JOURNAL OF NEUROTRAUMA 30:775–788 (May 1, 2013)

DOI: 10.1089/neu.2012.2391

Detection of Structural and Metabolic Changes in Traumatically Injured Hippocampus by Quantitative Differential Proteomics

Ping Wu,[1] Yingxin Zhao,[2,6–8] Sigmund J. Haidacher,[2,5,6] Enyin Wang,[1] Margaret O. Parsley,[3] Junling Gao,[1] Rovshan G. Sadygov,[4,7,8] Jonathan M. Starkey,[4] Bruce A. Luxon,[4,7,8] Heidi Spratt,[4,7,8] Douglas S. DeWitt,[3] Donald S. Prough,[3] and Larry Denner[2,5–8]

Abstract

Traumatic brain injury (TBI) is a complex and common problem resulting in the loss of cognitive function. In order to build a comprehensive knowledge base of the proteins that underlie these cognitive deficits, we employed unbiased quantitative mass spectrometry, proteomics, and bioinformatics to identify and quantify dysregulated proteins in the CA3 subregion of the hippocampus in the fluid percussion model of TBI in rats. Using stable isotope ^{18}O-water differential labeling and multidimensional tandem liquid chromatography (LC)-MS/MS with high stringency statistical analyses and filtering, we identified and quantified 1002 common proteins, with 124 increased and 76 decreased. The Ingenuity Pathway Analysis (IPA) bioinformatics tool identified that TBI had profound effects on downregulating global energy metabolism, including glycolysis, the Krebs cycle, and oxidative phosphorylation, as well as cellular structure and function. Widespread upregulation of actin-related cytoskeletal dynamics was also found. IPA indicated a common integrative signaling node, calcineurin B1 (CANB1, CaNBα, or PPP3R1), which was downregulated by TBI. Western blotting confirmed that the calcineurin regulatory subunit, CANB1, and its catalytic binding partner PP2BA, were decreased without changes in other calcineurin subunits. CANB1 plays a critical role in downregulated networks of calcium signaling and homeostasis through calmodulin and calmodulin-dependent kinase II to highly interconnected structural networks dominated by tubulins. This large-scale knowledge base lays the foundation for the identification of novel therapeutic targets for cognitive rescue in TBI.

Key words: animal models; bioinformatics; mass spectrometry; proteomics; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is an important problem due to the serious pathophysiological processes that contribute tremendous health burdens to millions of patients, their relatives, and caregivers.[12,61,71,79] Clearly TBI also causes a broader spectrum of symptoms and permanent disabilities, including impaired motor, sensory, cognitive, mood, sleep, and other functions. Primary injury and secondary damages contribute to the loss of neurons and oligodendrocytes, degeneration of dendrites and axons, loss of synaptic connections, destruction of the vasculature and blood–brain barrier, and infiltration of immune cells. Since little can be done once the initial injury has occurred, efforts have been focused on finding ways to prevent or minimize secondary damage. The field now has a rich description of the events and processes that have been implicated in TBI, including inflammation, edema/ischemia, oxidative stress, excitotoxicity, neurogenesis, angiogenesis, synaptogenesis, endogenous stem cells, and glial scar formation using traditional reductionistic experimental approaches.[8,9,22,25,40,43,52,58,63,74,75]

Comprehensive and integrative approaches to investigate these diverse mechanisms have only been described recently and to a limited extent. The value of an unbiased, global, systems biology analysis of dysregulated protein expression is well-documented and often provides novel insights into information processing that occurs at a higher level of emergent principles than can be appreciated from the analysis of a handful of signaling molecules in isolation.[62,70] Mass spectrometry and 2D gel electrophoresis have been successfully employed to discover biomarkers in cerebrospinal fluid or

Departments of [1]Neuroscience and Cell Biology, [2]Internal Medicine, [3]Anesthesiology, and [4]Biochemistry and Molecular Biology, and [5]Stark Diabetes Center, [6]McCoy Stem Cells and Diabetes Mass Spectrometry Research Laboratory, [7]Sealy Center for Molecular Medicine, and [8]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas.

serum from adult and pediatric TBI patients.[7,11,13,19,23,24,26,37,53,77,83] While the identification of biomarkers is important to bring novel tools to the diagnosis and management of recovery after TBI, these markers rarely provide insight into the underlying pathophysiological mechanisms of TBI.

The learning and memory deficits consequent to TBI are well established.[6,14,15,45] The detailed mechanisms that subserve learning and memory include proper regulation and integration of synaptic structure and function to maintain efficacious information processing. Several integrative inter- and intracellular processes contribute to this complex phenomenon. The hippocampus is an essential region required for the acquisition and consolidation of learning before more permanent storage in other cortical brain structures. The molecular processes underlying these early events in memory formation are diverse and distributive, including metabolic sequelae, structural maintenance and reorganization, nuclear gene transcription, and protein synthesis, processing, and post-translational modification, as well as directed transport of cargo molecules into or out of the soma, axons, dendrites, and spines. Information processing, ultimately occurring through synaptic transmission between neurons, is finely regulated by other important cells in the central nervous system. These complex events are reflected in convergent regulation of signaling pathways and networks that determine how information is received, processed, and propagated, while ultimately affecting subsequent responses to additional information.

Among the key debilitating aspects of TBI are the learning and memory deficits due to the injury. Because the hippocampus has an essential role in learning and memory, proteomics studies in this area using gel-based analyses provide additional insights.[33,38] Previously, we reported that grafting human neural stem cells (hNSCs) into the injured hippocampus acutely improved cognitive function of TBI rats.[18] However, the underling mechanisms remain elusive, and require a basic knowledge of how TBI changes the overall systems biology of protein expression in the host hippocampus. Because of the varied signaling pathways, unique cellular architecture, and electrophysiological properties within each of the subregions of the hippocampus, in this study we focused particularly on the CA3 region, which is the gateway for information flow into and processing within the hippocampus. Our unbiased approach to delineate higher level signaling networks dysregulated by TBI in the CA3 region of the hippocampus has provided novel, previously unappreciated insights to the systemic level of dysfunction, and their underlying reductionistic mechanisms, in a brain region important for the initial stage of learning and memory that contribute to such challenging outcomes in TBI.[70]

Methodologically, stable isotope labeling combined with mass spectrometry provides a quantitative, sensitive, and accurate tool for the quantitative measurement of changes resulting from a targeted perturbation of a biological system,[49] and is becoming the gold standard for quantitative proteomics.[20,51,65] A variety of metabolic, chemical, and enzymatic methods have been developed for stable isotope labeling.[21,50] Here, we used stable isotope ^{18}O-water differential labeling,[27,78] 2D liquid chromatography-mass spectrometry/mass spectrometry (2D-LC-MS/MS), and bioinformatics tools to identify proteins that are unaffected, upregulated, and downregulated in the CA3 region of the hippocampus by TBI in a rat fluid percussion model. This systems biology approach provides a higher-level knowledge base of the integrative molecular mechanisms of TBI, insights into key regulatory networks and their regulatory nodes, and lays the groundwork for the discovery of novel therapeutic strategies.

Methods

Animals and fluid percussion traumatic brain injury

Fluid percussion was used to create TBI in rats according to our previous description with minor modifications.[18] All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, and were performed under aseptic conditions in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Ten male Sprague-Dawley (SD) rats (300 g; Charles River Laboratories International, Inc., Wilmington, MA) were randomly divided into two groups: sham and TBI, five rats per group.

Briefly, isoflurane-anesthetized rats were subjected to parasagittal fluid percussion TBI according to our previous descriptions.[4,16,18] Rectal and temporalis muscle temperatures were monitored throughout the surgery. Sham-injured rats were connected to the fluid percussion trauma device without injury. TBI rats were subjected to injury (2.0–2.1 atm) by a rapid injection of saline driven by the descent of a pendulum from a controlled height. The average time to recover the righting reflex in these injured rats was 17 min. To make a comparison of the current proteomics analysis with our previous transplantation studies, all animals received 1 μL vehicle injection into the hippocampal region on the injury side 1 day after TBI, and received cyclosporine in the drinking water as previously described.[18]

Tissue processing, protein extraction, tryptic digestion and ^{18}O-labeling

Four days after TBI the hippocampus ipsilateral to the injury was removed and the CA3 dissected from the remainder of the hippocampus under magnification with a dissecting microscope and processed as previously described.[57,62,67,70,81] The 4-day time point was chosen because: (1) it is between the peaks of injury-induced inflammation[60] and of reactive astrogliosis,[55] (2) there are metabolic changes during this time,[31,34] and (3) it allows settlement of grafted and host cells after surgery for future studies aimed to compare TBI-injured animals with and without cell transplantation. Given that fluid percussion injury affects both the ipsilateral and contralateral sides of an injured brain,[42] we focused on comparing the proteomic profile of the injury side to that of a sham control.

Briefly, Trizol-extracted and precipitated proteins were dissolved in guanidinium HCl. In each experimental group, 300 μg of protein from each of 5 sham rats and 5 TBI rats were pooled. The two pools, containing 1.5 mg of protein each, were separately reduced and alkylated followed by digestion with trypsin.

The ^{18}O-labeling was performed as previously described[57,62] with slight modifications. Briefly, the paired experimental peptide samples were resuspended in normal water ($H_2{}^{16}O$) for sham, or heavy water ($H_2{}^{18}O$) for TBI, containing ammonium bicarbonate and immobilized trypsin. The corresponding ^{18}O-labeled and ^{16}O-unlabeled samples were pooled, dried, and desalted.

Two-dimensional liquid chromatography with tandem mass spectrometry

Pooled differentially-labeled peptides were separated and analyzed as previously described.[57,62] Briefly, the pools were resolved into 60 fractions by strong cation exchange chromatography. Each fraction was then analyzed in triplicate using LC-MS/MS performed with an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanospray source with an on-line ProteomX® nano-HPLC system (ThermoFinnigan). The mass spectrometer was operated in the data-dependent triple play mode. The three most intense ions in each MS survey scan were automatically selected for Zoomscan and MS/MS.

Data processing

The acquired MS/MS spectra were processed as previously described using MassXplorer developed in our laboratory.[57] Briefly, spectra were searched against a composite target-decoy rat protein database consisting of the target and decoy (reversed) protein sequences downloaded from the SWISSPROT Protein Database with the SEQUEST algorithm using the Bioworks 3.2 platform (ThermoFinnigan).

The zoom scan data were used to calculate the relative abundance ratios of ^{18}O-labeled peptide/^{16}O-unlabeled peptide pairs using MassXplorer.[57] We used power spectrum transformation to remove high-frequency noise and contributions from co-eluting species, to determine the elemental composition of the sequences to compute theoretical isotopic distributions for determination of peak positions, and for curve fitting to calculate the ratios.

