Delving Deeper into the Neuroproteome using Quantitative and Non-Quantitative Protein Profiling

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OUTLINE: Delving deeper

- Protein Profiling at the Protein Level
  - utilizing DIGE with MALDI-Tof/Tof analysis

- Protein Profiling at the Peptide Level
  - utilizing Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) with LTQ-Orbitrap analysis

- Proteome and phosphoproteome
  - cataloging of the choroid plexus

- Summary
DIGE Protein Profiling and Protein Identification Workflow

Protein spots differentially regulated are robotically picked and digested with trypsin.

Protein identification is based on a Mascot search using a combined peptide mass fingerprint and MS/MS database search (AB GPS Explorer).

AB 4800 MALDI-Tof/Tof ~ 82% ID rate

NIDA Neuroproteomics DIGE Projects:
25% usage of the total DIGE analysis for the year

- Dr. Thomas Biederer
- Dr. Rajita Sinha: cocaine, alcohol
- Dr. Angus Nairn
- Dr. Zoran Zimolo: THC, smoking, Dex
- Dr. Pietro De Camilli: dynamin
- Dr. Sreeganga Chandra; substrates of the CSPα/Hsc70/SGT complex
The Challenge: Getting to the Lower Abundant Proteins in Serum/Plasma

The Answer: Immunoaffinity Partitioning

- 5% of our NIDA investigators work with Plasma
- IgY-14 LC column depletion of the highly abundant 14 proteins
- Supermix LC column depletion of the 77 moderately abundant proteins

Typical Range Abundances Courtesy of Plasma Proteome Institute
THC is the main cannabinoid responsible for the psychoactive effects of cannabis and related drugs derived from cannabis.

Plasma was taken 60 minutes prior to an infusion of 2.5mg THC (4 patients) or Placebo (same 4 patients- 2 weeks apart)

And taken again at 10 and 140 minutes after administration of THC

Pools of 4 patients at each time point were subjected to IgY14 and Supermix depletion

BVA analysis with triplicate gels

DeCyder determined down regulation of the indicated protein spots in red.

Tryptic digestion and MALDI-Tof/Tof analysis (4800) for protein ID

**Carbonic Anhydrase I** = \( \frac{Cy5}{Cy3} = -1.5 \)

**Carbonic Anhydrase II** = \( \frac{Cy5}{Cy3} = -1.8 \)

Hashish is known to reduce the activity of Carbonic anhydrase I and II

Stable isotope labeling with amino acids in cell culture (SILAC) Sample processing using MudPIT Analysis and the Mascot Quantitation tool box

SILAC labeling

Arginine \([U-^{13}C_6, ^{15}N_4]\)
Lys \([U-^{13}C_6]\)

Complex mixture

Tryptic digest

20 SCX fractions collected (on or off line with the MS)

Peptide Quantitation - Mascot Quantitation Tool Box

Each cation fraction is analyzed by LC MS/MS analysis on the LTQ Orbitrap

Mascot database searches for protein identification

YPED
Testing of Mascot Quantitation software on SILAC Labeled Human Endothelial Cells

- Endothelial cells grown in 'Light' and 'Heavy' (Lys-6) medium and cellular proteins extracted
- Mixing of L & H protein samples in 2:1, 4:1, 2:3 and 1:3 - 1D SDS-PAGE (6ug total)
  - tryptic digest followed by LC-MS/MS on an LTQ-Orbitrap
  - Protein ID and quantification - MASCOT distiller software

Heat shock protein (HSP 90-beta) Identified

B. Derakhshan; B. Sessa; T. Lam; K. Stone; M. LoPresti
SILAC Protein Profiling

- Utilized by Drs. Rick Lifton and Jesse Rinehart for quantitative phosphopeptide Analysis (Erol Gulcicek will describe this afternoon)
- Dr. Pietro De Camilli for determining which protein expression levels are altered by depleting dynamin fibroblasts

Dr. Pietro De Camilli’s Project

- Dynamin is responsible for endocytosis
- Dynamin has 3 isoforms in mammals
  - dynamin 1 enriched in the brain
  - dynamin 2 ubiquitously expressed
  - dynamin 3 enriched in the brain and testis.
- Dynamin 1 and 2 are expressed in fibroblast cells.
- The Cre-LoxP methodology was used on primary embryonic fibroblast cell cultures from dynamin1/dynamin 2 versus the knockout dynamin 2 (which is embryonic lethal) using adenovirus.

Dynamin assembles into spirals
Dynamin is a right handed helix with right handed twisting ability
## SILAC Results on the Dyn1 KO (light) versus Dyn 1,2 dynamin knock out (heavy, Lys only)

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<thead>
<tr>
<th>Accession</th>
<th>Score</th>
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<th>#</th>
<th>Description</th>
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</table>

Myosin light polypeptide 6 has been confirmed by a Western blot to be up-regulated in the dynamin knock out.
The choroid plexus (CP) is an important blood-cerebrospinal fluid barrier that is chiefly recognized for its role in cerebrospinal fluid (CSF) production.

- The CP acts as a filtration system, removing metabolic waste, foreign substances, and excess neurotransmitters from the CSF.
- Helps to maintain the delicate extracellular environment required by the brain to function optimally.

