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Post-acute Brain Injury Urinary Signature: A New Resource for Molecular Diagnostics

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Running Title (<45): TBI Urinary Diagnostics

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Abstract

Heterogeneity within brain injury presents a challenge to the development of informative molecular diagnostics. Recent studies show progress particularly in cerebrospinal fluid with biomarker assays targeting one or a few structural proteins. Protein-based assays in peripheral fluids, however, have been more challenging to develop in part due to restricted and intermittent barrier access. Further, a greater number of molecular variables may be required to inform on patient status given the multi-factorial nature of brain injury. Presented is an alternative approach profiling peripheral fluid for a class of small metabolic by-products rendered by ongoing brain pathobiology. Urine specimens were collected for head trauma subjects upon admission to acute brain injury rehabilitation and non-traumatized matched controls. An innovative data-independent mass spectrometry approach was employed for reproducible molecular quantification across osmolarity-normalized samples. The post-acute human traumatic brain injury urinary signature encompassed 2,476 discriminant variables reproducibly measured in specimens for subject classification. Multiple sub-profiles were then discerned in correlation with injury severity per Glasgow Comma Scale and behavioral and neurocognitive function per Patient Competency Rating Scale and Frontal Systems Behavioral Scale. Identified peptide constituents were enriched for outgrowth and guidance, extracellular matrix and post-synaptic density proteins, which were reflective of ongoing post-acute neuroplastic processes demonstrating pathobiological relevance. Taken together, these findings support further development of diagnostics based on brain injury urinary signatures using either combinatorial quantitative models or pattern-recognition methods. Particularly, these findings espouse assay development to address unmet diagnostic and theragnostic needs in brain injury rehabilitative medicine.

Keywords: urine, brain injury, rehabilitation, mass spectrometry, metabolomics, biomarker

Introduction

Providers cite brain injury variability as a primary challenge to accurate characterization of symptoms and progress of their patients.[1-4] Brain injury heterogeneity has also complicated the development of informative diagnostics, which must be sensitive and selective to an individualized trajectory, diversified by a varied set of factors including: mechanism, severity, and localization of injury; demographics; individualized pathobiological response; comorbidity with other trauma.[5-7] Effective diagnostics must then reflect injury metrics, target acute or chronic pathology and employ appropriate models that are robust to the degree of variance present within brain injury.

To this end, the field of brain injury diagnostics may benefit from new approaches. To date, brain injury research has produced several candidate molecular biomarkers based on quantifying one or a few target proteins in CSF or blood (see recent reviews).[8-12] While these groundbreaking assays are promising, the target-protein approach presents several diagnostic limitations: underpowered pathobiological factor specificity from too few variables, quantitative variability due to under-fitting individualized aspects of disease response, and restricted, intermittent access to peripheral fluids, particularly unfavorable in the post-acute period when brain barrier stability is restored.[8,10,13,14] Alternatively, molecular efflux into peripheral fluid (e.g., blood and urine) is enhanced for smaller, ionic by-products rendered by ongoing neurobiological processes.[15-17] Encompassing an abundant class of pathobiologically informative molecules portends use of pattern detection methods to develop more robust brain injury diagnostics.[8,13,18,19]

Post-acute brain injury rehabilitative care would particularly benefit from assays based on molecules with more consistent brain barrier efflux that are accessible in easily attained peripheral fluids. Clear advances have been made over the last two decades in the rehabilitation of traumatic brain injury (TBI) patients.[2,20] Rehabilitative therapy improves cognition, quality of life, and perceived competence.[2,3,21] Further, therapeutic intensity is predictive of improved function.[22] However, therapeutic

needs vary widely among brain injury patients, demanding individualized therapeutic strategies and assessments to provide maximal rehabilitative benefit.[20,23] Rehabilitation practitioners are challenged to quickly, yet precisely characterize their patient's cognitive and behavioral performance to facilitate effective treatment planning and long-term recommendations. Patient readiness for acute rehabilitation is particularly difficult to assess with conventional neuropsychological testing that often does not capture the capacity to participate meaningfully in therapies.[20,24,25] Rehabilitation readiness and therapeutic responsiveness diagnostics that are independent of human verbal or written responses would advance our ability to identify and improve individualized care for persons with brain injury.

