

# Proteomics in stroke research: potentials of the nascent proteomics

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## ABSTRACT

Among omics, the proteomics assumes a unique role in that it offers the effectors or actuators of a biological condition. This brief review attempts to summarize the development in a relatively new but important subdiscipline of proteomics, the so-called *nascent proteomics*, and its potential applications in stroke research. First, we will discuss a few examples of proteomics-led discoveries in stroke research, and challenges or unmet demands when using commonly practiced proteomics approaches. Then we will introduce nascent proteomics and its studying tools, followed by discussions on its potentials in stroke research.

## INTRODUCTION

Since the term ‘proteomics’ was first coined in 1995,<sup>1–3</sup> over the past two decades or so, this discipline has advanced from qualitatively *fishing* in the early days, to semiquantitative, to quantitative, to now that a complex proteome can be described with absolute quantitation. Today, by the combined use of nanoflow ultra performance liquid chromatography (UPLC), tandem or triple quadrupole mass spectrometry (MS), and quantitation using either label-free or multiplexing labeling with stable isotopes approaches, thousands of proteins in a mammalian proteome can be detected and quantified in a single MS run. In 2014, more than a decade after the publication of the human genome, the first map of a human proteome was published, enlisting proteins or peptides encoded by 17294 genes.<sup>4</sup> It is of little doubt that proteomics will become as informative as the genomics does or even more in biomedical research in the days ahead.

## PROTEOMICS-LED DISCOVERIES IN STROKE RESEARCH AND CHALLENGES

### Proteomics in stroke research and unmet demands

In parallel with developments in proteomics technologies, the use of proteomic approaches in stroke research has been evolving as well. Prior to the year 2000, there were few publications on stroke research using proteomics. A PubMed search on the topic yielded only one hit—a 1995 study on mitochondrial encephalomyopathies, in which one of the seven study participants was noted under ‘stroke-like episodes’, and in which a two-dimensional electrophoresis (2-DE) technique was used.<sup>5</sup> Two-DE

used to be the method of choice for protein separation in proteomic studies at a time when a standard UPLC run could not offer satisfactory resolution. Fast forwardly, to date, essentially all available MS-based, quantitative proteomic approaches have been applied in stroke research, either on animal models, or directly on specimen from human patients with stroke (for more comprehensive reviews, see refs. 6–8). The objectives of proteomic studies in stroke research can be divided into two major categories: mechanistic studies to reveal molecular mechanisms underlying stroke pathology and to develop novel treatments, or biomarker discoveries for stroke diagnosis or prognosis purposes. The latter (biomarker discovery) has had some marked successes, with the identification of a number of plasma proteins as promising biomarkers.<sup>8,9</sup>

There are unmet demands. *First*, few published proteomic works have revealed novel and validated molecular mechanisms in stroke research. Rather, the results are often suggestive in nature, based on lists of altered proteins without follow-up biological studies. This is not surprising, given the complexity and high cost associated with proteomic studies that can be prohibitive in conducting studies at desired scale and depth. Also, follow-up and validation studies can be time-consuming. *Second*, for the discovery of stroke biomarkers, the focus has been mostly on plasma. Plasma is definitely a very useful source for stroke biomarkers. Now with the availability of protein chip technologies,<sup>10</sup> research on plasma biomarkers for stroke will be increasingly robust. However, the complexity in the sources of plasma proteins makes it extremely daunting to interpret the proteomic characteristics of plasma in stroke. In other words, plasma protein biomarkers can be useful in assisting stroke diagnosis or prognosis,<sup>11,12</sup> but are limited in providing an understanding of stroke pathology or new directions in stroke therapy.

