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Primer

A Primer on Concepts and Applications of Proteomics in Neuroscience

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The enormous complexity of the central nervous system has impeded its systemic exploration for decades but powerful "omic" technologies are now pushing forward the frontiers of neuroscience research at an increasing pace. This Primer reviews the most recent progress in mass spectrometry (MS)-based proteomics, focusing on the analysis of whole proteomes, protein-based interactions, and post-translational modifications. We also discuss how advanced workflows help to unravel spatial, regulatory, and temporal aspects of neuronal systems. These tools and approaches have already led to detailed and quantitative proteomic maps of the brain and its signaling architecture, generating new insights into health and disease. We predict that these new approaches will also accelerate biomarker discovery and contribute to novel therapeutics for neurodegenerative and other brain-related diseases.

Introduction

The brain is arguably the most fascinating organ and also the most complex, as reflected in its hundred billion neurons and their several hundred trillions of interconnections (Koch and Laurent, 1999). Together, this enormous network of cells is responsible for receiving, processing, and executing cognition and perception, controlling motor activities, and storing memories over decades (Kandel, 2001). Harmful perturbations at the basic cellular level or in the neuronal networks of the central nervous system (CNS) can lead to a variety of developmental, degenerative, or psychological disorders. Assessing this complexity of the nervous system at the molecular level remains one of the central challenges in neuroscience. Over the last decades, widely diverse technologies such as electron microscopy, *in situ* hybridization, magnetic resonance imaging, positron emission tomography, and optogenetics have provided vitally important insights into the underlying principles of the CNS. The rapid technical developments in genomics and transcriptomic approaches have likewise been used to describe the CNS in a system-wide fashion (McCarroll et al., 2014; Shin et al., 2014).

In contrast, biochemical approaches—especially large-scale ones—have had less of an impact on neuroscience research in recent years, which is unfortunate as all the functions of the brain are ultimately mediated by proteins. We believe that this is largely due to the lack of powerful tools to study proteins in an unbiased, quantitative, and sensitive manner, similar to the advent of next-generation sequencing (NGS) in the genomics field. We argue that these historical and conceptual limitations are now rapidly being removed due to the maturation of mass spectrometry (MS)-based proteomics into a powerful and versatile toolset, which can be used to investigate proteins and their functions in an unbiased manner (Aebersold and Mann, 2016; Altelaar et al., 2013). Although a plethora of different MS-based approaches have been described, the field has converged on a generic workflow that has been continuously refined over the years and that is applicable to a remarkable variety of questions. As shown in the workflow in Figure 1, proteins from any source are extracted in a form suitable for enzymatic digestion (Figure 1). The resulting peptides are then separated on high-performance liquid chromatography (HPLC) systems, which are coupled online to the mass spectrometer via electrospray ionization (Figure 1). Peptide mass and intensity are measured in MS scans that alternate between peptide selection and fragmentation, enabling peptides to be identified via comparison of the fragment or MS/MS spectra to a sequence database (Figure 1) (Savaryn et al., 2016). Ideally, this workflow provides the identity and quantity of all expressed proteins in the sample. There are different variations of this "bottom-up" workflow, including different MS and MS/MS scan regimes, as well as strategies for absolute or relative quantification, each with their own strengths and weaknesses (Ebhardt et al., 2015; Picotti and Aebersold, 2012). It is even possible to fragment intact proteins without digesting them into peptides, but this "top-down" approach is much more challenging and not the subject of this Primer (Toby et al., 2016).

Apart from differential protein expression, MS-based proteomics can be combined with up-front enrichment of organelles or of other compartments of the cell, or with immunoprecipitation of one or all proteins of interest (called organellar and interaction proteomics, respectively) (Larance and Lamond, 2015). Likewise, an enrichment step for peptides bearing specific post-translational modifications (PTMs) enables the study of these modifications at a systems-wide scale, with phosphoproteomics as a prominent example (Choudhary and Mann, 2010). Proteomics technologies can also be combined with each other, yielding an information-rich and quantitative picture of protein copy numbers, occupancies of PTMs and their combinations, and even conformational and structural features. Thus,

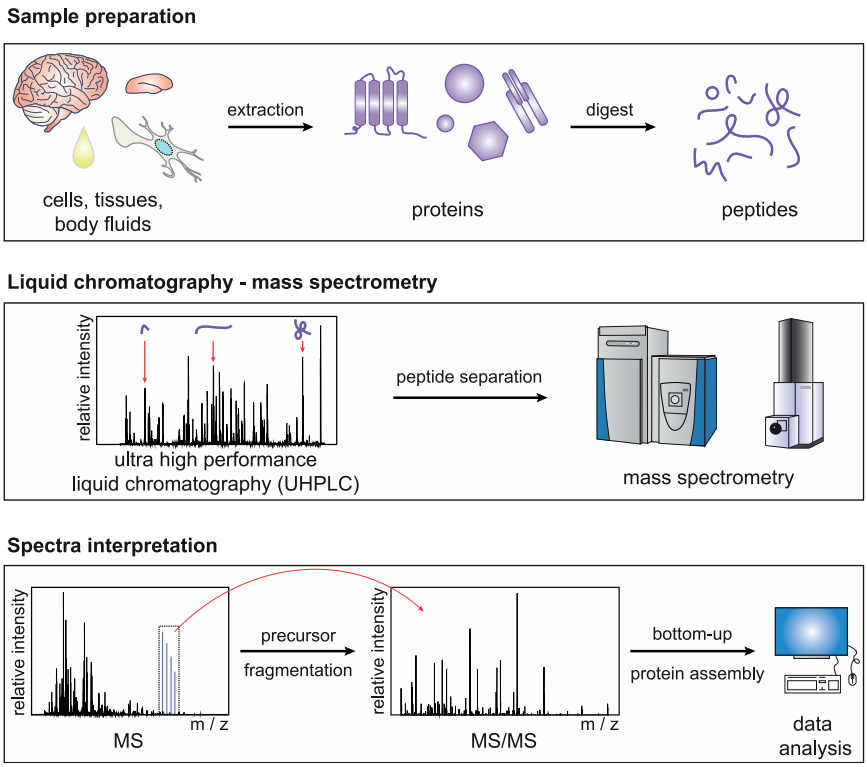

Figure 1. Universal Neuroproteomics Workflow

A schematic of sample preparation. Proteins can be extracted from any biological source, such as from whole tissues, cells, or body fluids. Bottom-up MS requires proteins to then be digested into peptides. Peptides are then separated by liquid chromatography systems to reduce the complexity of the sample. MS analysis is performed by selecting individual peptides, or small mass ranges (precursors) in the mass spectrum. They are then fragmented by collisions with nitrogen gas molecules at low pressure. This generates tandem mass spectra (MS/MS), which contain peptide sequence information. In the final step, organism-specific databases are searched to identify proteins from the peptide sequence information, to support "bottom-up" protein assembly. Further statistical analyses are all automatically done *in silico*.