Thus, there is a growing call for molecular diagnostics in brain injury rehabilitative medicine.[20,21] Four particular goals have been set: (1) to aid admitting and stratifying patients for customized therapy; (2) to monitor therapeutic progress and guide treatment course; (3) to reflect underlying pathobiology in evaluating new treatments; and (4) to predict outcome. Structural protein biomarkers under study for brain injury reflect on acute degenerative pathobiology, and thus may be less informative on post-acute regeneration.[21,26,27] Alternative diagnostics and theragnostics would preferentially reflect neuroplastic processes ongoing weeks to months after injury in order to target and optimize neurological and functional recovery.[23,28,29] Confronting all of the above, this study reports proof-of-principle for a new diagnostic approach assessing small metabolic brain injury by-products released into patient urine in the early rehabilitative phase of recovery. Complexity of the urinary TBI metabolomic signature was assessed along with the capacity to classify subjects and stratify based on clinical metrics of injury and function. Discriminant variables were probed for relevance to regenerative pathobiology substantiating a basis for further development into brain injury rehabilitation diagnostics.

Methods

A controlled demographic of young adult Caucasian male subjects was recruited with informed consent and approval by the Virginia Commonwealth University Institutional Review Board. TBI subjects were enrolled upon admission to inpatient rehabilitation at a mean 17 days post-injury (n=5; 26±6 years old; 5±3 initial Glasgow Coma Scale (GCS) score assessed acutely after injury). Non-traumatized matched control subjects were then recruited (n=5; 26±5 years old). Criteria excluded subjects with noncranial bone fractures, renal dysfunction at time of rehabilitation admission, and a positive history for past brain injury or neurological disease. Admission to the Brain Injury Rehabilitation Unit was based on standards of care for demonstrating readiness, with required medical stability and capacity to progress in an acute rehabilitation program. Consent was obtained within 48 h of admission to the unit. Beginning at 72 h on unit, three midstream urine specimens were acquired within a 48 h window. Urine specimens were placed at 4°C following collection and centrifuged at 1,500 xg and 4°C for 15 min. Aliquots were then stored at -80°C.

Specimens (three per subject) were load standardized to an osmolarity of 130 mOsm/kg with Nanopure water. Balanced specimens (100 μL) were filtered with 0.1 μm pore Ultrafree-MC units (Millipore, Billerica, MA), with the supernatants transferred to vials for direct injection (8 μL on column) in a group interspersed order. Reversed-phase separation was performed with a nanoAcquity chromatography system, using a Symmetry C18 trapping column (2 cm x 180 μm i.d.) and an HSS T3 nanoAcquity (15 cm x 75 μm i.d.) capillary column (Waters, Milford, MA). Components were gradient separated using 0.1% formic acid modified acetonitrile and water. Eluting peptides were electrosprayed into a Synapt G2 hybrid ion mobility - mass spectrometer (Waters) operated in a data-independent analysis mode as described previously.[30] All analytical work was performed within a climate controlled clean room.

Data were processed using PLGS software v.2.5.2 (Waters). Precursor and product ion measures exceeding 150 and 20 counts, respectively, were extracted, deisotoped, and charge state collapsed.

Accurate mass and retention time (AMRT) tables for the triplicate specimens were merged to generate a single composite molecular profile per subject that accounted for intradaily variance. All subject profiles were then aligned by AMRT values (±7 ppm mass accuracy; ±0.5 min retention time) using Expressions software (v.2.5).[31] Non-reproducing AMRT measures (< 3 /grp.) were removed. Values from a simulated Gaussian distribution randomized about the limit of quantification were imputed for left-censored data denoting a non-random, group-specific level below the detection limit.[30] Intersubject normalization (median intensity, 1,000 most intense ions) and log(2) transformation procedures were performed.

Aligned composite molecular profiles (one per subject) were statistically tested using the MultiExperimentViewer v.4.8.1 informatics package for array data.[32] Principle component analysis and Welch's t-test methods were applied with alpha corrected for multiple measures using a q-value false discovery rate method.[33] Pearson's analysis tested for correlation between TBI responsive molecular variables and Pavlidis templates of subject GCS, Galveston Orientation and Amnesia Test (GOAT), Disability Rating Scale (DRS), Frontal Systems Behavioral Scale (FrSBe), Neurobehavioral Rating Scale (NRS), and Patient Competency Rating Scale (PCRS) total scores. A one-sample t-test method assessed for chance correlation relative to a set of random templates; the significance level was adjusted with the Bonferroni correction method. Correlation between subject clinical scores was assessed by Pearson's analysis using SPSS v.20 (IBM, Armonk, NY).

