Characterization of postsynaptic density protein enrichment using targeted quantitative mass spectrometry methods

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Abstract

The postsynaptic density (PSD) is a specialized protein complex at the synaptic junction of glutamatergic excitatory synapses. The protein components of the PSD, including neurotransmitter receptors, cytoskeletal proteins, and signaling molecules, can be altered by synaptic activity and drug exposure. Therefore, methodologies to quantify the changes in the abundance of PSD proteins should help our understanding of the molecular basis of synaptic plasticity. In this study, 112 proteins in PSD fractions prepared from rat brain were initially selected for analysis using multiple reaction monitoring (MRM) mass spectrometry based on the number of peptides detected, peak distribution and signal/noise ratio. However considerable variation in the levels of a sub-set of proteins was observed that was dependent on sample preparation. To produce more consistent data, we applied fraction-enrichment analysis and analyzed the levels of a larger number of proteins than initially targeted by the MRM approach. Crude synaptoneurosome (P2) and PSD fractions were prepared systematically and analyzed by SWATH LC-MS/MS, a novel data-independent acquisition technique. We examined the levels of ~1,700 proteins by SWATH that were differentially enriched in PSD compared to the P2 fraction. Bioinformatic analysis revealed classes of proteins that were enriched or excluded from the PSD fraction compared to the P2 fraction, and identified factors that contributed to higher levels of technical and biological variance for identified PSD proteins. The results from these studies will be helpful in defining proteins that exhibit robust association with the PSD, and that can be reproducibly analyzed by targeted mass spectrometric methods.

Overview of our studies

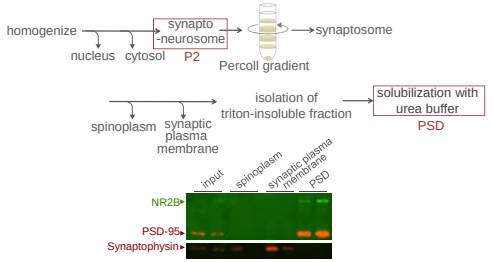
Development of MRM method for PSD proteins

Identification of PSD core proteins selection of 'appropriate' MRM target

Characterization of the differences in the PSD proteome following cocaine treatment

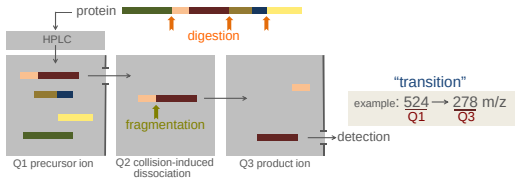
We originally started this project to characterize the differences in the PSD proteome following cocaine treatment. For this purpose, we designed a comprehensive workflow for the development of large scale (>1000 transitions/run) label-free LC-MRM proteome assay. However, the MRM data raised some issues related to sample variability in PSD proteins. To address these issues, we have examined in more detail the basis for altered PSD protein levels that might be caused by preparation conditions.

PSD preparation



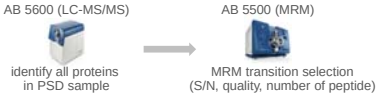
Rat brain tissue was homogenized using a Dounce tissue grinder. Nuclear and unhomogenized cell contaminants were removed by low-speed centrifugation, followed by a high-speed centrifugation to obtain the pellet containing synaptoneurosomes (P2). This was applied to a Percoll gradient and ultracentrifuged. The synaptosome fraction was collected and subjected to hypotonic lysis. Subsequently, the synaptic plasma membrane fraction was collected by ultracentrifugation. Following a Triton extraction, the PSD fraction was collected by ultracentrifugation.

MRM - multiple reaction monitoring



MRM is highly specific assay method for detecting analytes of interest utilizing, most predominantly, a triple quadrupole-based mass spectrometer. Q1 is set to transmit only the parent m/z of the peptide, the fragmentation via collisional induced dissociation occurs in Q2, and Q3 is set to transmit this a single diagnostic fragment. The specific combination of m/z associated with the parent and fragment ions selected is referred to as a "transition".

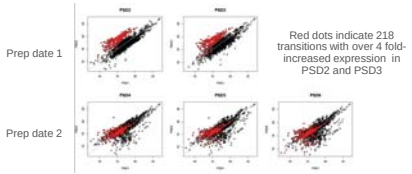
Discovery proteomics to MRM transition selection



	requirement	our study
protein	right target for each purpose	proteins associated with PSD
peptide	observability - high ionization efficiency - unique sequence	>3 peptides/protein >10 S/N >0.75 quality
transition	observability increased throughput -balance the number of transitions with retention time	3 peptides/protein 5 transitions/peptide

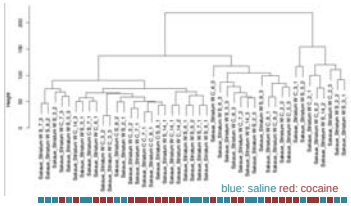
Approximately 1,200 proteins were initially identified in the PSD fraction by discovery runs on a TripleTOF 5600 MS. From the protein list, 112 proteins were selected as target analytes (M112) based on the number of peptides detected, peak distribution and signal/noise ratio. The data were translated into a QTRAP 5500 LC-MRM assay enabling rapid quantitation of 112 proteins. 3 peptides per protein were quantified by measuring 5 transitions for each peptide.