MS-based proteomics now offers a wide-ranging toolbox for investigating the dynamic and functional organization of individual proteins and the entire proteome.

In this Primer, we present the fundamental concepts of MS-based shotgun proteomics, discuss the applications and current limitations of the technology, and present recent methodological advances of particular interest to the neuroscience community. Our goal is to equip readers with a sufficient background to judge the applicability of proteomics to their specific research questions and to the neuroscience field in general.

Sample Preparation and LC-MS/MS Analysis

Evidently, the functional and morphological heterogeneity of cells in the CNS poses an immense challenge for MS sample preparation. Cell-type-specific isolation can in principle be performed by fluorescence-activated sorting or by laser capture microdissection (LCM), as is common practice in other tissues (Datta et al., 2015). However, the CNS' highly interconnected neurons are generally too fragile for these procedures and might disintegrate or only be captured in part. Isolation of single neurons by LCM from snap-frozen or formalin-fixed, paraffin-embedded tissue has nevertheless recently become feasible (Drummond et al., 2015; Molina et al., 2015).

In specialized laboratories, it is now routinely possible to quantify more than 10,000 proteins in cell lines and tissues (Kulak et al., 2017; Riley et al., 2016), and latest-generation workflows rival the depth of NGS in coverage of gene expression (Bekker-Jensen et al., 2017). However, the scarcity of certain cell types across brain regions limits the available input material and thus the detection of very low abundant proteins in these cell types. In the past, proteomics has required milligram amounts of cellular material, but recent efforts have used several hundred thousands of cells or even less to produce low or sub-microgram quantities of total protein. For instance, in-depth proteomic analyses of brain-derived immune cells or of inflammatory cytokine signaling have been challenging to perform but valuable information even from low cell numbers can now be obtained (Meissner et al., 2013). Methods for characterizing PTMs at a systems-wide level still generally require starting amounts of protein of one milligram or more, due to the initial peptide enrichment step (Humphrey et al., 2015; Svinkina et al., 2015). While this is easily achieved using whole tissues (Lundby et al., 2012), cell-type-specific detection of PTMs at a global scale remains challenging. Primary cell culture can bypass these limitations and can additionally allow cells to be labeled with stable isotopes to investigate even small quantitative changes in protein expression (Zhang et al., 2014). Nevertheless, the cultivation of primary neurons requires high purity to minimize contamination by other cell types (Bouchut et al., 2015) and might also cause changes in protein expression relative to cells derived directly from the CNS (Shimizu et al., 2011). Thus, it is crucial to carefully assess the quality and composition of the sample, for instance, by evaluating the characteristic expression of well-described markers and by bioinformatic analysis of cell-type-specific functionalities and pathways (Hornburg et al., 2014).

Many CNS proteins are involved in membrane-associated processes, such as neurotransmitter receptor signaling, docking and release of synaptic vesicles, or cell-cell adhesion. Given the low abundance and the amphipathicity of most membrane-associated proteins, special care has been taken in selecting proper lysis conditions to ensure proper solubilization of membrane proteins. Several studies have addressed the extraction and digestion properties of proteins associated with bilayer membranes in order to enhance their identification (Shevchenko et al., 2012; Wang and Liang, 2012). For instance, several membrane fractionation steps have been used to characterize the

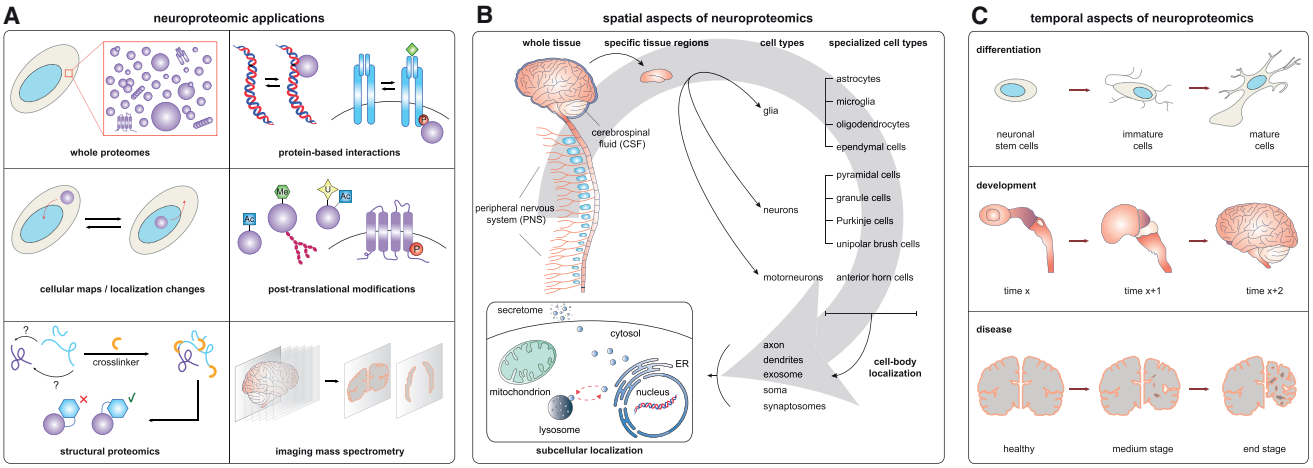

Figure 2. Complexity of Neuroproteomic Samples

(A) Neuroproteomics can be applied, as shown here, to quantify entire proteomes, and to identify protein-based interactions, cellular maps, and post-translational modifications, as well as for structural proteomics and for imaging mass spectrometry.

(B) Proteomics can also be used to interrogate the spatial diversity of proteins at whole tissue level, and in specific tissue regions and cell types, as well as protein localization to distinct parts of the cell body and to subcellular organelles.

(C) Neuroproteomics can also investigate temporal changes to the proteome, such as during cellular differentiation, tissue development, or in response to disease progression.

microglia membrane proteome, and this approach can even be combined with the selective enrichment of glycated peptides for glycoproteome analysis (Han et al., 2014b). Although the above studies are valuable for isolating intact membrane compartments for functional studies, in our experience, the identification and quantification of membrane proteins with standard sample preparation workflows have proven quite adequate, as these proteins almost always produce a sufficient number of soluble peptides. Another potential issue related to sample preparation is the handling time of nervous tissue during the dissection of specific brain regions or cell types. Enzymes such as proteases and phosphatases should be inactivated as soon as possible to preserve the modified *in vivo* state of a protein. Using flash freezing, we have not encountered problems even at timescales of seconds (Humphrey et al., 2015); other effective procedures include rapid heat inactivation (Lundby et al., 2012; Secher et al., 2016). Focused microwave irradiation of rodent brains also results in the rapid heat inactivation of PTM-modifying enzymes, such as phosphatases, *in situ* (O'Callaghan and Sriram, 2004). Overall, we highly recommend testing specific adaptations to MS sample preparation to obtain maximum coverage of the protein and peptide complement, given the tremendous heterogeneity of the CNS.