Sequence search analysis was performed on TBI positive responding variables (PLGS v.2.5.2) against a Human UniprotKB fasta database (2012_10 release). Parameters selected for: no restrictive enzyme; variable methionine oxidation; neutral loss of ammonia or water; MS tolerance of 5 ppm; MSMS tolerance of 15 ppm. Results (raw peptide score) were controlled to a 10% false sequence identification rate using a reversed decoy database method. Identified peptide products were matched to their parent protein or protein family. Enrichment analysis was performed with corresponding protein

symbols against GO annotation terms (molecular function, biochemical process and cellular component), biochemical pathways, and protein-protein interaction clusters using a Fisher's inverse chi-square method with a Bonferroni correction of alpha (ToppGene v.9.56.45).[34] TBI responsive results were further analyzed using protein-protein interaction network analysis (STRING v.9.0, action view)[35] with a minimum interaction confidence score of 0.6 and ten added interactor nodes.

Results

Using innovative data-independent mass spectrometry analysis, 10,929 distinct molecular measures were reproducibly quantified across group subjects. This urinary metabolome was comprised of small molecules with a median mass of 1,274 Da, and only 5% exceeding 5 kDa. Supervised statistical testing revealed 3,897 TBI-responsive measures (**Fig. 1A**) with a false discovery rate (FDR) of 2% (76 false detections). Measures reproducibly discriminated TBI from control subjects (**Fig. 1B**). A 64% majority of the measures (2,476) were significantly increased in TBI urine (TBI urinary signature). A non-supervised orthogonal transformation into principal components unambiguously classified TBI subjects from matched controls **(Fig. 1C**) supporting utility in diagnostic model building. Principal component 1 with a dominant eigenvalue of 17.5 represented over 66.4% of total variance and effectively bisected the subjects into two distinct clusters (x-axis of **Fig. 1C**). Secondary, intra-group variability was accounted for in principal components 2 (y-axis of **Fig. 1C**) through 4, where a definitive Scree plot breakpoint was identified. Eigenvalues for these components were appreciably smaller (3.1 to 1.2), accounting for an additional 21.7% of total variance.

To further evaluate diagnostic potential, the TBI urinary signature was assessed for correlation to templates of scalar clinical metrics of interest to rehabilitation practitioners. The number of correlative molecular variables (Pearson's $R > 0.95$) was not significantly different from random template matching for GOAT, DRS and NRS total scores once corrected for repeated measures ($p>0.004$). However, three

significant sub-profile factors were found in correlation with the following clinical metrics (Table 1): GCS, with a subset of 379 molecules; ($p=1.91e^{-8}$); PCRS, with a subset of 385 molecules ($p=2.23e^{-7}$); FrSBe, with a subset of 360 molecules ($p<9.07e^{-9}$). Relationships between the three sub-profiles are summarized using a Venn diagram (**Fig. 1D**). Total scores for PCRS and FrSBe were found to be correlative (Pearson's R=0.91, p=0.034). Thus, it fit that there was a two-thirds overlap in PCRS and FrSBe correlative molecular variables. In contrast, the GCS factor was largely distinct (294, or 77% unique) from the other two sub-profiles. In agreement, subject GCS scores were not predictive of either rehabilitation assessments (GCS to PCRS, Peasron's R=0.63, p=0.251; GCS to FrSBe, Pearson's R=0.62, p=0.261; see Table 1). Findings suggest that the TBI urinary signature comprises multiple clinically informative factors.

To assess a pathobiological connection, tandem mass spectra from the TBI urinary signature were matched with peptide sequences, which were then examined for their biomolecular relevance. Using accurate mass measurement and selective fragmentation spectra, 238 sequences were identified at a 10% FDR. These endogenous peptides had an average mass of 2,223±755 Da (ranging from 812 Da to 3,948 Da) and were of sufficient sequence length to discriminate 144 source proteins (or protein families). A 57% majority of proteins had only one corresponding endogenous peptide, with only 16% having three or more matched peptides. These findings suggested that most peptides were select metabolic products excreted from circulation rather than from non-specific protein catabolism in urine.