### Proteomics-led discoveries and new challenges in stroke research

In pursuit of *protein effectors and actuators* of ischemic brain injury and its prevention, we employ an experimental paradigm of focal cerebral ischemia, modeled on mice, that includes three principal brain ischemic conditions as follows: injured (animals are subjected to prolong brain ischemia, resulting in



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hemispheric infarction), preconditioned (short ischemia, resulting in limited injury), and tolerant (prolong ischemia preceded by the preconditioning ischemia, resulting in a great decrease in injury).<sup>13</sup> In other words, by prior exposure to brief, non-injurious ischemia, the brain can be made resistant to injurious ischemia. There is considerable therapeutic potential in dissecting the biological mechanisms that produce tolerance as the protection is robust. By means of label-free, quantitative MS, we analyzed and compared proteomes of mouse brains under the three aforementioned brain ischemic conditions. We found that a group of transcriptional repressor proteins, namely polycomb group (PcG) proteins, are enriched only in ischemic-tolerant brains but not in injured or preconditioned brains.<sup>13</sup> This proteomic finding was followed by a series of cell biology, molecular biology and electrophysiology studies, and eventually led to the discovery of a PcG protein-mediated, epigenetic mechanism of neuroprotection against neuronal ischemic injury.<sup>13</sup> Unexpected at that time, the mechanism involves potassium channel genes as potential PcG protein targets (this topic will be discussed in greater detail elsewhere). The study (proteomics and follow-up biological experiments) was published on *Science Signaling* in 2010, and was noted as one of the breakthrough studies published on the journal in the year (*Sci Sign* podcast). Later, the PcG protein-mediated neuroprotection was also reported for seizure brains modeled to resist epileptic injury.<sup>14</sup>

As we set out to investigate in detail the PcG protein-mediated neuroprotection, one of the first questions we asked was what would be the molecular mechanisms that underlie increased levels of PcG proteins in ischemic tolerance. We analyzed gene transcripts for several PcG proteins by qPCR or in situ hybridization. To our surprise, no increase in mRNA levels has been detected for any of the PcG proteins of interests in ischemic-tolerant brains (Zhou *et al*, unpublished data). Among possible mechanisms, such as regulated changes in the metabolism and/or usage efficiency of PcG proteins and their respective mRNAs at pre-translational, cotranslational, or post-translational steps we suspect that changes in PcG protein biosynthesis may have accounted for their increased levels in ischemic tolerance. This argument is favored by the understanding that genomic changes in ischemic-tolerant brains are predominantly transcriptional downregulation,<sup>15</sup> and yet it is known that the development of brain ischemic tolerance requires new protein synthesis.<sup>16</sup> Regulated changes in protein synthesis is one of the primary cellular responses to stress. Even under harmful conditions such as ischemia, with energy deprivation, dysregulation of metabolic pathways, and severe attenuation of protein biosynthesis, cells may still be able to produce selected proteins as a survival instrument.<sup>17–22</sup> Sustained expression of genes encoding ‘stress proteins’ in ischemic brains was documented in the early 1990s.<sup>22</sup> The questions have become: In ischemic tolerance, with widespread transcriptional suppression, what would be the changes in protein biosynthesis proteome wide? With a speculated, overall reduction in protein biosynthesis due to energy deprivation, will the biosynthesis of selected PcG proteins be sustained or even increased? If so, how should this (regulation of protein biosynthesis in brain ischemia) be studied? What would be the appropriate

methodologies for studying proteins that are newly made—those that constitute a *nascent proteome*? Worth mentioning here is that, in the report by the 2012 NINDS Stroke Progress Review Group, on reviewing the progress made since year 2007, ‘Kinetics of proteomic changes during stroke injury development or the exhibition of injury resistance, *focusing on the nascent proteomes* to capture the initiation of must-have or must-stop events’ was noted as one of the ‘new stroke research opportunities, emerging topics, and undersolved areas since 2007.’<sup>11</sup> Next, we will introduce *nascent proteomics* and its studying tools.

## NASCENT PROTEOMICS AND STUDYING TOOLS

### Nascent proteomics

The *nascent proteome* refers to the proteome consisting of proteins newly synthesized during a certain period of time, for example, in the brain, during the first few hours following a transient ischemic attack, or in cultured neuronal cells, during or after simulated ischemia (by oxygen-glucose deprivation). As we will discuss below, nascent proteomics introduces a novel source for disease biomarkers and mechanistic understanding of molecular events that are difficult to reveal by studying the *total proteome* (all existing proteins). Results from our own studies show encouraging prospects.

