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Proteomics: in pursuit of effective traumatic brain injury therapeutics

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Summary

Effective traumatic brain injury (TBI) therapeutics remain stubbornly elusive. Efforts in the field have been challenged by the heterogeneity of clinical TBI, with greater complexity among underlying molecular phenotypes than initially conceived. Future research must confront the multitude of factors comprising this heterogeneity, representing a big data challenge befitting the coming informatics age. Proteomics is poised to serve a central role in prescriptive therapeutic development, as it offers an efficient endpoint within which to assess post-TBI biochemistry. We examine rationale for multifactor TBI proteomic studies and the particular importance of temporal profiling in defining biochemical sequences and guiding therapeutic development. Lastly, we offer perspective on repurposing biofluid proteomics to develop theragnostic assays with which to prescribe, monitor and assess pharmaceutics for improved translation and outcome for TBI patients.

Keywords

proteomics; TBI; temporal; therapeutics; brain injury; inhibitory; synapses; theragnostics; informatics; post-translational modification

Advancement in the therapeutic care of traumatic brain injury (TBI) has been slow to come, despite growing recognition of over 2 million incidents annually in the U.S. [1,2]. Drugs found effective in the laboratory have failed repeatedly in clinical trials [3–12]. Upon reflection, we have been ineffective in matching a drug's pharmacology within the heterogeneous human TBI population [13]. TBI pathobiology varies in its evolution and complexity based on individualized injury-related, demographic and genetic factors [14,15]. Thus, a more targeted approach to TBI therapeutics is desired, one that translates an expanded understanding of a drug's biochemical actions after injury into individualized therapy that provides the right compound to the right patient at the right time.

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Proteomics stands to influence how we select and evaluate drugs and develop objective theragnostic endpoints for more effective TBI treatment. Pharmaceutics often work by manipulating protein processes (e.g., enzyme inhibitors, receptor antagonists, etc.) responsible for a wide range of biological activities (e.g., synthesis, enzymatic/metabolic, scaffolding and morphologic, trafficking, cell-to-cell communication, etc). We discuss how proteomics allows us to delve deeper into the protein-mediated biochemistry underlying TBI and the subsequent action of candidate drugs when instrumenting non-targeted, large-scale mass spectrometric analysis in multifactor study designs. We examine how proteomics can inform on novel therapeutic windows of opportunity and provide us with biochemical endpoints with which to evaluate candidate therapeutics in model systems. Further, biofluid proteomic methods engineered for biomarker discovery (reviewed elsewhere; e.g., [2,16–26]) can be repurposed to develop theragnostic assays with which to prescribe, optimize administration, and assess drug efficacy. In the next five years we expect proteomics to play a more decisive role in the development and translation of impactful TBI therapeutics.

Modeled TBI proteomics as a pharmaceutical test-bench

Ineffective translation of drug candidates has propelled the need to augment therapeutics research in TBI [14,27–30]. The latest large-scale proteomic technologies promise fundamental change in how we select and study drugs for TBI, providing the capacity to assess tens-of-thousands post-translational endpoints for TBI relevance and drug actions in a high-throughput, non-targeted analysis. TBI model systems serve as requisite test platforms with which to assess proteomic change, allowing control over cross-species populations, injury modality and severity, and intervention variables that require more rigorous pre-clinical evaluation ahead of costly clinical trials. Take the recent work of Mehan et al. in which they applied proteomics to assess subject age as a confounding factor in TBI outcome [31]. Proteomic results revealed that TBI increased the transmission of serum albumin proteins (SAPs) into the injured juvenile and young adult brain; however, the SAP response was absent in a geriatric cohort (21 month old rats). Thus, in addition to a more porous blood-brain barrier (BBB), the aged brain is now found to be defective in SAP-mediated transport after TBI. SAPs transport important neuroprotective factors (steroids, fatty acids, etc.); thus, their deficiency represents an important aged-associated vulnerability to TBI that would benefit from a targeted intervention to improve outcome specifically for older TBI patients.

