A Quantitative Screen of Post-Synaptic Density Proteins in Human Brain Tissue Fractions by nanoLC-SRM

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Overview

Purpose: To monitor a panel of post-synaptic density specific proteins in order to verify purity of biochemical fractionation using a triple quadrupole mass spectrometer.

Methods: A panel of 325 peptide targets were monitored in the endogenous and [$^{13}C^{15}N$]-labeled forms. This represented a total of 2575 individual SRM transitions monitoring the presence of 181 proteins. Relative quantification was possible using labeled peptides derived from a SILAC labeled proteome, while several proteins were absolutely quantified using synthetically generated forms.

Results: We demonstrated the ability to conduct absolute and relative peptide quantification on hundreds of peptide targets within a single LC-SRM experiment. The assay confirmed the purity of the post-synaptic density isolated from human brain tissue.

Introduction

The Postsynaptic density (PSD) is a thickening of the dendritic membrane rich in glutamate receptors and signaling molecules at which presynaptic signals are integrated to govern important neuronal functions from neuroplasticity to learning and memory. As these processes are implicated in a number of neuropsychiatric diseases it will be important to develop methodologies capable of assaying the nonlinear activities present at this microdomain. To that end, we have developed a LC-SRM method capable of multiplex PSD protein quantification that will likely serve as a platform to assess PSD protein composition, post translational modification and interactions.

Methods

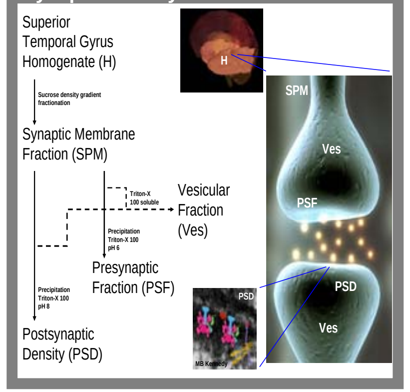
Sample: 600 mg of postmortem Human superior temporal gyrus tissue was subject to a recently validated biochemical fractionation method (Hahn 2009), yielding synaptic membrane (SPM), vesicular (Ves), pre-synaptic membrane (PSF), and Post-synaptic density (PSD) fractions (Fig. 1). Each fraction was mixed 1:2 with SILAP proteins. Stable Isotope labeled synthetic peptides were added at between 200 and 1500 fmol to all samples. Following enzymatic digestion, de-salting, and lyopholization, samples were redissolved to 0.333 µg/ µL endogenous protein.

SRM method creation: 2DLC-MS/MS was performed on the PSD fraction using the LTQ mass spectrometer. MS2 spectra of 325 peptides for 181 target proteins from this discovery experiment were imported into Pinpoint software. Heavy isotope labeled versions of all peptides were included in the analysis, and SRM transitions automatically assigned to the top 4 most intense y-ions based on the reference library (LTQ MS2 spectra).

Assay: A TSQ-Vantage was employed for LC-SRM analysis of all fractions and whole tissue homogenate. Relative quantification of PSD proteins with SRM transitions for 181 selected PSD proteins was relied on a SILAC labeled proteom developed from a human cell line, while absolute quantification of key proteins utilized synthetic peptide standards.

Nano-LC: All LC was accomplished using an Eksigent NanoLC-Ultra™ 2D equipped with an AS-2 autosampler. A Michrom packed HALO™ C18 column (0.075 X 150 mm, 3 µm) was used at a flow rate of 500 nL/ min. Buffer [A] was 100% H20 with 0.1% FA and [B] was 100% ACN with 0.1% FA.. Gradient conditions were 98:2 [A]:[B] at 0 min to 60:40 [A]:[B} at 80 min.

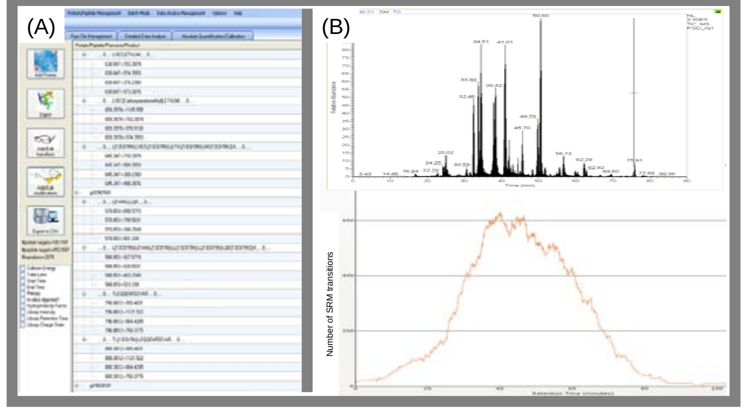
FIGURE 1. Isolation of the post-synaptic density from human brain



Samples were loaded directly on column at initial gradient conditions and the column was washed for 6 µL.