- The CP is understudied and poorly understood.
- The “kidney of the brain”
- Goal: to determine (at least in part) CP function using the Proteomics data.
Approaches used to catalogue the Rat Choroid Plexus

Pre-Fractionation

Proteins:
- FPLC (RPC3 column)
- C18 reverse phase
- 2D LC - 1st dimension chromatofocusing followed by non-porous reverse phase
- 1D SDS PAGE

Peptides:
- strong cation exchange/MudPIT
- Titanium dioxide phospho peptide enrichment

Mass Spectrometric Analysis
- UPLC and LTQ-Orbitrap
- Dionex capLC and QSTAR XL
- UPLC and QSTAR Elite
Preliminary results on proteins identified in the Choroid Plexus

**Mascot Database Search Information**

1) IPI Rat database
2) ≥2 peptides matched
3) significant Mascot score (32)
4) >95% probability
5) Search parameters varied based on the LC-MS/MS system
6) 1449 proteins ID’d
7) comparing the protein and microarray results

**Molecular Function—PANTHER**

- Cytoskeletal protein
- Nucleic Acid Binding
- Oxidoreductase
- Molecular function Unclassified

1449 proteins identified by using Mascot Distiller and the Mascot search algorithm
<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Peptide Score</th>
<th>Peptide Sequence</th>
<th>Protein Description</th>
<th>Peptide Score</th>
<th>Peptide Sequence</th>
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<tr>
<td>Prkra Interferon-inducible double stranded RNA-dependent protein kinase ac</td>
<td>33</td>
<td>HRAEAPLQREDGTFSLG</td>
<td>RGD1304816_predicted similar to Nucleoprotein TPR</td>
<td>37</td>
<td>HSQDSOHCSVSQDE EDELFK</td>
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<tr>
<td>Nucks Nuclear ubiquitou casein and cyclin-dependent kinases substrate</td>
<td>25</td>
<td>KTSASAPPLEK</td>
<td>LOC689421 similar to Beta-2-synthrophin (59 kDa dystrophin-associated prote</td>
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<td>GLQPPSSPPAPP</td>
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<tr>
<td>Gsk3a Glycogen synthase kinase-3 alpha</td>
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<td>GEPNVSYICSR</td>
<td>Spag1 Sperm-associated antigen 1</td>
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<td>Ace Angiotensin-converting enzyme testis-specific isoform precursor</td>
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<td>NoID8_predicted nuclear protein 8</td>
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<tr>
<td>Dck1c HiACA ribonucleoprotein complex subunit 4</td>
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<td>Bcl213_predicted BCL2-like 11</td>
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<tr>
<td>Ppp1r1a Protein phosphatase 1 regulatory subunit 1A</td>
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<td>STLSMSPR</td>
<td>Ncpl1 Nuclear cap-binding protein subunit 1</td>
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<tr>
<td>G3bp similar to Ras-GTPase-activating protein binding protein 1</td>
<td>25</td>
<td>RPPSPEPSAK</td>
<td>Spnb2 Non-erythroid spectrin beta</td>
<td>36</td>
<td>RPPSPEPSAK</td>
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<tr>
<td>Ppig Peptidyl-prolyl cis-trans isomerase G</td>
<td>27</td>
<td>DDKYNK</td>
<td>Tmx1c Thioredoxin domain containing 1</td>
<td>59</td>
<td>KVkeeGEEDEVDSE EETENREGES</td>
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<tr>
<td>Nolc1 Nucleolar phosphoprotein p130</td>
<td>48</td>
<td>AGKESEEESEDEETQNK</td>
<td>Sppgl Sphingosine-1-phosphatase phosphatase 1</td>
<td>19</td>
<td>RNSLTGEEELAK</td>
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<tr>
<td>Prkar2a cAMP-dependent protein kinase type II-alpha regulatory subunit 3</td>
<td>25</td>
<td>RVSVCAETFNPDEEDNDP</td>
<td>Bokdha branched chain ketoacid dehydrogenase E1, alpha polypeptide</td>
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<td>- 34 kDa protein</td>
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<tr>
<td>Canx Calnexin precursor</td>
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<td>AEEDEILNRSPT</td>
<td>Cdc65 Ribonucleoprotein-bound phosphoprotein 5</td>
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<tr>
<td>SIC3a3 Sodium-coupled neutral amino acid transporter 3</td>
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<td>GFLQSSKQSSKEHFTDFEGK</td>
<td>Spnb2 Non-erythroid spectrin beta</td>
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<td>EALVPEASESPRPALAR</td>
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<td>Hnrdp Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0</td>
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<td>Clic6 Chloride intracellular channel 6 protein</td>
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<tr>
<td>Goras1p Golgi reassembly-stacking protein 1</td>
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<td>Zfp265 Zinc finger Ran-binding-domain-containing protein 2</td>
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<td>Hdg1 Hepatoma-derived growth factor</td>
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<td>AGMDLEDSKPKRK ASPASQHOLSQDEEAA DHGR</td>
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<tr>
<td></td>
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<td>Ace Angiotenins-converting enzyme, isomeric isoform precursor</td>
<td>24</td>
<td>GPQFGSEVELR</td>
</tr>
</tbody>
</table>
DIGE continues to make contributions to protein profiling- iTRAQ analysis is also done in parallel
SILAC Protein Profiling has now been added to our proteome tool box
Mapping of the choroid plexus proteome and phosphoproteome is nearly complete
Acknowledgements

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