Once the proteome is reduced to peptides, subsequent analysis steps are largely identical between neurobiological and other application areas. When aiming for a very deep expression proteomic analysis of cells, tissues, or body fluids, and when sufficient material is available, peptide mixtures can be further separated by fractionation to reduce sample complexity. Strong ion exchangers have been the method of choice for a long time, because they fractionate peptides "orthogonal" to HPLC; high pH reversed-phase fractionation combined with concatenation fulfills the same function with higher resolution and is becoming popular for both whole-proteome and PTM analysis (Batth et al., 2014; Kulak et al., 2017). That said, single-run analysis of brain regions has recently been shown to quantify more than 10,000 proteins, a considerable part of the total proteome (Sharma et al., 2015). Due to the time and sample savings involved, single-run or multiplexed workflows allow more conditions to be compared in a given project and are thus widespread in many application fields (Christoforou and Lilley, 2012; Rauniyar and Yates, 2014). The latter typically involve chemical labeling of the samples with isobaric tags that resolve into different isotope channels upon fragmentation, reflecting the relative abundances of each fragmented peptide in the mixed samples.

The LC-MS/MS analysis itself is done using very narrow chromatographic columns packed with micrometer-sized C18 beads and run at very high pressure and nanoliter flowrates. Increasingly powerful mass spectrometers are playing a central role in powering the advances of proteomics. The technology generates large amounts of data—comparable to NGS. However, bioinformatic data analyses are advancing to keep pace with technical advances; as such, data analysis no longer presents a bottleneck in the MS workflow (Hoopmann and Moritz, 2013; Tyanova et al., 2016).

MS-Based Proteomic Applications: An Overview

Recent advances in MS-based quantitative proteomics have made possible a large variety of potential applications (Figure 2A). To begin with, these advances enable the unbiased characterization of all expressed proteins, the proteome, sometimes including the detection of alternatively spliced isoforms of neuronal gene products (Abascal et al., 2015). Such proteome catalogs can help to uncover processes that are specifically associated with neurological diseases. For instance, two recent proteomics and transcriptomics studies employed co-expression network analyses to identify potential markers that correlate with the progression of either Alzheimer's or Huntington's

disease in mouse models or in human tissue samples (Langfelder et al., 2016; Seyfried et al., 2017).

Proteins engage in different types of functionally important contacts, and the investigation of protein-protein, protein-DNA, or protein-small-molecule interactions is fundamental to understanding cellular communication. Proteins often translocate to distinct compartments or are restricted to certain organelles. In addition to microscopy-based techniques, the dynamic localization of proteins can also be assessed through MS-based correlation or fractionation profiling (Dunkley et al., 2004; Itzhak et al., 2016). Moreover, proteomics has shown that PTMs modulate the functions of almost all proteins. MS is clearly the method of choice for the systems-wide characterization of PTMs in an unbiased manner, disentangling cellular signaling networks and crosstalk between different PTMs (Choudhary and Mann, 2010; Hunter, 2007). We will discuss neuro-specific PTM analysis by MS later in this article.

Fueled by recent advances in data analysis methods, structural proteomics based on crosslinking-mass spectrometry (XL-MS) has started to provide valuable insights into conformational aspects not only of individual protein complexes but also of the proteome (Liu et al., 2015; Lössl et al., 2016). For example, XL-MS has been used to characterize dynamic receptor interactomes and to fine-map isoform-specific protein-peptide interactions, such as binding of the amyloid beta peptide to all three major apolipoprotein E isoforms (Deroo et al., 2015).

In addition to standard bottom-up workflows, specialized MS approaches can address additional questions of interest in neurobiology. For instance, imaging mass spectrometry (IMS) permits the direct analysis of tissue sections with high spatial resolution (Gessel et al., 2014). In combination with atmospheric pressure ionization, this approach can aid surgical decision making for brain tumors without the need for sample preparation (Calligaris et al., 2013). Although clearly useful in this context, IMS is generally limited to the low mass range and can suffer from lower reproducibility and sensitivity (Hanrieder et al., 2013). Finally, mass cytometry (CyTOF) is an exciting technology for single-cell proteomics that relies on labeling the sample with rare metal-encoded antibodies; it has already been used to investigate neuroimmunological questions (Bendall et al., 2011). In summary, neuroproteomic approaches can now be applied to address broad questions, as well as many specific ones. Partnering these distinct proteomic tools with each other and with complementary "omics" technologies can provide unique insights that provide a systems-wide understanding of neuronal circuits and pathways.

Spatial Resolution

The CNS is spatially organized at different levels (Figure 2B). For proteomic analysis, whole-brain tissue can be dissected into specific anatomical regions, such as the hippocampus or the striatum, and further sub-divided into major cell types like glia, neurons, or endothelial cells. Specialization at the cell-type level gives rise to a large number of cells that have been classified according to their unique morphology, function, or specific staining. Due to the substantial length of many neurons, protein localization within the cell body or across different organelles is more pronounced than in other cell types and further increases spatial complexity. These features should be taken into account in order to address a research question with the correct level of spatial resolution.

Proteomic analyses of brain regions can create in-depth and quantitative tissue-specific proteome resources for many defined brain areas, in a manner that is analogous to mapping projects, such as the Allan Brain Atlas (Sunkin et al., 2013) or the Connectome project (Glasser et al., 2016). Recently, we reported such a proteomic brain atlas, which consists of about 13,000 different proteins in ten mouse brain regions, as well as the major cell types (Sharma et al., 2015). Much can be learned from the quantitative co-expression patterns revealed by tissue-specific proteomic analysis, as exemplified by the identification of limbic system-associated membrane protein (Lsamp) as a negative regulator of myelination in this study. There are now several studies that provide insights into individual cell proteomes, including those of neurons, glial cells, astrocytes, oligodendrocytes, and their progenitors (Chaerkady et al., 2009; Han et al., 2014a; Iwata et al., 2013). An interesting approach to investigating the interactions of proteomes in a cell-type-specific manner is to label them differentially with isotopes that can be distinguished in the mass spectrometer (Jørgensen et al., 2009). Along these lines, CNS cells have been co-cultured using metabolic labeling with non-canonical amino acids combined with the cell-type-specific expression of modified tRNA synthetases able to bind to these amino acid analogs (Müller et al., 2015). This cell-type-selective protein labeling provides a promising tool for assessing proteome dynamics in heterogeneous cellular systems, as long as cell-type-specific protein expression can be assured. This approach might in the future be applied *in vivo* via the use of genome engineering.

