

Applying Proteomics in Brain Injury Studies



Kevin K.W. Wang, PhD

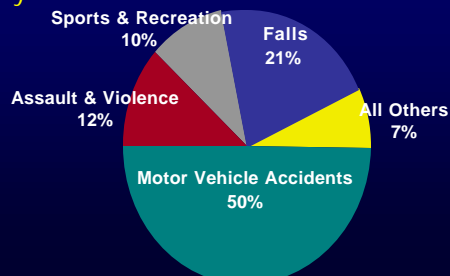
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Impact of Head Injury in the U.S.

Annual U.S. traumatic brain injury (TBI) statistics

- 2 million TBI cases
- 500,000 hospitalizations
- 100,000 deaths
- 70,000-90,000 people with long-term disabilities
- 2,000 survive in permanent vegetative state
- Medical costs of over \$48 Billion annually in US
- Costly MRI (\$1,100-1,500) or CT (\$700-1,000) scans
- 30% Battlefield injuries are head injury



Need for Diagnostic Biochemical Markers

Diagnostic markers could improve TBI patient management and outcome today

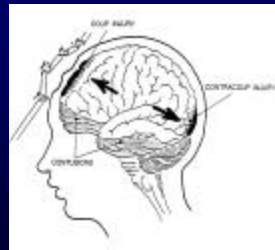
- Objective, quantitative index of mild TBI
- Acute patient management: admit vs. discharge:
further diagnostics

Biomarkers are necessary for the development and introduction of new TBI therapy

- Reduce costs/risks of clinical trials – surrogate marker
- Early assessment of treatment efficacy

Need for Diagnostic Biochemical Markers

- New proteomic-based technologies are making possible rapid discovery exciting and unanticipated diagnostic markers



Neuronal injury biomarkers are also highly relevant to other acute CNS injuries and psychiatric diseases

- Acute CNS injuries

- TBI, Stroke, Invasive neurosurgical procedures, Subarachnoid hemorrhage, Seizure

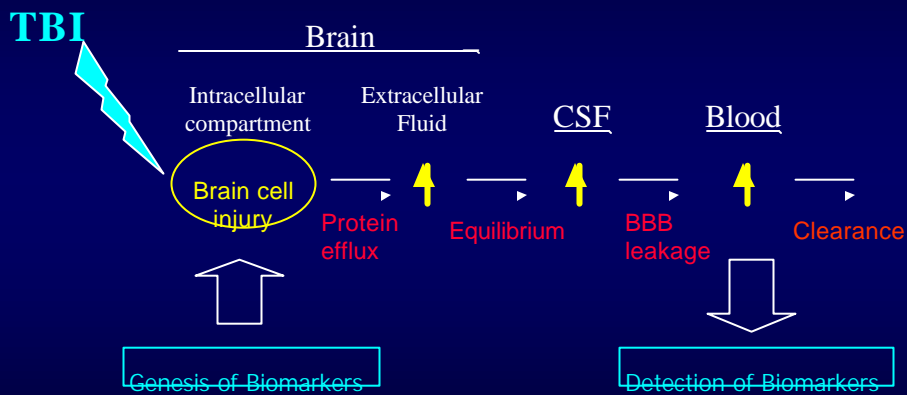
- Neurodegenerative diseases

- Alzheimer's disease, ALS
- Parkinsonism

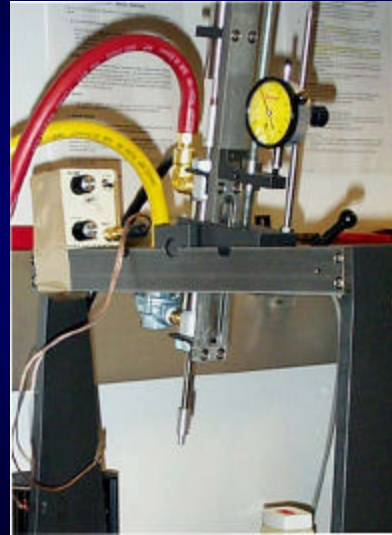
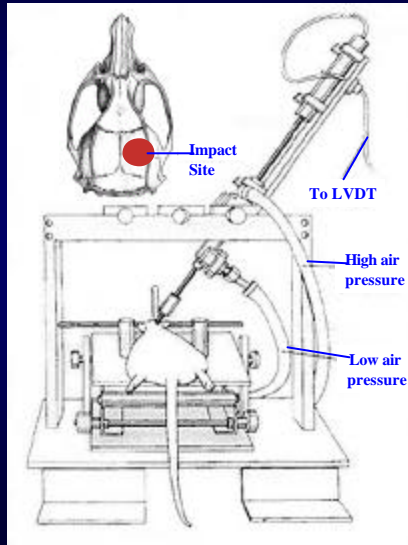
- Psychiatric disorders

- Alcoholism, Binge drinking
- Substance abuse (Methamphetamine, Ecstasy)

Fate of brain injury biomarkers



Rat model of TBI: Controlled Cortical Impact (CCI)



Integrated Proteomic Approach to TBI Biomarker Discovery

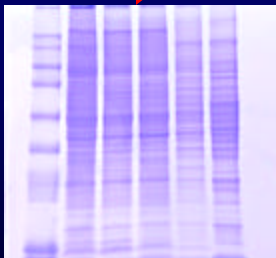
- (a) Differential 2D-gel electrophoresis
- (b) 1D-Gel Electrophoresis-LC-MS/MS
- (c) Liquid Chromatography/ 1D-Gel Electrophoresis-MS/MS
- (d) Degradomics and High Throughput Immunoblotting (HTPI) System



What is Neuroproteomics?

- Neuroproteomics is the application of multiple proteomic techniques to elucidate protein level changes, posttranslational modifications and protein-protein-interactions and protein pathways/networks of the nervous system at an organism-wide scale.
- Human neuroproteomics encompasses the central nervous system (CNS) (i.e. brain and spinal cord) and the peripheral nervous system (PNS).
- Neuroproteomics studies also have important significance in biochemical mechanism elucidation, diagnosis and therapy development for various neural diseases.

Separations



1D-SDS/PAGE

Why Separate?

