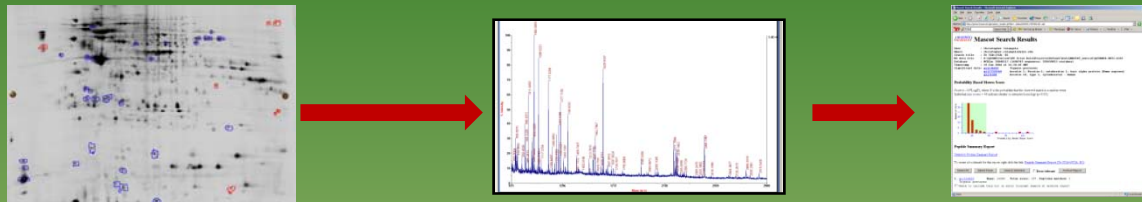


Delving Deeper into the Neuroproteome using Quantitative and Non-Quantitative Protein Profiling

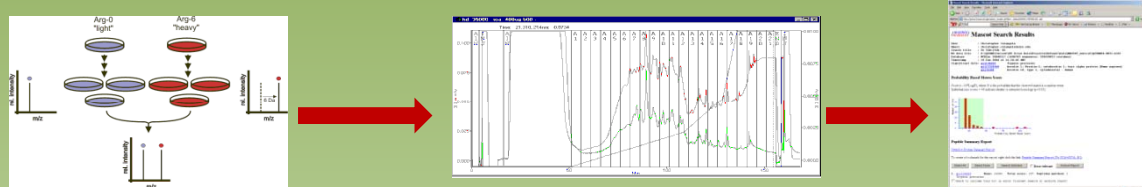
Kathy Stone
Director of MS & Protein Chemistry
Co-Director DIGE
Wm Keck Foundation Mass Spectrometry &
Proteomic Resources
Yale University
New Haven, CT

OUTLINE: Delving deeper

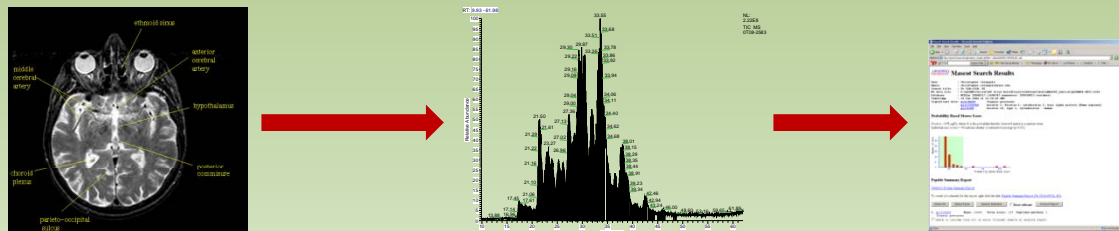
- Protein Profiling at the Protein Level
 - utilizing DIGE with MALDI-Tof/Tof analysis