In vitro models are also called for in deciphering inter- and intra-cellular aspects of the proteomic response to TBI. For example, Loov et al. employed a co-culture scratch model to assess the neuroproteomic response following a neuronal transection injury in the presence of supporting astrocytes, but not infiltrating inflammatory cells or commingled vascular pathology [32]. Applying non-targeted proteomics to this culture system, they identified novel factors secreted from deformed cells into the media, which otherwise would have been indistinguishable from intracellular proteomic change. They found that 28% of the secreted proteins were actin-interactors, such as the astrocyte-associated proteins ezrin and moesin. Live cell imaging revealed that these proteins are critical to astrocytic engulfment of dying cells, as later validated in an *in vivo* TBI model. Modulating those factors may prove beneficial in enhance debris clean-up after TBI. In all, TBI model systems provide the

workbench with which to test the effect of population and injury variables on the pathobiology of TBI and inform on biochemical targets for selective intervention.

Multifactor designs are necessary to interpret the TBI proteome

Future proteomics research needs to capitalize on multi-factorial study designs in order to better account for anatomical, cellular and temporal dimensionality. Interpreting these datasets will be biased by where and when changes take place. For example, Mehan et al. report that CRMP2 levels increased in abundance within neocortex but decreased within hippocampus at three days post-TBI [31]. CRMP2 is responsible for establishing neurite polarity during synaptogenesis; thus, there is a region specific propensity and/or timing of synaptic degeneration and remodeling that must be considered in evaluating systemic therapeutic interventions. Injury modality and severity must also be considered per their effect on the biochemical and neurobiological response to TBI. We recently reported differential pro-survival responses between traumatic and ischemic-only modalities of brain injury, despite a proportional burden of cell death [33]. Chaperone (Hsp70 and bound 14-3-3's) and antioxidant (Prdx) protein levels increased in cortical tissue two days following ischemic injury, while levels decreased in the same region following focal TBI. The proteomic response for proteins associated with cell survival, metabolic and synaptic dysregulation, were further correlated with the magnitude of injury. All together, the multifaceted influence of injury and subject variables must be accounted for in order to address how interventions will respond when challenged by the heterogeneity of clinical TBI.

TBI proteomics must also address complexity from sub-cellular translocation, post-translational modification and alternative isoform translation. Resolving the TBI proteome into soluble and membrane-insoluble fractions [34], we were able to deduce protein shifts from membrane-bound to matrix pools. For example, we discerned membrane-dissociation of vinculin after TBI, an integrin complexing protein relevant to synaptic destabilization and process retraction. We further found that translocated proteins were also post-translationally modified. Vinculin, for instance, exhibited increased phosphorylation at serine 721 [35]. Such investigations are now possible with careful analysis of individual peptide measures, in contrast with traditional peptide-to-protein roll-up analysis. Peptide-level assessment also divulges isoform-specific changes after TBI. For example, a unique peptide from a developmental isoform of neurofascin (NF125) was selectively increased in spared neocortex after TBI. In contrast, peptides common to mature NF155 and NF186 isoforms showed no change at the same time point. Immunoblot results affirmed that only the NF125 isoform was significantly upregulated. NF125 is transiently expressed during development to secure oligodendrocyte-axon contact [36]. Peptide-level data revealed that NF125 is also repurposed following TBI, perhaps to affirm axon-myelin contact. We have identified a multitude of uncommon protein isoforms within the TBI-responsive proteome, portending an important, yet underappreciated role for alternative translation in TBI pathobiology that can now be assessed with TBI proteomics.

