Commentary

MOLECULAR PAIN

Molecular Pain
Volume 12: 1–5

DOI: 10.1177/1744806916679658
mpx.sagepub.com
SAGE

Exploring novel paths towards protein signatures of chronic pain

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Abstract

Pain is a major symptom of many medical conditions and the worldwide number one reason for people to seek medical assistance. It affects the quality of life of patients and poses a heavy financial burden on society with high costs of treatment and lost productivity. Furthermore, the treatment of chronic pain presents a big challenge as pain therapeutics often lack efficacy and exhibit minimal safety profiles. The latter can be largely attributed to the fact that current therapies target molecules with key physiological functions throughout the body. In light of these difficulties, the identification of proteins specifically involved in chronic pain states is of paramount importance for designing selective interventions. Several profiling efforts have been employed with the aim to dissect the molecular underpinnings of chronic pain, both on the level of the transcriptome and proteome. However, generated results are often inconsistent and non-overlapping, which is largely due to inherent technical constraints. A potential solution may be offered by emerging strategies capable of performing standardized and reproducible proteome analysis, such as *d*ata-*i*ndependent *a*cquisition-*m*ass spectrometry (DIA-MS). We have recently demonstrated the applicability of DIA-MS to interrogate chronic pain-related proteome alterations in mice. Based on our results, we aim to provide an overview on DIA-MS and its potential to contribute to the comprehensive characterization of molecular signatures underlying pain pathologies.

Keywords

Chronic pain, proteomics, data-independent acquisition-mass spectrometry, mass spectrometry, protein signature, mouse models of chronic pain

Date received: 21 June 2016; revised: 1 September 2016; accepted: 13 October 2016

Overview of current profiling approaches in pain research

While acute pain is an evolutionary adaptive response, chronic pain is considered a pathology of the nervous system. In vertebrates, a painful stimulus is detected by specialized peripheral sensory neurons like those of dorsal root ganglia (DRG). These DRG neurons serve as primary cellular detectors of noxious stimuli.[1] Their activity ultimately shapes the afferent message transmitted via the spinal cord to different regions of the central nervous system where the sensation of pain is generated. From a molecular point of view, the function of each relay station of this pain axis relies on dedicated protein machineries that are fine-tuned and highly regulated.[1–3]

During the past decade, enormous efforts have been made towards the identification and characterization of key molecular pathways that orchestrate processes underlying chronic pain. Excellent previous studies documented pain-related transcriptome variations using microarrays and deep sequencing methods (RNA-Seq).[4,5] Although these findings yielded important insights, the extent to which mRNA alterations account for protein variability is limited.[6] It is thus desirable to directly assess chronic pain-induced proteome changes, as the proteome represents the composite readout of gene expression, translation, and posttranslational modulation.[6] Many investigators have employed MS in order to reveal proteome changes in tissues from both, rodent models[7–12] and patients suffering from chronic pain.[13,14] Despite the body of knowledge on potentially regulated proteins generated by these studies, the

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sparsity of overlapping identifications, even for the same experimental model, is striking.

A common factor of these proteome profiling schemes is the use of so-called *d*ata-*d*ependent *a*cquisition MS (DDA; commonly referred to as shotgun MS). When a sample (a mixture of diverse peptides) enters the mass spectrometer in an average DDA experiment, the most abundant peptides (i.e., the most intense signals) are selected for subsequent fragmentation and identification, while the rest of the peptides are excluded from analysis.[15] This results in "under-sampling," with the average DDA-MS experiment identifying only 10% to 20% of peptides of a given sample.[15–17] Furthermore, the selection of low- and medium-abundant peptides is very variable, leading to limited reproducibility of detection between technical replicates (40% to 70%)[15]— a relevant problem, especially in complex samples such as tissue lysates. Consequently, this ambiguity and bias towards detection of the most abundant proteins,[15] paired with differences in sample preparation, are major obstacles in the quest to monitor the dynamics of cellular protein networks across different pain states in a comprehensive and reproducible manner.

Emerging DIA-MS-based proteomics

In the past years, MS has significantly advanced in the search of novel solutions to circumvent these commonly encountered problems of classical DDA experiments.

Highly optimized and extended DDA profiling has successfully achieved extensive proteome coverage.[18] Still, limitations hinder its broad implementation in the pain research community: on one hand, the need of long instrument times and on the other hand, the lack of tools that can be shared to standardize the detection of these large sets of proteins.