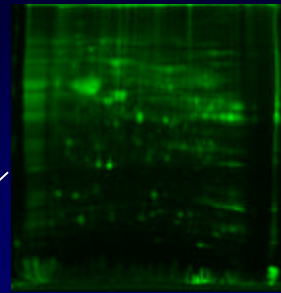
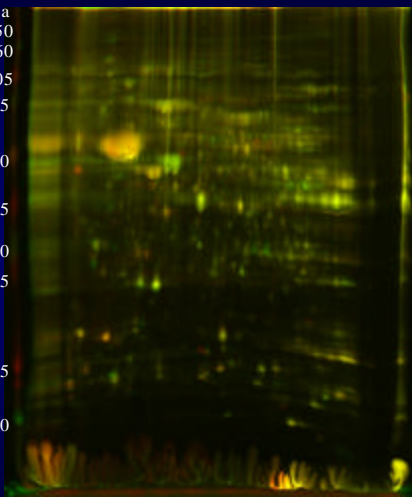
- > 10,000 proteins encoded in humans
- Multitude of Post-Translational Modifications
- 7-Order Dynamic Range in Concentration
- Proteins Mix Together In Biological Samples

2-D Differential In gel electrophoresis (DIGE)

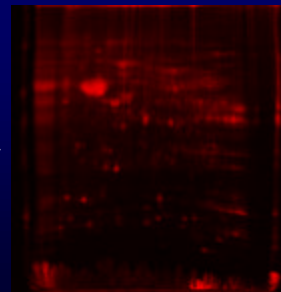
More and More Separations

2D-PAGE

kDa
250
160
105
75
50
35
30
25
15
10



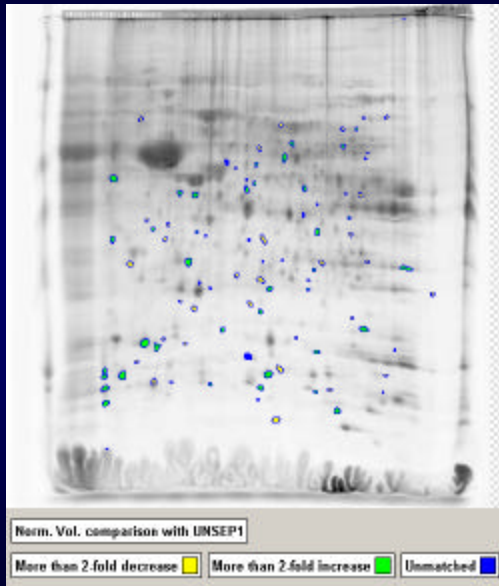
Cerebellum (Cy3, Green)



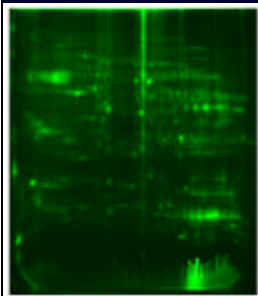
Cortex (Cy5, Red)

DIGE of 325ug of Cerebellum (Cy3, Green) and of Cortex (Cy5, Red) Protein Extract with 18cm pH 3-10NL IPG Strip for IEF and Pre-cast 8-16% Tris-HCl gel in Tris-Glycine Running Buffer for SDS-PAGE

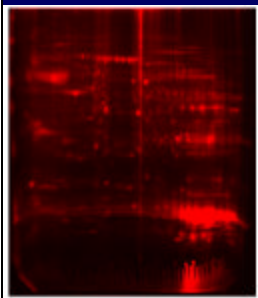
Differential Expression Analysis



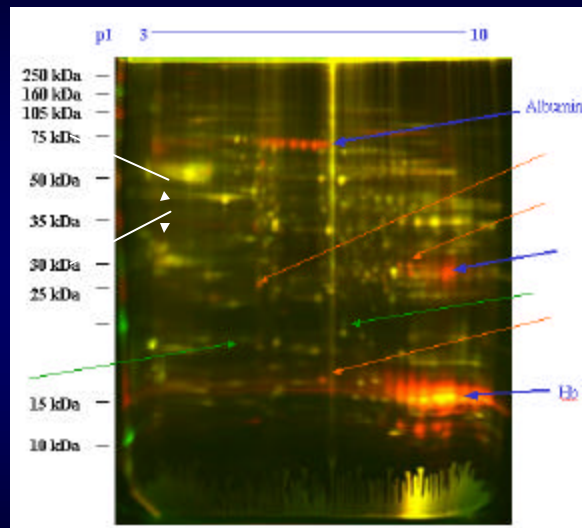
- Total protein difference: 320
- Unmatched proteins: 7
(3 cerebellum vs 4 cortex)
- More than 2 fold increase in cerebellum: 42
- More than 2 fold increase in cortex: 33
- 238 protein spots differ by less than 2 fold



Naive (Cy3, green)

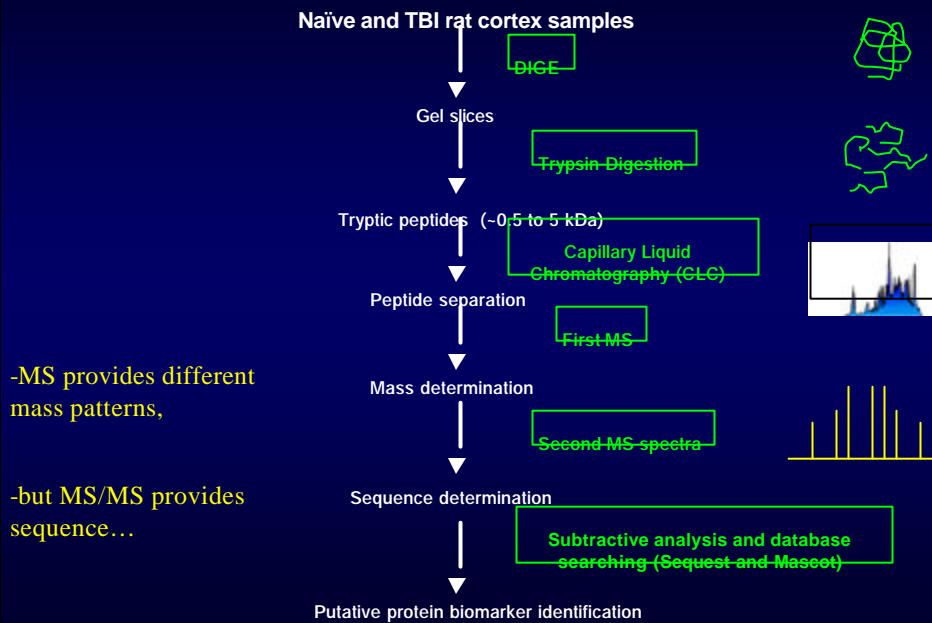


TBI (Cy5, red)

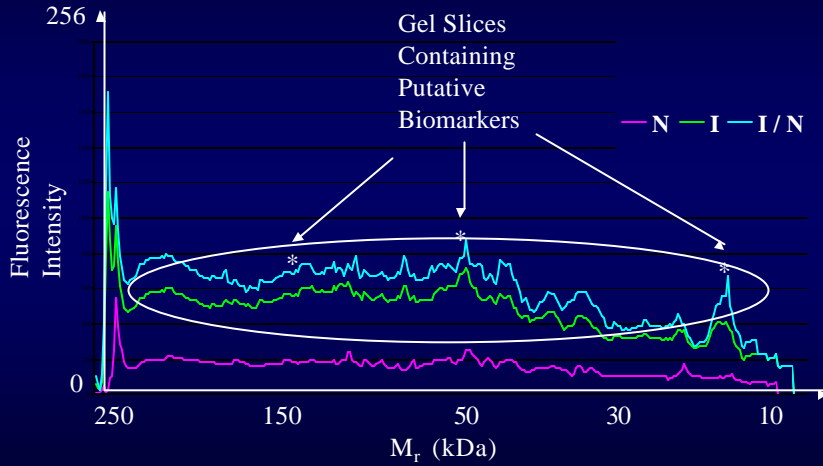


1D-Gel Electrophoresis- LC-MS/MS

From Samples to 1D-DIGE to CLC/ MS/MS



Putative Biomarkers Revealed by Fluorescence Line Plot Ratios: Injured-Cy3 (I) HC Vs. Naïve-Cy5 (N) HC