Time as critical factor in TBI proteomic studies

The temporal evolution of TBI pathobiology may be well recognized by researchers (conceptualized in Figure 1); yet, few large-scale proteomic studies have incorporated time in their design, hampering our understanding of the data. A recent study by Wu et al. helps illustrate how profiling the proteomic response to TBI resolves ambiguity in interpreting results [37]. With one of the most comprehensive TBI proteome datasets published to date, Wu et al. employed sophisticated informatic methods to identify calcineurin B1 (CANB1) as a central node in the down-regulation of the actin-tubulin structural network within injured hippocampus. However, as with most TBI proteomic studies, their data were generated at a single 4-day post-injury time point. Thus, the results lacked the temporal detail needed to interpret the observed CANB1 modulation, which could be associated with the tail-end of acute neuroprotection from calcium-initiated degeneration or the beginning of a post-acute impairment of neuronal outgrowth. After considering data from Bales et al., they were able to determine that their results aligned with an almost immediate CANB1 reduction (by two hours) in protecting against calcium dysregulation [38]. Interestingly, CANB1 was then upregulated two weeks after TBI suggesting a post-acute neurotrophic function. Further support for TBI proteomic profiling can be found in a proteomic study by Evans et al. where they identified an inverse temporal relationship between the loss of mature MBP and initiation of myelin-associated glycoprotein (MAG) over three month period after injury [39]. We further observed that MAG undergoes transient dephosphorylated at Y611 and Y620 between one and two weeks following TBI (2.8-fold relative to naïve control; p=8.3E-7, n=6 per group), two key L-MAG signaling motifs for initiating myelination [40]. Yet, signaling mechanisms can easily be missed with a static or under-sampled study design. While we have just scratch the surface, such data divulge the temporal complexity surrounding molecular events underlying pathophysiological processes such as myelination dynamics, and further reflects the complexity to which therapeutics must be applied.

Temporal proteomics captures the sequence of TBI molecular mechanisms

TBI proteomic profiling allows proper characterization of evolving biochemical processes. While temporal proteomics represents a resource intensive, big-data challenge, we appreciate, reflecting on Figure 1, how a static view of TBI dynamics will omit critical information that ultimately prevents us from effectively understanding what occurs. Consider, for example, how protein-protein network analysis (informatics now commonly used in proteomics) results are easily skewed when using data from one time point to interpret biochemical processes that unfold across time. Too often we miss much of the story simply by under-sampling the dynamic events of TBI. Addressing this limitation, we recently assessed 23674 biologically reproduced peptide measures from 2243 proteins (n=6/time point) between 2, 4, 7 and 14 days following moderate controlled cortical impact in the rat. Evaluating these data, we observed substantial evolution within the TBI proteome (from spared neocortical tissue, outside the glial scar [34]). At two days after injury we observed 6209 TBI-responsive peptides; yet, by day 14, over half (52%) of the TBI-responsive peptides were novel. All told, 65% of measured proteins (1472) responded at some point within two weeks following TBI, significantly expanding our understanding of just how complex the proteomic response to injury happens to be.

Yet, these data provide us with novel insight into the sequential processes occurring after injury. For instance, we uncovered a post-TBI molecular sequence that echoed a mechanism employed in inhibitory network formation during neurodevelopment [41]. We observed (Figure 2A): (1) ubiquitination of neuroligin 2 (NL-2) at lysine K749 on day two and (2) NL-2 dephosphorylation at serine S714 by day four. NL2 is a selective inhibitory trans-synaptic anchor that also mediates axon guidance during synaptogenesis. These modifications correlated with NL-2 dissociation from cell soma by day two and then from distal synapses by day four (Figure 2B). (3) Synaptic membrane localization of the chloride symporter KCC2 declined significantly starting at day four (Figure 2A), causing GABAergic transmission to become excitatory (depolarizing) as in development [42]. KCC2 remained dislocated through day seven in our study, consistent with an earlier report of reduced KCC2 levels in hippocampus at one week post-TBI [43], while NL-2 S714 phosphorylation recovered by this time. (4) By day seven, there was also a reduction in membrane-associated polymerized gephyrin (Figure 2A) attributable to synapse destabilization. The revealed molecular sequence (Figure 2C) tracks with the molecular mechanism for inhibitory network formation and likely is repurposed after TBI for dematuration and remodeling of the inhibitory network. While our present study is far from exhaustive, it illustrates the future potential of large-scale proteomics in elucidating post-TBI temporal processes that may then be targeted for therapeutic intervention.