Proteomes are highly dynamic. Individual proteins may differ greatly in their post-translational modifications and metabolisms in an organelle-specific, cell type-specific, tissue-specific, or organ-specific manner. In general, proteins in high abundance have slower turnover rates than those in low abundance.<sup>23</sup> Function wise, under normal conditions, proteins involved in ribosome biogenesis, cell cycle control, and DNA repair are found to show faster turnover rates than those involved in intracellular transports and protein complex assembling.<sup>23</sup> In mice, the turnover of proteome as a whole is faster in the liver than in the brain.<sup>24</sup> Importantly, proteome dynamics is subject to change, which ultimately will lead to phenotypic changes. While protein modification and degradation each contributes to proteome dynamics, protein biosynthesis plays a pivotal role in establishing a proteome. In this regard, in the contest of cellular response to stress, the nascent proteome can be viewed as a snapshot of *response-in-action*.

### Studying tools

Studying the nascent proteome involves two steps that are not needed in studies on the total proteome—metabolic labeling of living cells with a labeled amino acid, and detection of labeled, newly synthesized proteins. Traditionally, metabolic labeling for the purpose of studying protein synthesis involves the use of radioisotope-labeled amino acid(s) (eg, [<sup>35</sup>S]-methionine). Labeled proteins, with their identities unknown and remaining mixed with pre-existing proteins, are detected and quantified with either radiation counting of protein extracts, or radioautography of tissue sections or gels of fractionated protein extracts. This method has been used effectively for measuring changes in overall protein synthesis in brain ischemia.<sup>25</sup> The method, however, is not applicable for proteomic studies, in which the identities of all detected proteins are a must have.

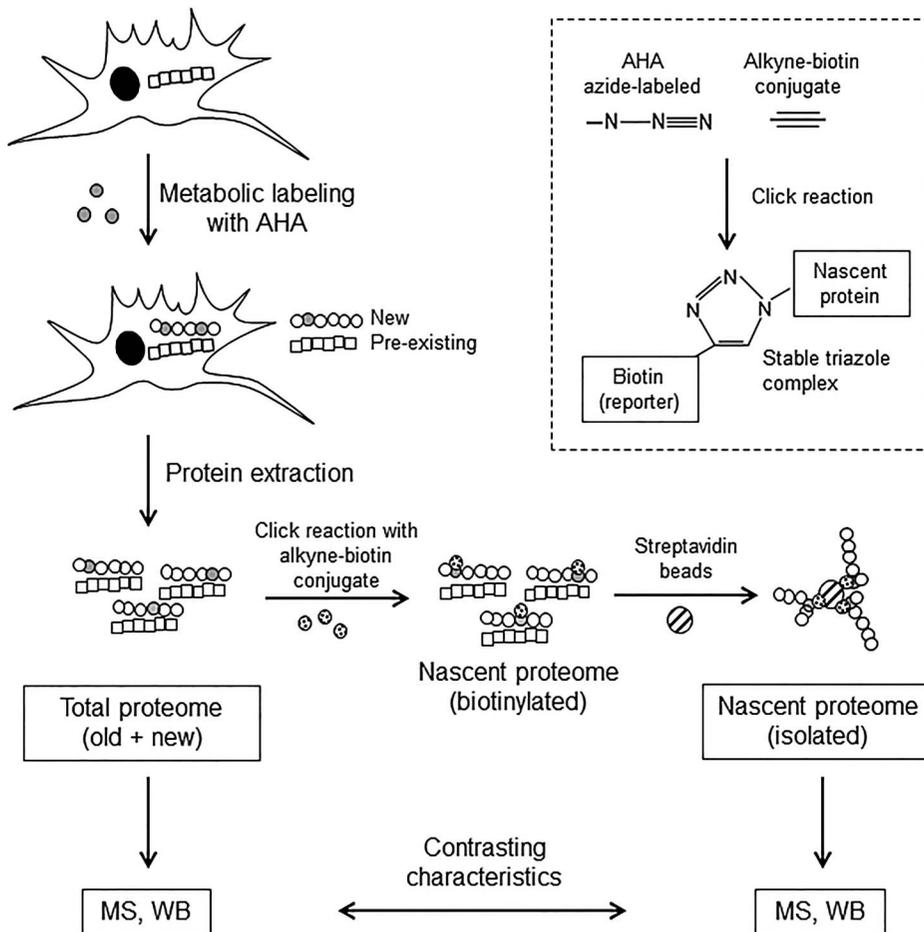
In recent years, the use of stable isotope-labeled amino acids, in combination with MS quantitation, has gained