Statistical analysis

Peptides with charge > 3, false-discovery rate $> 3\%$, ^{18}O:^{16}O ratios < 0.1 or > 10, and reversed sequences, were removed from further analysis. Calculated peptide ratios were $\log_2$ transformed and mean centered prior to statistical analysis. Significance was determined by the Wilcoxon rank-sum test (due to concerns regarding data distribution), with Benjamini-Hochberg false-discovery rate correction for multiple testing comparisons as indicated.[5] Finally, the data were analyzed through the use of Ingenuity Pathway Analysis (IPA®; Ingenuity Systems, Redwood City, CA), with a significance cutoff of $p \leq 0.05$ and 20% change in protein expression. The lines between proteins in Figures 2, 3, and 5 represent relationships derived from IPA Network Explorer and Canonical Pathways in the Ingenuity Knowledge Base, with further definitions available at www.ingenuity.com.

These studies were performed to identify the most robust, consistent changes that emerge in a population of animals (TBI or sham) without the idiosyncratic differences of individual animals. Thus we used five biological replicates per group to generate results that are reflective of the populations of animals with and without TBI. Further, the small amount of material in the CA3 of an individual animal, in conjunction with the extensive sample handling and mass spectrometry analyses, prevented the comparison of individual animals. To minimize the false-positive identification of differential expression, we required high-stringency filtering limits of mass spectrometry data, multiple peptide hits per protein with multiple measures, and highly rigorous statistical treatment of peptide and protein expression, as well as differential expression values. Similar rigorous approaches have been successfully used in several other valuable and important neuroproteomics studies.[37,53]

Western blot analysis

Further validation was carried out to quantitatively detect the protein expression levels of four calcineurin subunits and isoforms. Western blot analysis was performed as previously described.[66] Briefly, 5 or 50 μg of protein samples were probed with isoform and subunit specific primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), including polyclonal goat anti-PP2B-Aα (PP2BA; 1:200), anti-PP2B-Aβ (PP2BB; 1:200), anti-PP2B-B1 (CaNB1; 1:200), and anti-PP2B-B2 (CaNB2; 1:200). Blots were probed simultaneously with β-actin (1:20,000; Sigma-Aldrich, St. Louis, MO) as a loading control. Following stripping (Restore™; Pierce Biotechnology, Rockford, IL), the blots were reprobed for a second control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1,000; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were used at dilutions of 1:5000–1:10,000 (GE Healthcare, Hertfordshire, U.K.). ECL hyperfilms (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) were subsequently scanned, and densitometry analyses were performed with AlphaEase FC Software (Alpha Innotech, Santa Clara, CA). All data were normalized against β-actin or GAPDH and compared among blots. Statistical significance between groups was determined using Student's t-test.

Results

Quantitative proteomics strategy

In these studies, hippocampal CA3 tissue was extracted from TBI rats at ~5 months of age. Differential stable isotope labeling was performed on tryptic peptides from TBI samples labeled with ^{18}O-water, while peptides from control samples contained normal ^{16}O. Using our recently developed spectral analysis program for quantification of ^{18}O:^{16}O peptide ratios, MassXplorer,[57] we quantified expression of 38,135 peptide ratios (Supplemental Table S1; see online supplementary material at http://www.liebertonline.com). After filtering, 33,303 peptides were used for calculation of relative protein expression. To increase the quality of the proteins subjected to further analysis, we used the additional criteria of at least 2 distinct peptides and 3 total measurements to identify and quantify 1002 proteins common to the sham and TBI samples, the majority of which were unchanged by TBI (Supplemental Table S2; see online supplementary material at http://www.liebertonline.com). Determination of significance yielded 200 proteins dysregulated by TBI. The 124 upregulated proteins (Table 1) were clustered into groups of the actin-related cytoskeleton, neuronal structure and development/reorganization, neuronal transport and neurotransmitter action, and other. Of the 76 proteins significantly downregulated by TBI (Table 2), many were related to energy metabolism (Fig. 1). Nearly all the enzymes of the glycolytic pathway were downregulated. Enzymes regulating flux to and within the Krebs cycle were downregulated, as were many key intermediates of oxidative phosphorylation in the electron transport chain. Large-scale disruption of ATP synthesis and transport was also evident. Finally, key enzymes in calcium signaling and homeostasis were downregulated (Table 2).

Bioinformatics studies

IPA was used to analyze the literature-based interconnectivity relationships among the 1002 identified and quantified proteins.

Associated network functions. The top associated network functions for all 1002 proteins were Cell Morphology, Cellular Assembly and Organization, and Genetic Disorder, with a score of 41, as well as those of Protein Synthesis, Cancer, and Gastrointestinal Disease, also with a score of 41, where score $= -\log(p)$ value (Table 3). For the subset of 124 upregulated proteins, the top associated network functions were Cellular Assembly, Organization, and Function; and Nervous System Development and Function; with a score of 41. For the 76 downregulated proteins, the top scoring networks were for Energy Production, Nucleic Acid Metabolism, and Small Molecule Biochemistry, with an extremely high score of 64.

Biological functions. Analysis of the molecular and cellular functions (Table 4, upper portion) showed that the highest probability upregulated proteins were all related to cells, particularly those related to cell structure and function. These were led by the functional groups of Cellular Assembly and Organization (50 proteins) and Cell Morphology (43 proteins). The highest-scoring individual network of Cellular Assembly (Fig. 2) was predominated by connectivity with actin, neurofilament heavy, medium, and light (NEFH, NEFM, and NEFL), and vimentin (VIM).

Table 1. Proteins Upregulated by Traumatic Brain Injury

IPA name	*Ratio*	*Accession no.*	*Uniprot name*	*Protein name*
Actin-related				
CAPG	4.01	Q6AYC4	CAPG	Macrophage-capping protein
HLA-C	3.49	P16391	HA12	RT1 class I histocompatibility antigen; AA alpha chain precursor
TPM4	3.23	P09495	TPM4	Tropomyosin alpha-4 chain
HSPB1	2.27	P42930	HSPB1	Heat shock protein beta-1
EVL	2.08	O08719	EVL	Ena/VASP-like protein
CNN3	1.73	P37397	CNN3	Calponin-3
MARCKS	1.73	P30009	MARCS	Myristoylated alanine-rich C-kinase substrate
SEPT5	1.51	Q9JJM9	SEPT5	Septin-5
PPP1R9A	1.42	O35867	NEB1	Neurabin-1
PPP1R9B	1.40	O35274	NEB2	Neurabin-2
MYH9	1.37	Q62812	MYH9	Myosin-9
ACTN4	1.26	Q9QXQ0	ACTN4	Alpha-actinin-4
ACTA2	1.26	P62738	ACTA	Actin; aortic smooth muscle
ACTR2	1.24	Q5M7U6	ARP2	Actin-related protein 2
MYH10	1.15	Q9JLT0	MYH10	Myosin-10
ACTB	1.10	P60711	ACTB	Actin; cytoplasmic 1
Neuronal structure and development/reorganization				
BCAS1	2.39	Q3ZB98	BCAS1	Breast carcinoma-amplified sequence 1 homolog
PRPH	2.22	P21807	PERI	Peripherin
VIM	1.93	P31000	VIME	Vimentin
ODF4	1.74	Q6AXT9	ODFP4	Outer dense fiber protein 4
MAG	1.66	P07722	MAG	Myelin-associated glycoprotein precursor
APP	1.61	P08592	A4	Amyloid beta A4 protein precursor
GAP43	1.56	P07936	NEUM	Neuromodulin
ERMN	1.54	Q5RJL0	ERMIN	Ermin
DPYSL3	1.47	Q62952	DPYL3	Dihydropyrimidinase-related protein 3
BCAN	1.44	P55068	PGCB	Brevican core protein precursor
NEFL	1.42	P19527	NFL	Neurofilament light polypeptide
CADM2	1.41	Q1WIM2	CADM2	Cell adhesion molecule 2 precursor
NEFH	1.41	P16884	NFH	Neurofilament heavy polypeptide
NRCAM	1.39	P97686	NRCAM	Neuronal cell adhesion molecule precursor
BASP1	1.33	Q05175	BASP	Brain acid soluble protein 1
NEFM	1.29	P12839	NFM	Neurofilament medium polypeptide
MBP	1.27	P02688	MBP	Myelin basic protein S
INA	1.24	P23565	AINX	Alpha-internexin
MAP1A	1.24	P34926	MAP1A	Microtubule-associated protein 1A
CNTN1	1.23	Q63198	CNTN1	Contactin-1 precursor
MAP1B	1.17	P15205	MAP1B	Microtubule-associated protein 1B
MAP2	1.15	P15146	MAP2	Microtubule-associated protein 2
PLEC	1.11	P30427	PLEC1	Plectin-1
Neuronal transport and neurotransmitter action				
CHGB	3.11	O35314	SCG1	Secretogranin-1 precursor
TMED10	2.46	Q63584	TMEDA	Transmembrane emp24 domain-containing protein 10 precursor
DYNC1I2	1.98	Q62871	DC1I2	Cytoplasmic dynein 1 intermediate chain 2
CPNE9	1.83	Q5BJS7	CPNE9	Copine-9
USO1	1.77	P41542	USO1	General vesicular transport factor p115
HOMER3	1.49	Q9Z2X5	HOME3	Homer protein homolog 3
PACSIN1	1.20	Q9Z0W5	PACN1	Protein kinase C and casein kinase substrate in neurons protein
BSN	1.20	O88778	BSN	Protein bassoon
HSPA5	1.18	P06761	GRP78	78 kDa glucose-regulated protein precursor
NSF	1.15	Q9QUL6	NSF	Vesicle-fusing ATPase
SYN1	1.08	P09951	SYN1	Synapsin-1
Other				
LONP2	4.67	Q3MIB4	LONP2	Peroxisomal Lon protease homolog 2
NPTXR	3.54	O35764	NPTXR	Neuronal pentraxin receptor
HBB	3.19	P02091	HBB1	Hemoglobin subunit beta-1
SLC6A6	3.06	P31643	SC6A6	Sodium- and chloride-dependent taurine transporter
unmapped	2.86	P01836	KACA	Ig kappa chain C region; A allele
PRSS2	2.65	P00763	TRY2	Anionic trypsin-2 precursor
PZP	2.24	Q63041	A1M	Alpha-1-macroglobulin precursor
C3	2.21	P01026	CO3	Complement C3 precursor

(*continued*)

Table 1. (Continued)