TBI proteomics as a framework to develop and optimize interventions

Proteomic data provides a roadmap with which to target and evaluate candidate TBI therapeutics. For instance, the molecular sequence discussed above defines multiple intervention prospects (Figure 3). Transient KCC2 destabilization between days 4 and 14 suggests the use of compounds such as 2,5-dimethoxy-4-iodoamphetamine (DOI) that promote KCC2 expression and stabilization of the inhibitory network [44–46]. In modeled spinal cord injury, DOI treatment restored KCC2 levels and recovered chloride homeostasis resulting in a functional reduction in spasticity. In tandem with targeting KCC2, drugs like dihydromyricetin (DHM) [47] may also assist in stabilizing the inhibitory network after injury by maintaining gephyrin polymerization [48,49], which holds together the post-synaptic specialization [50–52]. Thus, we may look to molecular sequences revealed from temporal proteomics as guides for when and what interventions may be effective after TBI.

Recently Wang et al. published a study that illustrates translation of proteomic findings into a pre-clinical intervention with demonstrated functional efficacy [53]. Their proteomics study examined the molecular mechanisms underlying stem cell therapy in TBI [53]. They indentified that an unexpected smooth muscle-associated isoform of actin, SMA, was responsive to stem cell therapy after TBI. Both *in vivo* and *in vitro* models revealed that injury induced alpha-SMA, an isoform not normally found in the brain, as part of the mechanism regulating stress fiber formation, which in-turn inhibits neurite regeneration. They deduced that stem cell-released GDNF blocked TBI-induced RhoA signaling and subsequent alpha-SMA production, which enhanced neurite outgrowth and improved functional recovery. They later affirmed that they could pharmaceutically block RhoA induced alpha-SMA expression to effectively enhance neurite outgrowth after modeled TBI.

TBI proteomics can also inform on as-yet unknown actions of pharmaceutics already under evaluation. Strides in this direction have already appeared within the stroke research community. Campos-Martorell et al. reported on a non-targeted proteomic study to investigate the mechanism by which simvastatin provides neuroprotection following rat embolic brain injury [54]. Their results show that oxidative stress pathways were attenuated by simvastatin, helping to preserve BBB integrity and minimize inflammation. Similarly, Zgavc et al. investigated the effect of hypothermia as a treatment in an endothelin-1 rat model of cerebral ischemia [55]. Proteomics revealed a positive effect on cellular assembly and organizational processes in the cortical penumbra. Specifically, BAIAP2L1 and A1AT were found to be novel mediators of hypothermic neuroprotection. The former is a brain specific 14-3-3 suppressor of p53-mediated apoptosis, while the latter inhibits neutrophil elastase to reduce vasogenic edema and BBB disruption. Proteomics is also conducive to testing candidate TBI drugs across species. Mass spectrometric analysis, unlikely immunological-based assays, can be easily ported from rodent to larger mammals for targeted and non-targeted assessments of inter-species pharmaceutical efficacy prior to the move into humans.

TBI proteomics in theragnostic biofluid assay development

Objective biofluid measures are sought to assist in identifying for whom and when to administer a given drug and assess biochemical efficacy. Existing biofluid proteomic methods and practices used for TBI biomarker discovery, validation and optimization (see Shen et al. for a recent update [56]) can be repurposed in devising theragnostic assays. Biofluid assays, when performed under carefully controlled conditions particularly with regard to clinical sampling parameters [57,58], promise a minimally invasive means to detect and monitor ongoing pathobiology for therapeutic management and prognosticating outcome [59–63]. Yet, to our knowledge, biofluid proteomic has yet to be used to develop TBI theragnostic assays. Again, looking to the stroke literature, we find an example from Campos-Martorell et al. [54]. After identifying that simvastatin helped preserve BBB integrity and minimize inflammation by attenuating oxidative stress, they then employed biofluid proteomics and resolved a correlation between HSP75 levels in plasma and simvastatin administration relative to placebo. Interventions for TBI may similarly induce biofluid changes that correlate with TBI biochemistry. Supporting this possibility, Boutte et al. recently demonstrated CSF measures track protein changes within injured neocortex CSF [64]. After modeled penetrating brain injury, syntaxin, tyrosine hydroxylase, UCH-L1, protein phosphatase 2C and cullin-1 proteins all increased proportionally in brain parenchyma and CSF, with a modest deviation in their temporal presentation.