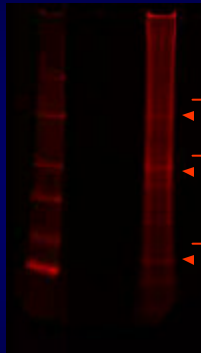
Proof-of-Principle: -Distinct peaks can be identified and quantified

1D-Differential In Gel Electrophoresis (1D-DIGE)

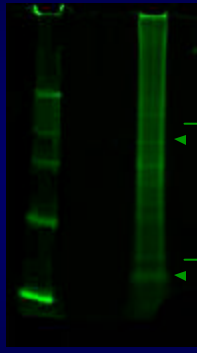
Images:

Injured-Cy3 (I) HC Vs. Naïve-Cy5 (N) HC

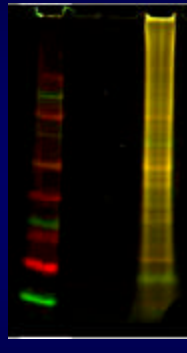
M I N I+N M I N I+N M I N I+N M I N I+N



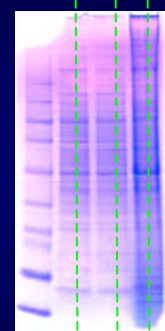
Naïve: Cy5-labeled
(red) image



TBI: Cy3-labeled
(green) image



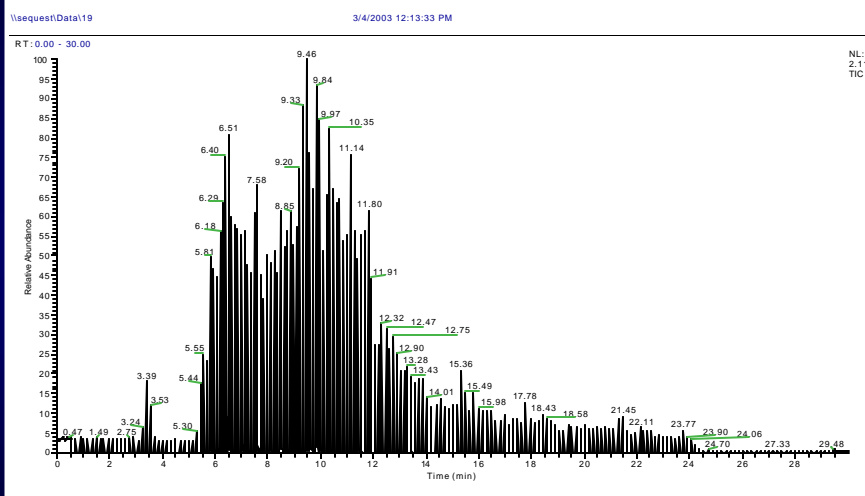
Combined Cy3+Cy5
labeled (yellow) image



Coomassie blue
stain image

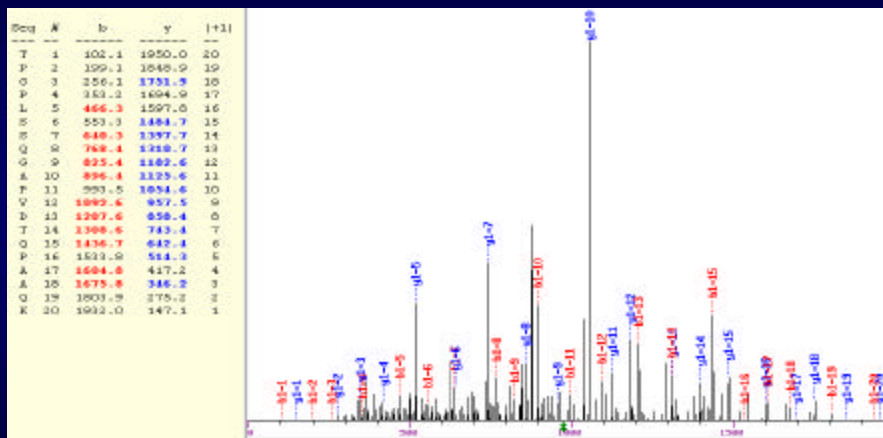
-Individual unlabeled (TBI, Naïve) and Combined Cy3-Cy5 labeled samples run on 1D-GE

Capillary LC separation of tryptic peptides and MS determination of exact MW



Representative CLC chromatogram of tryptic peptides from the unlabeled injured (I) sample in gel slice 19.

1st-ranked Tryptic Peptide MS-MS analysis in Gel Slice 22 (Synaptojanin): Injured-Cy3 (I) HC



Tryptic peptide ID: TPGPLSSQGAPVDTQPAQK

Tryptic Peptides Observed in the 1st- ranked protein (Synaptojanin) in Gel Slice 22: Injured-Cy3 (I) HC

MAPSKGPRIV HKLDPPPPSL IVETRHKEBC LMFESGAVAV LSSAEKEAK GTYAKVLDAY GLLGVLRNL GDTMLHYLV
 VTQCMSTVCKI QSEKVFRTS TEFISLRVIDA SDEDRISEVR KVLNQNPFV AWSASQVSLD LSLNAHRSMQ ERTTDRFPFW
 NQSLHLHLKH YGVNCDWLL RLMCOQVEIR TIYAAHEQAK ACLISRLSCE RAGTRFNVRG TNDQCHVANF VETEQVIYLD
 DQVSSFIQR GSVPLFWEQP GLQVGSRRFR MRRGFEANAP AFDRHFFTLK DLYCKQVYN LLSKKEGERM LSKAFQSHLK
 ASREHABDGM WFDYHQMYK COKAKELHSV LKPCVQKFLD YGFFYDQSA VQRQZQTVR TINGLGLDRF NSVQAFLGLE
 MIAKQLEALG LAEKPQLVTR FQRVFRMWS VMLGSKKY AGTGALBOKA KLDQARSVT RTIQNNRFLS SKQEADVLL
 LGNTLNSDLA DKARALLITG SLRVSEQLQ SASSKYLENM CENFYKYSKP KIKRVCVOTW NYNOQKQFES IAFKNQILID
 WLLDAPKLAQ IQEFDKRSK PTDFAIDFE EMVELMAONI VNASTTNOKL WAVELOKTS RDNKYVLLAS EQLVQWCLFV
 FIRPQLAFPI RQVAVDTRKT GIMGGATGNLQ AVAJRMLFHT TSLCPTCSHF AAGQSQVEKER NEDFVELARK LSPFMGRMLP
 SHDYVFWOD FNYRIDLPNE EVKELRQQN WDSLIAQDL INQKNAQQIF RQPLEKQVTF APTKYDILFS EDYDTSEKCR
 TPAWTDRELVW RRRKWWPDRS AEDLLINAS PQDESKLYT WTPGTLHHYG RAEIKTSDHR PVAALIDID FEVEAERQK
 IVICEYAVQG PFDGTVLVSI KESAQENTFP DDALIDELLQ QPAHPGEVIL IRFVEDKMWV TPLBOSSALN VLSLNGKLL
 NRTITTLKS PDWIKTLEB MSLEKISVTL PESTSGLLQKQAPISAQED MTCNNDQYSA EWEELLQHL QPSSSSQLGT
 SPSSSPRTSP CQSPTAPEYS APSLRFRSR AFSF IPGPLS SQGAPVDTQP AAQKE ISQTI EPKRPFPPRP VAPPARPAPP
 QRFPFFQAR SPAPARKERG AFKSPGTARK DMLGRNQSP QAGLAGPFS GTGAARPTIP ARAGVISAPQ SQARVSAQRL
 TPESQSKFLB TSKGPAVLE PLKPAAFFP QPSLPTFAQL LQDPLYPAA PMFFSIFQSN LETPFLPFRSRSSQSLPSD
 SSPQLQEQP TQ