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

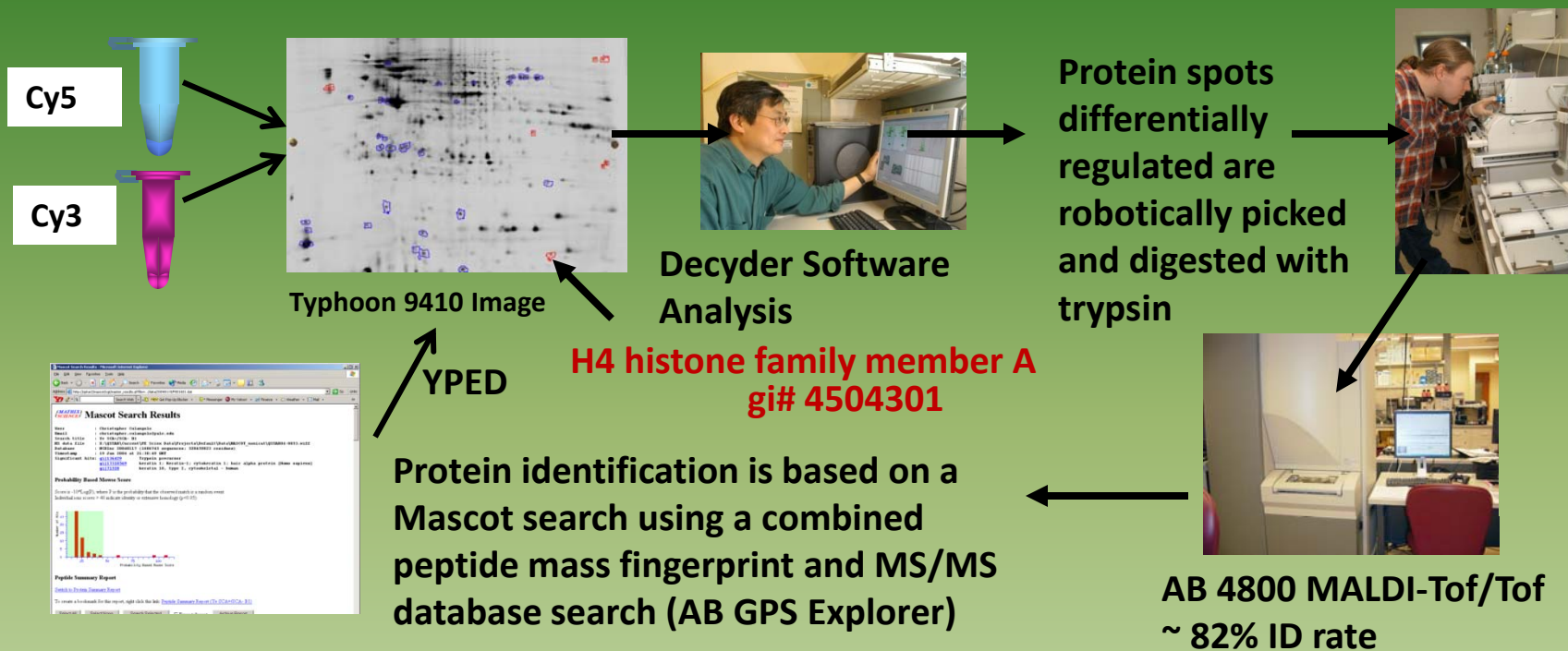


- Proteome and phosphoproteome
 - cataloging of the choroid plexus



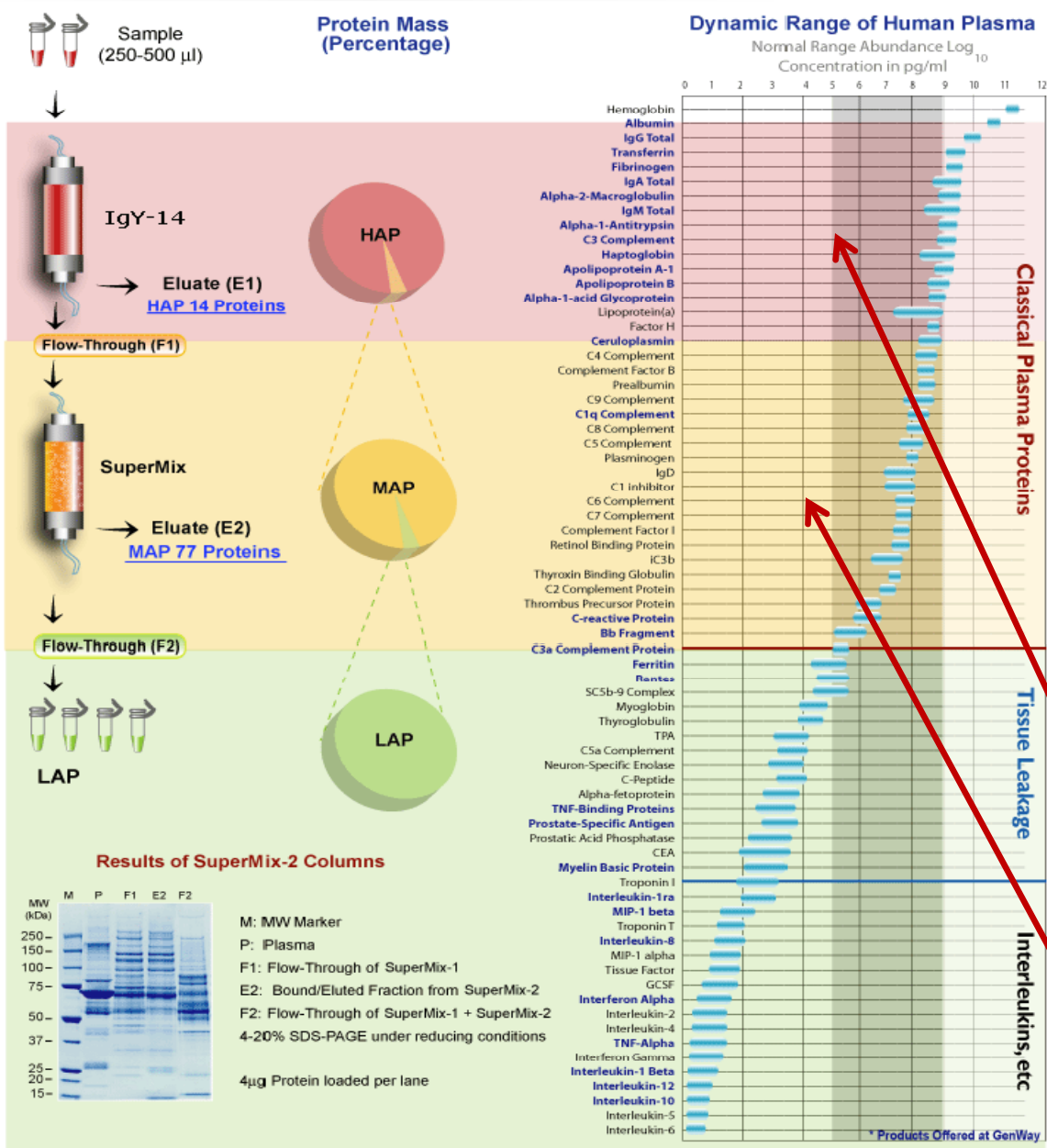
- Summary

DIGE Protein Profiling and Protein Identification Workflow



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 CSP α /Hsc70/SGT complex