Of the 144 source proteins, 119 were annotated in molecular ontology databases and were mined for enriched biomolecular associations (**Fig. 2**). The identified peptides were largely fragments of extracellular matrix (ECM) and membranous proteins that are known to or may likely shed peptides during synaptic reorganization.[36,37] Four distinct activity networks of interest were related to: outgrowth, guidance and morphogenesis; the ECM; transcriptional activity; post-synaptic density interaction. This later network included peptides from several major components of the excitatory glutamate receptor complex including, glutamate receptor 2A & B (Grin2A/B), synaptic Ras GTPase activating protein

1(Syngap1) and SH3 ankyrin repeat domain 1 (Shank1). Taken together, these results provide a mechanistic link with neuroplastic dynamics.

Discussion

Presented are results supporting an alternative approach for brain injury molecular diagnostics espoused particularly to address needs in post-acute care. Results unveil the discriminant capacity, pathobiological relevance, and diagnostic potential of a TBI urinary signature. Findings suggest applicability for multivariate analyses of small molecular by-products released into peripheral fluids to assess brain injury pathobiological and functional status.

Results newly reveal that human urine specimens contain several thousand TBI discriminant measures (**Fig. 1A**). Urine has largely been overlooked as a brain injury biomarker source, with proteins of interest generally excluded through normal renal function.[9] However, urine is a non-invasive, readily attainable and stable biofluid – preferable attributes for longitudinal monitoring in rehabilitative care (including outpatient) or with sensitive populations such as pediatric TBI.[38] The extensive molecular diversity (10,929 reproducible measurements) uncovered in urine is an advantageous finding. The present approach capitalizes on renal barrier function in excreting small metabolites from circulation, providing filtration enrichment for by-products of interest here. In particular, barrier permeable small ionic metabolites, such as proteolytically shed peptides, are also amenable to mass spectrometric analysis. **Fig. 1C** provides a reduced multivariate projection of the extensive factor space objectively classifying TBI from control subjects (component 1, x-axis). The factor space included 834 molecular variables that were over three-fold more abundant in TBI specimens relative to non-traumatized controls. These measures could provide for sensitive and robust multivariate diagnostic models to address the multifactor heterogeneity in TBI.

Further study assessed whether the TBI urinary signature consisted of clinically informative subset factors (**Fig. 1D**). Pearson's analysis revealed molecular factors correlated with subject PCRS and FrSBe rehabilitation scores (Table 1). The two sub-profiles were, however, confluent with a two-thirds overlap in variables. In explanation, PCRS and FrSBe instruments reflected upon similar aspects of behavioral and neurocognitive competencies at the same post-acute period, with significant correlation between their total scores. GCS, conversely, was found in correlation with an independent sub-profile. A measure of conscious state, GCS was assessed acutely after TBI as used to gauge injury severity; thus, a different metric and period in the pathobiology. It rationally followed that GCS related to a distinct subset of biochemical products. Taken together, these results denote multiple distinct molecular factors within the TBI urinary signature in correlation with different clinical metrics.

Sample collection was standardized with admission to rehabilitation assessed with standard-of-care examination. This in-part accounted for variable recovery rates among individuals. However, more precise diagnostic indicators of maximal readiness may be possible using longitudinal assessment of measures associated with regenerative pathobiology. Readiness may be linked with priming of neuroplastic processes underlying regeneration and influenced by rehabilitative activities.[28,29] Timing is all too critical as early intervention may negatively impact regeneration and functional recovery.[39,40] Post-acute neuroplastic reorganization involves changes to brain matrix and release of extracellular signaling factors (e.g., peptides). The TBI urinary signature demonstrated a significant overrepresentation of peptide by-products of proteins involved in neuroplastic processes (**Fig. 2**). Included were peptide growth factors and matrix components connected with Ncam signaling for neurite out-growth and axon guidance as well as an enrichment of post-synaptic density interacting components. These findings underscore the neuroplastic relevance of the TBI urinary signature supporting potential utility in brain injury rehabilitative medicine. Subsequent studies are needed, however, to examine temporal biokinetics of the TBI urinary signature in association with rehabilitative care and outcome.[7]

