IDENTIFICATION OF ALTERATIONS IN SYNAPTIC PROTEIN COMPOSITION IN CORTICO-LIMBIC-STRIATAL BRAIN REGIONS AFTER CHRONIC COCAINE EXPOSURE IN MICE

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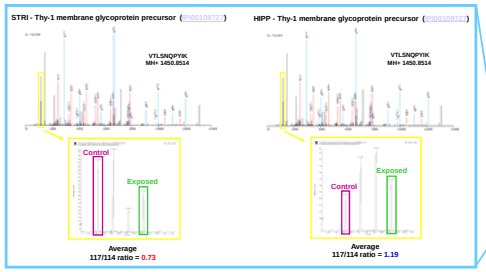
INTRODUCTION

Long-lasting neuroadaptations in intracellular signaling pathways and synaptic morphology are thought to underlie drug-induced plasticity in addiction. Such changes resemble those implicated in learning and memory. Consequently, it has thus been suggested that drugs of abuse may usurp the molecular machinery required for learning in brain reward centers, resulting in an aberrant form of plasticity. In animal models, chronic exposure to a variety of drugs of abuse can produce locomotor activity hyperactivity (for review, Robinson, T.E. & Berridge, K.C. 2000). The induction of behavioral sensitization is associated with cocaine-induced neuroplasticity in brain regions known to be involved in addiction (Li *et al*. 2004). This behavioral plasticity may contribute to the increased drive and motivation for drug, a core symptom of addiction.

Persistent morphological alterations as well as number of dendritic spines are associated with long-lasting changes at the molecular level (Robinson, T.E. & Kolb, B. 1999). Repeated exposure to drugs of abuse also appears to alter the amount and even types of genes expressed in several brain regions known to be involved in drug addiction (for review, Nestler, E.J. 2004). However, large-scale studies of drug-induced molecular alterations at the protein level are lacking.

This study aims to look at protein changes associated with chronic exposure to cocaine which underlie both the behavioral and structural plasticity. We hypothesized that the postsynaptic density (PSD), which is a dynamic multi-protein complex that links neurotransmission with intracellular signaling molecules, is critical for these persistent cocaine-induced synaptic alterations. Previous characterizations of the PSD proteome from whole brain reveals a complex organelle which consists of between 250 and 500 proteins (Li *et al*. 2004, Jordan *et al*. 2004, Yoshimura *et al*. 2002, Collins *et al*. 2005). In our study, we have begun the characterization of brain region-specific differences in the PSD proteome following cocaine exposure.



Summary of iTraq analysis of cortical, striatal, and hippocampal PSDs from chronic cocaine exposed mice