The Challenge:
Getting to the Lower Abundant Proteins in Serum/Plasma

The Answer:
Immunoaffinity Partitioning

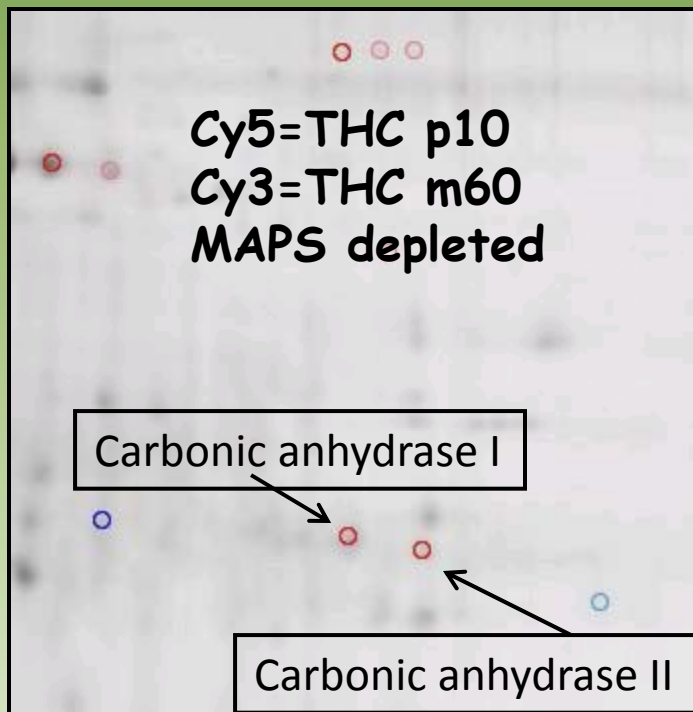
- 5% of our NIDA investigators work with Plasma
- IgY-14 LC column depletion of the highly abundant 14 proteins
- Supermix LC column depletion of the 77 moderately abundant proteins