Mitochondria related proteins are susceptible to the preparation conditions



MRM log 2 scatter plots between PSD 1 and five other PSD biological replicates. The red dots indicate transitions that are four fold up-regulated in PSD 2 vs. PSD 1. These same transitions were then mapped onto the other four scatter plots.

Effect of preparation date and cage compared to cocaine treatment on the M112 proteins



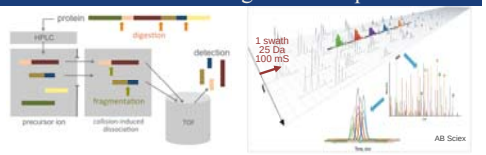
Peak area clustering analysis for 112 PSD proteins from control (blue square) or cocaine (red square) treated rat. The control and cocaine treated groups could not be distinguished. Samples prepared at same day or from same cage tend to form clusters.

Evaluation of variability from preparation conditions for the reselection of MRM targets using SWATH

cage	rat ID	tissue	frozen or unfrozen	prep-date	fractions
A	1	half cortex	unfrozen	day 0	P2 and PSD
		half cortex	frozen	day 0	P2 and PSD
	2	half cortex	unfrozen	day 0	P2 and PSD
		half cortex	frozen	day 0	P2 and PSD
B	3	half cortex	frozen	day 1	P2 and PSD
		half cortex	frozen	day 2	P2 and PSD
	4	half cortex	frozen	day 1	P2 and PSD
		half cortex	frozen	day 2	P2 and PSD
C	5	half cortex	frozen	day 1	P2 and PSD
		half cortex	frozen	day 2	P2 and PSD
	6	half cortex	frozen	day 1	P2 and PSD
		half cortex	frozen	day 2	P2 and PSD

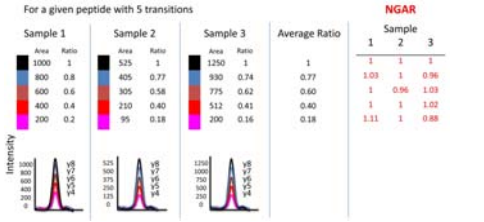
The PSD fraction might be susceptible to variability in preparation condition. To analyze the variability among sample preparations, both the P2 and PSD fractions were prepared in different conditions and compared for ALL proteins by SWATH analysis.

SWATH - sequential windowed acquisition of all theoretical fragment-ion spectra



In the MRM workflow, a fixed number of analytes are targeted and high resolution MS/MS spectra are collected across an LC run. On the other hand, in SWATH, a wider Q1 window containing more analytes is passed. This produces a more complex MS/MS spectrum which is a composite of all the analytes within that Q1 m/z window. The Q1 quadrupole is stepped at 25 amu increments across the mass range of interest, passing a 25 amu window through into the collision cell. The transmitted ions are fragmented and the resulting fragments are analyzed in the TOF MS Analyzer at high resolution.

Normalized Group Area Ratio (NGAR)

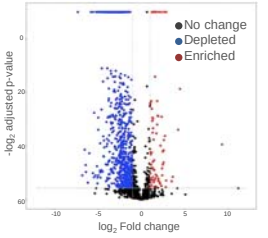


The NGAR divides by the average of this ratio for all samples (for a given transition). The net result was that the reported value should be close to 1.0 if the ratio of a transition to the first is constant across the samples. If not, one (or other) of the peaks was not integrated well or had some other interference.

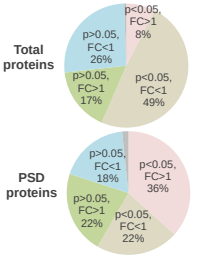
Enrichment analysis

Biostatistics method
-all transitions
-without normalization
-with NGAR to all (P2 and PSD)

PSD/P2 enrichment analysis were performed using MSstats without normalization and with NGAR. Right dot plot: Volcano plots by protein level analysis. $-\log_2$ scaled adjusted p-value (y-axis) against $\log_2$ fold change (x axis). According to the adjusted p-values and fold change, they are labeled in black (no change), blue (depleted) or red (enriched).