Theragnostic assays are likely to employ a panel of biofluid measures, given the innate complexity of the biochemical response to TBI. Panel assays are espoused for their robust tolerance of inter-subject variability, yet to be effective in a theragnostic application, the selected measures should optimally correlate with the targeted pharmacology. Crawford et al. recently proposed employing protein-protein network analysis for panel assay development, whereby measures are selected based on their inter-relation through a common biochemical process [65]. In a similar fashion, network analysis could be adopted to develop a therapy-responsive biofluid assay. Another important finding in their study, Crawford et

al. observed a cluster of plasma proteins that varied in their temporal presentation in response to the severity of injury. A cohort of measures were present in blood one day following mild TBI but did not appear until three months after severe TBI, suggesting that a common biochemical process, perhaps linked with recovery, was delayed in onset with more severe TBI. Intervention windows are expected similarly to shift in time, particularly across the heterogeneous human TBI population, providing rationale for theragnostics. Siman et al. observed considerable inter-subject TBI variability in human CSF with their 11-protein assay [66,67]. The set of neuronal-derived CSF measures, developed with pre-clinical TBI proteomics, proved highly selective for TBI patients when assessed against normal pressure hydrocephalus and aortic surgical patients owed to its correlation with modeled neuronal injury. Yet, more work may prove valuable in linking these measures with specific biochemical processes that may be relevant to neuroprotective mechanisms. In this way, their bench-to-bedside proteomics approach may prove further beneficial in evaluating candidate drugs. Yet, theragnostic assays also need to correlate intervention with positive outcomes. Trajectory analysis as first demonstrated by Berger et al. offers a promising means with which to evaluate panel assay data against clinical outcome [68]. In their study, they showed that high-risk and low-risk trajectories were characterized by specific blood protein trajectories (S100B, NSE, MBP) in predicting better and worse outcomes, respectively. Drug-responsive TBI proteomic measures may similarly undergo trajectory model building to correlate intervention with positive outcomes.

Expert commentary and five-year view

The neurotrauma community recognizes a need to re-think how interventions are developed and translated effectively into the clinic. Current proteomic technology provides us with a novel, broad-scope window into the complex molecular processes underlying TBI, and stands to enable our ability to assess the influence of population- and injury-associated variables underlying the heterogeneity of clinical TBI. Thus, TBI proteomics will fundamentally change how we devise, test and translate TBI therapeutics over the next five years, as already begun in stroke. To achieve such impacts, proteomic study design and informatic approaches need significant attention. We are just now at the point where resources and technology allow for the temporal assessment of the large-scale proteomic response to TBI, yet the developing tools to resolve protein trajectory-maps and decipher patterns remains a fertile area of research. Methods will be devised to better track isoform-selective dynamics and the range of post-translational signaling events that govern TBI-altered biochemistry and function. Much as our society moves to address big-data challenges, we will gain the capacity to address the intricate nature of the TBI proteome and use that knowledge to advance development of TBI therapeutics to improve TBI patient care.

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Key Issues

- Despite growing public recognition and increased research attention, advances in TBI therapeutics have failed to materialize.
- Large-scale proteomics offers a forward-leap in our capacity to study the molecular impact of candidate drugs, both positive and negative, and how their action can be tailored across the injury and population factors that give rise to the heterogeneity of clinical TBI.
- Temporal characteristics of the neuroproteomic response to TBI are critical to understanding the sequential nature of the molecular processes governing secondary insults, recovery, and regeneration.
- Accounting for the breadth of translational and post-translational proteomic responses to TBI represents a monumental big-data challenge, requiring improved molecular characterization and informatic technologies.
- Objective biofluids measures of ongoing biochemical change are needed to assist translation of novel therapeutics. Theragnostic assays are needed for subject selection and as objective outcome measures in future clinical trials.