Typical Range Abundances Courtesy of Plasma Proteome Institute
The human plasma proteome: History, character, and diagnostic prospects.
Anderson, N.L. and Anderson, N.G., Molecular and Cellular Proteomics, 1.11, 845-867 (2002)

Understanding the Proteome effects of Δ -9-tetra-Hydrocannabinol (THC)

Dr. Zoran Zimolo

- 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 = $Cy5/Cy3 = -1.5$

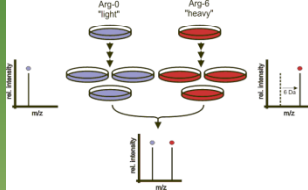
Carbonic Anhydrase II = $Cy5/Cy3 = -1.8$

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

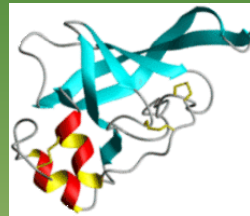
(Pharm. Bio, 2004, 42,p434-439)

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

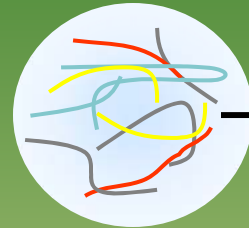
SILAC labeling



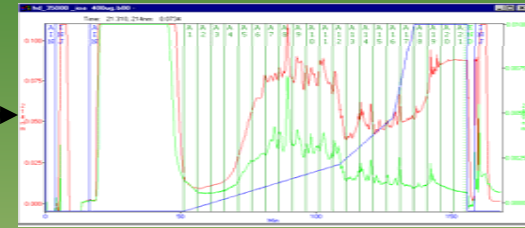
Arginine [$U-^{13}C_6, ^{15}N_4$]
Lys [$U-^{13}C_6$]