In contrast to NGS-based methods, proteomics is inherently capable of subcellular resolution; however, this spatial information is often sacrificed by homogenization after tissue or cell lysis. In any case, interrogating subcellular compartmentalization in sufficient quantities of material is challenging in the CNS. For example, to enrich for active zone, post-synaptic densities, or synaptosomes, various sorting tools were combined and the starting material was pooled and then fractionated (Bayés et al., 2012; Biesemann et al., 2014; Boyken et al., 2013). Nevertheless, the ongoing lack of purification strategies for many interesting subcellular structures or specific tissues limits their in-depth analysis by MS. The recent combination of quantitative proteomics with proximity-labeling approaches, such as APEX or BioID, in engineered cells is very promising because these approaches do not need extensive fractionation (Rhee et al., 2013; Roux et al., 2012). These methods have already been used to elucidate protein complexes, pathways, and subcellular organelles and have even enabled the discrimination of excitatory and inhibitory synaptic clefts via proteomic characterization in living neurons (Loh et al., 2016). Finally, several studies have reported an analysis of the secretome of various CNS cell types. Recent advances in MS now allow secreted proteins to be directly quantified from activated immune cells or from neuron-dependent astrocytic secretion (Eichelbaum et al., 2012; Kuhn et al., 2012; Meissner et al., 2013; Stiess et al., 2015; Thouvenot et al., 2012).

Given its direct contact with the CNS, and in the absence of viable CNS biopsy procedures, the cerebrospinal fluid (CSF) has been a focus of attention for many years. The CSF is routinely accessed in the context of disease and represents a closer snapshot of changes in the CNS secretome compared to blood. While human or rat CSF can be extracted in adequate volumes, the collection of murine CSF is more challenging but it can still be obtained with adequate purity by puncture of the cisterna magna (Dislich et al., 2015; Liu and Duff, 2008). Proteomic CSF analyses have primarily aimed to discover diagnostic or prognostic biomarkers for multiple conditions, such as neurodegenerative diseases, psychiatric disorders, or autoimmune diseases (Häggmark et al., 2016). However, very few, if any, of the numerous postulated CSF biomarkers have been independently reproduced, and consequently they have not made their way into clinical practice (Lleó et al., 2015). Several factors have contributed to this analytical variability, including the lack of a standardized CSF collection procedure, improper sample handling, the extremely high dynamic range of the CSF proteome, and variable protein contamination from blood or surrounding tissue cells (Teunissen et al., 2009). However, recent advances in MS-based proteomics can be applied to CSF as well and promise to revive biomarker discovery (Geyer et al., 2016, 2017). In our laboratory, a single-run analysis of CSF quantifies more than 1,000 proteins in less than 1 hr of measurement time, making large-scale studies realistic. Regardless, an important aspect of CSF biomarker discovery is the validation of results in multicenter studies. Traditionally, such validation studies have been performed on just a few ''biomarker candidates'' using targeted proteomics methods (Heywood et al., 2015; Percy et al., 2013). The high throughput and coverage possible today now make biomarker discovery and validation feasible at greater proteomic depth (Geyer et al., 2017).

Temporal Resolution

Cells adapt to environmental conditions over time by regulating their repertoire of proteins. As such, the proteome has been investigated in time series experiments during three main temporal processes: cell differentiation; tissue development; and during perturbations, including disease progression (Figure 2C). For instance, studies in mice have shed new light on the multiple activation states displayed by microglia, which range from neurotrophic to neurotoxic. Using stable isotope labeling with amino acids in cell culture (SILAC)-based proteomic analysis of immortalized mouse microglia, a novel activation marker was found that reveals differences and similarities among these distinct states (Bell-Temin et al., 2015). Similarly, a quantitative phosphoproteomic study has revealed a key role for protein kinase A in neural stem cell differentiation via its phosphorylation of downstream Wnt signaling pathway components (Wang et al., 2016). Information processing in the brain critically depends on proper synaptic connectivity mediated by cell adhesion molecules. Several leucine-rich repeat transmembrane (LRRTM) proteins have been linked to excitatory synapse development by affinity purification coupled to mass spectrometry (AP-MS), highlighting its potential to identify relevant players in neuronal development (de Wit et al., 2013; O'Sullivan et al., 2012). The above-mentioned mouse proteomic brain atlas also contains a rich resource of global proteome changes during postnatal tissue development, as well as comparisons of the proteomes of immature and mature CNS cell types (Sharma et al., 2015). Such studies are becoming increasingly feasible and accessible, and our systems-level understanding of evolutionary brain development will be aided by the imminent availability of the in-depth brain proteomes of other species.

Importantly, the quantitative assessment of protein changes during disease progression has the potential to uncover potential biomarkers or therapeutic targets. A recent study of progressive Alzheimer's disease focused on alterations in the proteome of the cornu ammonis and the subiculum, disease-relevant hippocampal subregions (Hondius et al., 2016). All these examples also highlight how the implementation of quantification strategies have fundamentally changed proteomics from its once purely qualitative and descriptive nature toward a powerful technology that allows thousands of proteins and PTMs to be characterized in parallel, making proteomics well suited for answering questions with a spatiotemporal dimension.

Protein Interactions

Direct physical interactions with proteins are required for almost all biological processes (Charbonnier et al., 2008). Many methods have been developed to investigate these protein-based interactions—mainly for cases where both interaction partners are already known. In contrast, MS-based methods are very efficient at discovering the identity of interacting proteins by affinity purification of the bait followed by MS (Schmidt and Robinson, 2014). The AP-MS method has recently been renamed affinity enrichment-mass spectrometry (AE-MS) to reflect the fact that proteomics determines binding partners by their enrichment over controls, rather than from their absence or presence (Keilhauer et al., 2015). Importantly, baits are not restricted to proteins but include (modified) peptides, nucleic acids, lipids, polysaccharides, or small molecules.

The assessment of protein-protein interactions (PPIs) is one of the most useful applications of proteomics (Figure 3A, left), particularly on a global scale, as this allows large-scale interactomes to be constructed and underlying protein networks characterized (for a recent example, see Huttlin et al., 2017). Furthermore, if preys are expressed at endogenous levels and quantitative information is available, the ratio of bait to prey and of both to the cellular proteome can be inferred, greatly aiding biological interpretation and allowing the extraction of true network properties (Figure 3A, middle) (Hein et al., 2015; Smits et al., 2013).

Genetic pathologies can exert their effects through aberrant PPIs, for instance via single nucleotide polymorphisms (SNPs), alterations to the coding sequence, or to splice sites, affecting the choice of splicing isoforms to change protein interaction surfaces (Schuster-Böckler and Bateman, 2008). The resulting differences in the protein-binding behavior of genetic variants can be systematically mapped by interaction proteomics to reveal gain- or loss-of-function interactions or privileged prey binding to disease-associated baits (Figure 3A, right). Such studies have revealed important information about mutations associated with cancer or neurodegenerative diseases (Hosp et al., 2015; Lambert et al., 2013b).