IPA name	*Ratio*	*Accession no.*	*Uniprot name*	*Protein name*
CST3	2.18	P14841	CYTC	Cystatin-C precursor
LXN	2.10	Q64361	LXN	Latexin
DDX46	1.96	Q62780	DDX46	Probable ATP-dependent RNA helicase DDX46
EIF3B	1.95	Q4G061	EIF3B	Eukaryotic translation initiation factor 3 subunit B
DUSP4	1.95	Q62767	DUS4	Dual specificity protein phosphatase 4
RAP1B	1.93	Q62636	RAP1B	Ras-related protein Rap-1b precursor
ATP4A	1.92	P09626	ATP4A	Potassium-transporting ATPase alpha chain 1
SOX10	1.88	O55170	SOX10	Transcription factor SOX-10
HIST1H1D	1.85	P15865	H12	Histone H1.2
LMNA	1.81	P48679	LMNA	Lamin-A
RPS20	1.80	P60868	RS20	40S ribosomal protein S20
PTGES3	1.78	P83868	TEBP	Prostaglandin E synthase 3
GFAP	1.75	P47819	GFAP	Glial fibrillary acidic protein
LRRC8E	1.74	Q3KRC6	LRC8E	Leucine-rich repeat-containing protein 8E
ADCY8	1.73	P40146	ADCY8	Adenylate cyclase type 8
CLU	1.73	P05371	CLUS	Clusterin precursor
ALB	1.69	P02770	ALBU	Serum albumin precursor
NDUFS4	1.68	Q5XIF3	NDUS4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4; mitochondrial
ITM2B	1.67	Q5XIE8	ITM2B	Integral membrane protein 2B
SSRP1	1.66	Q04931	SSRP1	FACT complex subunit SSRP1
PPP5C	1.64	P53042	PPP5	Serine/threonine-protein phosphatase 5
GSK3B	1.61	P18266	GSK3B	Glycogen synthase kinase-3 beta
RPL17	1.59	P24049	RL17	60S ribosomal protein L17
PGRMC1	1.59	P70580	PGRC1	Membrane-associated progesterone receptor component 1
PDIA4	1.55	P38659	PDIA4	Protein disulfide-isomerase A4 precursor
GM10117	1.55	P17077	RL9	60S ribosomal protein L9
NME2	1.54	P19804	NDKB	Nucleoside diphosphate kinase B
HBA1	1.52	P01946	HBA	Hemoglobin subunit alpha-1/2
KHSRP	1.52	Q99PF5	FUBP2	Far upstream element-binding protein 2
PTK2B	1.51	P70600	FAK2	Protein tyrosine kinase 2 beta
RPLP2	1.51	P02401	RLA2	60S acidic ribosomal protein P2
PDIA6	1.50	Q63081	PDIA6	Protein disulfide-isomerase A6 precursor
PIP4K2B	1.49	O88377	PI42B	Phosphatidylinositol-5-phosphate 4-kinase type-2 beta
STIP1	1.49	O35814	STIP1	Stress-induced-phosphoprotein 1
GPD1	1.46	O35077	GPDA	Glycerol-3-phosphate dehydrogenase [NAD+]; cytoplasmic
AKAP12	1.45	Q5QD51	AKA12	A-kinase anchor protein 12
RNPEP	1.41	O09175	AMPB	Aminopeptidase B
DBI	1.40	P11030	ACBP	Acyl-CoA-binding protein
ANXA3	1.40	P14669	ANXA3	Annexin A3
HCG 25371	1.39	Q5M9I5	QCR6	Cytochrome b-c1 complex subunit 6; mitochondrial precursor
HNRNPH1	1.39	Q8VHV7	HNRH1	Heterogeneous nuclear ribonucleoprotein H
ABI1	1.39	Q9QZM5	ABI1	Abl interactor 1
ACSBG1	1.39	Q924N5	ACBG1	Long-chain-fatty-acid CoA ligase ACSBG1
APOE	1.38	P02650	APOE	Apolipoprotein E precursor
PLCB1	1.37	P10687	PLCB1	1-phosphatidylinositol-4;5-bisphosphate phosphodiesterase beta
CA2	1.36	P27139	CAH2	Carbonic anhydrase 2
THY1	1.35	P01830	THY1	Thy-1 membrane glycoprotein precursor
HSP90AB1	1.33	P34058	HS90B	Heat shock protein HSP 90-beta
IDH3B	1.32	Q68FX0	IDH3B	Isocitrate dehydrogenase [NAD] subunit beta; mitochondrial precursor
PPIA	1.30	P10111	PPIA	Peptidyl-prolyl cis-trans isomerase A
ENO3	1.30	P15429	ENOB	Beta-enolase
ATP5C1	1.29	P35435	ATPG	ATP synthase subunit gamma; mitochondrial
ATP6V1E1	1.29	Q6PCU2	VATE1	Vacuolar proton pump subunit E 1
GOT1	1.29	P13221	AATC	Aspartate aminotransferase; cytoplasmic
NDUFV2	1.28	P19234	NDUV2	NADH dehydrogenase [ubiquinone] flavoprotein 2; mitochondrial
HSPA2	1.27	P14659	HSP72	Heat shock-related 70 kDa protein 2
UCHL1	1.26	Q00981	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1
YWHAH	1.25	P68511	1433F	14-3-3 protein eta
PRKCA	1.25	P05696	KPCA	Protein kinase C alpha type
TKT	1.24	P50137	TKT	Transketolase
HYOU1	1.24	Q63617	HYOU1	Hypoxia up-regulated protein 1 precursor
CCT5	1.22	Q68FQ0	TCPE	T-complex protein 1 subunit epsilon
PRDX6	1.21	O35244	PRDX6	Peroxiredoxin-6
SPTAN1	1.21	P16086	SPTA2	Spectrin alpha chain; brain
CCDC92	1.17	P09606	GLNA	Glutamine synthetase
PEA15	1.12	Q5U318	PEA15	Astrocytic phosphoprotein PEA-15

Table 2. Proteins Downregulated by Traumatic Brain Injury

IPA name	*Ratio*	*Accession no.*	*Uniprot name*	*Protein name*
Glycolysis				
HK1	0.88	P05708	HXK1	Hexokinase-1
ALDOC	0.68	P09117	ALDOC	Fructose-bisphosphate aldolase C
TPI1	0.89	P48500	TPIS	Triosephosphate isomerase
PGAM1	0.82	P25113	PGAM1	Phosphoglycerate mutase 1
ENO1	0.85	P04764	ENOA	Alpha-enolase
PKM2	0.93	P11980	KPYM	Pyruvate kinase isozymes M1/M2
Krebs cycle				
LDHA	0.89	P04642	LDHA	L-lactate dehydrogenase A chain
PDHB	0.62	P49432	ODPB	Pyruvate dehydrogenase E1 component subunit beta; mitochondrial
ACO2	0.93	Q9ER34	ACON	Aconitate hydratase; mitochondrial precursor
Oxidative phosphorylation/electron transport chain				
NDUFA10	0.76	Q561S0	NDUAA	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10
NDUFA9	0.89	Q5BK63	NDUA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9
SDHA	0.88	Q920L2	DHSA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit; mitochondrial
UQCRC2	0.84	P32551	QCR2	Cytochrome b-c1 complex subunit 2; mitochondrial precursor
ATP synthesis and transport				
SLC25A4	0.67	Q05962	ADT1	ADP/ATP translocase 1
ATP5O	0.39	Q06647	ATPO	ATP synthase subunit O; mitochondrial precursor
ATP5F1	0.67	P19511	AT5F1	ATP synthase subunit b; mitochondrial precursor
ATP5A1	0.85	P15999	ATPA	ATP synthase subunit alpha; mitochondrial precursor
ATP5B	0.89	P10719	ATPB	ATP synthase subunit beta; mitochondrial precursor
ATP1A1	0.66	P06685	AT1A1	Sodium/potassium-transporting ATPase subunit alpha-1 precursor
ATP1A3	0.85	P06687	AT1A3	Sodium/potassium-transporting ATPase subunit alpha-3
Calcium signaling and homeostasis				
PPP3R1	0.58	P63100	CANB1	Calcineurin subunit B isoform 1
CAMK2D	0.49	P15791	KCC2D	Calcium/calmodulin-dependent protein kinase type II delta chain
CALM1	0.56	P62161	CALM	Calmodulin
ATP2B2	0.73	P11506	AT2B2	Plasma membrane calcium-transporting ATPase 2
ATP2B3	0.74	Q64568	AT2B3	Plasma membrane calcium-transporting ATPase 3
SLC8A1	0.67	Q01728	NAC1	Sodium/calcium exchanger 1 precursor
Other				
ZNF22	0.21	Q9ERU2	ZNF22	Zinc finger protein 22
MAPRE1	0.26	Q66HR2	MARE1	Microtubule-associated protein RP/EB family member 1
DYNC2H1	0.27	Q9JJ79	DYHC2	Cytoplasmic dynein 2 heavy chain 1
RAB1B	0.31	P10536	RAB1B	Ras-related protein Rab-1B
RPL23	0.34	P62832	RL23	60S ribosomal protein L23
RALA	0.43	P63322	RALA	Ras-related protein Ral-A precursor
RPL22	0.48	P47198	RL22	60S ribosomal protein L22
DPYSL4	0.48	Q62951	DPYL4	Dihydropyrimidinase-related protein 4
SLC1A2	0.50	P31596	EAA2	Excitatory amino acid transporter 2
SFXN5	0.51	Q8CFD0	SFXN5	Sideroflexin-5
PLS3	0.52	Q63598	PLST	Plastin-3
CKMT1B	0.53	P25809	KCRU	Creatine kinase; ubiquitous mitochondrial precursor
SFXN1	0.55	Q63965	SFXN1	Sideroflexin-1
DSTN	0.56	Q7M0E3	DEST	Destrin
PRDX2	0.57	P35704	PRDX2	Peroxiredoxin-2
ANXA5	0.58	P14668	ANXA5	Annexin A5
VDAC3	0.58	Q9R1Z0	VDAC3	Voltage-dependent anion-selective channel protein 3
ALDH5A1	0.59	P51650	SSDH	Succinate-semialdehyde dehydrogenase
ASRGL1	0.59	Q8VI04	ASGL1	L-asparaginase
HDHD2	0.62	Q6AYR6	HDHD2	Haloacid dehalogenase-like hydrolase domain-containing protein
VAMP2	0.63	P63045	VAMP2	Vesicle-associated membrane protein 2
TUBB3	0.65	Q4QRB4	TBB3	Tubulin beta-3 chain
EEF1A2	0.66	P62632	EF1A2	Elongation factor 1-alpha 2
ACAT1	0.67	P17764	THIL	Acetyl-CoA acetyltransferase; mitochondrial precursor
RHOB	0.67	P62747	RHOB	Rho-related GTP-binding protein RhoB precursor
PHB2	0.74	Q5XIH7	PHB2	Prohibitin-2

(*continued*)

Table 2. (Continued)