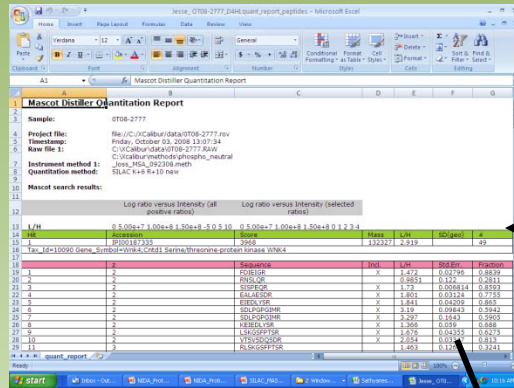
Complex mixture



Tryptic digest

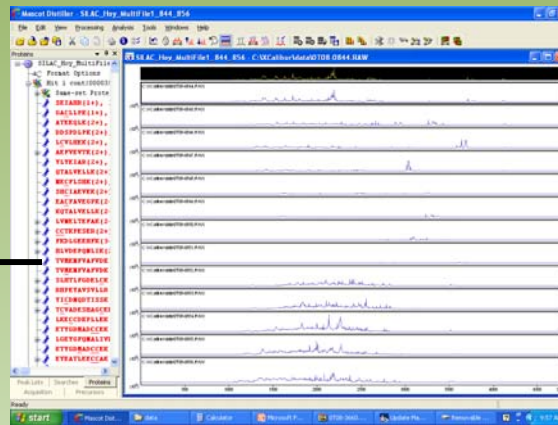


20 SCX fractions collected (on or off line with the MS)



Peptide Quantitation-
Mascot Quantitation
Tool Box

YPED

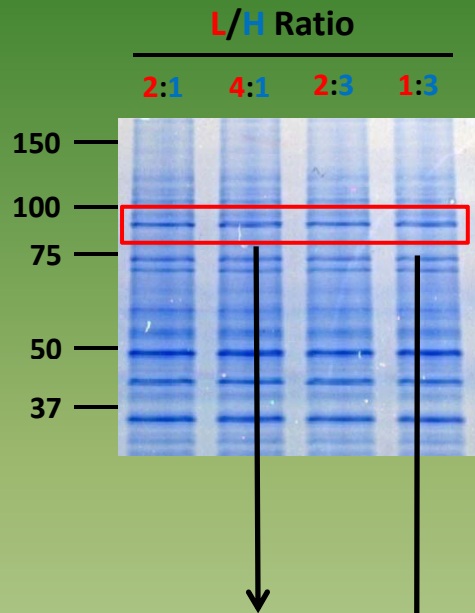


Mascot database
searches for protein
identification

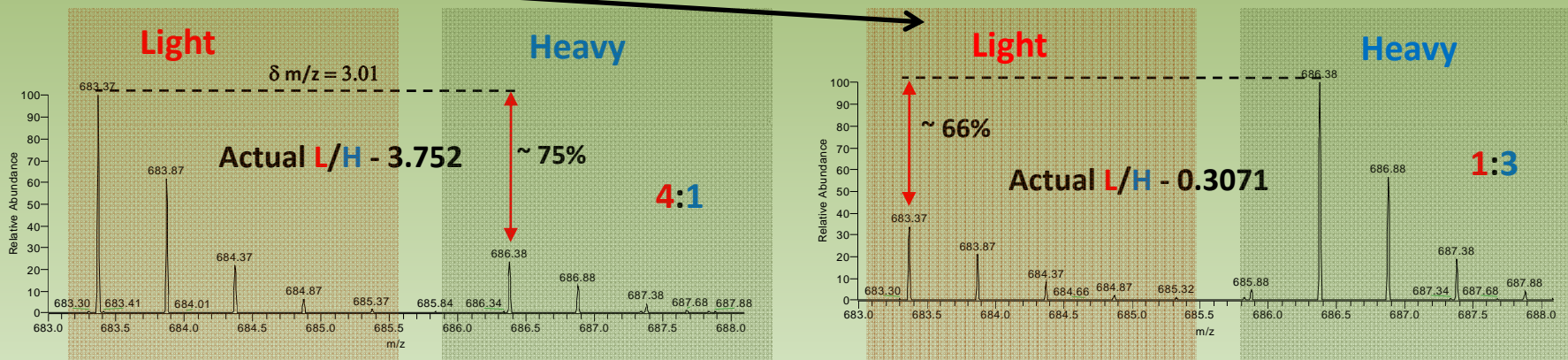


Each cation fraction
is analyzed by LC
MS/MS analysis on
the LTQ Orbitrap

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

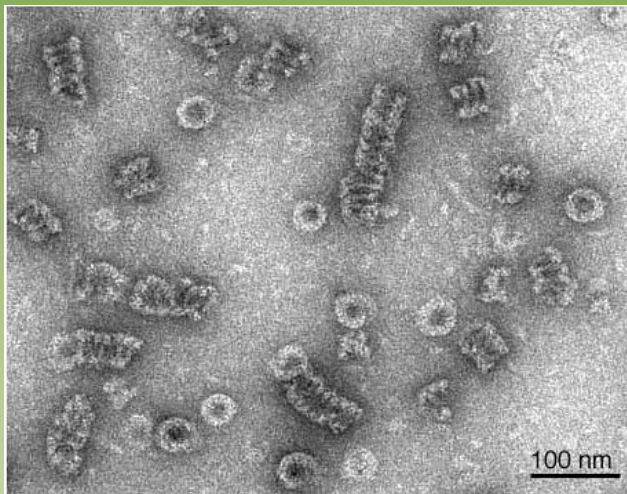


Heat shock protein (HSP 90-beta) Identified

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 assembles into spirals

Dynamin is a right handed helix with right handed twisting ability

- **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.

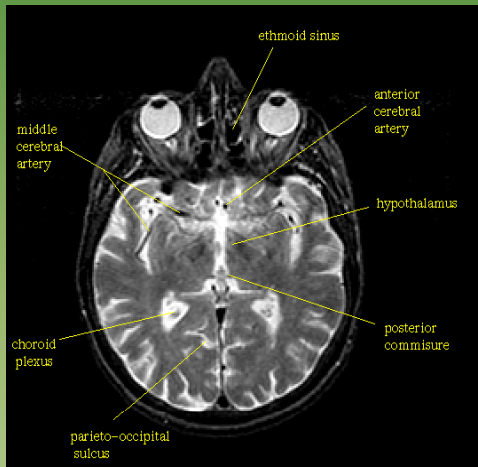
SILAC Results on the Dyn1 KO (light) versus Dyn 1,2 dynamin knock out (heavy, Lys only)