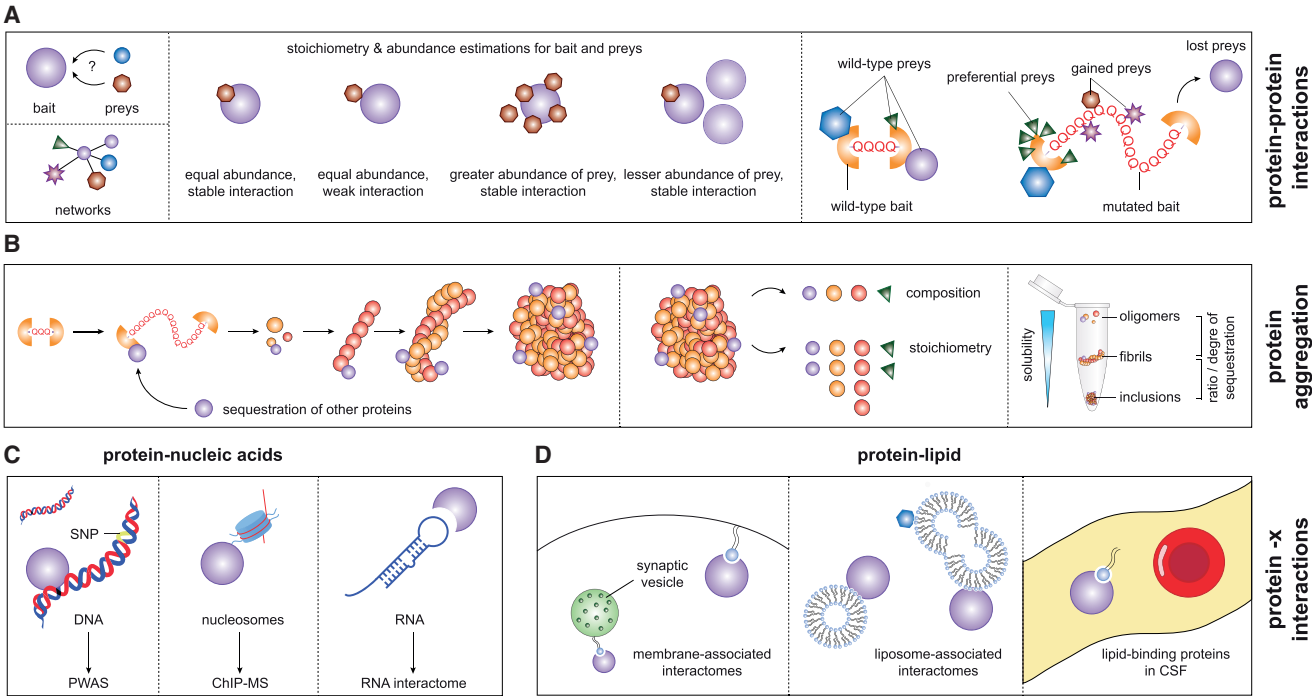

Figure 3. Investigation of Protein-Based Interactions Using Neuroproteomics

(A) Quantitative assessment of protein-protein interactions and protein interaction networks. Estimation of protein abundance and stoichiometries for baits and preys provides detailed quantitative information about protein complexes. Comparing the interactome of wild-type and mutated bait proteins enables preferential interactors to be detected, as well as the gain or loss of protein interactions.

(B) MS analysis of insoluble proteome fractions reveals the composition and stoichiometry of protein aggregates involved in disease. Integration of both soluble and insoluble proteomic data even enables the degree of protein sequestration to be determined over time.

(C) Characterization of protein interactions with oligonucleotides. Proteome-wide analysis of SNPs (PWAS) allows the rapid screening of differential DNA-binding, which results from subtle changes in DNA sequence. Chromatin immunoprecipitation combined with mass spectrometry (ChIP-MS) is used to elucidate sequence-specific transcription factor binding complexes. RNA probes can interrogate the RNA-bound protein interactome.

(D) Protein interactions with lipids can also be characterized to reveal membrane-bound interactomes, liposome-associated interactomes, and proteins that bind to lipids in body fluids for diagnostic purposes.

Although not a classical bait-prey interaction, the cellular aggregates that form in most neurodegenerative diseases can be seen as a pathological form of PPI. Aggregate formation proceeds via the incremental sequestering of proteins, forming soluble oligomers to insoluble end-stage inclusion bodies (Figure 3B). Despite their clinical relevance, our knowledge of the composition of these aggregates has been hindered by a lack of methods that can combine high-resolution proteomics with preparation methods capable of disrupting these extremely insoluble entities (Pedersen and Heegaard, 2013). Recently, starting with formic acid digestion (Hazeki et al., 2000), we have successfully solubilized such neurotoxic aggregates from disease models *in vitro* and *in vivo*, enabling the quantitative analysis of their constituent proteins and their degree of sequestration, in comparison to the total proteome (F.H. et al., unpublished data) (Kim et al., 2016).

Protein-based interactions with nucleic acids, for instance in the form of transcription factor complexes, regulate a wide range of biological functions, and investigation of their interplay is essential for understanding numerous gene regulatory processes (Wierer and Mann, 2016). DNA pull-down assays combined with MS (termed ChIP-MS in analogy to ChIP-seq) identifies proteins bound to any DNA or RNA sequence, including differential SNP binding (Figure 3C). Proteome-wide analysis of SNPs (PWAS) has been used to explain the differential muscle content of European versus Asian breeds of pigs and is an appealing strategy for assessing allele-specific transcription factor binding because it is unbiased, i.e., does not presuppose knowledge of binding candidates (Butter et al., 2010; Markljung et al., 2009). As many neurodegenerative diseases have been linked to altered transcription factor activity or binding, PWAS can also shed new light on transcriptional dysfunction in pathological contexts (Butter et al., 2012). With the emergence of robust genome-wide association methodologies and their capability to reveal the genetic susceptibility of complex neurological diseases (Lambert et al., 2013a), the outcome of such studies can also be combined with PPI data to link disease genes and phenotypes (Hosp et al., 2015; Rossin et al., 2011). Furthermore, modified histone peptides alone or in nucleosomes can be used as baits in chromatin immunoprecipitation (ChIP) experiments. ChIP can be followed by MS to identify proteins that read specific histone PTMs, which can regulate the expression of nearby genes (Bartke et al., 2010; Vermeulen et al., 2010). One such example is the recent linking of aberrant gene expression to the DNA damage-induced loss of heterochromatin in tau-mediated neurodegeneration (Frost et al., 2014). Together with other

transcriptional profiling approaches, ChIP-MS provides an excellent tool to further explore the epigenetic landscape of CNS-related diseases.