IPA name	*Ratio*	*Accession no.*	*Uniprot name*	*Protein name*
SYN2	0.74	Q63537	SYN2	Synapsin-2
AK1	0.75	P39069	KAD1	Adenylate kinase isoenzyme 1
TUBB2C	0.77	Q6P9T8	TBB2C	Tubulin beta-2C chain
RAB3C	0.78	P62824	RAB3C	Ras-related protein Rab-3C
PFN1	0.78	P62963	PROF1	Profilin-1
AP2A2	0.78	P18484	AP2A2	AP-2 complex subunit alpha-2
S100A1	0.79	P35467	S10A1	Protein S100-A1
HCG 2023776	0.80	P04256	ROA1	Heterogeneous nuclear ribonucleoprotein A1
YWHAZ	0.80	P63102	1433Z	14-3-3 protein zeta/delta
EIF5A	0.81	Q3T1J1	IF5A1	Eukaryotic translation initiation factor 5A-1
GDA	0.81	Q9WTT6	GUAD	Guanine deaminase
TNR	0.81	Q05546	TNR	Tenascin-R precursor
HSPD1	0.84	P63039	CH60	60 kDa heat shock protein; mitochondrial precursor
TUBA1B	0.85	Q6P9V9	TBA1B	Tubulin alpha-1B chain
RAB3A	0.86	P63012	RAB3A	Ras-related protein Rab-3A
GNB1	0.86	P54311	GBB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta
YWHAE	0.87	P62260	1433E	14-3-3 protein epsilon
GNAI1	0.89	P10824	GNAI1	Guanine nucleotide-binding protein G(i); alpha-1 subunit
TUBB2A	0.89	P85108	TBB2A	Tubulin beta-2A chain
YWHAB	0.89	P35213	1433B	14-3-3 protein beta/alpha
CNP	0.90	P13233	CN37	2′;3′-cyclic-nucleotide 3′-phosphodiesterase
ENO2	0.92	P07323	ENOG	Gamma-enolase
TUBA1A	0.94	P68370	TBA1A	Tubulin alpha-1A chain
STXBP1	0.95	P61765	STXB1	Syntaxin-binding protein 1

The molecular and cellular functions (Table 4, upper portion) of the highest-probability downregulated proteins were centered on metabolic dysfunction, including Carbohydrate Metabolism (11 proteins), Energy Production (13 proteins), and Small Molecule Biochemistry (37 proteins). The individual network that illustrated these properties included proteins important for ATP synthesis and transport (Fig. 3, lower portion), which are largely connected through the 14-3-3 adaptor proteins (YWHAZ, YWHAE, and YWHAB) to structural proteins, predominantly tubulins (Fig. 3, upper portion).

Analysis of physiological system development and function (Table 4, lower portion) indicated that the highest probabilities were in Nervous System Development and Function for both upregulated proteins (46 proteins) and downregulated proteins (15 proteins).

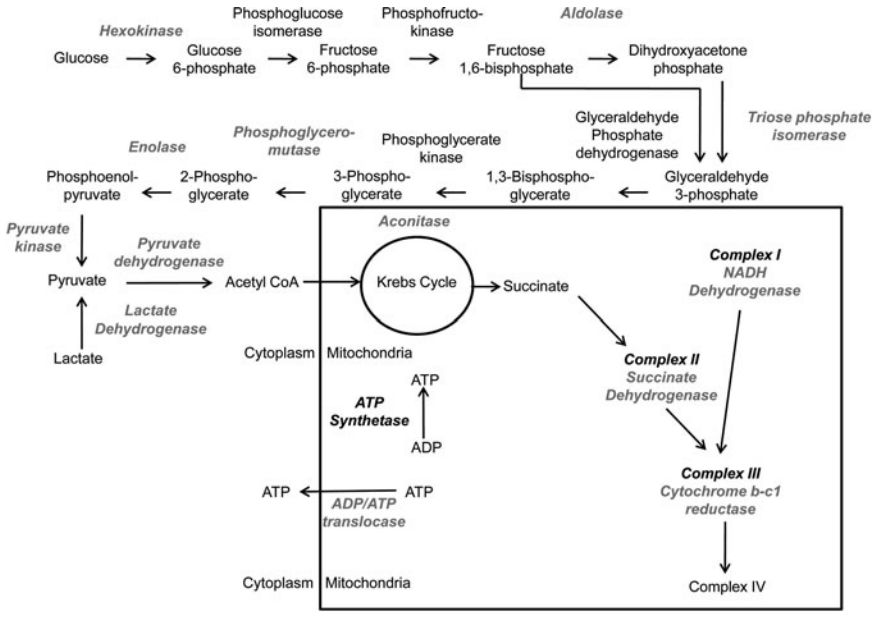

FIG. 1. Global metabolic dysfunction induced by traumatic brain injury (TBI). Differential stable isotope mass spectrometry led to the determination that many enzymes related to energy metabolism were coordinately downregulated by TBI compared to sham controls (Modified from Lehninger, 1970[41]).

Table 3. Associated Network Functions

All 1002 identified and quantified proteins	*Score*[a]
Cell morphology, cellular assembly and organization, genetic disorder	41
Protein synthesis, cancer, gastrointestinal disease	41
Molecular transport, small molecule biochemistry, cellular assembly/organization	38
Cell signaling, cellular assembly and organization, cellular function/maintenance	38
Cellular assembly and organization, genetic disorder, metabolic disease	38
124 Upregulated proteins	
Cellular assembly, organization, function; nervous system development	41
Cardiovascular disease, neurological disease, amino acid metabolism	40
Cellular assembly, organization, function; neurological disease	37
Nervous system development and function, cell death, cellular development	30
Nucleic acid metabolism, small molecule biochemistry, molecular transport	29
76 Downregulated proteins	
Energy production, nucleic acid metabolism, small molecule biochemistry	64
Cell death, energy production, molecular transport	46
Carbohydrate metabolism, infectious disease, inflammatory disease	37
Cell death, cell signaling, molecular transport	21

[a]Rank of networks according to their degree of relevance to the total network eligible molecules in the dataset of proteins taking into account the total number of molecules in the Ingenuity Knowledge Base that could potentially be included in networks.

Table 4. Top Biological Functions of Differentially-Regulated Proteins

Name	p *Value*[a]	*No. proteins*[b]
Molecular and cellular function		
Upregulated		
Cellular assembly and organization	1.16E-13–7.95E-03	50
Cell morphology	6.58E-11–7.95E-03	43
Cellular function and maintenance	1.88E-08–7.95E-03	31
Cellular compromise	1.70E-07–7.95E-03	23
Cell-to-cell signaling and interaction	4.89E-07–7.95E-03	29
Downregulated		
Carbohydrate metabolism	7.34E-08–3.90E-02	11
Energy production	2.83E-07–3.90E-02	13
Nucleic acid metabolism	2.83E-07–4.85E-02	19
Small molecule biochemistry	2.83E-07–4.96E-02	37
DNA replication, recombination, and repair	2.04E-06–3.68E-02	7
Physiological system development and function		
Upregulated		
Nervous system development and function	1.45E-13–7.95E-03	46
Behavior	2.84E-04–3.21E-04	8
Organismal survival	3.65E-04–1.98E-03	7
Cardiovascular system development and function	3.72E-04–7.95E-03	9
Tissue development	3.72E-04–7.95E-03	18
Downregulated		
Nervous system development and function	2.20E-04–4.85E-02	15
Cardiovascular system development and function	5.01E-04–3.90E-02	6
Organ morphology	5.01E-04–3.42E-02	4
Hematological system development and function	4.96E-03–3.90E-02	4
Skeletal, muscular system development, function	4.96E-03–3.90E-02	8

[a]Likelihood that the association between the proteins in our entire dataset and a related function is due to random association.
[b]Number of proteins within each subcategory of functions.

Canonical pathways. The highest-ranked canonical pathways (Table 5) for upregulated proteins were signaling by protein kinase A and RhoA, in addition to endocytosis. All five top downregulated canonical pathways had very high *p* values, and were focused on metabolic control, including those for Glycolysis and Gluconeogenesis, Oxidative Phosphorylation, 14-3-3-Mediated Signaling, and Pyruvate Metabolism.

Downregulation of calcineurin B1 (CANB1 or PPP3R1) in TBI

Using an unbiased, quantitative, differential stable isotope labeling approach, we found the ratio of the CANB1 in TBI animals compared to sham animals was 0.58, with a *p* value of < 1E-14, corresponding to a 42% decrease (Table 2, UniProt Name CANB1, Accession no. P63100, calcineurin subunit B isoform 1; see Supplemental Table S3 for additional calcineurin nomenclature; see online supplementary material at http://www.liebertonline.com). CANB1 was identified by five peptides (Table 6 and Supplemental Table S1; see online supplementary material at http://www.liebertonline.com), that were entirely unique to CANB1, thus allowing unambiguous assignment to this subunit of the calcineurin subunits. Each of these five peptides, whose positions within the protein are indicated (Table 6), was identified multiple times with a total of 197 quantifications. PP2BA, the catalytic subunit that binds the CANB1 regulatory subunit to form a functional calcineurin enzyme, was slightly reduced by TBI to a ratio of 0.88 (12% decrease), but with a *p* value of 0.052, which was slightly below significance (Supplemental Table S2, UniProt Name PP2BA, Accession no. P63329).

Western blot analysis confirmed a 55.2% downregulation of CANB1 in TBI (Fig. 4A). Since Western blots are more sensitive to small changes than mass spectrometry, we were able to determine that the CANB1 catalytic partner, PP2BA (Fig. 4B), was downregulated by 23.8%. Finally, the regulatory subunit CANB2 and its catalytic partner PP2BB were unaffected by TBI (Fig. 4C and D).

Numerous lines of evidence indicate that these effects are specific and unrelated to the administration of FK506 and cyclosporine (CsA) to the animals. First, the half-life of FK506 is far shorter than the 4 days that passed between administration and sacrifice.[69] Second, CsA does not directly affect PP2BA levels in CA3.[82] Our recent publication also supports the hypothesis that changes in calcineurin are independent of FK506 or CsA.[70] Third, transplantation of neural stem cells into rats treated with these two agents rescued the TBI-induced decrease. Fourth, in an *in vitro* model of traumatic axonal injury that emulates many of the characteristics of

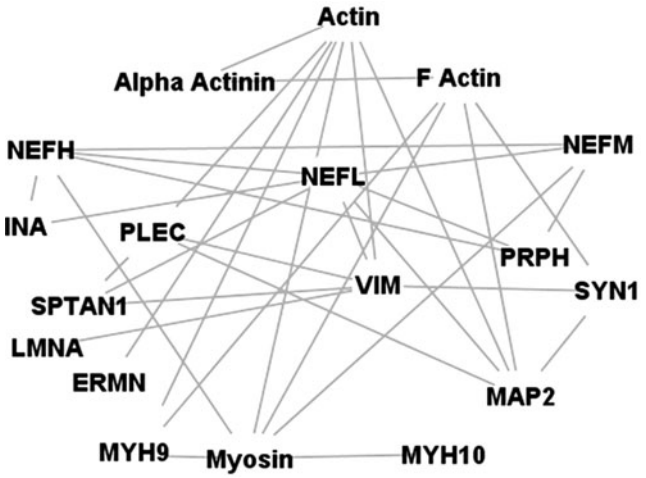

FIG. 2. Highest scoring upregulated network: Cellular Assembly. Ingenuity Pathway Analysis of upregulated proteins shows extensive dysregulation of cytoskeletal proteins in traumatic brain injury (TBI). All listed proteins are significantly changed by TBI compared to sham controls ($p \leq 0.05$ by Benjamini-Hochberg rank-sum statistical analysis). Lines between proteins represent relationships derived from the IPA Network Explorer and Canonical Pathways in the Ingenuity Knowledge Base (ERMA, ermin; INA, alpha internexin; LMNA, lamin-A; MAP2, microtubule associated protein 2; MYH9, myosin-9; MYH10, myosin-10; NEFH, NEFM, NEFL, neurofilaments heavy, medium, and light; PLEC, plektin-1; PRPH, peripherin; SPTAN1, spectrin alpha chain, brain; SYN1, synapsin-1; VIM, vimentin);