popularity in characterizing nascent proteomes. A widely used method is the so-called SILAC method—*stable isotope labeling by amino acids in cell culture*.<sup>26</sup> In SILAC, two cell cultures (eg, a drug-treated culture and its respective control) are metabolically labeled with, respectively, light-stable and heavy-stable isotope-labeled amino acids (eg, [<sup>12</sup>C]-lysine or [<sup>13</sup>C]-lysine). Proteins extracted from light-labeled and heavy-labeled cultures are mixed together prior to MS analysis. During MS analysis, for each identified and quantified protein, the ratio between light and heavy forms is determined. The SILAC method is straightforward and robust. However, it does not satisfy the need of capturing all nascent proteins produced within a narrow time window, when they constitute only a small fraction of the total proteome, preventing effective detection. To this challenge, the click chemistry-based metabolic labeling offers a solution.<sup>27, 28</sup> This approach, as schematically illustrated in figure 1, allows labeling AND isolation (ie, enrichment) of newly synthesized proteins prior to MS analysis. The most widely used surrogate amino acid is an azide-containing methionine substitute—azidohomoalanine

(AHA). AHA can be covalently joined to a reporter-conjugated alkyne through the so-called click reaction (figure 1). The method was first used in bacteria cultures in vitro.<sup>29</sup> Until now, a wide variety of ‘clickable’ reagents are commercially available, and being used in studies on the biosynthesis of proteins, as well as in nucleic acids (DNA and RNA), lipids, and carbohydrates. The application has also been expanded from cultured cells in vitro to live animals in vivo.<sup>30–34</sup>

**NASCENT PROTEOMICS IN STROKE RESEARCH**  
**Feasibility**

Nascent proteomics in neuronal disorders is at its infant stage, more so in stroke research. As indicated earlier, there are unmet demands in understanding molecular mechanisms underlying neuronal ischemic tolerance, demands that cannot be met without knowledge of protein biosynthesis under different brain ischemic conditions, either proteome wide or for individual proteins of interests such as PcG proteins. A unique challenge in studying protein biosynthesis in stroke is the ability to capture newly synthesized



**Figure 1** Workflow of a typical click chemistry-based nascent proteomics study. Briefly, cells are incubated with AHA for a desired period of time (eg, 30 min). Proteins are extracted from cells. The extracted proteins can be directly subjected to analysis either by MS for proteomics or WB for individual proteins of interest, or to the click reaction to biotinylate AHA-labeled, newly synthesized proteins. The insert in the dashed box explains the principle of the click reaction. Biotinylated, AHA-labeled proteins are then isolated from the rest of the protein extracts by incubation with streptavidin beads, and subjected to MS or WB analysis. The same workflow can be applied to whole animal labeling for in vivo studies with adjustments of AHA concentration and duration of labeling. AHA, azidohomoalanine; MS, mass spectrometry; WB, western blotting.

proteins, when their amounts are greatly reduced in ischemic cells. In this regard, click chemistry-based metabolic labeling does have its advantages by affording isolation (enrichment) of labeled proteins (figure 1).