Mass (mono): 14289.8 Identifier: gij166375 Database: C:\Coalbuzz\database\ref.fasta
 Protein Coverage: 262/1292 = 20.3% by amino acid count, 26247/14289.8 = 19.8% by mass

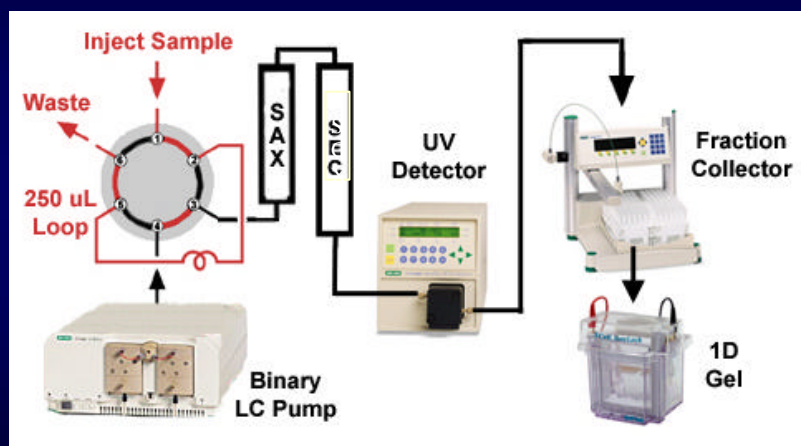


Putative TBI Biomarkers

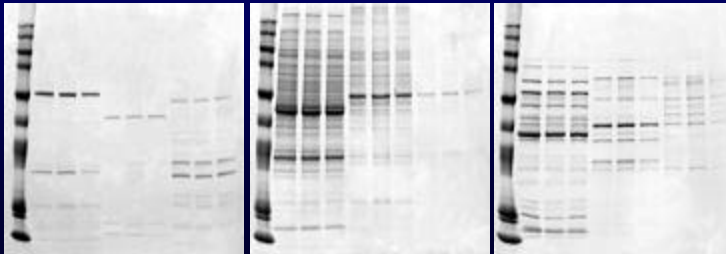
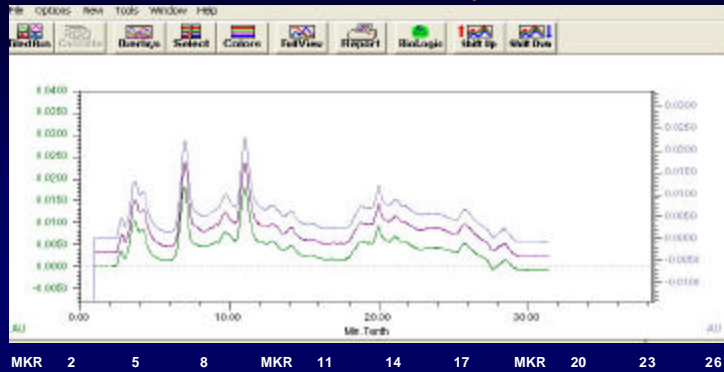
- 322** Unique proteins Identified in rat hippocampus
- 88** Found in both Injured and Naïve tissue
- 125** Found only in Injured tissue
- 89** Found only in Naïve tissue
- 20** Contaminants (keratins, hemoglobin, and trypsin)

Liquid Chromatography/ 1D-Gel Electrophoresis-MS/MS

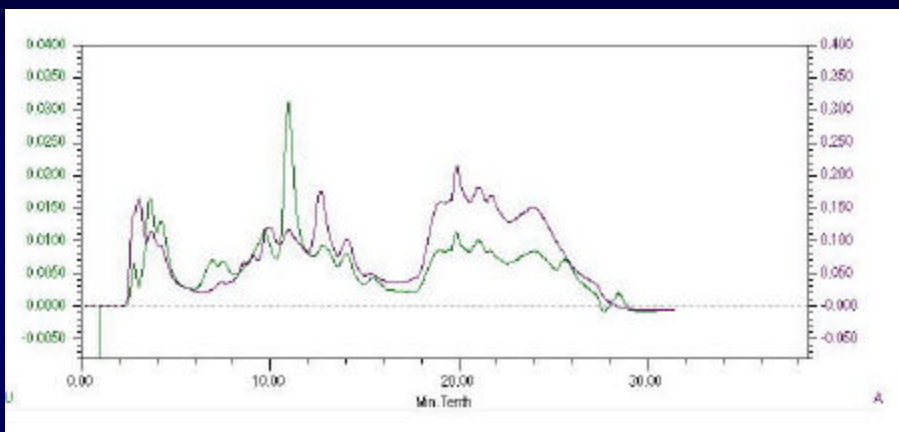
Tandem Liquid Chromatography/ 1D-Gel Electrophoresis



Cerebellum tissue Chromatograms – even protein distribution, low run-to-run variability

