks after the last PCP injection.

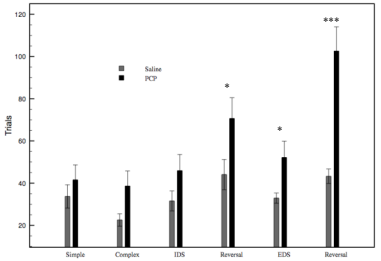


Figure 3: ***Table of proteins identified as regulated following chronic PCP administration in Caudate/Putamen, Orbitofrontal cortex, and Dorsolateral cortex.*** Numbers represent the fold regulation in PCP-administered animals and are classified according to function.

		Caudate/Putamen	Orbitofrontal cortex (11/12, 13/14)	Dorsolateral cortex (46, 9)
Metabolic	Creatine Kinase		↑	
	Cytochrome C 1		↓	
	Cytochrome C oxidase subunit 4	↑(Cau) ↓(Put)		
	Enolase	↑		↑
	Glyceraldehyde-3-phosphate-dehydrogenase	↑(Cau) ↓(Put)		
	Phosphoglycerage mutase		↓	
Cytoskeletal	Glial fibrillary acidic protein	↑	↓	
	Tubulin beta	↑(Cau) ↓(Put)	↓	
PKC/CaM signaling	14-3-3	↓	↓	
	Brain abundant signal protein 1	↓	↑	↑
	Neurogranin	↓		
	Neuromodulin (GAP-43)	↓	↑	
	S100		↓(11/12) ↑(13/14)	
Other	Myelin basic protein	↑	↑	↑
	Neural cell adhesion molecule		↓	
	Synaptobrevin-2	↑		

Figure 4: ***Sp1 Transcription Factor pathway interactions.***

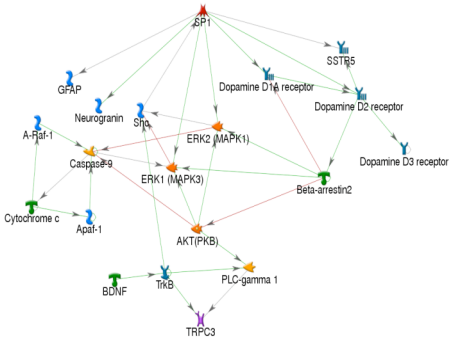


Figure 5: ***Secondary confirmation of target proteins and pathways using Western blotting.*** Antibodies were obtained for several targets of interest, and total protein samples were run on Western blots. Results are displayed for each brain region analyzed. Quantification is expressed as percentage of saline control fluorescence. (#, p=0.09-0.051 trend; *, p=0.05-0.01; **, p<0.01)

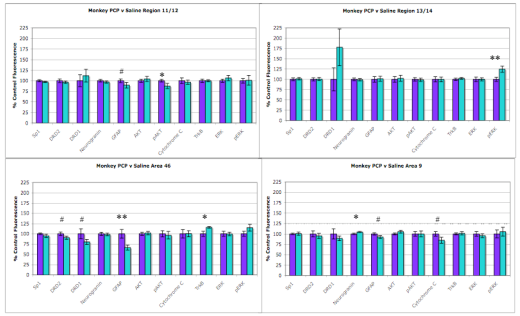
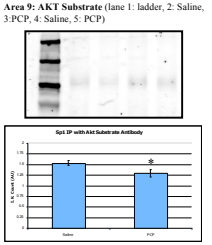


Figure 6: ***Immuno-precipitation of Sp1 probed with kinase substrate antibodies.*** Immunoprecipitated Sp1 was probed with kinase substrate antibodies to identify modification site differences. The level of AKT substrate phosphorylation of Sp1 was significantly decreased in PCP-exposed monkeys (p=0.017). A representative blot and graph showing raw arbitrary values are shown.

Area 9: AKT Substrate (lane 1: ladder, 2: Saline, 3:PCP, 4: Saline, 5: PCP)



CONCLUSIONS

• Chronic PCP administration leads to altered expression of a large number of synaptic proteins in the cortico-striatal network. Analyses confirmed the greatest enrichment of proteins involved in metabolic processes, cytoskeletal arrangement, protein kinase and CaM-regulated intracellular signaling.

•Changes in synaptic proteins identified by iTRAQ predicted changes in total protein changes identified by Western blotting for a selected number of proteins, while additional targets could not be conclusively confirmed. A comprehensive analysis of all targets identified by iTRAQ will assess the overall relationship and reproducibility of changes in protein expression identified using iTRAQ and alternative methodologies.

•Transcriptional analyses with bioinformatics techniques identified Sp1 as highly integrated in the proteomic map. Sp1 has previously been implicated in Schizophrenia (Ben-Shachar and Karry, 2007). While Sp1 expression was not changed, the PCP-induced effects on kinase and/or phosphatase activity identified here may result in altered Sp1 phosphorylation and change the transcriptional activity of Sp1. Such changes in Sp1 function may play a role in the differential regulation of many downstream targets of Sp1, such as GFAP, Dopamine D1 and D2 receptors, and neurogranin. Indeed, we found reduced Akt-dependent Sp1 phosphorylation in the dorsolateral PFC of PCP-exposed rats. The relationship between such alterations in Sp1 phosphorylation and the transcriptional regulation of downstream targets, as well as their relationship to the behavioral alterations observed, is currently being explored.

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