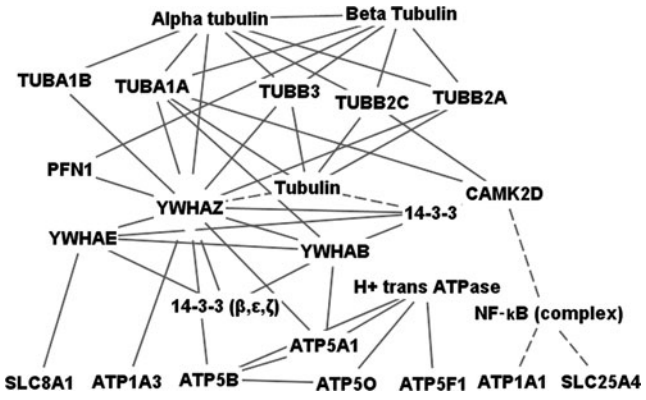

FIG. 3. Highest-scoring downregulated network: Energy Production, Metabolism and Cell Structure. Ingenuity Pathway Analysis of downregulated proteins shows substantial dysregulation of ATP-related enzymes, the 14-3-3 (YWHA proteins), signaling intermediates, and tubulins, in traumatic brain injury (TBI). All listed proteins are significantly changed by TBI compared to sham controls ($p \leq 0.05$ by Benjamini-Hochberg rank-sum statistical analysis). Lines between proteins represent relationships derived from IPA Network Explorer and Canonical Pathways in the Ingenuity Knowledge Base (ATP1A1 and ATP1A3, sodium/potassium-transporting ATPase subunits alpha-1 and alpha-3; ATP5A1, ATP5B, ATP5F1, ATP5O, ATP synthase subunits alpha, beta, b, and O; CAMK2D, calcium/calmodulin-dependent protein kinase type II delta chain; PFN1, profilin-1; SLC-8A1, sodium/calcium exchanger 1; SLC25A4, ADP/ATP translocase 1; TUBA1A, TUBA1B, TUBB2A, TUBB2C, TUBB3, tubulin alpha-1A, alpha-1B, beta-2A, beta-2C, and beta-3; YWHAB, YWHAE, YWHAZ, 14-3-3 β, ε, and ζ; NF-κB, nuclear factor-κB).

Table 5. Canonical Pathways of Differentially-Regulated Proteins

Name	p *Value*[a]	*Ratio*[b]
Upregulated		
Protein kinase A signaling	1.35E-04	10/319 (0.031)
RhoA signaling	2.09E-04	6/110 (0.055)
Caveolar-mediated endocytosis	3.03E-04	5/85 (0.059)
Downregulated		
Glycolysis/gluconeogenesis	4.70E-11	10/142 (0.07)
Oxidative phosphorylation	7.40E-07	8/165 (0.048)
14-3-3-Mediated signaling	1.17E-06	7/114 (0.061)
Pyruvate metabolism	4.70E-05	5/149 (0.034)

[a]Measure of the likelihood that the association between the functional analysis proteins in this experiment and a given pathway is due to random chance.
[b]Number of proteins that participate in a canonical pathway divided by the total number of proteins in the IPA Knowledge Base of Canonical Pathways.

TBI, similar injury-dependent decreases in calcineurin protein and catalytic activity were found in the absence of FK506 or CsA.[70] Fifth, the decrease is subunit-specific, since only CANB1 and PP2BA, but not CANB2 and PP2BB, were affected by TBI. Taken together, these data suggest that TBI-dependent changes in calcineurins are specific and unrelated to the exposure of animals to FK506 or CsA.

To further understand the role of CANB1 downregulation in TBI, IPA of downregulated proteins was performed. CANB1 was a central node in a network of downregulated calcineurin and protein phosphatases identified by mass spectrometry (Fig. 5, top). This network was connected through downregulated calmodulin and calcium calmodulin-dependent protein kinase 2 delta (CAMKII) with the highly interconnected structural networks predominated by tubulins (Fig. 5, bottom).

Discussion

Using a rigorous quantitative systems biology approach to understand the complex pathophysiology of traumatic brain injury in the CA3 subregion of the rat hippocampus, we revealed extensively interrelated proteins and networks that reflect widespread downregulation of metabolism, and calcium signaling and homeostasis, with upregulation of proteins related to synaptic and neuronal structure and function. In contrast to previous global hippocampal proteomics studies, we focused on CA3 because of its central role in information flow through the hippocampus, where acquisition and consolidation of memories occur, and disruption of these processes underlies cognitive dysfunction in TBI. While many of the dysregulated proteins reported here have previously been

Table 6. Mass Spectrometry Identification of Calcineurin B1

Peptide sequence	*Position*	*No. hits*
LDLDNSGSLSVEEFMSLPELQQNPLVQR	29–57	130
VIDIFDTDGNGEVDFK	58–73	50
DGYISNGELFQVLK DTQLQQIVDK	104–117	11
DTQLQQIVDK	126–135	2
ISFEEFCAVVGGLDIHK	138–164	4

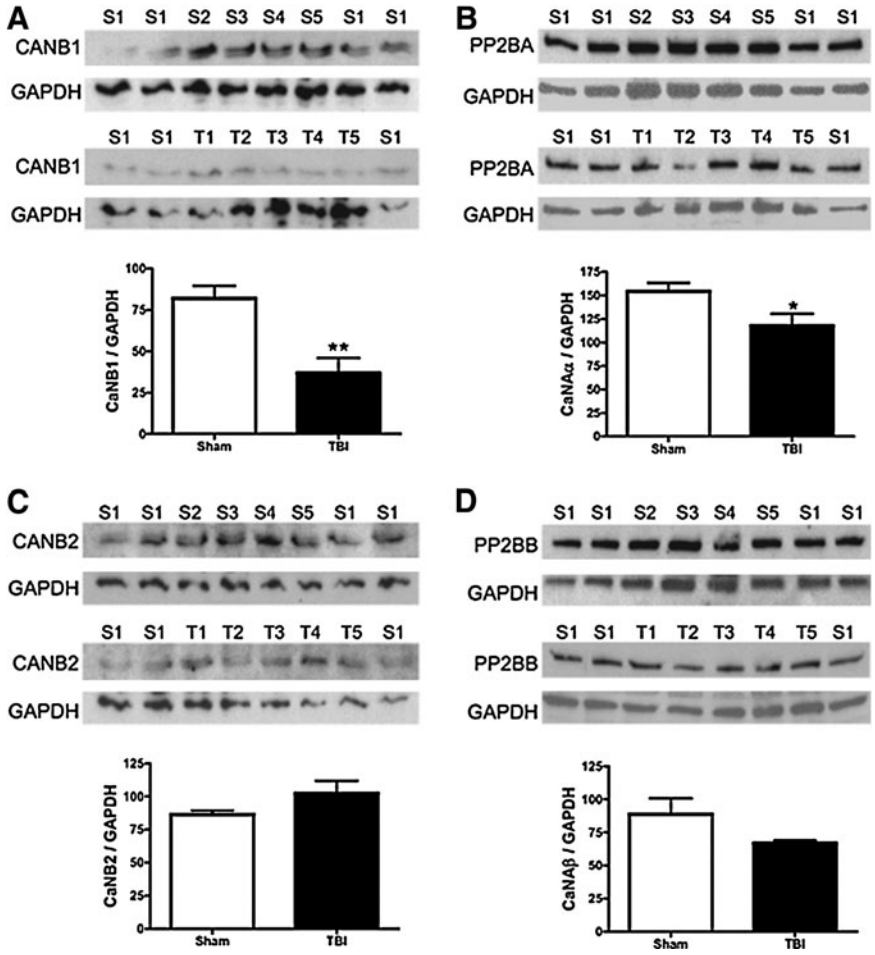

FIG. 4. Expression of calcineurin family members in traumatic brain injury (TBI). Calcineurin expression was validated using GAPDH as an internal control in injured hippocampi by quantitative Western blot analysis. (**A**) CANB1. (**B**) PP2BA. (**C**) CANB2. (**D**) PP2BB. Gel images show five individual samples from sham (S1–S5) and five from TBI (T1–T5) animals. Graphs show the quantitative densitometry analyses of specific signals, with data expressed as mean ± standard error of the mean; $*p < 0.05$ and $**p < 0.01$ significantly different from sham animals by Student's t test; $n = 5$; see Supplemental Table S3 for additional calcineurin nomenclature; GAPDH, glyceraldehyde 3-phosphate dehydrogenase).

identified in larger areas of the brain,[33,38] focusing on a small region like CA3 allows more confidence that the observed dysregulated proteins are closely related in controlling information processing. Our results also revealed key features of the importance of independent validation studies, since several differences from previous reports[33] could be accounted for by different models of TBI, ages, time post-injury, subregional analysis, and proteomics techniques. Nonetheless, unbiased large-scale proteomics studies require confirmation of our results with reductionist studies. We have shown here with calcineurin, and in another recent report,[70] that several of the novel observations in the present proteomics study revealed by pathway analysis have indeed been confirmed by traditional reductionist strategies, thus exhibiting the validity and value of our approach. The agreement of these independent observations also attests to the credibility of stringent statistical rigor to generate high-confidence, unbiased mass spectrometry data.

Based on this comprehensive study, we propose the following sequelae outlined in Figure 6. The metabolic consequences of TBI we found in the CA3 are widespread and far-reaching, including glycolysis, the Krebs acid cycle, and oxidative phosphorylation. The reduction of several key regulatory glycolytic enzymes would severely compromise flux through this pathway, resulting in low levels of the end product, pyruvate. But with decreased pyruvate dehydrogenase (ODPB), combined with decreased lactate dehydrogenase-mediated (LDHA) production of pyruvate from lactate, the amount of pyruvate available for conversion to acetyl-CoA to enter the Krebs cycle would be very limited. Low aconitase (ACON) would further compromise Krebs cycle production of reducing equivalents in the form of NADH, and the important product from this cycle, succinate.