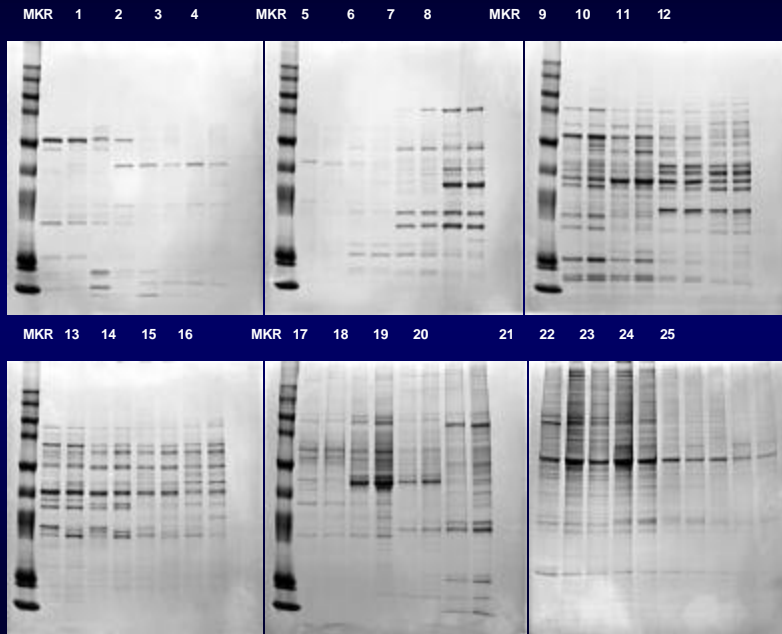


Chromatograms Overlay from Cortex and Cerebellum Brain Samples

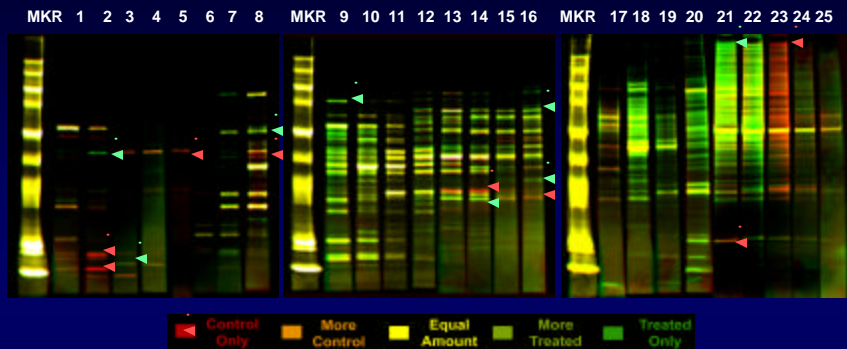


Cortex A 280nm and 214nm 1mg, comparable peaks, wide spreading within 30 fractions

Side-by-Side Cerebellum (1st) and Cortex (2nd) Fraction comparison by 1D-SDS-PAGE



Differential protein analysis by lane overlay with pseudo-color



Information to Maintain

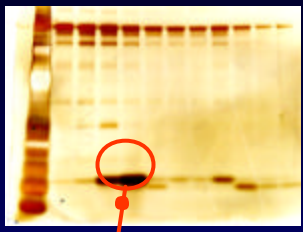
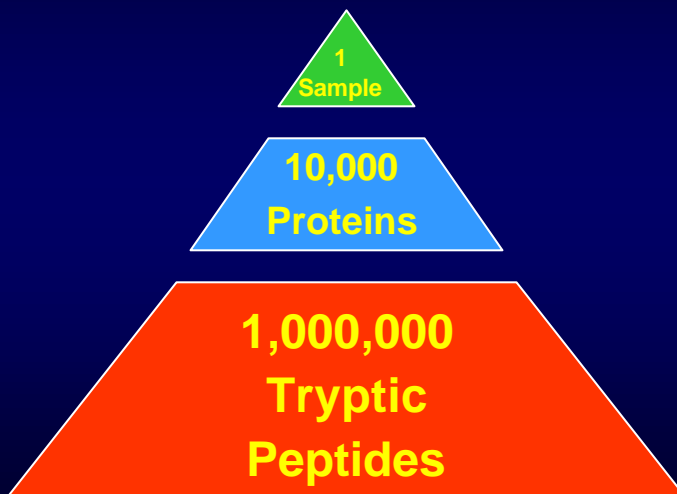
- Fraction (time) Number
- Mass at Band Location
- Color Shift Value
- Band Intensity

Steps to Identification

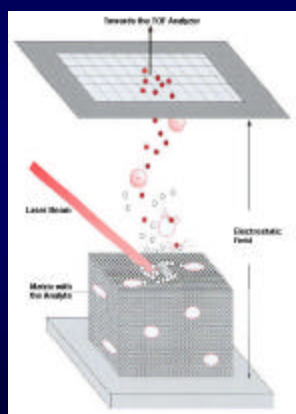
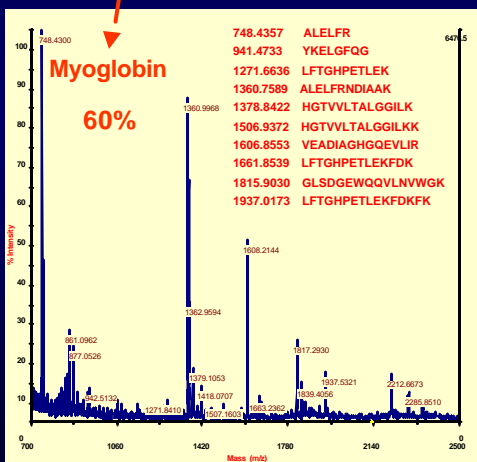
- Excise Bands of Interest
- Digest Protein with Enzymes
- Perform Mass Spectrometry
- Search Protein Database