Neurons have developed distinctive mechanisms to regulate their RNA metabolism. This includes the unique expression of several RNA-binding proteins in neurons, the differential processing of RNAs in the CNS relative to other tissues, and the regulation of transcripts localized to specific subcellular compartments (Ule et al., 2005). Many neurological disorders are subject to dysfunctional RNA-processing and RNA-binding events (Modic et al., 2013). Systems-wide approaches to understanding the RNA-bound interactome commonly involve cross-linking-immunoprecipitation and variations thereof, such as UV crosslinking coupled to AP-MS (Baltz et al., 2012; Castello et al., 2012). However, the identification of active RNA-binding proteins in living cells, especially the comparison of mRNA interactomes in response to differential environmental situations, remains a challenge for the future.

Protein interactions with lipids remain to be extensively studied in the CNS. However, given the high abundance of membrane-associated proteins and their important role in signaling pathways, their systematic analysis is essential for understanding basic neurobiological processes. Of particular interest are integral and peripheral membrane-associated proteins, liposome-associated binders, as well as soluble proteins that bind to lipids outside of cellular membranes or in body fluids like the CSF (Figure 3D). Recent examples of proteomic studies include interaction screens with immobilized lipids (Jungmichel et al., 2014), global profiling of protein-lipid interactions using bifunctional lipid precursors (Haberkant et al., 2013), as well as click chemistry-based investigations of cholesterol-specific interactors (Hulce et al., 2013). Surrogate membranes, such as liposomes, have been employed to systematically and quantitatively characterize protein recruitment to bilayer membranes (Saliba et al., 2014); clearly, such approaches could also be performed in a proteome-wide manner. Given the propensity of amyloids to interact with lipid bilayers, thereby disturbing the integrity of membranes, there is a clear need to further expand protein-lipid interactome studies in the CNS.

Post-translational Modifications

PTMs constitute an essential layer of regulation for almost any cellular process. Their highly dynamic nature enables fast adaptations to environmental perturbations in an efficient manner. The enormous landscape of different PTMs, their substoichiometric nature, and the fact that different PTMs can be present at the same sites makes their proteomic analysis enormously challenging. Advances in PTM enrichment and detection by MS-based proteomics are constantly improving the unbiased identification and quantification of PTMs, resulting in global studies of signaling processes that can survey thousands of phosphorylation, ubiquitination, and acetylation sites *in vitro* and *in vivo* (Huttlin et al., 2010; Kim et al., 2011; Lundby et al., 2012). However, a remaining limitation is the high amount of protein input material required, generally in the milligram range, which has so far prevented the in-depth cell-type-specific detection of PTMs in the CNS. Nevertheless, streamlined workflows now considerably simplify PTM analyses, and several different PTM classes can be detected in the same experiment via serial enrichment from the same biological sample (Humphrey et al., 2015; Mertins et al., 2013). A holy grail of proteomics is to detect the entire proteome and its modifications without specific enrichments, and impressive first steps toward this goal are already being taken (Bekker-Jensen et al., 2017). Furthermore, unbiased methods for PTM detection have been developed that do not require the modified amino acids to be specified in the database search (Lassak et al., 2015; Savitski et al., 2006). In addition to PTM identification and quantification, the determination of individual PTM site occupancies, i.e., the fraction of proteins being modified at a given site, is now possible on a large scale (Olsen et al., 2010; Wu et al., 2011). Recent findings have highlighted the value of PTM stoichiometries as a key to understanding the role of various modifications. Increases in acetylation occupancies at specific sites on tau can reduce its binding to microtubules and directly raise its aggregation propensity, whereas an increase in tau methylation occupancy has the opposite effect and can protect against pathological tau aggregation *in vitro* (Cohen et al., 2011; Funk et al., 2014).

State-of-the-art proteomics facilitates the investigation of many PTM-associated cellular regulatory mechanisms, as summarized in Figure 4. For example, proteomics can be applied to study the PTM-mediated regulation of protein stability, such as the abnormal modification of tau protein and the resulting dissociation of microtubules in several forms of dementia, including Alzheimer's or Parkinson's disease (Figure 4A) (Morris et al., 2015).

Sirtuins are either deacylases or ADP-ribosyltransferases and this enzymatic activity is itself tightly controlled by PTMs (Figure 4B). Given their key role in physiology and their implication in aging and neurodegeneration, sirtuins have been a prime target in the search for enzymatic inhibitors (Donmez and Outeiro, 2013; Fang et al., 2016). An in-depth MS study of lysine acetylation inhibitors, combining genetic and chemical approaches, revealed that the commonly used pan-sirtuin inhibitor nicotinamide primarily targets SIRT1 and, surprisingly, that many chemical inhibitors tended to be more specific *in vivo* than *in vitro* (Schölz et al., 2015).

Conformational changes in proteins brought about by allosteric regulation or the modulation of protein-protein interactions are often mediated by phosphorylation (Figure 4C). Such regulation has been extensively studied for protein tyrosine kinases and appears to be regulated by dynamically linked amino acids (Foda et al., 2015). Phosphorylation can also control receptor dimerization, such as in the human adenosine A_{2A} receptor C terminus, where it mediates receptor heteromerization with an intracellular loop of the dopamine D_2 receptor (Ciruela et al., 2004). PTMs often function as a docking site for both intra- and inter-molecular protein interactions (Figure 4D), thereby regulating biomolecular binding and downstream signaling cascades (Nagai et al., 2016). PTM-mimetic mutations can also be introduced to functionally characterize the behavior of particular modification sites. Furthermore, the quantification of acetylation sites regulated by the deacetylase SIRT3 indicates that selective pressure maintains positively charged residues at substrate sites across species (Rardin et al., 2013).