MS: Nano-ESI spray was achieved using a Michrom ADVANCE source equipped with a 20 µm tip supplied with 950 V. The capillary temperature was 275 °C. Both Q1 and Q3 resolution were set to 0.7 Da (FWHM). All 2575 transitions were timed (± 6 min) allowing for a 2 sec cycle time (dynamic dwell time). At maximum, 610 transitions were monitored in a single cycle, meaning the dwell time ~ 3 msec (Fig. 2). Collision energies were automatically assigned to transitions using Pinpoint and the collision gas (argon) in Q2 was 1.5 (mtorr).

FIGURE 2. Timed SRM method to monitor 2575 transitions over a 90 min LC elution gradient. Pinpoint software was used to generate the method (A). Transitions were timed to optimize dwell time (B).



Results

Analytical reproducibility for most peptides was < 25% CV. These % CVs held across all elution times, even when the dwell time was less than 3 msec, as exemplified here by the medium intensity peptide LLLLGAGESGK (e^4 signal intensity, R_t = 40.82 min) and by the low intensity peptide LSQMGVTTDGVPAQQLR (e^2 signal intensity, R_t = 39.38 min) (Fig. 3).

FIGURE 3. Reproducibility of peptides quantified at time region of minimum dwell time. Bar graphed intensities represent peak area processed in Pinpoint.

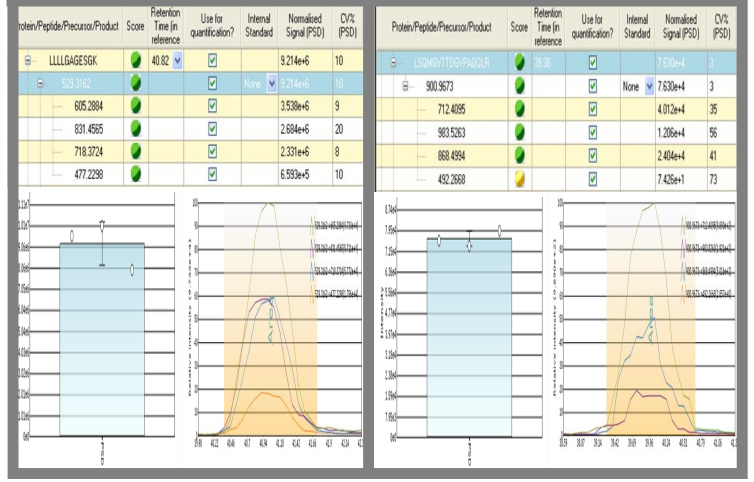
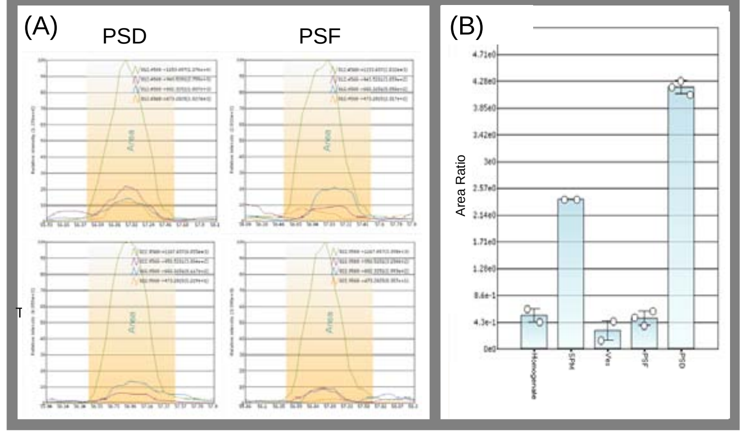
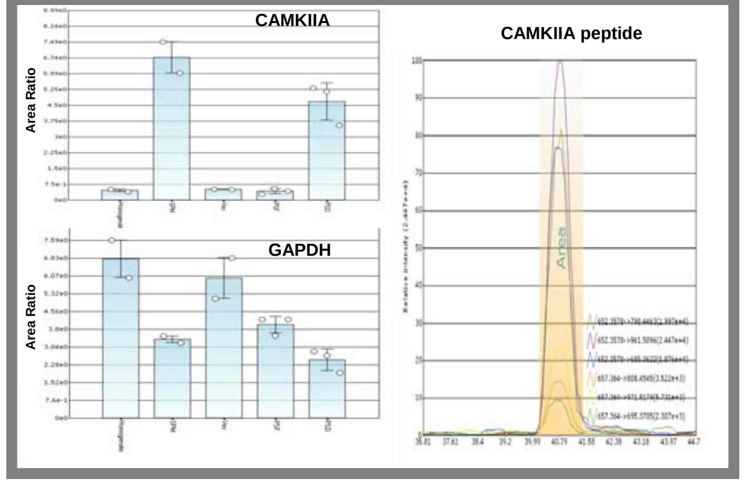