Accession	Score	L/H	#	Description	Accession	Score	L/H	#	Description
IPI00123181	324	0.66	7	Myosin-9	IPI00124771	57	2.07	2	Phosphate carrier protein, mitochondrial
IPI00354819	89	0.77	3	Isoform Smooth muscle of Myosin light polypeptide 6	IPI00118413	60	2.54	2	Thrombospondin 1
IPI00127942	146	0.81	2	Destrin	IPI00131138	292	8.28	2	Isoform 1 of Filamin-A
IPI00110827	178	0.82	3	Actin, alpha skeletal muscle	IPI00308324	101	17.28	2	Rdx Radixin
IPI00457898	102	1.25	2	Phosphoglycerate mutase 1	IPI00119149	40	17.76	2	Isoform 1 of A-kinase anchor protein 4
IPI00319994	94	1.29	3	L-lactate dehydrogenase A chain	IPI00411115	46	22.11	2	Ras-related protein Rab-8B
IPI00117352	276	1.31	2	Tubulin beta-5 chain	cont 000023	125	140.40	1	trypsin (enzyme used for digestion)
IPI00169463	276	1.31	2	Tubulin beta-2C chain	cont 000035	519	155.20	15	serum albumin [Bos taurus (contaminant)]
IPI00122450	181	1.32	2	caldesmon 1					>20% down regulated
IPI00311344	162	1.32	2	89 kDa protein					>20% up regulated
IPI00553798	57	1.47	5	AHNAK nucleoprotein isoform 1					contaminant
IPI00319992	90	1.66	2	78 kDa glucose-regulated protein					
IPI00118384	60	1.72	2	14-3-3 protein epsilon					

Myosin light polypeptide 6 has been confirmed by a Western blot to be up regulated in the dynamin knock out

Cataloging the Proteome and the Phosphoproteome of Rat Choroid Plexus

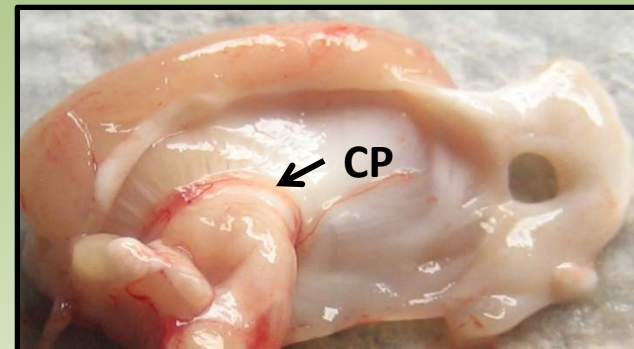
Dr. Samuel Sathyanesan



www.braininfo.rprc.washington.edu

- 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

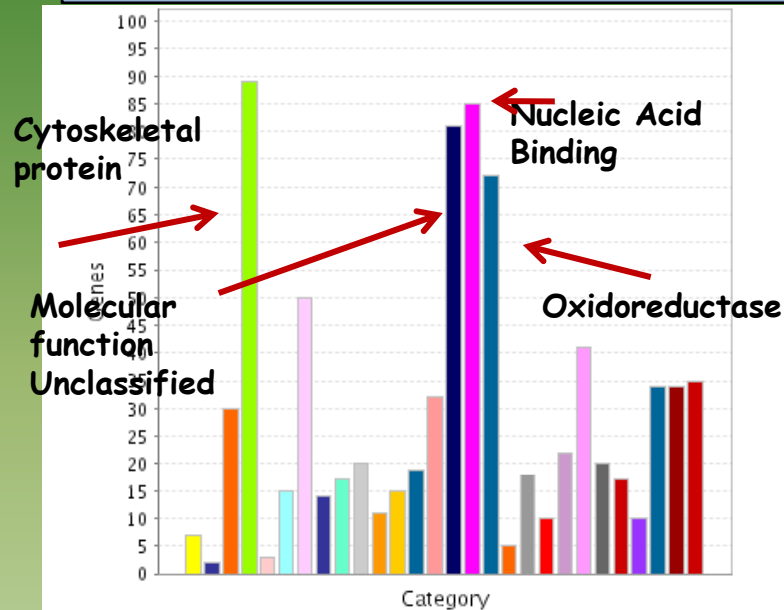
- UPLC and LTQ-Orbitrap
- Dionex capLC and QSTAR XL
- UPLC and QSTAR Elite