Several limitations of the present study also need to be addressed in future studies. Foremost, the present findings provide support for larger cohort studies. Enrollment criteria for these initial studies aimed to minimize confounders by restricting subject demographics, injury severity and exclusion of other major organ trauma.[7,41] However, follow-up studies are needed to evaluate the impact of these relevant factors on the TBI urinary signature. Individualized factors associated with patient symptomology and associated pharmacology and therapeutic care no doubt add further variability across subsets of molecular variables. While our study design focused on factors reproducibly responsive across all subjects, there are likely other molecules that remain to be explored within the TBI urinary signature that reflect such individualized aspects. Acknowledging these shortcomings, these findings provide proof-of-principle to support further research. Innovation was enabled by data-independent quantitative mass spectrometry, providing for reproducible measurement of endogenous biofluid constituents across subjects (**Fig. 1B**). Conventional data-dependent methods lack sufficient duty-cycle to provide consistent observation and precise quantification as necessary for these studies without employing molecular labeling methods, which are not suited to metabolomic analysis. The present approach used high-frequency and accurate precursor and product ion mass measurements to assess a large array of molecules with femtomolar detection and a general dynamic range on par with singular target ELISA kits. Given the stability of urine specimens, daily mass spectrometric assessment of the post-acute TBI urinary signature is feasible through properly equipped regional clinical service laboratories.

Conclusion

Findings reveal a diverse class of molecular products present within human urine that effectively classify TBI subjects from matched non-traumatized controls. The encompassed TBI urinary signature provided a reproducible pattern across a controlled cohort of severe TBI study subjects. Measures are directly linked with neuroplastic processes with relevance to brain injury pathobiology during the post-

acute rehabilitative phase. Further, the TBI urinary signature comprises multiple subsets found to correlate with clinical metrics of acute injury severity and post-acute behavioral and neurocognitive function. These results support further development of pattern-based urinary metabolite diagnostics and theragnostics to assess rehabilitation readiness and efficacy of intervention applicable broadly to brain injuries from trauma, ischemic and hemorrhagic insults.

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Authors Disclosure Statement

No competing financial interests exist.

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Table 1. Details of Correlation Analyses with TBI Clinical Metrics

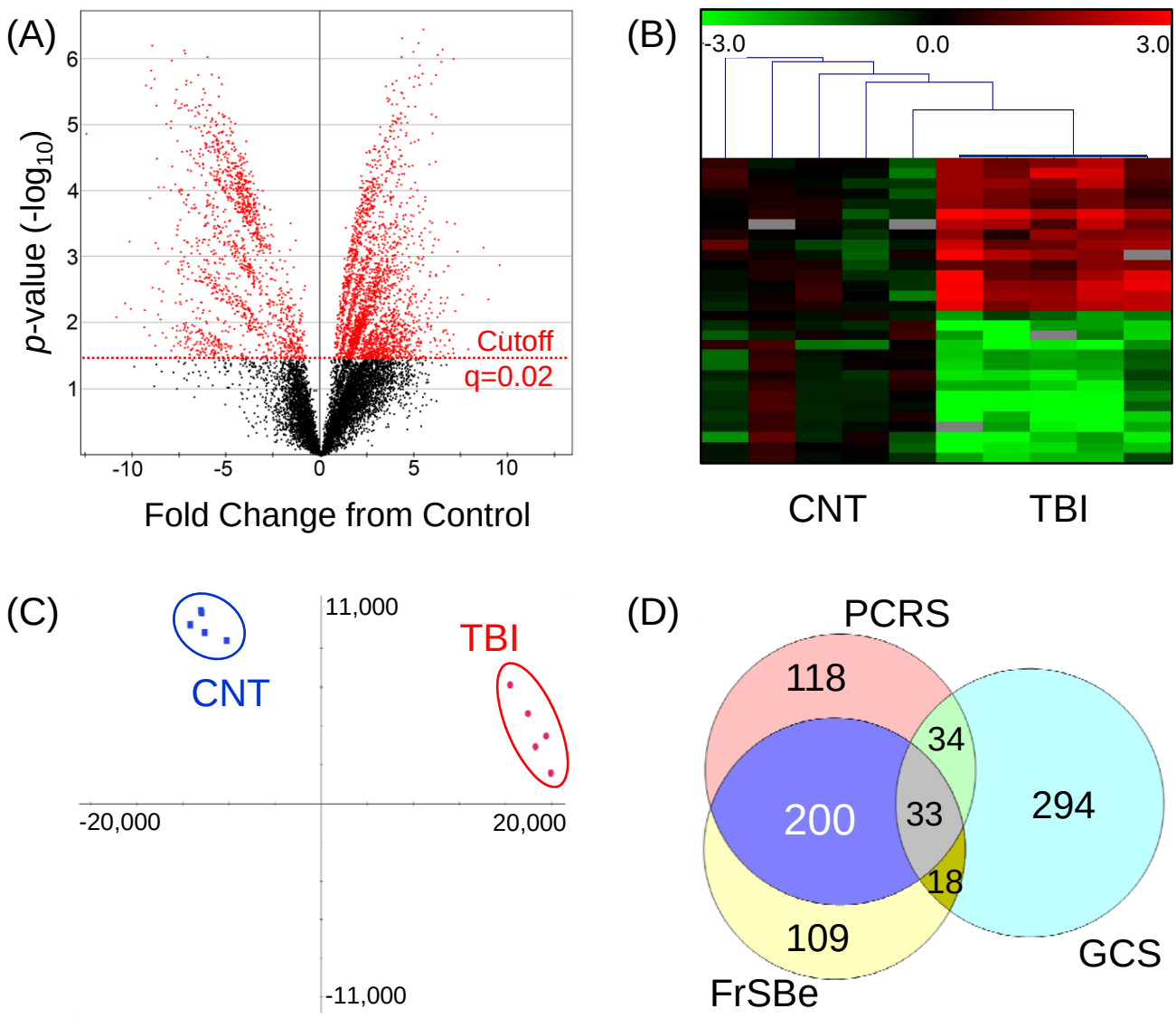
			TBI Clinical Assessments		
			GCS	PCRS	FrSBe
Subject Metrics	1		5	128	67
	2		3	141	119
	3		3	124	74
	4		6	137	85
	5		8	149	188
Correlated Sub-Profile Results	Pearson's R		>0.95	>0.95	>0.95
	# Corr. Var.		379	385	360
	t-score		7.787	8.114	6.752
	p-value		1.91E-08	9.07E-09	2.23E-07
Correlation between Clinical Metrics	GCS	R	1	0.634	0.624
		p		0.251	0.261
	PCRS	R	0.634	1	0.906
		p	0.251		0.034
	FrSBe	R	0.624	0.906	1
		p	0.261	0.034	