FIGURE 4. Relative Quantification using SILAC labeled proteom. Example shows quantification of Ubiquitin-activating enzyme. An Example Peptide in the PSD and PSF fraction is shown in (A), while the averaged area ratio of all three Ubiquitin-activating enzyme peptides monitored across all biological samples are shown in (B).



Relative quantification was possible using SILAC derived peptides. The example shown is for Ubiquitin-activating enzyme (Fig. 4). Results show the protein is enriched in the synaptic membrane (SPM), and Post-synaptic density (PSD) fractions. Triplicate injections showed the protein enriched 10-fold in the PSD, as compared to the PSF (at 2 and 18 %CV respectively).

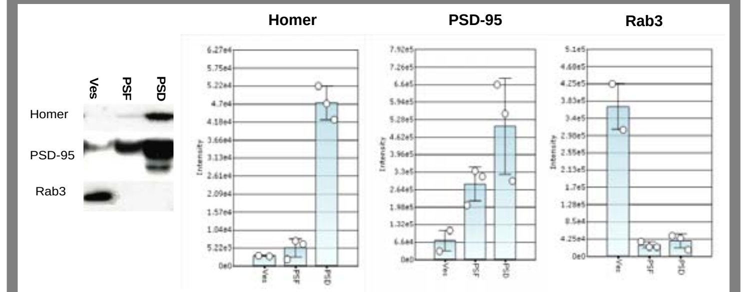
FIGURE 5. Absolute quantification of Ca2+/calmodulin-dependent protein kinase IIA (CAMKIIA) relative to GAPDH



In addition to relative quantification, several proteins were targeted for absolute quantification using stable isotope labeled synthetic peptides. The example shown is for CAMKIIA, which was calculated at 31 fmol/ µg PSF protein and 345 fmol/ µg PSD protein (20 and 17 %CV respectively). Since many of the signaling proteins at the PSD are known to have altered expression and PTM, house keeping proteins such as GAPDH were included in the analysis. These "control" proteins will possibly serve to normalize future biological experiments. Compared to the amount of CAMKIIA enriched in the PSD, these house keeping proteins, as exemplified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed little preference for localization (Fig. 5).

Lastly, the SRM analysis was able to confirm immunochemical detection of several proteins previously analyzed in the Hahn lab (Hahn, 2009). Similar to the western blot, peptide intensities for proteins Homer and PSD-95 were shown to be enriched in the PSD compared to the vesicular fraction. Conversely, Rab3 was found more than a 1000-fold more enriched in the vesicular fraction (Fig 6.).

FIGURE 6. Confirmation of Western blot by LC-SRM demonstrating the purity of PSD isolation



Conclusions

A quantitative nano-LC/SRM screen of 181 proteins, corresponding to 652 peptides (2575 SRM transitions), was carried out using the TSQ-Vantage triple quad. The method was created and data was processed using Pinpoint software. Results showed the ability to reproducibly quantify peptides, even at dwell times below 3 msec. Protein amounts were in agreement with anticipated results, while several were confirmed using western blotting. Future experiments will further refine this screen and apply it to the study of disease states were PSD function is implicated. One exciting possibility is the use of this assay in the systematic study of PSD protein interactions in the normal and schizophrenic brains.

References

Hahn CG, *et al. PLoS ONE. 2009;4(4):e5251. Epub 2009 Apr 16.*