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



**Chart tooltips are read as: Category name (Accession): # genes; Percent of gene hit against total # genes; Percent of gene hit against total # Function hits

1449 proteins identified by using Mascot Distiller and the Mascot search algorithm

Click to get gene list for a category:

- [Cell adhesion molecule \(MF00040\)](#)
- [Cell junction protein \(MF00276\)](#)
- [Chaperone \(MF00077\)](#)
- [Cytoskeletal protein \(MF00091\)](#)
- [Defense/immunity protein \(MF00173\)](#)
- [Extracellular matrix \(MF00178\)](#)
- [Hydrolase \(MF00141\)](#)
- [Ion channel \(MF00024\)](#)
- [Isomerase \(MF00166\)](#)
- [Kinase \(MF00107\)](#)
- [Ligase \(MF00170\)](#)
- [Lyase \(MF00157\)](#)
- [Membrane traffic protein \(MF00267\)](#)
- [Miscellaneous function \(MF00197\)](#)
- [Molecular function unclassified \(MF00208\)](#)
- [Nucleic acid binding \(MF00042\)](#)
- [Oxidoreductase \(MF00123\)](#)
- [Phosphatase \(MF00113\)](#)
- [Protease \(MF00153\)](#)
- [Receptor \(MF00001\)](#)
- [Select calcium binding protein \(MF00188\)](#)
- [Select regulatory molecule \(MF00093\)](#)
- [Signaling molecule \(MF00016\)](#)
- [Synthase and synthetase \(MF00118\)](#)
- [Transcription factor \(MF00036\)](#)
- [Transfer/carrier protein \(MF00087\)](#)
- [Transferase \(MF00131\)](#)
- [Transporter \(MF00082\)](#)