One emerging group of MS strategies that have the potential to overcome these bottlenecks is so-called *d*ata-*i*ndependent *a*cquisition (DIA)-MS.[19–21] DIA-MS is not only capable of detecting and fragmenting the majority of peptides present in a complex sample,[19–21] but it also does so with high reproducibility enabling standardization among laboratories. In contrast to DDA-MS, DIA-MS performs concurrent selection and fragmentation of nearly all peptides entering the mass spectrometer on the basis of their mass/charge (m/z) ratio and irrespective of their intensity (as long as their intensity is above the detection limit of the mass spectrometer). The measured fragmentation data (composed of retention time, intensity, and m/z data from each fragmented ion) for each generated spectrum are stored in DIA digital maps, which are unique for a given sample.[19–21] However, such digital maps are highly complex as each generated spectrum contains information from different peptides (referred to as a chimeric spectrum); this prevents the use of conventional database search tools to identify unique peptides. Hence, reference spectral libraries are employed to query these DIA digital maps in a targeted way.[22] A reference spectral library is built from several highly optimized DDA-MS runs on the sample of interest in order to achieve the most extended number of peptide identifications possible.[23,24] This compendium of peptides contains the necessary physicochemical information to uniquely identify them in the aforementioned chimeric spectra of DIA digital maps, which are produced by subsequent analysis of the same sample in DIA-MS mode. The use of these reference spectral libraries permits the quantification of thousands of proteins across different samples with unprecedented reproducibility.[19–21] Another interesting feature of DIA-MS is that DIA digital maps can be queried in silico any time using new/extended spectral libraries thereby opening the opportunity to test novel hypotheses without the need to perform laborious new experiments. These characteristics, together with the availability of both DIA digital maps and reference spectral libraries in growing public repositories (PeptideAtlas, http://www.peptideatlas.org), positions DIA-MS as a promising tool towards highly reproducible and standardized protein profiling.[21] Indeed, several reports highlighted the potential of DIA-MS to interrogate pathology-related proteomes, such as signatures for tumor characteristics[25,26] or drug-related protein–protein interactions.[23,27]

DIA-MS-based proteomics in pain research

We have recently demonstrated the applicability of DIA-MS to pain research and its potential to reach standardization in future pain-related profiling projects.[28] In our study, we defined and compared changes in the membrane-enriched proteome of DRG in two commonly used mouse models of chronic pain with different etiology: inflammatory pain induced by injection of Complete Freund's Adjuvant (CFA; Vehicle injections served as controls) and neuropathic pain using the spared nerve injury (SNI) model (control groups received sham surgery). A methodological outline of our workflow and experimental design is shown in Figure 1. After biochemical preparation of the samples, we first generated a DRG-specific spectral library including samples from each condition (CFA vs. Vehicle and SNI vs. Sham). The final spectral library contains information on 3,067 proteins exhibiting diverse molecular functions across many cellular compartments as assessed by gene ontology analysis.[28] This spectral library provides the first available resource of its kind for future DIA-MS experiments in any tissue such as regions of the pain axis (the spectral library is deposited in PeptideAtlas; http://www.peptideatlas.org, No. PASS00826, username: PASS00826, password: ZE5945at).

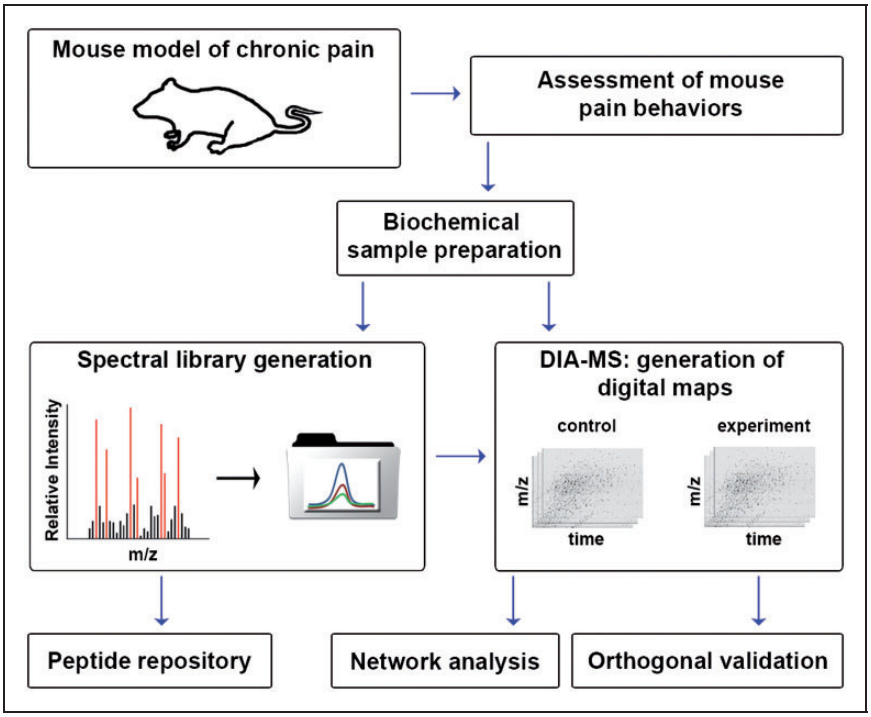

Figure 1. Schematic representation of an experimental workflow suitable for standardized and comparative protein profiling in any tissue of interest across mouse models of chronic pain.