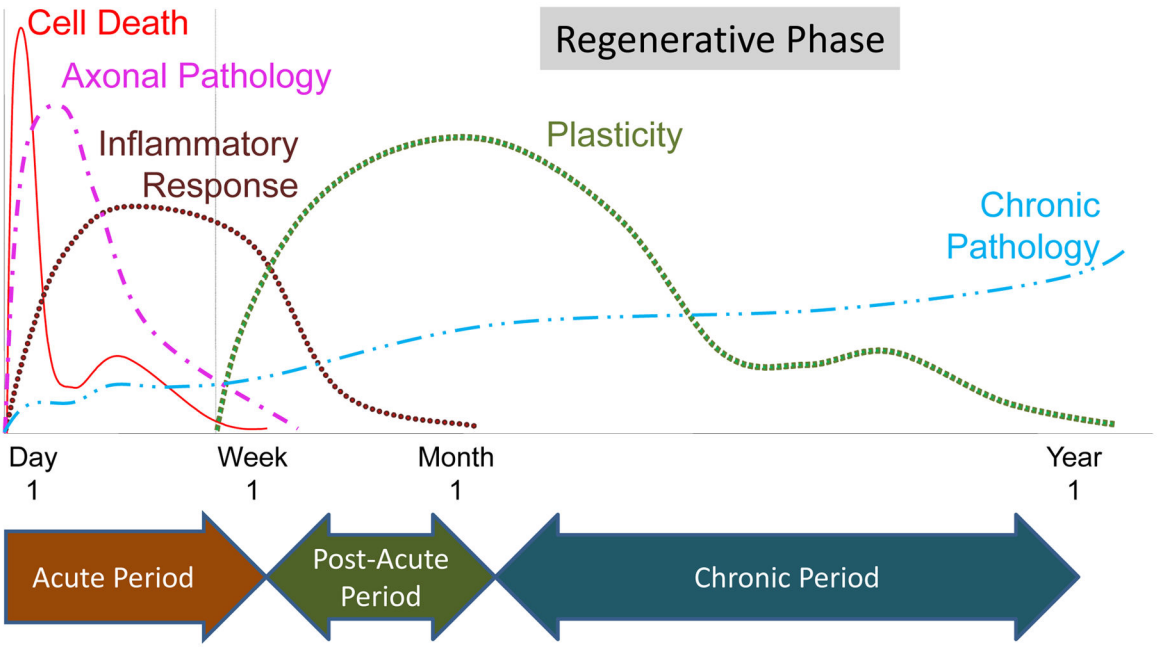

Figure 1. TBI pathobiology comprises a series of biochemical and physiological events that initiate and evolve in an individualized fashion

The response to TBI is dynamic and individualize based on a host of population- and injury-associated variables; yet, too often proteomic studies invoke static endpoint analysis. We must strive toward temporal analysis of the TBI proteome to effectively resolve the underlying processes of TBI and the extent to which they vary with key study factors.