Summary of Choroid Plexus Phosphoproteome: 59 Phosphopeptides Identified

Protein Description	Peptide Score	Peptide Sequence	Protein Description	Peptide Score	Peptide Sequence	Protein Description	Peptide Score	Peptide Sequence
Prkra Interferon-inducible double stranded RNA-dependent protein kinase ac	33	HRAEAPPLQREDSGTFSLGK	RGD1304816_predicted similar to Nucleoprotein TPR	37	HSQDSQHCVSVDGEDELDFK	Slc7a10 System asc amino acid transporter Asc-1	34	RDSDMTGHIQQPGGR
Nucks Nuclear ubiquitous casein and cyclin-dependent kinases substrate	25	KTSASPPLEK	LOC689421 similar to Beta-2-syntrophin (59 kDa dystrophin-associated prote	33	GLGPPSPAPP	Serbp1 Isoform 2 of Plasminogen activator inhibitor 1 RNA-binding protein	42	SKSEEHAEDSVMMDH HFR
Gsk3a Glycogen synthase kinase-3 alpha	31	GEPNVSYICSR	Spag1 Sperm-associated antigen 1	18	LDCDKALQIDSK	Pgrmc1 Membrane-associated progesterone receptor component 1	28	EGEPTVYSDDEEPPK EAAR
Ace Angiotensin-converting enzyme, testis-specific isoform precursor	31	GPQFGSEVELR	Nol8_predicted nucleolar protein 8	24	DTETNGPQNVSHCKFDTTSK	Spnb2 Non-erythroid spectrin beta	36	RPPSPEPSAK
Dkc1 H/ACA ribonucleoprotein complex subunit 4	22	RKRDSDSDADEATPTTTPR	Sec61b_predicted Sec61 beta subunit	19	PGPTPSATNVGSSGRSPSK	Rbmxrt_predicted Heterogeneous nuclear ribonucleoprotein G	23	RSTPSGPVR
Ppp1r1a Protein phosphatase 1 regulatory subunit 1A	40	STLSMSPR	Ncbp1 Nuclear cap-binding protein subunit 1	35	RRHSYENDGGQPHK	Myh8 similar to myosin, heavy polypeptide 8, skeletal muscle, perinatal is	28	TKDELAKSEAK
G3bp similar to Ras-GTPase-activating protein binding protein 1	25	SASPAPADVAPAQEDLR	Bcl2l13_predicted BCL2-like 13	43	SHTGEAIAAR	RGD1561119_predicted 79 kDa protein	21	EANARCGILKSSPFSR
Ppig Peptidyl-prolyl cis-trans isomerase G	27	DDKYNK	Txndc1 Thioredoxin domain containing 1	59	KVEEEQEAEEDVSE EETENREGESK	Cspg3 Neurocan core protein precursor	23	GTVLCGPPPAVENASL VGVRKVYNVHATVR
Nolc1 Nucleolar phosphoprotein p130	48	AGKESEEEEEDETEQNKK	Sgpp1 Sphingosine-1-phosphate phosphatase 1	19	RNSLTGEEGELAK	Espn 93 kDa protein	25	RSSSSTGKVR
Prkar2a cAMP-dependent protein kinase type II-alpha regulatory subunit	25	RVSVCAETFPDEEEDNDPR	Bckdha branched chain ketoacid dehydrogenase E1, alpha polypeptide	49	IGHHSTSDSSAYR	Hsph1 Protein	32	IESPKLER
- 34 kDa protein	28	KKEPAISSQNSPEAR	Cetn2 similar to centrin 2	30	RMSPKPELTTEEKQK	RGD1566201_predicted similar to ADAMTS-9 precursor	33	QFFIGSEK
Canx Calnexin precursor	35	AEDEILNRSR	Raly HnRNP-associated with lethal yellow	40	GRLSPVVP	LOC686980 similar to arsenate resistance protein 2	24	ERFSPRHLSPPQK
Slc38a3 Sodium-coupled neutral amino acid transporter 3	21	GFLQSSSKEPHFTDFEGK	Spnb2 Non-erythrocyte beta-spectrin	32	RPPSPEPSAK	Myh8 223 kDa protein	28	TKDELAKSEAK
Slc9a3r1 Ezrin-radixin-moesin-binding phosphoprotein 50	45	EALVEPASESPRALAR	Tloc1 Ab2-292	29	SDSEEKSDSEKKEDEEGK	RGD1309870_predicted hypothetical protein LOC289778	25	HQGPLSQMVTTVKPY NPFNRK
Hnrpd Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	33	IDASKNEEDEGHSSNSR	Yap1 52 kDa protein	32	QASTDAGTAGALTPQHVR	Slc16a1 Monocarboxylate transporter 1	31	SKESLQEAGK
Myh3 Myosin-3	24	TKDELAKSEAK	Clic6 Chloride intracellular channel 6	23	APDGLDGEAAK	Gpr101_predicted G protein-coupled receptor 101	19	MPSSCTNSTQENSS R
Gorasp1 Golgi reassembly-stacking protein 1	22	KPPSASSGTPAK	Cdc42bpb Serine/threonine-protein kinase MRCK beta	33	HSTPSNSSNPSGPPSPNSPHR	Atp2b3 Isoform XC of Plasma membrane calcium-transporting ATPase 3	62	SGSFQGA VR
Myh9 Myosin-9	25	GTGDCSDEEVDGKADGADAK	LOC306766 Isoform 2 of UPF0498 protein KIAA1191 homolog	40	HPASAQSSPSSTPHSPK	Ace Angiotensin-converting enzyme, somatic isoform precursor	24	GPQFGSEVELR
Dnajc5 DnaJ homolog subfamily C member 5	32	SLSTSGESLYHVLGLDK	Whsc1_predicted similar to Wolf-Hirschhorn syndrome candidate 1 protein is	19	RSTGSASRSR	Hdgf Hepatoma-derived growth factor	46	AGDMLEDSKPRPK ASPASGHQLSDQEEA DHGR
			Zfp265 Zinc finger Ran-binding domain-containing protein 2	32	SRPSSPA VR	Tjp3_predicted tight junction protein 3	30	

Summary

- **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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Kei Cheung

NIDA Investigators

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Pietro De Camilli

Samuel Sathyanesan

Thomas Biederer