GCS, Glasgow Comma Scale; PCRS, Patient Competency Rating Scale; FrSBe, Frontal Systems Behavioral Scale; R, Pearson's R; *p*, *p*-value.

Figure Legends

FIG. 1. TBI urinary signature discriminates post-acute TBI subjects from controls. (**A**) Volcano projection of fold-change relative to control (CNT) plotted against statistical probability (*p*) values for 10,929 reproducing molecular measures in human urine specimens. Adjusting the significance level to an FDR of 2% (q=0.02), 3,897 measures (red) were found statistically responsive to TBI. (**B**) Heatmap plot of 30 representative molecular measures detected across CNT and TBI subjects (n=5/grp.). Measures are plotted as fold-change from control, scaled between -3.0 and 3.0, with gray fields denoting absent values. K-means hierarchical clustering results are illustrated by leader lines at top, with TBI data clustered together apart from CNT data. (**C**) Multivariate presentation of TBI (red) and CNT (blue) subjects by factor scores across principal components 1 (x-axis) and 2 (y-axis) with ellipsoids demarking eigenvector covariance. The maximum proportion of variance (PC1) comprised of discriminate molecular variables effectively resolved TBI subjects from CNT. (**D**) Venn diagram presentation of confluence between sub-profiles found in correlation with subject GCS (379), PCRS (385) and FrSBe (360) scores.

FIG. 2. The TBI urinary signature reflects on an ongoing neuroplastic response to TBI during the post-acute rehabilitative phase of care. Identified peptide fragments found increased within TBI urine were metabolized products of proteins associated with the post-synaptic density complex (DLG4 interactions), neurite out-growth, guidance cues and projection morphogenesis factors (growth factors, morphogenesis), extra-cellular matrix components (ECM) and transcriptional activity. (Top) Biological classes significantly enriched among represented proteins with selective relevance to a neuroplastic response following TBI. (Bottom) Protein-protein interaction network of proteins metabolized to form by-product peptide detected within the TBI urinary signature (nodes, with protein symbols).