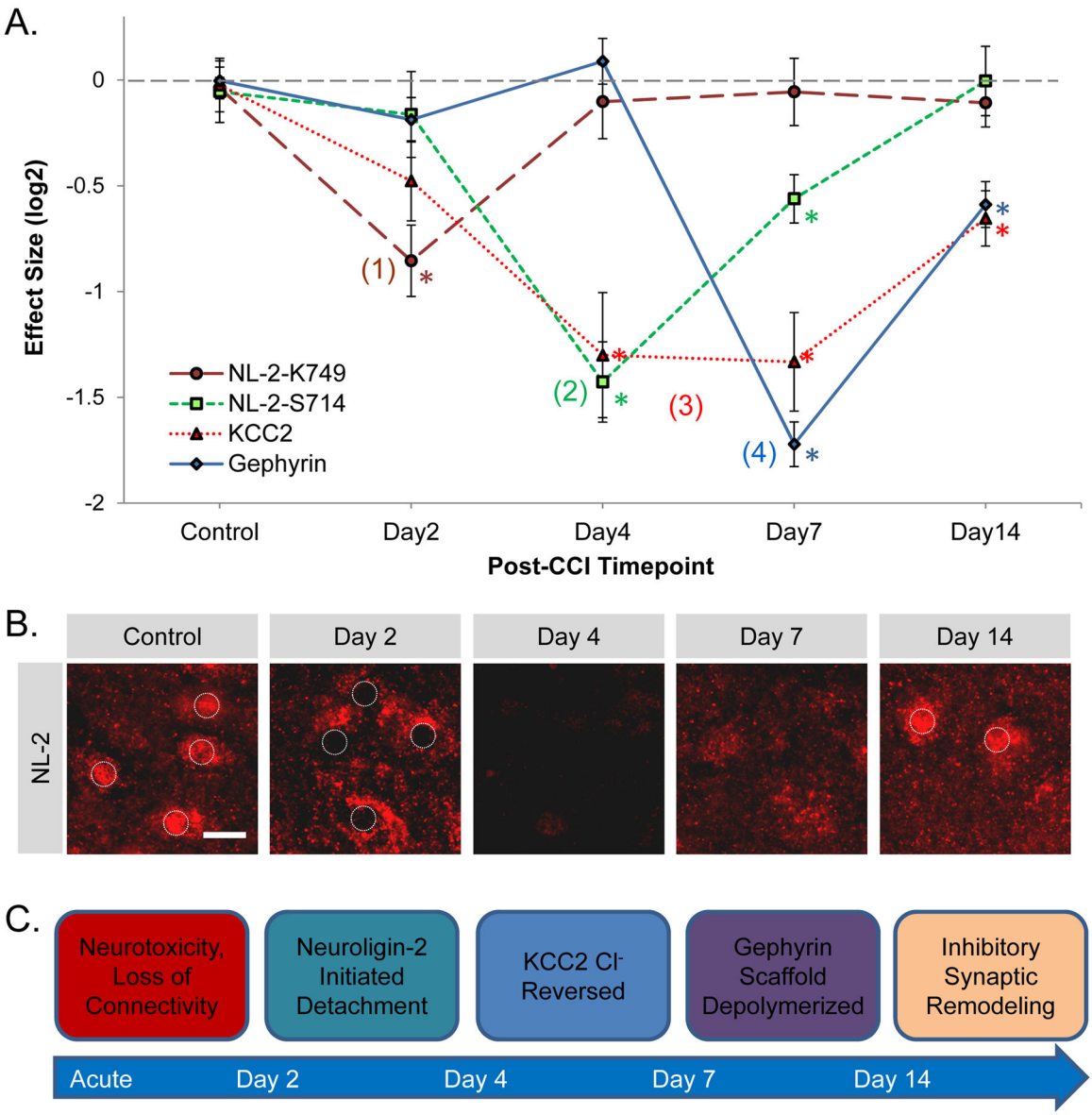

Figure 2. Decoding the temporal proteomic response to TBI: uncovered initiation cascade in remodeling the inhibitory synaptic network

Future applications of proteomics to TBI must consider the temporal dimensionality of the molecular response to TBI. For example, **A.** the initiation of inhibitory synaptic remodeling involves a series of post-translational events: (1) transient NL-2 ubiquitin signaling on K749 by day two; (2) transient NL-2 dephosphorylation on S714 by day four; (3) dissociation of KCC2 symporters from the membrane between days four and 14; (4) depolymerization of membrane-tethered gephyrin by day seven through 14. Mean±SE, n=6 per time point. **B.** NL-2 immunofluorescence depicting loss of somatic NL-2 staining (centered at dashed circles) at day two coinciding with K749 ubiquitin signaling, followed by synaptic NL-2 loss (red puncta) coinciding with S714 dephosphorylation at day four. NL-2 staining then recovers between seven and 14 days after TBI in time with recovery of S714 phosphorylation. Representative images of layer 4 somatosensory cortex; bar = 10 μm. **C.**

Trajectory of molecular events in initiating inhibitory synaptic remodeling after TBI. CCI: controlled cortical impact; KCC2: potassium-chloride co-transporter 2; NL-2: neuroligin-2.