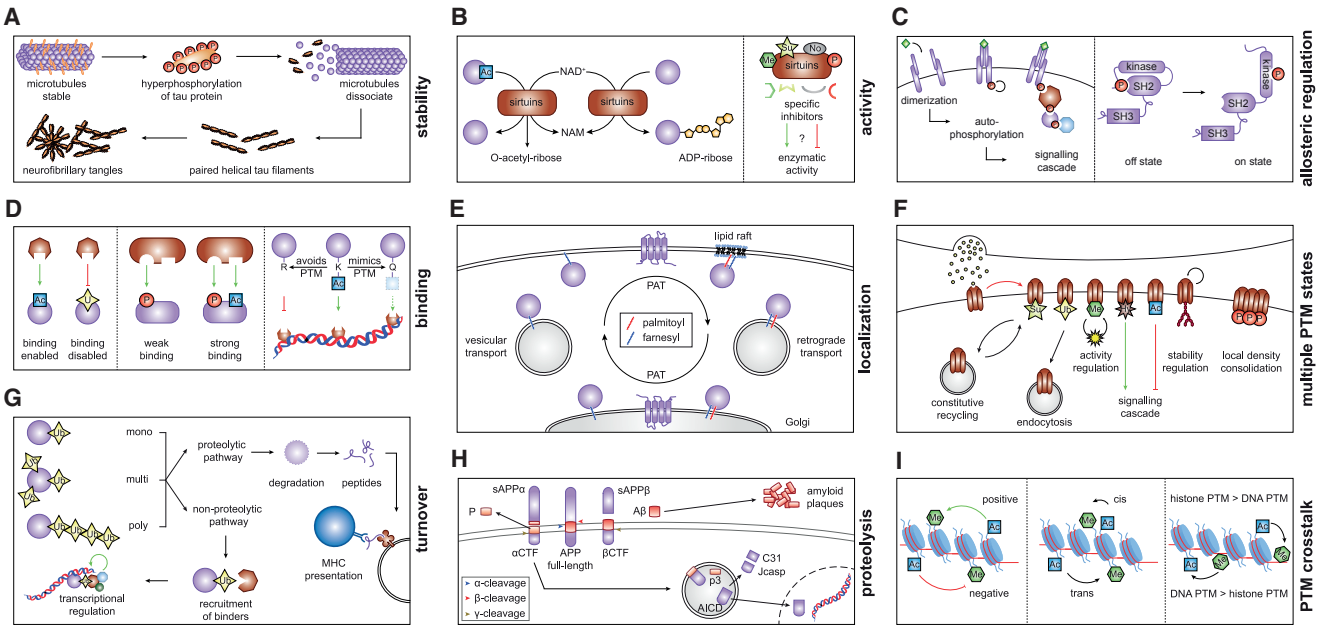

Figure 4. Global Investigation of Post-translational Modifications with Neuroproteomics

(A) Tau proteins stabilize microtubules. The hyperphosphorylation of tau causes microtubule destabilization, leading to the formation of neurofibrillary tangles, a central feature of tauopathies.

(B) Sirtuin activity is regulated by post-translational modifications (PTMs) such as acetylation or phosphorylation. Site-specific or pan-sirtuin inhibitors are attractive drugs to modulate the acetylation (Ac) state of substrate proteins.

(C) Receptor auto-phosphorylation enables dimerization, initiating subsequent signaling cascades. Phosphorylation of kinase domains can result in the allosteric regulation of proteins, thus changing their structure and function.

(D) PTMs may enable or block binding sites that mediate protein-protein interactions, affecting the strength of a given interaction site. PTM-mimetic mutations can be utilized to investigate binding site functionality.

(E) Palmitoylation of membrane proteins is commonly involved in protein trafficking and altered protein localization.

(F) Receptor activation can result in multiple PTMs states at the same time, leading to distinct fates of differentially modified protein molecules.

(G) Ubiquitination targets proteins for proteolytic degradation or recruits ubiquitin-specific binders to regulate other cellular processes, such as transcription.

(H) Proteolysis of full-length proteins can give rise to a number of smaller, bioactive fragments. In the case of amyloid precursor protein (APP), pathological mutations affect the cleavage process and generate toxic amyloid beta fragments.

(I) Crosstalk between distinct PTMs, such as between histone and DNA PTMs as shown here, might influence the further modification of the protein or of distinct biomolecules in different ways.

PTMs can affect the subcellular localization of proteins, as exemplified by reversible palmitoylation on integral and peripheral membrane proteins, a modification that influences protein trafficking (Figure 4E). One of the first in-depth palmitoylation studies by MS revealed that the brain-specific Cdc42 splice variant is palmitoylated, in contrast to the prenylated canonical Cdc42 form (Kang et al., 2008). The same study also showed dynamic regulation of palmitoylation in response to different drugs, affecting the localization and neuronal activity of several proteins. Palmitoylation of the receptor anchoring protein gephryin is crucial for normal postsynaptic clustering and for potentiating GABAergic synaptic transmission (Dejanovic et al., 2014).

Distinct PTMs often affect the same protein or even the same amino acid residue. Hence, the total pool of a specific protein can harbor multiple PTM states at the same time (Figure 4F). Interestingly, proteins that undergo multiple PTM events have more intrinsically disordered regions and are more prone to be involved in disease, likely due to their central role in regulating molecular recognition processes (Huang et al., 2014).

Ubiquitin is best known for its role in protein turnover by mono-, multi-, or poly-modification of target proteins (Figure 4G), which is particularly important in many protein misfolding disorders (Hipp et al., 2014). More broadly, the ubiquitin-dependent recruitment of protein binders and the subsequent transcriptional regulation via the non-proteolytic ubiquitin pathway has been implicated in chromatin dynamics, DNA repair, kinase activation, and membrane trafficking (Chen and Sun, 2009). For instance, ubiquitin receptor protein ubiquilin-1 can stimulate K63-linked polyubiquitination of the amyloid precursor protein (APP) and thus regulate its degradation and trafficking (El Ayadi et al., 2012). Proteomics can resolve both the presence and the type of ubiquitin linkage and is increasingly able to provide quantitative values for entire pathways and substrates (Ordureau et al., 2015).

In addition to removing proteins, proteolysis can also generate cleavage products with distinct functionalities. A classic example of this is the proteolytic processing of APP, which generates several peptide fragments that have important roles in neuronal plasticity, survival, and—for the amyloid beta peptides—in disease progression (Figure 4H) (O'Brien and Wong, 2011). Likewise, cleavage of full-length huntingtin by various proteases releases smaller N-terminal fragments, which are centrally involved in the pathogenesis of Huntington's disease (El-Daher et al., 2015).