Protein Name	CTX Exp/Cont	STRI Exp/Cont	HIPP Exp/Cont	Category
Splice Isoform 1 of Tenascin-R precursor	1.25	0.99	1.00	Cell Adhesion
Contactin 1 precursor	1.21	1.17	0.98	
Tubulin alpha-4 chain	1.27	0.97	0.97	Cytoskeletal
Tubulin beta-5 chain	0.80	0.78	1.05	
Tubulin polymerization-promoting protein	0.96	0.75	1.01	
Cytoskeleton-assoCiated protein 4	0.85	1.38	1.04	
Neurofilament triplet L protein	0.92	0.78	1.12	
Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2	0.70	1.24	1.04	GPCR Signaling
Guanine nucleotide-binding protein G(z), alpha subunit	1.31	1.09	1.12	
Ras-related protein Ral-A	0.97	0.77	1.07	GTPase Regulation
RAS-related C3 botulinum substrate 1	1.30	1.16	0.92	
BMB8 antigen	0.85	1.03	1.01	Immune
P32-RACK	0.98	0.72	0.97	
Thy-1 membrane glycoprotein precursor	1.13	0.73	1.20	Lipid Raft
Brain acid soluble protein 1	1.06	0.81	1.23	
Prohibitin	0.51	1.04	0.88	
Mitochondrial glutamate carrier 1	0.63	1.05	0.84	Metabolic
ATP synthase e chain, mitochondrial	0.54	0.92	0.89	
Cytochrome c oxidase polypeptide VIIa	1.02	1.03	1.44	
Dihydrolipoyl dehydrogenase	0.91	1.23	0.99	
ATP synthase O subunit	0.66	0.90	0.96	
ATPase, H+ transporting, V1 subunit B	1.21	1.18	1.04	
cytochrome c oxidase, subunit Va	1.08	0.58	1.43	
NADH-ubiquinone oxidoreductase 19 kDa subunit	1.12	0.53	1.29	
NADH-ubiquinone oxidoreductase PDSW subunit	1.04	0.80	1.19	
Voltage-dependent anion-selective channel protein 2	0.76	1.29	0.93	
Voltage-dependent anion channel 3	0.62	1.27	0.93	
Phosphate carrier protein	0.52	1.18	0.76	
ATP synthase coupling factor 6	0.62	0.85	1.08	
NADH-ubiquinone oxidoreductase MLRQ subunit	0.77	1.10	1.00	
Splice Isoform 1 of Sideroflexin-3	0.75	0.82	0.86	
ADP/ATP translocase 2	1.00	1.28	0.82	
NADH-ubiquinone oxidoreductase 49 kDa subunit	0.99	1.25	0.99	
Ubiquinol-cytochrome c reductase complex 11 kDa	0.66	0.67	1.01	
ATP synthase alpha chain, mitochondrial precursor	0.62	1.05	0.87	
NADH-ubiquinone oxidoreductase subunit B14.5a	1.19	0.95	1.23	
Cytochrome c oxidase subunit 2	0.90	1.42	1.16	
Cytochrome c oxidase polypeptide VIc	1.02	1.07	1.23	
Pyruvate dehydrogenase E1 component beta subunit	0.69	0.99	0.99	
Ubiquinol-cytochrome c reductase binding protein	1.22	0.73	0.99	
Acyl carrier protein, mitochondrial precursor	0.96	0.73	1.06	
NADH-ubiquinone oxidoreductase B12 subunit	1.28	1.15	1.00	
Acetyl-CoA acetyltransferase	1.42	0.83	1.05	
Amine-oxidase [flavin-containing] A	1.18	1.02	0.66	
NADH-ubiquinone oxidoreductase 23 kDa subunit	1.28	0.96	0.79	
Ubiquinol-cytochrome c reductase complex ubiquinone-binding protein QP-C	1.11	0.71	0.93	
Cytochrome c oxidase subunit VIb isoform 1	0.75	0.96	1.34	
NADH-ubiquinone oxidoreductase 18 kDa subunit	1.04	0.74	1.15	
NADH-ubiquinone oxidoreductase B16.6 subunit	1.02	1.05	1.20	
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	0.78	0.99	0.90	
Cytochrome b5 outer mitochondrial membrane	1.06	0.93	0.76	
NADH-ubiquinone oxidoreductase 13 kDa-B subunit	1.27	1.02	1.16	
Mitochondrial import receptor subunit TOM22	0.84	0.73	0.86	
ATP synthase B chain	0.55	0.91	1.00	
ATPase, H+ transporting, V1 subunit A, isoform 1	1.40	1.16	1.04	
ATP synthase delta chain	0.39	0.58	0.92	
ATP synthase beta chain	0.50	0.89	0.83	
Splice Isoform HK1-SA of Hexokinase type I	0.57	1.00	0.91	
Glutamine synthetase	0.89	0.70	1.07	
solute carrier family 25	0.92	1.20	0.82	
Oligodendrocyte-myelin glycoprotein precursor	0.94	0.93	1.27	Myelin/Glial
peroxiredoxin 6	1.02	0.73	0.95	Neuroprotection
60S ribosomal protein L28	0.73	1.42	1.15	Protein Synthesis
Na+/K+-ATPase alpha 3 subunit	0.78	0.99	0.88	Receptor/Channel
Sodium/potassium-transporting ATPase alpha-1	0.80	1.02	0.96	
Shank3	1.22	0.94	0.97	Scaffolding
Vacuolar ATP synthase subunit E	1.26	1.10	0.95	Trafficking
Isoform SNAP-25b of Synaptosomal-associated protein 25	0.87	0.73	1.01	
Splice Isoform A1-II of Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1	1.39	1.14	1.09	
Vacuolar ATP synthase subunit H	0.95	0.79	0.90	
Septin 5	1.23	1.14	1.16	
Splice Isoform 2 of Septin-11	1.32	1.06	1.11	
septin 6	1.35	1.13	1.19	
Vacuolar ATP synthase subunit d	1.14	1.23	1.00	
Bcl-2 inhibitor of transcription	0.69	1.14	1.01	Transcriptional Reg.
hypothetical protein	0.68	0.92	0.85	Unknown
Protein C10orf70 homolog	0.80	0.74	0.85	
42 kDa protein	0.68	0.93	1.08	

Figure 1: Cortical, striatal, and hippocampal PSDs were isolated from animals either exposed to cocaine (30 mg/kg) (n=8, pooled) or saline (n=8, pooled) for 22 days. Using MALDI-LC-MS/MS, 271 proteins were confidently identified in cortical PSDs, while 482 and 471 were identified in the hippocampal and striatal PSDs, respectively. Summarized are proteins found in all three regions with decreases of 0.8 fold or less following cocaine exposure verses saline in red and increases of 1.20 fold or more in blue. Protein identification, cortical PSD, striatal PSD, and hippocampal PSD results as well as categorization of proteins are shown.