The Mass Spec Approach: What's Involved

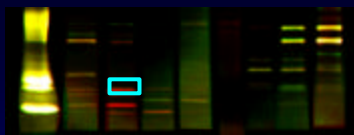
1: Address Sample Complexity



Mass Spectrometers MALDI / MS



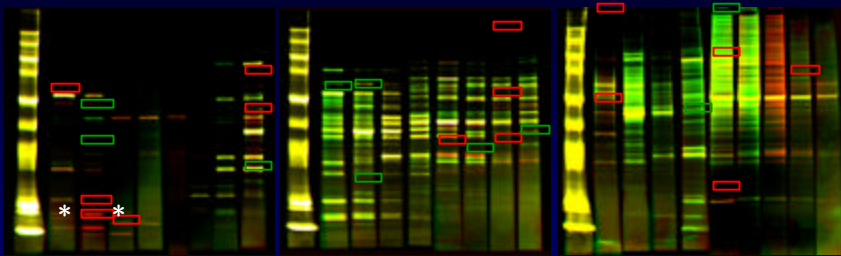
More Data More Numbers



File: single_001-033

	Reference	Score	Accession	Peptides (Hits)
49	Q9L127(209) parvalbumin, parvalbumin cytosolic (vertebrates) [Pisces]	14.1	1136004	14 (14 0 0 0)
50	NC_003021(1) keratin 1, keratin 1, cytoskeletal, hair shaft protein [Homo sapiens]	14.0	11210004	14 (14 0 0 0)
51	Q9L127(1) parvalbumin (Parvalbumin cytosolic) [Pisces]	13.9	11360039	14 (14 0 0 0)
52	Q9L127(2) keratin 2a (Keratin cytosolic) gene 2 [Homo sapiens]	94.3	452703	7 (5 2 0 0)
53	Q9L127(2) keratin 1, profilin-1 [Homo sapiens]	42.3	452698	6 (1 4 0 0)
54	Q9L127(1) keratin 1b, keratin-1b [Homo sapiens]	40.3	452697	4 (4 0 0 0)
55	Q9L149(2) heavy chain-binding protein 5, epidermal [Homo sapiens]	38.2	20324394	4 (2 1 0 0)
56	NC_003427(1) hypothetical protein [Palaemonetes pugio] [Arthropoda]	34.8	20321118	6 (0 1 1 2)
57	Q9L118(3) heavy chain-binding protein 5, epidermal keratinocyte [Homo sapiens]	36.1	454445	5 (2 0 0 0)
58	Q9L127(2) keratin 2 [Homo sapiens] [Homo sapiens]	28.2	452705	5 (2 1 0 0)
59	Q9L127(2) keratin 2 [Homo sapiens] [Homo sapiens]	28.1	3079436	3 (2 1 0 0)
60	NC_003015(1) L-lysine hydroxylase cytosolic [Homo sapiens] [Mollusca]	28.3	20304345	3 (0 1 0 0)
61	NC_003015(1) DNA replication origin [Homo sapiens] [Mollusca]	22.1	18053871	2 (1 0 2 0)
62	Q9L127(2) parvalbumin [Palaemonetes pugio] [Arthropoda]	20.2	4526339	2 (2 0 0 0)
63	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	14.4	11360040	2 (1 1 0 0)
64	NC_003428(1) hypothetical protein [Palaemonetes pugio] [Arthropoda]	16.3	18006180	2 (0 0 0 0)
65	Q9L127(2) keratin 5, keratin-5, 5S-like cytoskeletal, keratin [Homo sapiens]	18.2	452696	2 (1 0 0 0)
66	NC_003148(1) Regulator of G-protein signaling 1 [Homo sapiens]	14.5	6326124	2 (0 1 0 0)
67	Q9L127(2) putative protein family member, with a calmodulin domain [Homo sapiens]	16.1	11360041	2 (0 1 0 0)
68	NC_003428(1) histone acetyltransferase [Homo sapiens] [Homo sapiens]	12.8	20512952	2 (0 1 0 0)
69	NC_003428(1) hypothetical protein [Palaemonetes pugio] [Arthropoda]	12.8	1814403	2 (0 0 1 0)
70	NC_003017(1) conserved hypothetical protein [Palaemonetes pugio] [Arthropoda]	12.2	11430417	2 (1 0 0 1)
71	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.3	11360042	1 (1 0 0 0)
72	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.3	6079643	1 (1 0 0 0)
73	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.2	4526708	1 (1 0 0 0)
74	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.2	11360043	1 (1 0 0 0)
75	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.2	11360044	1 (1 0 0 0)
76	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.2	20325219	1 (1 0 0 0)
77	Q9L127(2) keratin 1b, keratin-1b [Homo sapiens] [Homo sapiens]	10.2	4526711	1 (1 0 0 0)
78	NC_003018(1) putative enzyme [Palaemonetes pugio] [Arthropoda]	10.1	20320331	1 (1 0 0 0)
79	Q9L127(2) heavy chain-binding protein 5 (parvalbumin-associated) [Homo sapiens]	10.1	452697	1 (1 0 0 0)
80	NC_003018(1) conserved hypothetical protein [Palaemonetes pugio] [Arthropoda]	10.1	20320327	1 (1 0 0 0)

MKR 1 2 3 4 5 6 7 8 MKR 9 10 11 12 13 14 15 16 MKR 17 18 19 20 21 22 23 24 25



Frac.	Band	Protein 1	Protein 2
1	1	Enolase 1, phosphopyruvate (47kDa)	α -Enolase 1 (47 kDa)
2	1	Creatine kinase (42 kDa)	Aldolase A (39 kDa)
2	2	Carbonic anhydrase (29 kDa)	--
* 2 *	* 3 *	Parvalbumin Ca Binding (12 kDa)	Profilin (14 kDa)
2	4	Calbindin 2 (29 kDa)	--
3	1	Ubiquitin & Ribosomal L40 (14 kDa)	Parvalbumin (12 kDa)
8	1	Pyruvate Kinase (57 kDa)	--
8	2	Aldo-keto Reductase 1 (36 kDa)	Transaldolase 1 (37 kDa)
8	3	ADP-ribosylation Factor (21 kDa)	Phosphatidyl. Binding (21 kDa)
9	1	Pyruvate kinase muscle (58 kDa)	Aldehyde dehydrogenase 2 (56 kDa)
10	1	Pyruvate kinase muscle (58 kDa)	Aldehyde dehydrogenase 2 (56 kDa)
10	2	3-oxoacid CoA transferase (56 kDa)	--

TBI Degradomics

TBI “Degradome”: A unique biomarker opportunity

TBI Degradome - The complete set of protein substrates that are subjected to protease degradation during and after traumatic brain injury

“Protease over-activation is a major theme in traumatic and ischemic brain injury”

- Calpain,
- Caspases,
- Cathepsins,
- MMP's & Proteasome

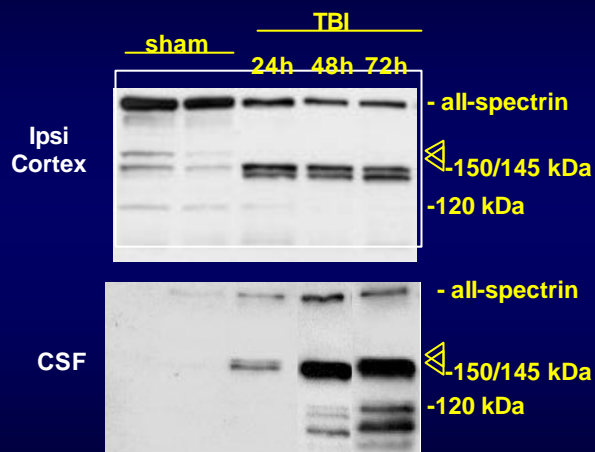
“Degradomics” Overall et al., Nature Reviews 3:509 (2002)

(a) TBI Proteolytic Markers

Protease over-activation is a major theme in traumatic and ischemic brain injury

Protein fragments become markers for brain injury

all-SPECTRIN BREAKDOWN PRODUCTS (SBDPs) AS A PROTOTYPICAL BIOCHEMICAL MARKER



TBI Degradomic Markers: What's Next

- **TBI degradomic markers identified :**

- Provide not only surrogate endpoints but mechanistic analysis of injury, but also powerful insight into subcellular localization of injury

- **Next:**

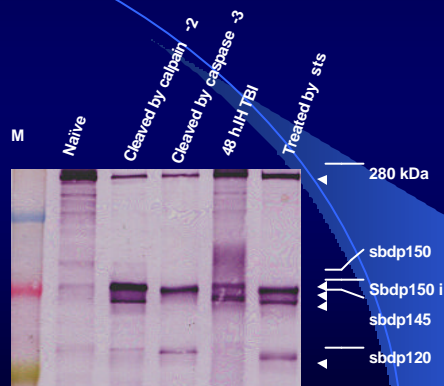
- “Fragment-specific” Antibodies
- Sandwich ELISA for CSF and blood sample analysis

High Throughput Immunoblotting (HTPI) System

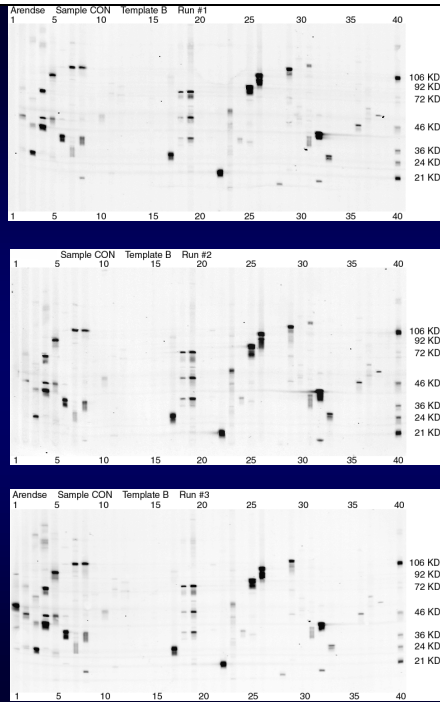
High Throughput Immunoblotting (HTPI) System