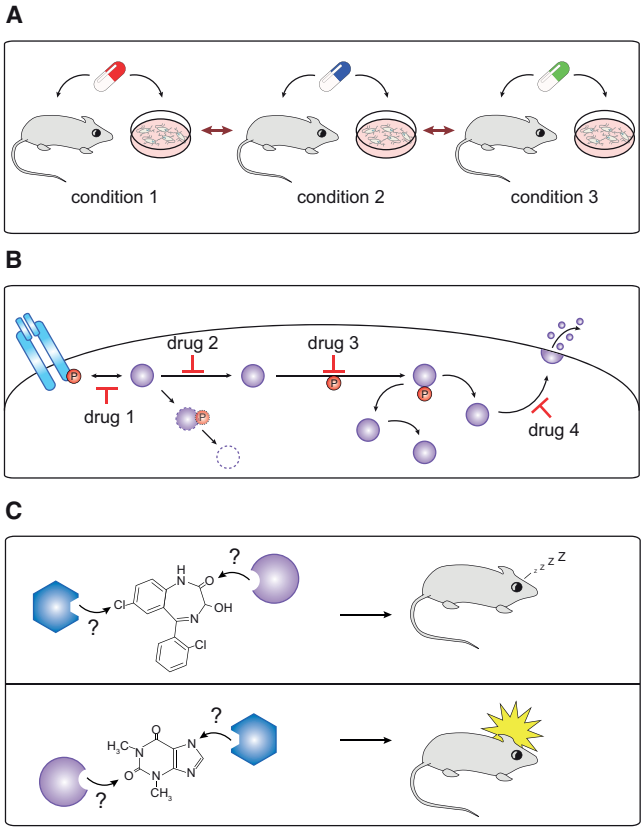

Figure 5. Proteomics of Drug-Based Perturbations
(A) MS-based quantitative assessment of drug perturbations *in vitro* and *in vivo*.
(B) Proteomics and phosphoproteomics unravel signaling networks to enable the precise modulation of pathways.
(C) Characterization of protein interactions with small molecules. Target discovery of small molecules by affinity purification-mass spectrometry.

The fact that many proteins can be modified by several different PTMs, simultaneously, gives rise to crosstalk between them, with the above-discussed histone modifications as a prime example (Figure 4I). PTM crosstalk can act directly on the same protein in *cis* or on spatially distant proteins in *trans* and can even regulate the interplay between protein or DNA modifications (Cedar and Bergman, 2009). A different type of PTM crosstalk is involved in protein degradation, such as the prolyl hydroxylation-dependent ubiquitination of the beta(2)-adrenergic receptor (Xie et al., 2009).

Gene and Drug-Based Perturbation

The etiology of many neurological diseases is highly complex, usually involving multiple genes and a strong environmental contribution. While genetic linkage analysis has identified many disease associations, the functional characterization of these associations lags behind. Often the biological functions of the proteins encoded by the altered genes are not fully understood, particularly concerning how they affect cellular function in the brain and how this manifests at the organismal level. In this context, proteomics offers a rich toolkit for investigating the biological functions of disease-associated genetic variants, as well as of their wild-type counterparts, and provides an unbiased strategy with which to bridge the gap between disease gene discovery and associated phenotype. The most frequently mutated gene in familial Parkinson's disease is *PARK8*, which encodes LRRK2, a protein of unknown cellular function. Mutations stimulate its kinase activity, but its verified substrates remain elusive (Schapansky et al., 2015). Exemplifying the power of modern proteomics methods, a combination of phosphoproteomics, genetics, and newly developed LRRK2 inhibitors recently revealed that a subset of Rab GTPases are key LRRK2 substrates *in vivo*. Pathogenic LRRK2 variants reduced the affinity of these Rab GTPases to regulatory proteins, linking aberrant cellular trafficking mediated by these GTPases to Parkinson's disease (Steger et al., 2016).

Drugs affect brain activity by tapping into neuronal transmission, and their targets are usually embedded in cellular networks that interconnect many other proteins and biomolecules. Proteomics can elucidate both the effects of drugs on CNS cells and their targets simultaneously, unraveling selective signaling pathways involved in neurological disorders (Figures 5A and 5B). In preclinical drug discovery, MS-based proteomics has become a common tool, often in chemoproteomic approaches to characterize binding affinities or the selectivity of biosimilars and small molecules. Drug on- and off-target identification in living cells has also been addressed globally by so-called thermal protein profiling. This approach combines cellular thermal shift assays with quantitative MS to determine the proportion of proteins that remain in solution at elevated temperatures due to drug binding (Savitski et al., 2014).

Recent examples of the application of proteomics to drug-related questions in neurobiology include: the target discovery of stathmin-2 as a molecular effector of tetrahydrocannabinol-induced fetal cortical circuitry (Tortoriello et al., 2014); the biased phosphorylation of the serotonin 5-HT_{2A} receptor in response to hallucinogenic agonists by phosphoproteomics (Karaki et al., 2014); a combined chemoproteomics and lipidomics approach to elucidate the function of diacylglycerol lipases in coordinating synaptic plasticity (Ogasawara et al., 2016); and the role of mica-nozole and other drugs in oligodendrocyte maturation and re-myelination in the CNS (Najm et al., 2015). Over the past decades, many potent and selective small molecules have been generated that target G protein-coupled receptors (GPCRs), as GPCRs are involved in numerous key neurotransmitter processes and are affected in many neurological diseases (Lagerström and Schiöth, 2008; Thathiah and De Strooper, 2011). Given the prevalence of drugs targeting these GPCRs, ion channels, and other receptors, an exciting goal for future studies will be to disentangle their downstream signal transduction pathways in response to different stimuli using phosphoproteomics.

The blood-brain barrier represents a physical border that limits the permeability of antibodies or drugs to the CNS. As such, small molecules remain one of the most promising candidates for CNS drug targets, and their interaction with proteins has become an attractive target for drug development. MS-based proteomics offers a wide-range of approaches to identify and validate druggable targets (Figure 5C), including affinity purification of immobilized compounds for target identification, or for drug metabolism and pharmacokinetic feature detection (McFedries et al., 2013).

Conclusions and Perspectives

Neuroproteomics is an expanding field that has progressed tremendously over the past decade. This is mainly due to technological advances in all aspects of the proteomics workflow, from sample preparation, separation technology, MS instrumentation to algorithms for data analysis. Compared to 10 years ago, proteomics workflows have also become more streamlined and accessible. That said, much work remains to be done to ensure that proteomics becomes as ubiquitous as other standard tools regularly used by the neurobiological community. This is especially important for enabling multi-dimensional proteomic studies that address neuroscientific questions from several angles, as is already happening in cell culture-based studies (Larance and Lamond, 2015). We foresee continuing progress in up-front sample treatment, in data acquisition and in subsequent data interpretation, although the high costs of mass spectrometers remain a concern. Integration with other ''omics'' technologies will also become more routine and this will be essential for addressing both the systematic characterization of individual neurons and their mutual interplay at a systems level (Kitchen et al., 2014; Poulin et al., 2016).

A particular challenge in neurobiology is the tremendous cellular heterogeneity that exists in the CNS and the resulting small numbers of cells that are available for analysis. We predict that the above-mentioned technological improvements will create new synergies in the overall MS workflows, thus dramatically pushing the current detection limits.

This Primer has shown that proteomics is already an extremely powerful, and often a unique, tool with which to address protein-based, functional questions in a global manner. We envision that its impact in neuroscience will grow substantially, as researchers in the field become more acquainted with it. A particularly promising opportunity will be the translation of proteomics to clinical applications, such as investigating the biological basis of disease. This includes the large-scale analysis of body fluids in the context of biomarker analysis for disease stratification as a part of employing proteomics in precision therapeutics for neurobiological disorders.

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