Oxidative phosphorylation through the electron transport chain requires succinate to generate electrons to transport protons across the inner mitochondrial membrane. This process creates potential energy in the form of an electrochemical gradient, which allows protons to flow back across the membrane through ATP synthase, which converts ADP into ATP to produce energy. Any reduction in the NADH dehydrogenases (NDUAA and NDUA9), part of complex 1 or the entry enzymes into oxidative phosphorylation, would compromise the production of electrons to drive the electrochemical gradient, and ultimately ATP production. In addition to the low production of succinate from the Krebs cycle, low levels of succinate dehydrogenase (DHSA) would further compromise the function of

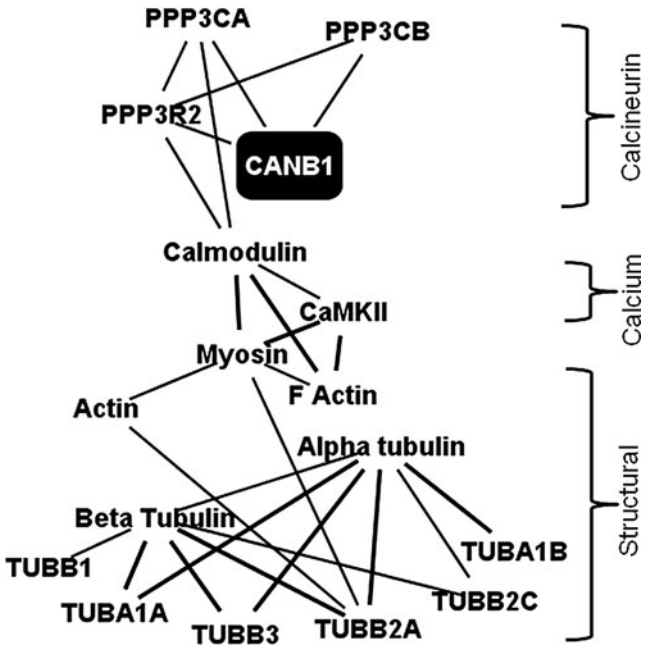

FIG. 5. Downregulated network of CANB1 in traumatic brain injury (TBI). Network analysis indicating coordinate dysregulation of calcineurins, calcium, and structural proteins. Lines between proteins represent relationships derived from Ingenuity Pathway Analysis (IPA) Network Explorer and Canonical Pathways in the Ingenuity Knowledge Base (CANB1, PPP3R1, calcineurin subunit B type 1; PPP3CA, PP2BA, serine/threonine-protein phosphatase 2B catalytic subunit alpha; PPP3CB, PP2BB, serine/threonine-protein phosphatase 2B catalytic subunit beta; PPP3R2, CANB2, calcineurin subunit B type 2; TUB-A1A, TUB-A1B, TUB-BB1, TUB-BB2A, TUB-BB2C, TUB-BB3, tubulins A1A, A1B, BB1, BB2A, BB2C, BB3; CaMKII, calcium calmodulin-dependent protein kinase 2 delta).

complex II, which converts succinate to fumarate. Downregulation of cytochrome b-c1 (QCR2) causes a reduction of flux through complex III of the electron transport chain. Finally, on top of all these steps reducing the production of ATP even if it is produced, diminished ADP/ATP translocase 1 (ADT1) decreases the ability to export the lower levels of ATP that are produced from the mitochondria to the cytoplasm. To exacerbate the metabolic imbalance even further, reduced levels of creatine kinase (KCRU; Table 2) lower the levels of the important energy store phosphocreatine, which is particularly important for organs with high energy consumption, such as the brain. In addition to these metabolic pathways producing less energy, dysregulated flux through the electron transport chain causes a premature release of electrons, which then increases the production of oxygen free radicals and the generation of reactive oxygen species, and also decreases the mitochondrial membrane potential that regulates ion transport. The widespread coordinated dysregulation of these many proteins would have profound effects on mitochondrial function and overall cellular metabolism, producing the reactive oxygen species and oxidative stress that are well-established hallmarks of TBI pathophysiology.[39]

From the standpoint of synaptic structure and function, multiple mechanisms reflect dysregulated ATP metabolism and calcium responses. Decreased plasma membrane sodium/potassium-ATPase activity (AT1A1 and 3) would reduce the functional activity of the excitatory amino acid transporter (EAA2), the protein levels of which are also decreased (Table 2). This would lead to sustained glutamate-mediated excitotoxicity, since glutamate removal from the synaptic cleft would be diminished. The decreased mitochondrial membrane potential would reduce the ability of the uniporters to mobilize calcium from the cytoplasm to the mitochondria. In combination with decreased ability of the plasma membrane calcium transporting ATPases 2 and 3 (AT2B2 and 3), and the sodium/calcium exchanger (NAC1) to diminish intracellular calcium, all of these mechanisms contribute to sustained intracellular calcium, protracting the associated damage.

The calcineurins, also known as protein phosphatase 2B, are phosphatases with well-established roles in regulating synaptic structure and function.[36] They respond to TBI through a wide variety of mechanisms, including mitochondrial dysfunction, actin-mediated synaptic reorganization, oxidative stress, and apoptosis.[1,30,32,56,59,68,76] The calcineurin family is composed of several isoforms, with the active form composed of two subunits each, one regulatory and one catalytic. We found statistically significant downregulation of both the regulatory subunit CANB1 (CaNBα), and its catalytic partner PP2BA (CaNAα), 4 days after injury with no changes in CANB2 (CaNBβ) or PP2BB (CaNAβ). These results reciprocally confirmed our unbiased quantitative mass spectrometry approach. Suggestions from a controlled cortical impact (CCI) model imply that calcineurin subunit expression shows additional temporal changes.[2,3] In particular, CCI results in a reduction of CANB1, similarly to our observations, in the CA3 region 2 h after injury, which returns to normal at 2 weeks post-injury. Further findings of regional and synaptic subfield-specific changes in the expression of calcineurin subunits also indicate the need for future mass spectrometry studies to identify specific fields and subcellular compartments within CA3.

Calcineurins dephosphorylate cytoskeletal and synaptic vesicle proteins to regulate synaptic structure and function both pre- and post-synaptically, and several of these processes are dysregulated in TBI. It is of particular note that CANB1 and PP2BA downregulation occurs with elevated calcium in response to injury, which on the surface appears paradoxical, since calcium prototypically leads to activation of calcineurins. Under normal circumstances transient elevations in calcium are important for the maintenance of synaptic transmission and structure. But excessive sustained calcium would drive these processes abnormally into a state of neuronal hyperexcitability.[54]

On one hand, the inhibition of calcineurins may be neuroprotective in TBI.[44] The coordinate downregulation of CANB1, calmodulin (CALM), and CaMKII (KCC2D), may be a compensatory response to diminish further damage mediated by calcium (Fig. 6). Reduced calcineurin would reduce dephosphorylation of cofilin,[56,72] resulting in net increased phosphorylation (inactivation), and shifting of the actin equilibrium from depolymerization toward actin filament polymerization, elongation, and spine synapse growth and reorganization.[73] At the same time, since elevated calcineurins lead to mitochondrial dysfunction,[1] downregulation would provide an additional compensatory mechanism to attempt to help protect mitochondria from further damage in light of the extensive dysregulation already occurring with respect to energy metabolism, ATP synthesis and transport, and the generation of oxygen free radicals and reactive oxygen species. Finally, downregulation of calcineurin would (1) maintain elevated phosphorylation and activation of myristoylated alanine-rich C-kinase substrate (MARCS), which we showed is elevated in TBI and is important for supporting neurotransmitter release;[46] (2) increase NMDA receptor phosphorylation to moderate glutamate-mediated

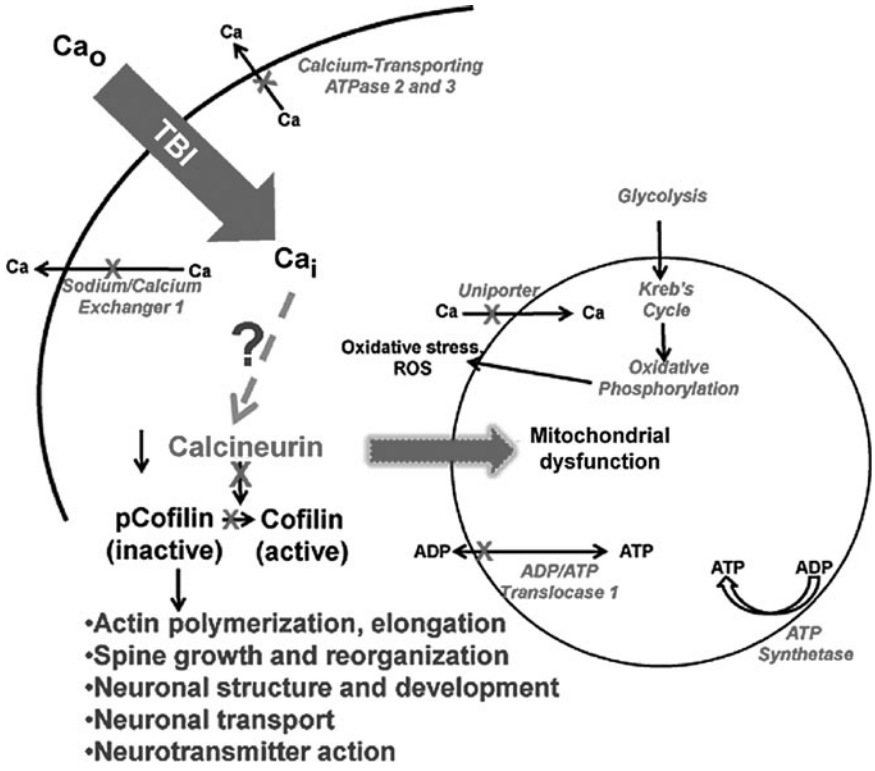

FIG. 6. Calcineurin compensation model. Traumatic brain injury (TBI) induces alterations in many intracellular functions regulated by calcineurins. Downregulation of calcineurins, potentially through dysregulation of intracellular calcium, leads to mitochondrial dysfunction and metabolic disturbances. These actions are coupled with alterations in synaptic structure and function that disrupt normal neuronal activity.

excitatory synaptic transmission;[47] (3) increase AMPA receptor phosphorylation to reduce synaptic depression;[64] and (4) diminish dispersal of GABA receptors from the synapse.[17] This calcineurin compensatory model is consistent with the lack of progression of our TBI model to apoptotic neuronal death, in spite of the massive homeostatic disturbances. It is further supported by the role of cyclosporine A and FK506, which inhibit calcineurins to provide neuroprotection and improve outcomes after TBI.[44,48]

On the other hand, TBI-induced downregulation of calcineurins may be destructive. Previously we reported that fluid percussion TBI significantly decreased the expression of glial cell line-derived neurotrophic factor (GDNF) in rat hippocampi, whereas grafted human neural stem cells produced GDNF and rescued the cognitive deficit.[18] This protective effect of GDNF was also reported by others.[35] Interestingly, GDNF protected neurons and promoted neurite outgrowth by increasing calcineurins in cultured embryonic midbrain neurons,[10] and increasing phosphorylation of cofilin,[80] which are precisely opposite to the effects of TBI. In this regard it is striking that several of the dysregulated proteins identified here have been reported to comprise part of the GDNF-responsive proteome in rat striatal progenitor cells,[28] and mouse striatal cells,[29] but had opposite effects from those seen after TBI. Furthermore, our recent study showed that grafted neural stem cells and GDNF reduced axonal injury by normalizing the level of CANB1 in both an *in vivo* fluid percussion injury model, and an *in vitro* stretch injury model, respectively.[70] It remains to be determined how grafted neural stem cells affect calcineurins and other proteins that are disturbed by TBI.