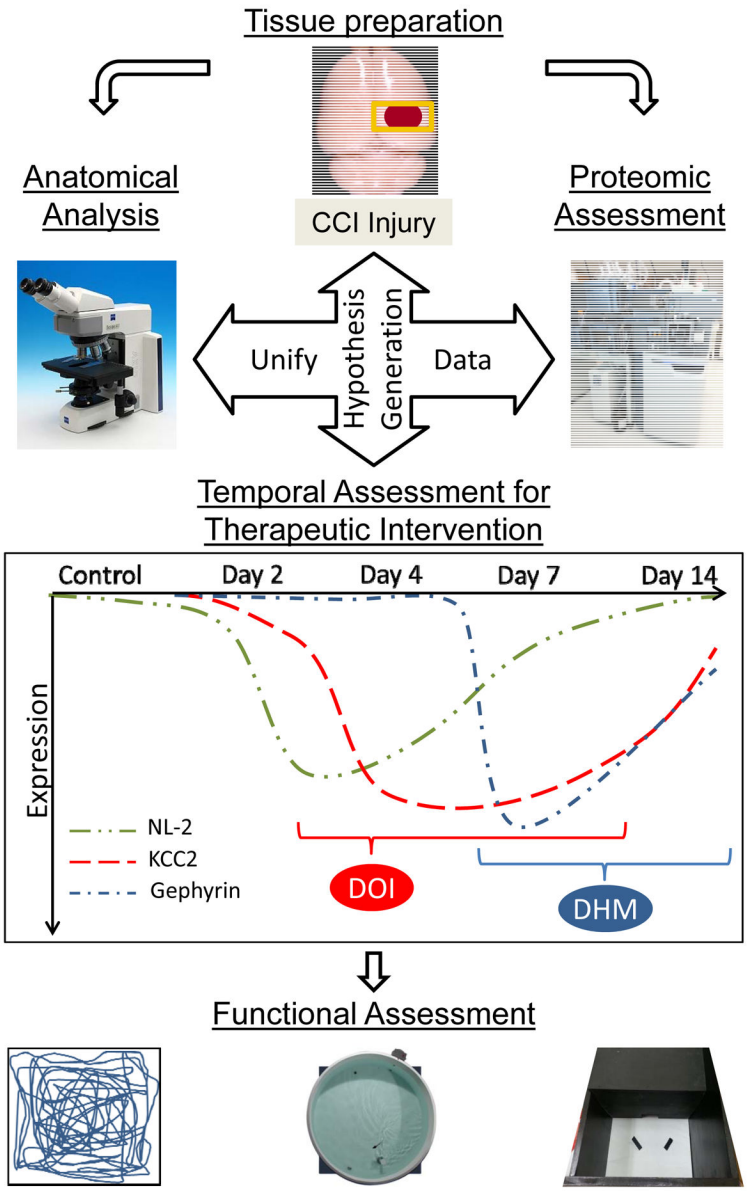

Figure 3. TBI proteomics guides novel intervention opportunities in a systems biology approach

Proteomics provides us with an efficient means to investigate the diverse biochemistry underlying TBI pathobiology. As a broad-scope endpoint measure, proteomics is conducive to discovery analysis when integrated with targeted anatomical, physiological and functional assessments as affirmatory evidence. Proteomics can inform, for example, on the molecular sequence underlying critical period inhibitory network remodeling within the first few weeks following injury. Pharmaceutics may then be selected based on the proteomic results. For example, DOI and DHM may be efficacious in maintaining the inhibitory synaptic network by mitigating TBI-induced declines in KCC2 symporters and polymerized gephyrin, respectively, when administered appropriately within the defined critical window.