Proteomic analysis of the effect of cocaine exposure on the proteins associated with the post synaptic density

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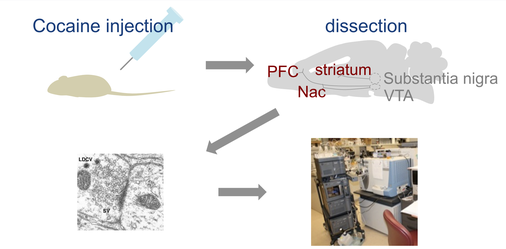
Introduction

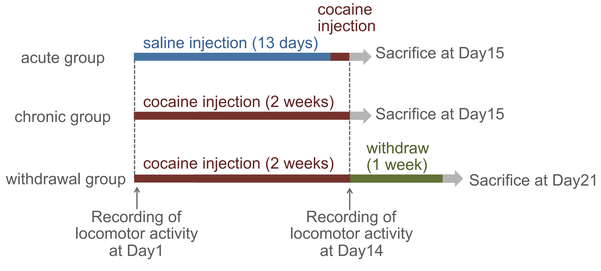
Exposure to cocaine is known to alter the number and structure of dendritic spines, the sites of excitatory neurotransmission. Several recent studies using protein profiling methods indicate that cocaine exposure alters the expression levels and post-translational changes of proteins associated with the postsynaptic density (PSD). We have hypothesized that the PSD which is a dynamic multi-protein complex that links neurotransmission with intracellular signaling molecules, might be critical for persistent cocaine-induced synaptic alterations. In our studies, we therefore characterized differences in the PSD proteome following three different conditions of cocaine treatment, acute, chronic and withdraw, using a large-scale LC-MS/MS with multiple reaction monitoring (MRM) assay.

Strategy



To investigate changes of PSD proteome, cocaine was injected to rats on three different conditions, acute, chronic and withdraw. Rat prefrontal cortex, striatum and nucleus accumbens were then isolated. PSD fractions were prepared using Percoll gradient. The samples were analyzed by MRM which is a targeted quantitative mass spectrometry method.

Cocaine sensitization



Male Sprague-Dawley rats (n=12 animals each group) were treated once daily with an i.p injection of saline or 15 mg/kg cocaine either acutely or for 14 days. Animals were sacrificed 24 hr after the final cocaine injection for the acutely and chronically treated animals. Another group was exposed for 14 days, but was sacrificed one week after the final cocaine injection. On the first day and last day of cocaine treatment, locomotor activity was measured.

Acknowledgements

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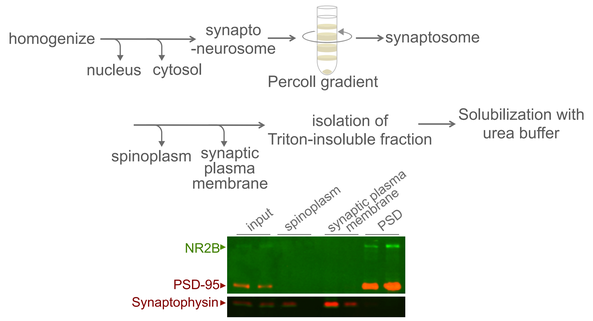
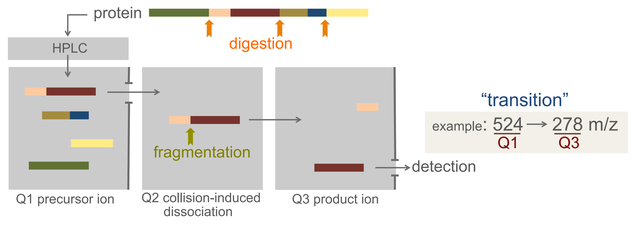
PSD preparation

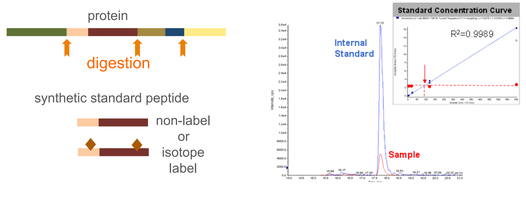


Tissue from the dorsal striatum, prefrontal cortex, Nacc was homogenized. Nuclear and unhomogenized cell contaminants were removed by low-speed centrifugation, followed by a high-speed centrifugation to obtain the pellet containing synaptoneurosomes. This was applied to a Percoll gradient and ultracentrifuged. 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 diagnostic fragment only. The specific combination of m/z associated with the parent and fragment ion selected are referred to as a "transition".



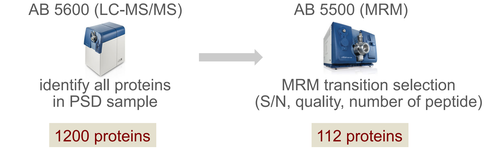
In absolute quantitation experiments, the analyte of interest is used to generate a calibration curve to characterize the MS response of each analyte. This can be done with the analyte alone or with an additional isotope labeled analyte that has identical MS response. A calibration curve is generated and the amount of analyte present in each sample is determined by comparing to the curve.