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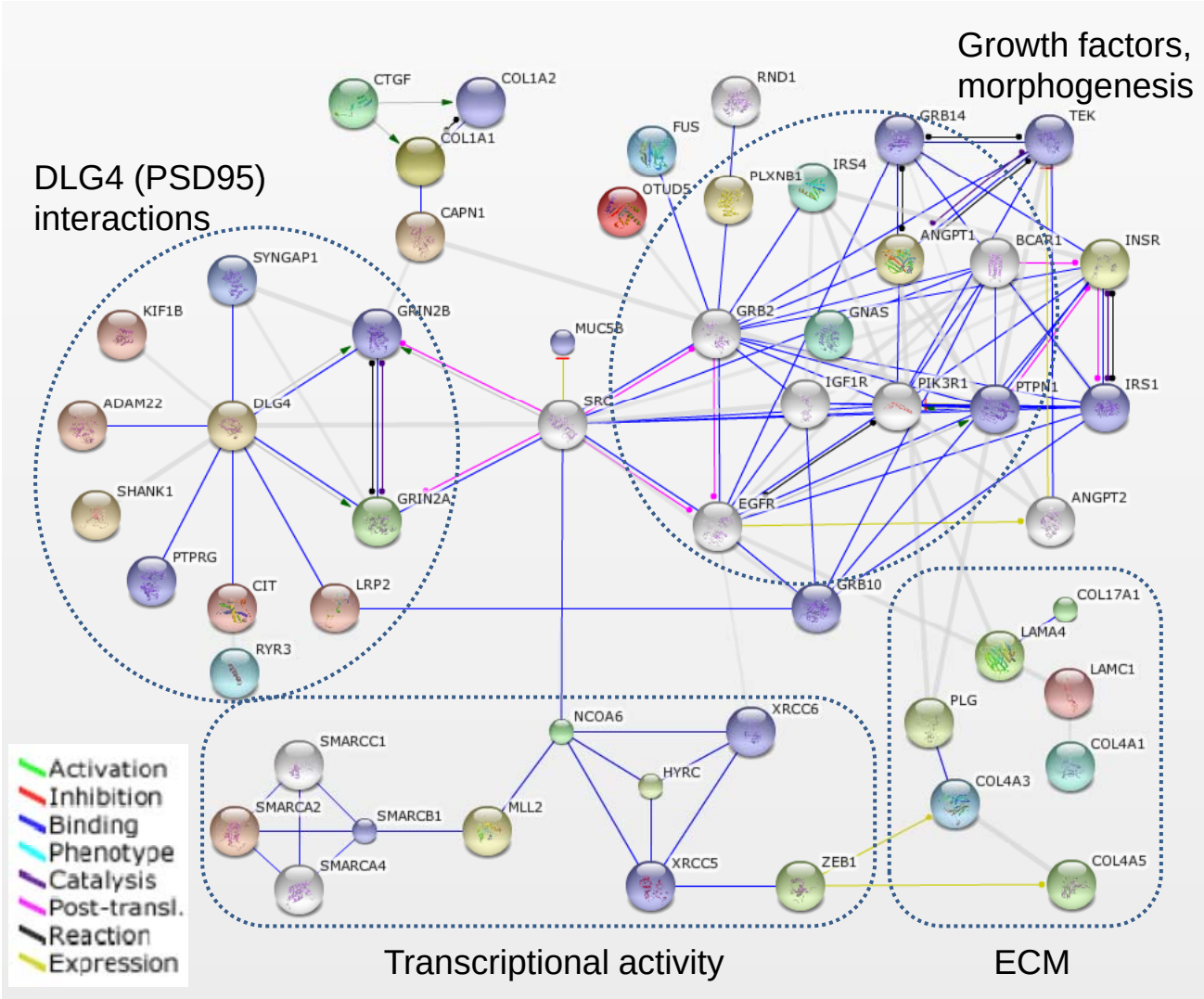
Name	Class	ID	Qty	P-value
DLG4 (PSD95) binding/activity partners	Interactions	Int:DLG4	8	$2.47e^{-3}$
Ncam signaling for neurite out-growth	Reactome	MSigDB:375165	7	$5.65e^{-5}$
Involved in axon guidance	Reactome	MSigDB:422475	8	$1.75e^{-3}$
Proteinaceous extracellular matrix	Cell Comp.	GO:0005578	18	$5.86e^{-9}$
Tissue development	Bio. Process	GO:0009888	24	$1.18e^{-3}$
Cell projection morphogenesis	Bio. Process	GO:0048858	15	$2.78e^{-2}$
Extracellular matrix structural constituents	Mol. Function	GO:0005201	10	$1.63e^{-8}$
Growth factor binding	Mol. Function	GO:0019838	6	$3.87e^{-2}$



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