In 2011, we published the first study on nascent proteomes of cultured, ischemic neuronal cells.<sup>35</sup> The results support the feasibility. Most encouragingly, results of bioinformatic analysis of regulated nascent proteins revealed changes in cellular processes that have not been seen from analysis of the total proteome of ischemic brains. Later, the success was extended to an *in vivo* study on ischemic mouse brains. In the study, AHA was administered to the animal via intracerebroventricular injection (after 1 hour reperfusion to allow some recovery of protein biosynthesis). Both the nascent and the total proteomes were analyzed. Comparisons were made between the nascent and the total proteomes of the same brain, and among brains under different ischemic conditions. The results showed that the characteristics of the nascent proteome and its changes are drastically different from that of the total proteome. The initial results were presented at the 2013 Annual Meeting of Society for Neurosciences (San Diego, California, USA. Bian F *et al*, Nascent proteomes in response to brain ischemia. Poster #487.19/MMM9). Most recent results from our laboratory showed that, in cultured neuronal cells, there is an overall decrease in protein biosynthesis in all three ischemic conditions (injured, preconditioned, and tolerant). Interestingly, in the same cultures, the biosynthesis of selected PcG proteins shows a trend of increase under the tolerant condition (Zhou *et al*, unpublished data). Hence, looking forwardly, we consider nascent proteomics a valid approach in stroke research in *in vitro* and *in vivo* experimental settings.

### Clinic applications

Can click chemistry-based nascent proteomics be applied to clinic research?

We noted earlier that plasma protein biomarkers for stroke, despite their potential usefulness, are difficult to interpret and offer limited understanding to stroke pathology, given the uncertainty of their sources. Remarkably, results from blood transcriptomics analyses, either on the whole blood or on isolated white blood cells, have shown close correlations between the patterns of gene expression and the types and severities of stroke.<sup>36–38</sup> This indicates the potential usefulness of nucleated cells in circulation as a source for stroke biomarkers with specification of brain conditions. Taken the advantages of our success in studying nascent proteomes in neuronal cultures, we piloted a study on nascent proteomes in peripheral blood mononuclear cells (PBMC) in human patients with stroke. In the study, fresh PBMC cultures were prepared from patients with stroke admitted to a local hospital in Atlanta, and their age-matched, sex-matched, and race-matched controls. Both nascent and total proteomes from the same cell preparations were determined. Again, we found changes in PBMC nascent proteomes that were not seen in total proteomes.<sup>39</sup> Since the study included only a small number of patients, we were cautious in considering the significance of proteins that showed a change in the PBMC nascent proteomes in patients with stroke. Nevertheless, the study has

demonstrated the applicability of nascent proteomics in human disease research.

### Potentials

With continuous developments in novel methodologies for protein labeling, isolation, and detection, this unique sub-discipline of proteomics is poised to be instrumental in stroke research in a broad range of topics. Most obviously, by characterizing proteins that are most responsive to stroke by changing their biosynthesis, nascent proteomics helps to capture cellular activities or signaling events that are actively regulated and can be potential targets of intervene. Click chemistry-based metabolic labeling of whole animals is extremely enticing, since it provides the possibility to assess protein biosynthesis proteome wide and systematically during and after a brain event. Within the scope of this brief review, we did not discuss the development in some other areas of proteomics, such as spatial and single cell proteomics. Theoretically, it is doable to analyze the nascent proteome to that detail, to bring forth a novel body of knowledge of molecular events in defined populations of brain cells. To translational stroke research, the nascent proteome of circulating blood cells can be a novel source for biomarkers, those that signature brain conditions, as well as reveal changes in the blood immune cells themselves. With ample choices of commercially available labeling reagents, multiple nascent omes can be studied simultaneously. An achievable goal would be to characterize PBMC nascent transcriptomes and proteomes simultaneously in human patients with stroke inclusive of different stroke types and severities. We can anticipate multiple novel panels of biomarkers for stroke, and revelation of molecular events in PBMC.

In summary, nascent proteomics offers a unique and in-depth perspective to our understanding of cellular response to stroke. It is applicable to basic and clinic stroke research.

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