- Protein samples subjected to a set of 5 blots
- Each blot has 39 usable lanes (manifold system)
- Each lane is developed with 5-6 different monoclonal antibodies (toward antigen with non-overlapping molecular weight)
- Total: probed with >1,000 monoclonal antibodies

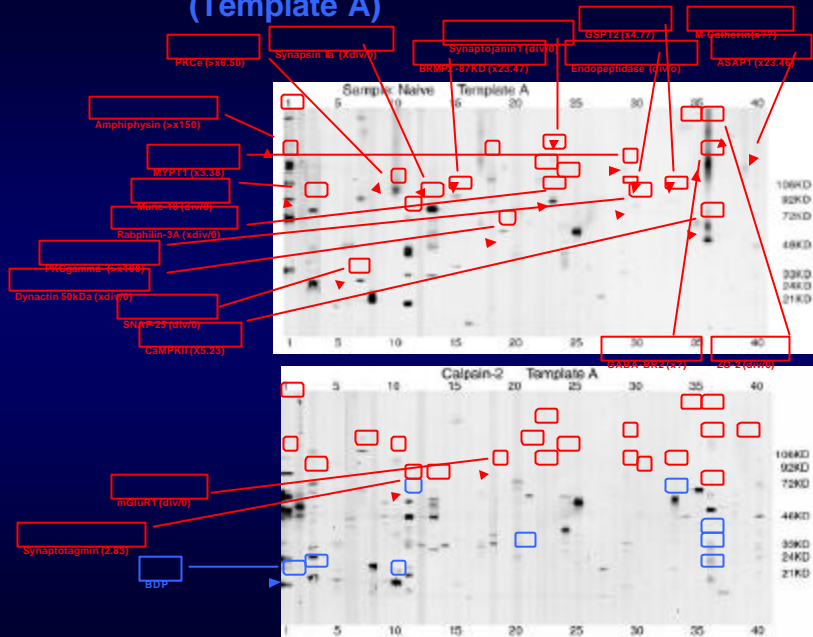
all-spectrin immunoblot as Quality Control before HTSI



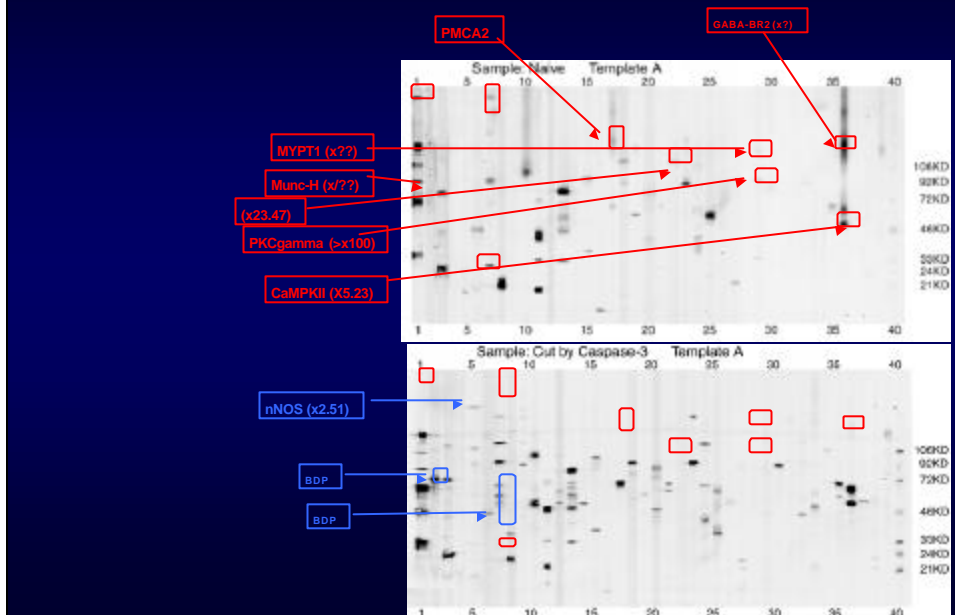
HTPI Run-to-Run variability is low



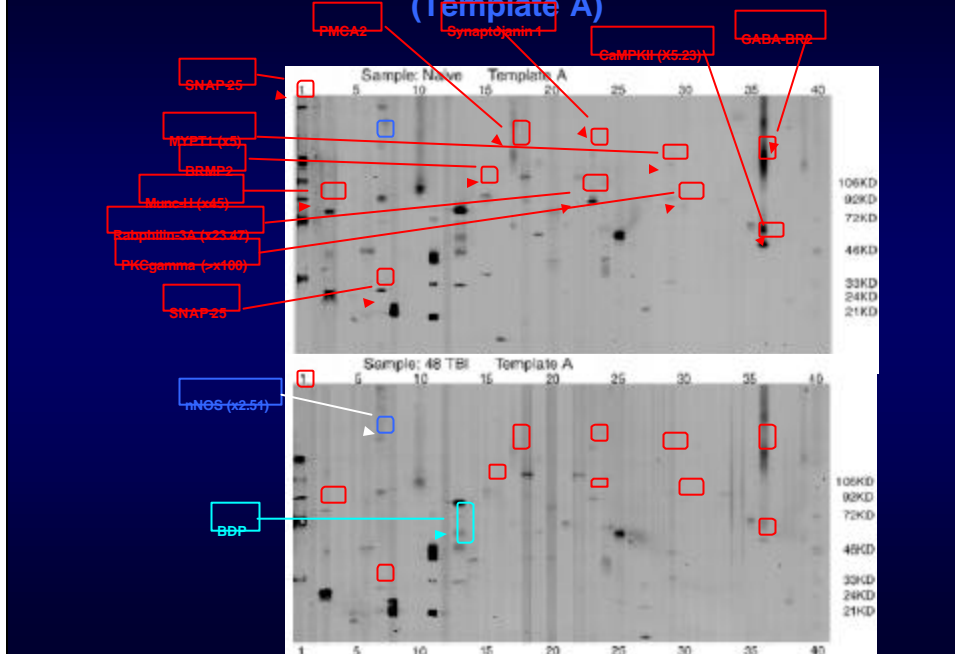
Example of Calpain Degradome (Template A)



Example of Caspase-3 Degradome (Template A)



Example of TBI Degradome (Template A)



Number of degradomic hits from each Template of PowerBlot

Template	A	B	C	D	E	TOTAL Hits
Calpain-2	22	14	8	3	9	56
Caspase-3	9	14	5	3	9	40
TBI	13	9	5	4	8	39

% Hits Rate :