	Total proteins	PSD proteins
p<0.05, FC>1	127	22
p<0.05, FC<1	823	13
p>0.05, FC>1	281	13
p>0.05, FC<1	442	11
not qualified	9	1
total	1682	60

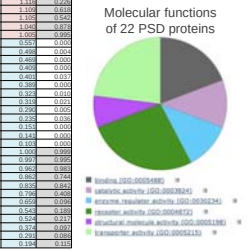


Upper table and right pie charts: Proteins were sorted depending on fold-change value and adjusted p-value. 60 PSD proteins were identified based on gene ontology.

gene name	protein name	FC	adj.pvalue
Synpaip1	Ras/Rap GTPase-activating protein SynGAP	21.760	0.000
Grin2b	Glutamate receptor ionotropic, NMDA 2B	4.840	0.000
Gria3	Isoform Flip of Glutamate receptor 3	4.010	0.000
Dlg3	Disks large homolog 3	3.310	0.000
Shank1	Isoform 3 of SH3 and multiple ankyrin repeat domains protein 1	3.030	0.000
Lrrc7	Leucine-rich repeat-containing protein 7	3.020	0.000
Pclo	Isoform 3 of Protein piccolo	2.610	0.000
Dlg4	Disks large homolog 4	2.500	0.000
Bsn	Protein bassoon	2.500	0.000
Grik2	Glutamate receptor ionotropic, kainate 2 (Fragment)	2.460	0.000
Shank3	SH3 and multiple ankyrin repeat domains protein 3	2.370	0.000
Srcin1	Protein Snip	2.340	0.000
Pkp4	Protein Pkp4	2.300	0.000
Shank2	Isoform 2 of SH3 and multiple ankyrin repeat domains protein 2	2.220	0.000
Grin2a	Glutamate receptor ionotropic, NMDA 2A	2.211	0.000
Gria2	Isoform Flip of Glutamate receptor 2	2.090	0.000
Erc2	ERC protein 2	1.970	0.000
Ctnnd2	Uncharacterized protein	1.880	0.000
Dlgap1	Disks large-associated protein 1	1.870	0.000
Gria1	Isoform Flip of Glutamate receptor 1	1.580	0.000
Dlg1	Disks large homolog 1	1.480	0.000
Homer1	Homer protein homolog 1	1.400	0.033
Grin1	Glutamate receptor ionotropic, NMDA 1	33.110	0.292
Chrm1	Muscarinic acetylcholine receptor M1	5.220	0.000
Cacng8	Voltage-dependent calcium channel gamma-8 subunit (Fragment)	1.391	0.151
Grik4	Glutamate receptor 4	1.290	0.103
Srgap2	SLIT-ROBO Rho GTPase-activating protein 2	1.260	0.835
Dbnl	Drebrin-like protein	1.240	0.529
Lztfl1	Leucine zipper putative tumor suppressor 1	1.140	0.942
Map1b	Microtubule-associated protein 1B	1.110	0.228
Fyn	Tyrosine-protein kinase Fyn	1.100	0.618
Sorbs2	Sorbin and SH3 domain-containing protein 2	1.100	0.542
Exoc4	Exocyst complex component 4	1.040	0.878
Grm5	Metabotropic glutamate receptor 5	1.000	0.995
Add1	Alpha-adducin	0.957	0.000
Lphn1	Latrophilin-1	0.498	0.004
Gap43	Neuromodulin	0.466	0.000
Akap5	A-kinase anchor protein 5	0.404	0.000
Syn1	Synapsin-1	0.403	0.037
Dnm3	Dynamin-3	0.380	0.000
Picalm	Phosphatidylinositol-binding clathrin assembly protein	0.323	0.014
Ptk2b	Protein-tyrosine kinase 2-beta	0.319	0.021
Ppp2ca	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	0.294	0.000
Rab8a	Ras-related protein Rab-8A	0.235	0.038
Nsf	Vesicle-fusing ATPase (Fragment)	0.151	0.000
Stin1	SCG10/I84 isoform CRA_a	0.143	0.000
Snap91	Isoform 2 of Clathrin coat assembly protein AP180	0.100	0.000
Sipa1l1	Signal-induced proliferation-associated 1 like protein 1	1.000	0.999
Src	Neuronal C-SRC tyrosine-specific protein kinase	0.997	0.999
Cnih2	Protein cornichon homolog 2	0.962	0.983
Ywhaz	14-3-3 protein zeta/delta	0.962	0.744
Epha4	Protein Epha4	0.835	0.842
Grm3	Metabotropic glutamate receptor 3	0.796	0.468
Bcas1	Breast carcinoma-amplified sequence 1 homolog	0.655	0.098
Park2	E3 ubiquitin-protein ligase parkin	0.543	0.188
Grik5	Glutamate receptor ionotropic, kainate 5	0.524	0.217
Cdk5	Cyclin-dependent kinase 5	0.374	0.087
Ntrk2	BDNF/NT-3 growth factors receptor	0.243	0.000
Pick1	PRKCA-binding protein	0.194	0.319

Left table: fold change value (pink: enriched, light blue: depleted) and p-value (grey: >0.05) of 59 PSD proteins. 22 PSD core proteins (FC≥1, p<0.05) are indicated by boldface.

Lower Pie chart: 22 PSD core proteins were classified by molecular function using Panther.



Acknowledgements

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