Workflow for development of MRM assay

- assay development
  - -protein identification
  - -parent fragment peak picking
  - -calibrate RT between AB 5600 and 5500 instruments
  - -schedule transition
- run established assays
  - -LC-sMRM assay to reorder primary transition
  - -Run replicate LC-xMRM assay
- quantitative data analysis
  - -peak picking and integration
  - -normalization
  - -cluster dendrograms
  - -quality assessment plot
  - -correlation plots
  - -fold change analysis

The TripleTOF 5600 MS was used to "sequence" peptides from a tryptic digest. Yale Protein Expression Database (YPED) was then used to translate the "learned" peptide sequences into a triggered LC-MRM (xMRM) assay that were run in triplicate on a QTRAP 5500 MS. The resulting LC-xMRM data was processed with MultiQuant software utilizing a newly developed SignalFinder Research algorithm. A novel xMRM assay (research version of Analyst software) is critical to maximize cycle and dwell times since only the most intense group transition is designated as the primary transition and monitored throughout its entire scheduled window. Secondary MRMs for each peptide are only monitored when the primary MRM exceeds a predetermined threshold, enabling such large scale MRM assays. For data analysis, metrics of quality assessment, method of data normalization, and peptide/protein fold change calculation for this study were developed to identify interesting targets whose expression changes significantly by cocaine exposure.

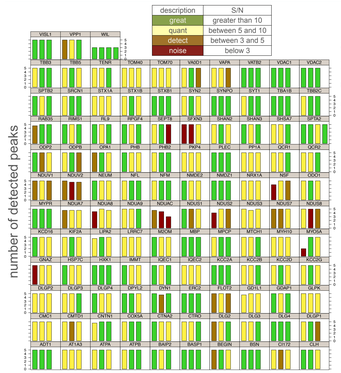
Discovery proteomics to MRM transition selection



	requirement	our study
protein	depend on aim	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

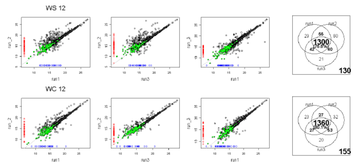
1,200 proteins were identified in the PSD fraction by discovery runs on a TripleTOF 5600 MS. From the protein list, 112 proteins were selected as a target based on the number of peptides detected, peak distribution and signal/noise ratio. The data were translated into a QTRAP 5500 LC-MRM assay enabling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

Development of metrics for quality control

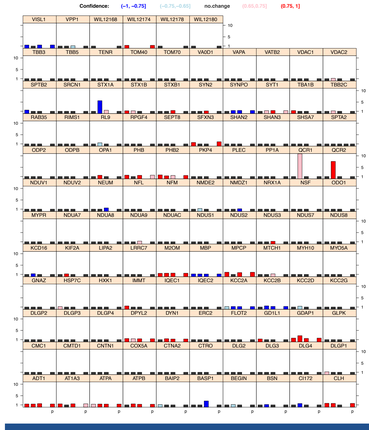


Metric Bar Graph of PSD xMRM assay quality. The plot is broken into 113 individual bar graphs for each protein and internal standard (WIL) in the PSD assay and each bar graph is further sub divided into individual bars which represents the data quality of single peptide with 5 transitions. The height of each bar corresponds to the number of MS/MS transitions observed for the corresponding peptide with all proteins potentially having 5 transitions as compared to the maximum of 3 transitions that were monitored for each of the internal standard peptides. Finally, the color of each bar depicts the average signal/noise ratio for the underlying, usually, 5 transitions as described in the table.

MRM analysis following cocaine exposure and withdrawal



MRM log2 scatter plots shows the reproducibility of MRM transitions across 3 technical replicates for rat striatal PSD after withdrawal. The blue and red indicate the peak detected only in the X-axis run and Y-axis run respectively. Green dots represent peak detected both runs but their S/N < 5. Venn diagram shows the number of transitions with S/N > 5 in each technical replicates. The bar charts show fold change of 112 proteins between saline and cocaine. 3 bars on the left and one bar on the right indicate fold change of each peptide and the average of 3 peptide respectively. The color of each bar depicts the fold change value as headings along the top of the chart. Only one example are shown here, fold change calculation across all animals needs the normalization to remove variability of data distribution from sample preparation.



Conclusions

- Optimized MRM AB5500 software
  - setting of time window - linking of 5 transition
- Listed 112 target PSD proteins for MRM assay
- Developed new data metrics for quality control
  - -automated R plots for S/N
- Developed data analysis method
  - -automated R plots for normalization and cluster analysis
- MRM technical replicate showed good reproducibility