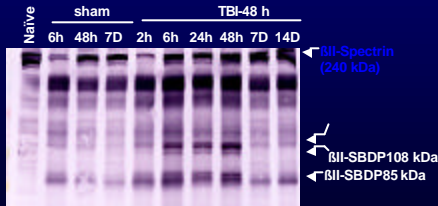
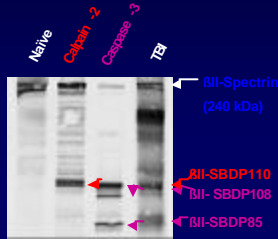
- Calpain-2:** 56 / 1,000 = 5.6%
- Caspase-3:** 40 / 1,000 = 4.0%
- TBI :** 39 / 1,000 = 3.9%

Table 2. Calpain-2, Caspase-3 and TBI degradomes

Protein name	Template + Lane	Swiss Pro ID	M.W. (kDa)	Calpain-1 degraded	Caspase-3 degraded	TBI degraded
AKAP220	A 1	Q62924	220	(*)	(*)	(*)
Amphiphysin	A 1	Q96163	125	(*)		
ASAP1	A 29	Q92WY8	130	(*)		
BRMP2	A 15	Q08539	96/89	(*)	(*)	
CaMKK1B	A 36	P11275	52	(*)	(*)	(*)
Dynactin	A 19	Q13561	50	(*)	(*)	(*)
Endopeptidase	A 29	P42676	80	(*)		
GABA-B α 2	A 36	Q08871	130	(*)	(*)	(*)
GSP12	A 33	Q88180	88	(*)		
M-Calpain	A 34	P10287	130	(*)		
MGluR1 (check)	A 19	P23385	133	(*)??		
Munc18	A 3	Q99P72	88	(*)		(*)
MYPT1	A 29	???	130			(*)
PKC- δ	A 18	P05771	80	(*)		
PKC- ϵ	A 19	Q05156	90	(*)		
PKC- ζ	A 30	P05129	80	(*)	(*)	(*)
PKC ζ 2	A 17	P11606	133		(*)	(*)
Rabphilin-3A	A 23	P47709	75	(*)		S
SNAP-25	A 7	P13795	25	(*)	(*)	(*)
Synapsin-IIa	A 13	Q63637	74	(*)	(*)	(*)
Synapsin-1	A 23	Q62910	140	(*)		(*)
Synaptotagmin	A 11	P51579	65	(*)		
<hr/>						
Autophagy	B 28	P17426	112	(*)		
Bad (check)	B 30	Q61337	23	(*)??	(*)	
B-Catenin	B 5	Q02248	92	(*)	(*)	(*)
Cathepsin L	B 25	P07711	43	(*)	(*)	(*)
Dynamin	B 26	P21575	100	(*)	(*)	(*)
Grb7	B 9	P46013	395	(*)	(*)	(*)
MEF2D	B 3	Q63943	70	(*)	(*)	(*)
NCK	B 2	P16333	47	(*)		
NSP1	B 24	Q9V2X4	72	(*)	(*)	(*)
P150Glued	B 26	P28023	150	(*)	(*)	(*)
PT190	B 3	Q13017	190	(*)	(*)	(*)
PKC- α	B 25	P17252	82	(*)	(*)	(*)
Rap-1 (check)	B 29	Q9NVS0	21	(*)?		
RhoGD1 (check)	B 3	P52566	28		(*)?	
SIRT1B	B 32	P55193	38	(*)		
SrinacDiablo	B 6	Q9NR28	22	(*)	(*)	(*)
TAF1170 or DRBP76	B 7	P49848; Q12906	72/90	(*)		
<hr/>						
Arp-3	C 18	P32391	50	(*)	(*)	(*)
CaMKK-IV	C 12	Q16566	60	(*)	(*)	(*)
c-Cbl	C 9	P22681	120	(*)		
HPH17	C 15	Q06508	86	(*)	(*)	(*)
Nck2	C 5	P51955	46	(*)		
P55 Cdc	C 8	Q98W56	55	(*)		
Profilin	C 28	???	15	(*)	(*)	
a- β -SNAP	C 21	P54920	35/36	(*)		(*)
Syntaxin	C 22	P71483	110	(*)		
TNRC	C 38	Q5UK55	150	(*)	(*)	(*)
<hr/>						
Catenin/yp120	D 32	P30999	120	(*)	(*)	(*)
PT90-B	D 28	Q13017	195	(*)	(*)	(*)
RC3N1	D 27	Q04912	40	(*)		(*)
Synapsin-Ia	D 38	P09951	80	(*)	(*)	(*)
<hr/>						
ATP Synthase a	E 21	??	55	(*)	(*)	(*)
BIT-Spectrin	E 8	??	240	(*)	(*)	(*)
B-Raf	E 25	??	95/72	(*)	(*)	(*)
CapZ a	E 8	??	37	(*)	(*)	(*)
Clathrin Heavy Chain	E 39	P11442	120	(*)	(*)	(*)
CIB1	E 26	Q91W16	48	(*)	(*)	(*)
JIP	E 13	Q90W19	112	(*)		(*)
NSF	E 13	P46459	82	(*)	(*)	(*)
SCAR-1	E 4	Q92588	80	(*)	(*)	(*)

HTPI approach allows rapid "hits" confirmation

Anti-βII-Spectrin

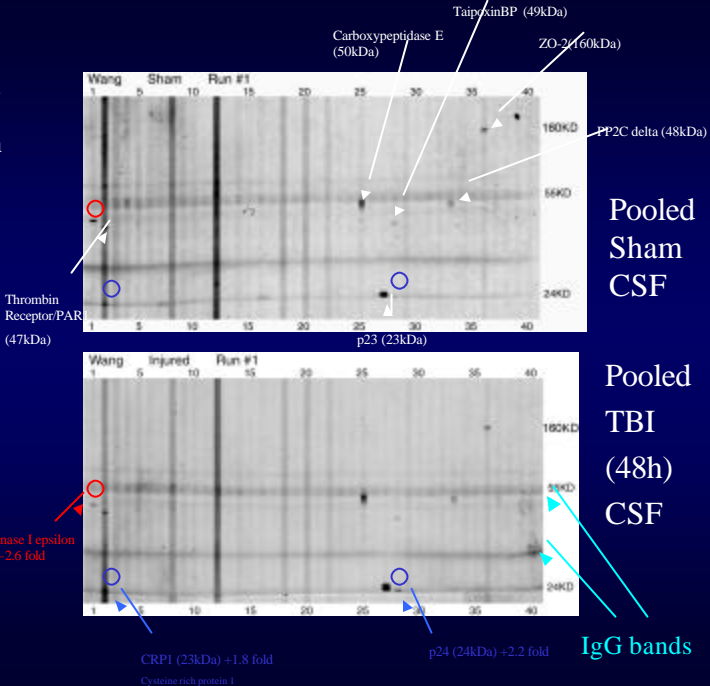


Rat CSF : TBI vs. Sham

-400 ug CSF protein loaded

Insufficient loading of CSF proteins as evidence of the IgG bands and the weak detection signal of most protein bands.

Yet, three proteins with significant increases or decreases in TBI are identified.



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