In summary, using an unbiased mass spectrometry and bioinformatics approach with confirmatory targeted reductionist analyses, we have shown that a coordinated, integrative dysregulation of proteins occurs in response to TBI. We thus propose a calcineurin compensatory model, mediated by CANB1 and PP2BA, that protects from the extensive downregulation of energy metabolism and upregulation of proteins responsible for synaptic structure, function, and reorganization, that normally occur during development, but are reinitiated in the response to injury.

Acknowledgments

This work was supported by the Department of the Army (W81XWH-08-2-0137 to P.W.), via the U.S. Army Medical Research Acquisition Activity, Fort Detrick, Maryland. The content of this work does not necessarily reflect the position or the policy of the U.S. government, and no official endorsement should be inferred. Other support for this study came from the Miriam and Emmett McCoy Foundation (L.D.), the Coalition for Brain Injury Research (P.W.), the Moody Foundation (P.W.), the TIRR Foundation (P.W.), and the John S. Dunn Research Foundation (P.W.).

Author Disclosure Statement

No competing financial interests exist.

References

1. Asai, A., Qiu, J., Narita, Y., Chi, S., Saito, N., Shinoura, N., Hamada, H., Kuchino, Y., and Kirino, T. (1999). High level calcineurin activity predisposes neuronal cells to apoptosis. J. Biol. Chem. 274, 34450–34458.
2. Bales, J.W., Ma, X., Yan, H.Q., Jenkins, L.W., and Dixon, C.E. (2010a). Expression of protein phosphatase 2B (calcineurin) subunit A isoforms in rat hippocampus after traumatic brain injury. J. Neurotrauma 27, 109–120.

3. Bales, J.W., Ma, X., Yan, H.Q., Jenkins, L.W., and Dixon, C.E. (2010b). Regional calcineurin subunit B isoform expression in rat hippocampus following a traumatic brain injury. Brain Res. 1358, 211–220.
4. Bedell, E.A., DeWitt, D.S., Uchida, T., and Prough, D.S. (2004). Cerebral pressure autoregulation is intact and is not influenced by hypothermia after traumatic brain injury in rats. J. Neurotrauma 21, 1212–1222.
5. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Royal Statistical Soc. Series B (Methodological) 57, 289–300.
6. Blanchet, S., Paradis-Giroux, A.A., Pepin, M., and McKerral, M. (2009). Impact of divided attention during verbal learning in young adults following mild traumatic brain injury. Brain Inj. 23, 111–122.
7. Burgess, J.A., Lescuyer, P., Hainard, A., Burkhard, P.R., Turck, N., Michel, P., Rossier, J.S., Reymond, F., Hochstrasser, D.F., and Sanchez, J.C. (2006). Identification of brain cell death associated proteins in human post-mortem cerebrospinal fluid. J. Proteome Res. 5, 1674–1681.
8. Chaichana, K.L., Pradilla, G., Huang, J., and Tamargo, R.J. (2010). Role of inflammation (leukocyte-endothelial cell interactions) in vasospasm after subarachnoid hemorrhage. World Neurosurg. 73, 22–41.
9. Chew, E., and Zafonte, R.D. (2009). Pharmacological management of neurobehavioral disorders following traumatic brain injury—a state-of-the-art review. J. Rehabil. Res. Dev. 46, 851–879.
10. Consales, C., Volpicelli, F., Greco, D., Leone, L., Colucci-D'Amato, L., Perrone-Capano, C., and di Porzio, U. (2007). GDNF signaling in embryonic midbrain neurons in vitro. Brain Res. 1159, 28–39.
11. Conti, A., Sanchez-Ruiz, Y., Bachi, A., Beretta, L., Grandi, E., Beltramo, M., and Alessio, M. (2004). Proteome study of human cerebrospinal fluid following traumatic brain injury indicates fibrin(ogen) degradation products as trauma-associated markers. J. Neurotrauma 21, 854–863.
12. Corso, P., Finkelstein, E., Miller, T., Fiebelkorn, I., and Zaloshnja, E. (2006). Incidence and lifetime costs of injuries in the United States. Inj. Prev. 12, 212–218.
13. Dayon, L., Hainard, A., Licker, V., Turck, N., Kuhn, K., Hochstrasser, D.F., Burkhard, P.R., and Sanchez, J.C. (2008). Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Anal. Chem. 80, 2921–2931.
14. De Beaumont, L., Theoret, H., Mongeon, D., Messier, J., Leclerc, S., Tremblay, S., Ellemberg, D., and Lassonde, M. (2009). Brain function decline in healthy retired athletes who sustained their last sports concussion in early adulthood. Brain 132, 695–708.
15. De Monte, V.E., Geffen, G.M., and Massavelli, B.M. (2006). The effects of post-traumatic amnesia on information processing following mild traumatic brain injury. Brain Inj. 20, 1345–1354.
16. DeWitt, D.S., Smith, T.G., Deyo, D.J., Miller, K.R., Uchida, T., and Prough, D.S. (1997). L-arginine and superoxide dismutase prevent or reverse cerebral hypoperfusion after fluid-percussion traumatic brain injury. J. Neurotrauma 14, 223–233.
17. Fukura, H., Komiya, Y., and Igarashi, M. (1996). Signaling pathway downstream of GABAA receptor in the growth cone. J. Neurochem. 67, 1426–1434.
18. Gao, J., Prough, D.S., McAdoo, D.J., Grady, J.J., Parsley, M.O., Ma, L., Tarensenko, Y.I., and Wu, P. (2006). Transplantation of primed human fetal neural stem cells improves cognitive function in rats after traumatic brain injury. Exp. Neurol. 201, 281–292.
19. Gao, W.M., Chadha, M.S., Berger, R.P., Omenn, G.S., Allen, D.L., Pisano, M., Adelson, P.D., Clark, R.S., Jenkins, L.W., and Kochanek, P.M. (2007). A gel-based proteomic comparison of human cerebrospinal fluid between inflicted and non-inflicted pediatric traumatic brain injury. J. Neurotrauma 24, 43–53.
20. Goshe, M.B., and Smith, R.D. (2003). Stable isotope-coded proteomic mass spectrometry. Curr. Opin. Biotechnol. 14, 101–109.
21. Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., and Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–999.
22. Hall, E.D., Vaishnav, R.A., and Mustafa, A.G. (2010). Antioxidant therapies for traumatic brain injury. Neurotherapeutics 7, 51–61.
23. Hanrieder, J., Wetterhall, M., Enblad, P., Hillered, L., and Bergquist, J. (2009). Temporally resolved differential proteomic analysis of human ventricular CSF for monitoring traumatic brain injury biomarker candidates. J. Neurosci. Methods 177, 469–478.
24. Haqqani, A.S., Hutchison, J.S., Ward, R., and Stanimirovic, D.B. (2007). Biomarkers and diagnosis; protein biomarkers in serum of pediatric patients with severe traumatic brain injury identified by ICAT-LC-MS/MS. J. Neurotrauma 24, 54–74.
25. Hardingham, G.E. (2009). Coupling of the NMDA receptor to neuroprotective and neurodestructive events. Biochem. Soc. Trans. 37, 1147–1160.
26. Haskins, W.E., Kobeissy, F.H., Wolper, R.A., Ottens, A.K., Kitlen, J.W., McClung, S.H., O'Steen, B.E., Chow, M.M., Pineda, J.A., Denslow, N.D., Hayes, R.L., and Wang, K.K. (2005). Rapid discovery of putative protein biomarkers of traumatic brain injury by SDS-PAGE-capillary liquid chromatography-tandem mass spectrometry. J. Neurotrauma 22, 629–644.
27. Heller, M., Mattou, H., Menzel, C., and Yao, X. (2003). Trypsin catalyzed 16O-to-18O exchange for comparative proteomics: tandem mass spectrometry comparison using MALDI-TOF, ESI-QTOF, and ESI-ion trap mass spectrometers. J. Am. Soc. Mass Spectrom. 14, 704–718.
28. Hoffrogge, R., Beyer, S., Hubner, R., Mikkat, S., Mix, E., Scharf, C., Schmitz, U., Pauleweit, S., Berth, M., Zubrzycki, I.Z., Christoph, H., Pahnke, J., Wolkenhauer, O., Uhrmacher, A., Volker, U., and Rolfs, A. (2007). 2-DE profiling of GDNF overexpression-related proteome changes in differentiating ST14A rat progenitor cells. Proteomics 7, 33–46.
29. Hong, Z., Liu, J., Xia, L., Pan, J., Xiao, Q., Lu, G., Liang, L., and Chen, S.D. (2009). Identification of glial-cell-line-derived neurotrophic factor-regulated proteins of striatum in mouse model of Parkinson disease. Proteomics Clin. Appl. 3, 1072–1083.
30. Hovda, D.A., Becker, D.P., and Katayama, Y. (1992). Secondary injury and acidosis. J. Neurotrauma 9 Suppl. 1, S47–S60.
31. Hovda, D.A., Yoshino, A., Kawamata, T., Katayama, Y., and Becker, D.P. (1991). Diffuse prolonged depression of cerebral oxidative metabolism following concussive brain injury in the rat: a cytochrome oxidase histochemistry study. Brain Res. 567, 1–10.
32. Huang, R.Q., and Dillon, G.H. (1998). Maintenance of recombinant type A gamma-aminobutyric acid receptor function: role of protein tyrosine phosphorylation and calcineurin. J. Pharmacol. Exp. Ther. 286, 243–255.
33. Jenkins, L.W., Peters, G.W., Dixon, C.E., Zhang, X., Clark, R.S., Skinner, J.C., Marion, D.W., Adelson, P.D., and Kochanek, P.M. (2002). Conventional and functional proteomics using large format two-dimensional gel electrophoresis 24 hours after controlled cortical impact in postnatal day 17 rats. J. Neurotrauma 19, 715–740.
34. Jiang, X.B., Ohno, K., Qian, L., Tominaga, B., Kuroiwa, T., Nariai, T., and Hirakawa, K. (2000). Changes in local cerebral blood flow, glucose utilization, and mitochondrial function following traumatic brain injury in rats. Neurol. Med. Chir. (Tokyo) 40, 16–28; discussion 28–19.
35. Kim, B.T., Rao, V.L., Sailor, K.A., Bowen, K.K., and Dempsey, R.J. (2001). Protective effects of glial cell line-derived neurotrophic factor on hippocampal neurons after traumatic brain injury in rats. J. Neurosurg. 95, 674–679.
36. Klee, C.B., Crouch, T.H., and Krinks, M.H. (1979). Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. USA 76, 6270–6273.
37. Kobeissy, F.H., Ottens, A.K., Zhang, Z., Liu, M.C., Denslow, N.D., Dave, J.R., Tortella, F.C., Hayes, R.L., and Wang, K.K. (2006). Novel differential neuroproteomics analysis of traumatic brain injury in rats. Mol. Cell Proteomics 5, 1887–1898.
38. Kochanek, A.R., Kline, A.E., Gao, W.M., Chadha, M., Lai, Y., Clark, R.S., Dixon, C.E., and Jenkins, L.W. (2006). Gel-based hippocampal proteomic analysis 2 weeks following traumatic brain injury to immature rats using controlled cortical impact. Dev. Neurosci. 28, 410–419.
39. Kochanek, P.M. (1993). Ischemic and traumatic brain injury: pathobiology and cellular mechanisms. Crit. Care Med. 21, S333–S335.
40. Laird, M.D., Vender, J.R., and Dhandapani, K.M. (2008). Opposing roles for reactive astrocytes following traumatic brain injury. Neurosignals 16, 154–164.
41. Lehninger, A.L. (1970). *Biochemistry: The Molecular Basis of Cell Structure and Function*. Worth Publishers: New York.
42. Lowenstein, D.H., Thomas, M.J., Smith, D.H., and McIntosh, T.K. (1992). Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. J. Neurosci. 12, 4846–4853.
43. Lu, D., Goussev, A., Chen, J., Pannu, P., Li, Y., Mahmood, A., and Chopp, M. (2004). Atorvastatin reduces neurological deficit and