Secondary analysis of iTraq results with Western blotting

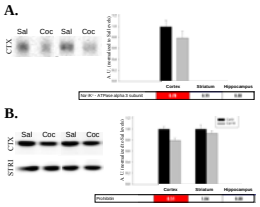


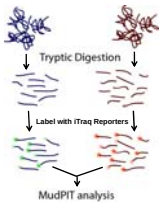
Figure 2: Proteins determined by iTraq analysis to change in the PSD following chronic cocaine exposure were confirmed by immunoblotting. (a) Na+/K+ –ATPase alpha 3 showed a 0.80 decrease in cocaine-exposed cortical PSDs (n=6) when compared to saline-treated cortical PSDs (n=6). (b) Prohibitin showed a 0.80 decrease in cocaine-exposed cortical PSDs (n=6) when compared to saline-treated cortical PSDs (n=6). There was no difference in prohibitin between cocaine-exposed and saline-treated striatal PSDs.

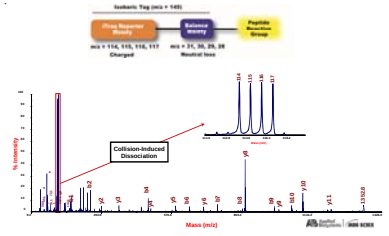
METHODS

Chronic Cocaine Exposure: Male C57 mice (n=6, each group) were treated once daily with an intraperitoneal (i.p) injection of saline or 30mg/kg cocaine for 22 days. Animals were sacrificed 24 hours after the final cocaine injection and cortical, hippocampal, and striatal regions were isolated. Locomotor activity was recorded following cocaine administration for the first ten days where sensitization was observed at this dose (data not shown).

PSD preparation: Postsynaptic density (PSD) from brain regions of interest were isolated as described previously with procedural modifications (Carlin *et al*. 1980). Briefly, tissue was homogenized using a Dounce tissue grinder in 0.32M sucrose, 20mM HEPES, pH 7.4 with protease and phosphatase inhibitors. Nuclear and unhomogenized cell contaminants were removed by low-speed centrifugation, followed by a high-speed centrifugation to obtain pellet containing synaptoneurosomes. This was applied to a Percoll gradient (3%, 10%, 23%) and ultracentrifuged. The interface between 10% and 23% was collected and subjected to hypotonic lysis (20mM HEPES, pH 7.4, 1.0mM DTT). Subsequently, the synaptic plasma membrane fraction was collected by ultracentrifugation. Following a detergent treatment (0.32M sucrose, 20mM HEPES, pH 7.4, 0.5% Triton), the PSD fraction was collected by ultracentrifugation and stored at -80°C.

Western blotting analysis: PSD and unfractionated samples from animals treated with either 30 mg/kg cocaine (n=6) and saline (n=6) were isolated from the cortex, striatum, and hippocampus. Samples were run on 8-16% gels and transferred onto nitrocellulose membrane. Antibodies against Na+/K+ - ATPase alpha 3 subunit and Prohibitin were used and blots analyzed and quantified using Odyssey Infrared Imaging System (LiCor, NE, USA). Samples were normalized using bands from post-transfer gels stained with SimpleBlue Safe Stain (Invitrogen, CA, USA).





iTraq analysis: iTraq technology uses a chemical tagging reagent to which allows multiplexing of four samples and produces identical MS/MS sequencing ions for all four versions of the same derivatized tryptic peptide. Quantitation is achieved by comparison of the peak areas and resultant peak ratios for the four MS/MS reporter ions, which range from 114 to 117 Da. For our experiments, 50 ug of control and treated samples from cortical, hippocampal and striatal PSDs were digested with trypsin and then labeled. After labeling the samples were combined and separated into 20 fractions via cation-exchange chromatography. Each fraction was dried and resuspended in 10 µl 0.1% formic acid in preparation for RP-LC with the LC Packing's Ultimate workstation allowing us to pre-concentrate the 10 µl samples on a Waters 5 mm C18 Symmetry 300 trap column. The individual peptides were separated at a flow rate of 450 nl/min on an in-line 100 µm x 15 cm Waters Atlantis C18 column equilibrated with 0.5% acetic acid, 5% acetonitrile and eluted with a 60 min acetonitrile gradient. Electrospray Ionization of the eluent was followed by data-dependent acquisition on a Applied Biosystems API Q-Star XL mass spectrometer. Data analysis was performed using MASCOT database search engine to determine protein identifications and ProQuant software to determine iTRAQ ratios. Data results were analyzed with the Trans Proteomic Pipeline developed at the Seattle NHLBI Proteomics Center.

CONCLUSIONS

- The PSDs obtained from the cortex, striatum, and hippocampus are composed of a wide variety of molecular species. Functionally, the most represented class of proteins are metabolic, followed by proteins involved in trafficking, as well as cytoskeletal proteins.
- There is an overrepresentation of metabolic proteins that change with chronic exposure to 30 mg/kg cocaine in cortical, hippocampal, and striatal PSDs.
- Increases in cytoskeletal proteins in the PSDs from cortical, hippocampal, and striatal neurons are consistent with structural alterations seen after chronic exposure to cocaine and may be associated with cocaine-induced neuroplasticity.

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