We applied this spectral library for the targeted search of DIA digital maps obtained from DRG after induction of inflammatory and neuropathic pain as well as respective controls (CFA vs. Vehicle and SNI vs. Sham). As a result, we obtained a quantitative comparison of 2526 proteins across all biological replicates of the four conditions tested. Interestingly, these experiments revealed significant and largely pain model-specific alterations of 129 proteins, among which are targets that have either previously been reported to be relevant for nociception or represent yet uncharacterized proteins. In addition, the high statistical power of our DIA-MS results allowed us to uncover global alterations in diverse cellular protein networks, such as protein maturation and mitochondrial function. A comparison with known biology, combined with our functional validation experiments using independent animal cohorts, demonstrates that DRG protein networks relevant for pain and somatosensory signaling were indeed identified.

DRG constitute a major peripheral part of the pain axis. Yet, pain is perceived and strongly modulated by an intricate interplay of many regions of the central nervous system along the pain axis. Elegant studies have documented prominent pain-induced plasticity in the dorsal horn of the spinal cord[29–32] as well as in diverse supraspinal sites. For example, several forms of synaptic plasticity in the anterior cingulate cortex are implicated in emotional-aversive aspects of pain including pain-related anxiety.[33,34] Moreover, distinct pain-associated modifications in neuronal activity were found in the prefrontal cortex[35] as well as in the amygdala.[36,37]

It is this complexity underlying pain perception and modulation that renders the search for molecular signatures of pain a challenging endeavor. Nonetheless, the features offered by DIA-MS towards the standardization of protein profiling provide novel opportunities. These include the generation of tissue-specific spectral libraries and the applicability of existing spectral libraries to explore proteome changes in any region of the pain axis.

Conclusions and outlook

In spite of its likely impact on molecular pain research, several limitations, which are currently preventing DIA-MS from reaching its full potential have yet to be solved.

DIA digital maps are very complex and reliable extraction of protein information with spectral libraries is demanding. In our study, for example, the spectral library (and thus our DIA-MS results) did not contain information on many ion channels known to be involved in nociception and pain such as Nav1.7. The reasons can be manifold, ranging from relative low expression levels (and consequently difficulties in detection by DDA-MS used for spectral library generation) to insufficient solubilization — factors known to render membrane protein analysis challenging.[38] Hence, optimization of biochemical sample preparation and spectral library generation will be key to obtain the most complete coverage of the

proteome. In addition, recent advances in both targeted (using spectral library information[22]) and untargeted approaches (using computational workflows[39]) offer improved strategies to extract information from DIA digital maps. Moreover, novel MS acquisition strategies[40] are designed to reduce the complexity of DIA maps and to simultaneously maximize the amount of protein information obtained. Further, while to date DDA-MS units are available in many research institutions, DIA-MS platforms are still in a nascent stage. We expect that the growing landscape of technological solutions together with the access to user-friendly DIA analysis software (e.g., Skyline, OpenSWATH) will facilitate the incorporation of DIA-MS into the researchers' toolbox.

Despite the vast amount of research, pain management still relies on the ''one drug fits all'' model. However, cellular signaling is diverse and it has long been known that the function of a single protein may be modulated by its assembly into dynamic multi-protein complexes and networks—a fact represented by the concept of cellular ''molecular machines.''[41] Therefore, evolving strategies aimed at correcting dysfunctional networks may provide promising tools for achieving analgesia.[42] Identifying the composition of such networks in a tissue-specific manner can greatly facilitate insights into functional misalignments of pathology-associated processes with the opportunity to modify them at distinct, critical hubs.[43] This may provide major benefits: (i) increase of specificity, (ii) possibilities for discrete tuning of cellular functions, and consequently, (iii) less side effects on key physiological functions.

The results from our study serve as a proof-of-principle for the generation of molecular portraits of signaling networks underlying specific chronic pain states. The availability of large and tissue-specific spectral libraries will open the possibility to perform longitudinal studies investigating the molecular plasticity along the whole pain axis or even in any tissue of interest. On the clinical front, DIA-MS has the potential to expedite the long-awaited stratification of chronic pain conditions through sensitive and specific diagnostics tools using minimally invasive liquid biopsy matrices (e.g., plasma). Consequently, more efficacious and personalized pain therapies may be developed.

We are confident that forthcoming DIA-MS-based research efforts of the pain-community will help to initiate new turns on the path towards pain relief.

Acknowledgments

We are grateful to Allison Barry (IMPRS Neurosciences, Göttingen) for proof reading the manuscript.

Author Contributions

Both authors contributed equally.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Emmy Noether-Program of the Deutsche Forschungsgemeinschaft (SCHM 2533/2-1 to MS), research grants of the Deutsche Forschungsgemeinschaft (GO 2481/3-1 to DGV; SCHM 2533/4-1 to MS) and the Max Planck Society. MS received a research award and travel support by the German Pain Society (DGSS), both of which were sponsored by Astellas Pharma GmbH (Germany). DGV received research support from Biognosys AG (Zurich, Switzerland) Neither funding from Astellas Pharma GmbH nor from Biognosys AG influenced the content of this article.

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