increases synaptogenesis, angiogenesis, and neuronal survival in rats subjected to traumatic brain injury. J. Neurotrauma 21, 21–32.
44. Marmarou, C.R., and Povlishock, J.T. (2006). Administration of the immunophilin ligand FK506 differentially attenuates neurofilament compaction and impaired axonal transport in injured axons following diffuse traumatic brain injury. Exp. Neurol. 197, 353–362.
45. Matser, E.J., Kessels, A.G., Lezak, M.D., Jordan, B.D., and Troost, J. (1999). Neuropsychological impairment in amateur soccer players. JAMA 282, 971–973.
46. McNamara, R.K., Hussain, R.J., Simon, E.J., Stumpo, D.J., Blackshear, P.J., Abel, T., and Lenox, R.H. (2005). Effect of myristoylated alanine-rich C kinase substrate (MARCKS) overexpression on hippocampus-dependent learning and hippocampal synaptic plasticity in MARCKS transgenic mice. Hippocampus 15, 675–683.
47. Ohmitsu, M., Fukunaga, K., Yamamoto, H., and Miyamoto, E. (1999). Phosphorylation of myristoylated alanine-rich protein kinase C substrate by mitogen-activated protein kinase in cultured rat hippocampal neurons following stimulation of glutamate receptors. J. Biol. Chem. 274, 408–417.
48. Okonkwo, D.O., Melon, D.E., Pellicane, A.J., Mutlu, L.K., Rubin, D.G., Stone, J.R., and Helm, G.A. (2003). Dose-response of cyclosporin A in attenuating traumatic axonal injury in rat. Neuroreport 14, 463–466.
49. Ong, S.E., and Mann, M. (2005). Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1, 252–262.
50. Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell Proteomics 1, 376–386.
51. Ong, S.E., Foster, L.J., and Mann, M. (2003). Mass spectrometric-based approaches in quantitative proteomics. Methods 29, 124–130.
52. Oshima, T., Lee, S., Sato, A., Oda, S., Hirasawa, H., and Yamashita, T. (2009). TNF-alpha contributes to axonal sprouting and functional recovery following traumatic brain injury. Brain Res. 1290, 102–110.
53. Ottens, A.K., Kobeissy, F.H., Fuller, B.F., Liu, M.C., Oli, M.W., Hayes, R.L. and Wang, K.K. (2007). Novel neuroproteomic approaches to studying traumatic brain injury. Prog. Brain Res. 161, 401–418.
54. Palop, J.J., Mucke, L., and Roberson, E.D. (2011). Quantifying biomarkers of cognitive dysfunction and neuronal network hyperexcitability in mouse models of Alzheimer's disease: depletion of calcium-dependent proteins and inhibitory hippocampal remodeling. Methods Mol. Biol. 670, 245–262.
55. Robel, S., Berninger, B., and Gotz, M. (2011). The stem cell potential of glia: lessons from reactive gliosis. Nat. Rev. Neurosci. 12, 88–104.
56. Rusnak, F., and Mertz, P. (2000). Calcineurin: form and function. Physiol. Rev. 80, 1483–1521.
57. Sadygov, R.G., Zhao, Y., Haidacher, S.J., Starkey, J.M., Tilton, R.G., and Denner, L. (2010). Using power spectrum analysis to evaluate (18)O-water labeling data acquired from low resolution mass spectrometers. J. Proteome Res. 9, 4306–4312.
58. Scheff, S.W., Price, D.A., Hicks, R.R., Baldwin, S.A., Robinson, S., and Brackney, C. (2005). Synaptogenesis in the hippocampal CA1 field following traumatic brain injury. J. Neurotrauma 22, 719–732.
59. Shapira, Y., Yadid, G., Cotev, S., and Shohami, E. (1989). Accumulation of calcium in the brain following head trauma. Neurol. Res. 11, 169–172.
60. Soares, H.D., Hicks, R.R., Smith, D., and McIntosh, T.K. (1995). Inflammatory leukocytic recruitment and diffuse neuronal degeneration are separate pathological processes resulting from traumatic brain injury. J. Neurosci. 15, 8223–8233.
61. Sosin, D.M., Sniezek, J.E., and Waxweiler, R.J. (1995). Trends in death associated with traumatic brain injury, 1979 through 1992. Success and failure. JAMA 273, 1778–1780.
62. Starkey, J.M., Zhao, Y., Sadygov, R.G., Haidacher, S.J., Lejeune, W.S., Dey, N., Luxon, B.A., Kane, M.A., Napoli, J.L., Denner, L., and Tilton, R.G. (2010). Altered retinoic acid metabolism in diabetic mouse kidney identified by O isotopic labeling and 2D mass spectrometry. PLoS One 5, e11095.
63. Stoica, B.A., and Faden, A.I. (2010). Cell death mechanisms and modulation in traumatic brain injury. Neurotherapeutics 7, 3–12.
64. Tan, S.E., Wenthold, R.J., and Soderling, T.R. (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. J. Neurosci. 14, 1123–1129.
65. Tao, W.A., and Aebersold, R. (2003). Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr. Opin. Biotechnol. 14, 110–118.
66. Tarasenko, Y.I., Yu, Y., Jordan, P.M., Bottenstein, J., and Wu, P. (2004). Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. J. Neurosci. Res. 78, 625–636.
67. Tilton, R.G., Haidacher, S.J., Lejeune, W.S., Zhang, X., Zhao, Y., Kurosky, A., Brasier, A.R., and Denner, L. (2007). Diabetes-induced changes in the renal cortical proteome assessed with two-dimensional gel electrophoresis and mass spectrometry. Proteomics 7, 1729–1742.
68. Tong, G., Shepherd, D., and Jahr, C.E. (1995). Synaptic desensitization of NMDA receptors by calcineurin. Science 267, 1510–1512.
69. Venkataramanan, R., Warty, V.S., Zemaitis, M.A., Sanghvi, A.T., Burckart, G.J., Seltman, H., Todo, S., Makowka, L., and Starzl, T.E. (1987). Biopharmaceutical aspects of FK-506. Transplant. Proc. 19, 30–35.
70. Wang, E., Gao, J., Yang, Q., Parsley, M., Dunn, T., Zhang, L., Dewitt, D., Denner, L., Prough, D.S., and Wu, P. (2011). Molecular mechanisms underlying effects of neural stem cells against traumatic axonal injury. J. Neurotrauma 29, 295–312.
71. Wang, K.K., Ottens, A., Haskins, W., Liu, M.C., Kobeissy, F., Denslow, N., Chen, S., and Hayes, R.L. (2004). Proteomics studies of traumatic brain injury. Int. Rev. Neurobiol. 61, 215–240.
72. Wang, Y., Shibasaki, F., and Mizuno, K. (2005). Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin. J. Biol. Chem. 280, 12683–12689.
73. Whiteman, I.T., Gervasio, O.L., Cullen, K.M., Guillemin, G.J., Jeong, E.V., Witting, P.K., Antao, S.T., Minamide, L.S., Bamburg, J.R., and Goldsbury, C. (2009). Activated actin-depolymerizing factor/cofilin sequesters phosphorylated microtubule-associated protein during the assembly of alzheimer-like neuritic cytoskeletal striations. J. Neurosci. 29, 12994–13005.
74. Xiong, Y., Mahmood, A., and Chopp, M. (2010a). Angiogenesis, neurogenesis and brain recovery of function following injury. Curr. Opin. Investig. Drugs 11, 298–308.
75. Xiong, Y., Mahmood, A., and Chopp, M. (2010b). Neurorestorative treatments for traumatic brain injury. Discov. Med. 10, 434–442.
76. Yang, S.A., and Klee, C.B. (2000). Low affinity Ca2 + binding sites of calcineurin B mediate conformational changes in calcineurin A. Biochemistry (Mosc.) 39, 16147–16154.
77. Yang, X., Yang, S., Wang, J., Zhang, X., Wang, C., and Hong, G. (2009). Expressive proteomics profile changes of injured human brain cortex due to acute brain trauma. Brain Inj. 23, 830–840.
78. Yao, X., Freas, A., Ramirez, J., Demirev, P.A., and Fenselau, C. (2001). Proteolytic 18O labeling for comparative proteomics: model studies with two serotypes of adenovirus. Anal. Chem. 73, 2836–2842.
79. Yi, J.H., and Hazell, A.S. (2006). Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. Neurochem. Int. 48, 394–403.
80. Yoong, L.F., Wan, G., and Too, H.P. (2009). GDNF-induced cell signaling and neurite outgrowths are differentially mediated by GFRalpha1 isoforms. Mol. Cell. Neurosci. 41, 464–473.
81. Zhao, Y., Denner, L., Haidacher, S.J., LeJeune, W.S., and Tilton, R.G. (2008). Comprehensive analysis of the mouse renal cortex using two-dimensional HPLC-tandem mass spectrometry. Proteome Sci. 6, 15.
82. Zhu, W.L., Shi, H.S., Wang, S.J., Wu, P., Ding, Z.B., and Lu, L. (2011). Hippocampal CA3 calcineurin activity participates in depressive-like behavior in rats. J. Neurochem. 117, 1075–1086.
83. Zuberovic, A., Wetterhall, M., Hanrieder, J., and Bergquist, J. (2009). CE MALDI-TOF/TOF MS for multiplexed quantification of proteins in human ventricular cerebrospinal fluid. Electrophoresis 30, 1836–1843.

Address correspondence to:
Larry Denner, PhD
Division of Endocrinology and Stark Diabetes Center
Department of Internal Medicine
8.138 Medical Research Building
The University of Texas Medical Branch
301 University Boulevard
Galveston, TX 77555-1060

E-mail: ladenner@utmb.edu