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Review

Neuroproteomics – the tasks lying ahead

The brain is unquestionably the most fascinating organ. Despite tremendous progress, current knowledge falls short of being able to explain its function. An emerging approach toward improved understanding of the molecular mechanisms underlying brain function is neuroproteomics. Today's neuroscientists have access to a battery of versatile technologies both in transcriptomics and proteomics. The challenge is to choose the right strategy in order to generate new hypotheses on how the brain works. The goal of this review is therefore two-fold: first we recall the bewildering cellular, molecular, and functional complexity in the brain, as this knowledge is fundamental to any study design. In fact, an impressive complexity on the molecular level has recently re-emerged as a central theme in large-scale analyses. Then we review transcriptomics and proteomics technologies, as both are complementary. Finally, we comment on the most widely used proteomics techniques and their respective strengths and drawbacks. We conclude that for the time being, neuroproteomics should focus on its strengths, namely the identification of posttranslational modifications and protein–protein interactions, as well as the characterization of highly purified subproteomes. For global expression profiling, emphasis should be put on further development to significantly increase coverage.

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1 Introduction

The brain is the most sophisticated and complex organ that nature has devised [1]. It controls most of the body activities and is responsible for perception, behavior, cognition, memory, and consciousness. As a consequence, there are more than 1000 disorders associated with dysfunction of the nervous system, such as neurological and psychiatric conditions [2]. Most of them have a huge social and economic impact. Dementia affects ~10% of US citizens above the age of 65 and 50% of those aged 85 or older, and over 2 million US-Americans suffer from schizophrenia [3]. In recognition of the importance of the nervous system for scientists, clinicians, and laymen alike, the 1990s were proclaimed the decade of the brain in the United States of America (Bush, G., *http://www. loc. gov/loc/brain/ proclaim. html* 2005.).

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Abbreviation: NMDA, *N*-methyl-D-aspartate

A major initiative put forward in these years was the Brain Molecular Anatomy Project (http://trans.nih.gov/bmap/) with two major goals: "(1) Gene discovery: to catalogue all the genes expressed in the nervous system, under both normal and abnormal conditions. (2) Gene expression analysis: to monitor gene expression patterns in the nervous system as a function of cell type, anatomical location, developmental stage, and physiological state, and thus gain insight into gene function" [4] (Tessier-Lavigne, M., Stryer, L., *http://trans. nih. gov/bmap/* 2002.). Such knowledge is expected to open new avenues of understanding development, maintenance, and function of the brain. In addition, it likely will result in the discovery of novel drug targets for the treatment of ailments such as dementia, stroke, brain tumors, schizophrenia, depression, anxiety disorders, and addiction.

Recent developments have made it possible to use approaches on a global scale to decipher the molecular bar code of the brain. One such system-based approach is neuroproteomics, *i.e.*, the large-scale profiling and functional annotation of brain proteins. Indeed, the characterization of the brain proteome under normal and disease conditions is one of five initiatives launched recently by the human proteome organization (HUPO) [5, 6].

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Neuroproteomics is currently on the move from an emerging to a mature technology platform [7]. It seems like a suitable time to recall the tasks lying ahead, and to draw lessons from the past, in order to accelerate the transition. This reflection might even be more appropriate in the light of recent data [8–10], which suggest that the molecular analysis of the brain is an even more daunting task than thought before. A careful weighing up of the most appropriate technology in the experimental design is required. Researchers need to be aware of different proteomic approaches, and complementary genomic technologies have to be considered in order to select the most promising combination for the question being addressed. This review will therefore first portray the tasks waiting for neuroproteomics. We then will review the current state of the art both in transcriptome and proteome research. Finally, conclusions will be drawn from the current achievements and limitations, followed by an outlook on potential future directions.

2 Complexity of the brain

In a behaving animal, the brain must integrate a number of different stimuli picked up by the connected sensory systems. It does not only process all this input but also evaluates it and initiates adequate responses. More importantly, the brain modifies behavioral responses based on experience. This requires learning and memory, which represent two complex brain functions. To fulfill these numerous distinct tasks, the nervous system is divided into subsystems on the anatomical, cellular, and chemical level, with each subsystem having its own function and molecular repertoire.

2.1 Complexity on the structural and functional level

A large degree of heterogeneity is observed in the nervous system on all levels analyzed. Numerous different histological regions, defined nuclei, subnuclei, and neuronal clusters can be distinguished, together with an immense number of connections and circuits. For many of these structures, specialized functions have been demonstrated that are reflected in the anatomical layout or molecular repertoire. For instance, acoustic information is processed in numerous auditory centers located in different brain regions. After signal transduction in the cochlea, auditory information is sent to the cochlear nucleus. Neurons there project to the superior olivary complex and, *via* the nuclei of the lateral lemniscus, to the inferior colliculus, all located in the brainstem. From there, auditory information reaches the medial geniculate body in the diencephalon and finally the auditory cortex in the telencephalon [11]. In each of these stations, auditory information is processed in a different manner. Basic features, such as amplitude and frequency are encoded early on, whereas higher order centers, such as the superior olivary complex or the inferior colliculus, use this information to perform more complex computational tasks, such as sound localization or echo suppression. Finally, auditory perception occurs in the cortex [11, 12].

Functional and structural heterogeneity continues on the cellular level. The human central nervous system consists of 10^{10}–10^{11} neurons and most likely a ten-fold higher number of glial cells [13]. Several thousand cell types can be distinguished, based on function, shape, the extend and complexity of their processes, and the identity of the transmitter they synthesize, release, and respond to (Tessier-Lavigne, M., Stryer, L., *http://trans. nih. gov/bmap/* 2002) [14]. Consider, for instance, neurons in the cochlear nucleus. At least five different types of neurons can be distinguished based on their morphology, such as bushy neurons, octopus neurons, pyramidal neurons, stellate neurons, and root neurons, and all display characteristic electrophysiological properties [15] (Fig. 1). In general, it can be assumed that each neuronal cell type in the brain has a distinct physiological role and their individual characterization is essential to any bottom-up understanding of brain function.

2.2 Complexity on the transcriptional level

A surprising result of the human genome project and other large-scale projects was the low gene number in mammals [16–18]. At present, there are 20 000–25 000 protein-coding genes predicted, which represent less than 2% of the total genome sequence [16]. However, recent expression analyses using tiling arrays or the comprehensive characterization of transcriptional start and termination sites added new facets to this apparently low complexity [19, 20]. Analysis of 1 000 000 expressed sequences in mouse revealed more than 181 000 individual transcripts, surpassing the number of predicted mouse genes by a factor of nearly 10 [10]. Over 56 000 transcripts analyzed code for proteins, including previously undetected ones. At least 65% of the transcriptional units analyzed were modified by splicing, and numerous new splice variants were detected. It will therefore be interesting to see whether the previously described more than 38 000 potential splice variants of the *Dscam* gene in *Drosophila melanogaster* [21] or the more than 1000 predicted splice variants of the neurexins [22] remain rare examples.

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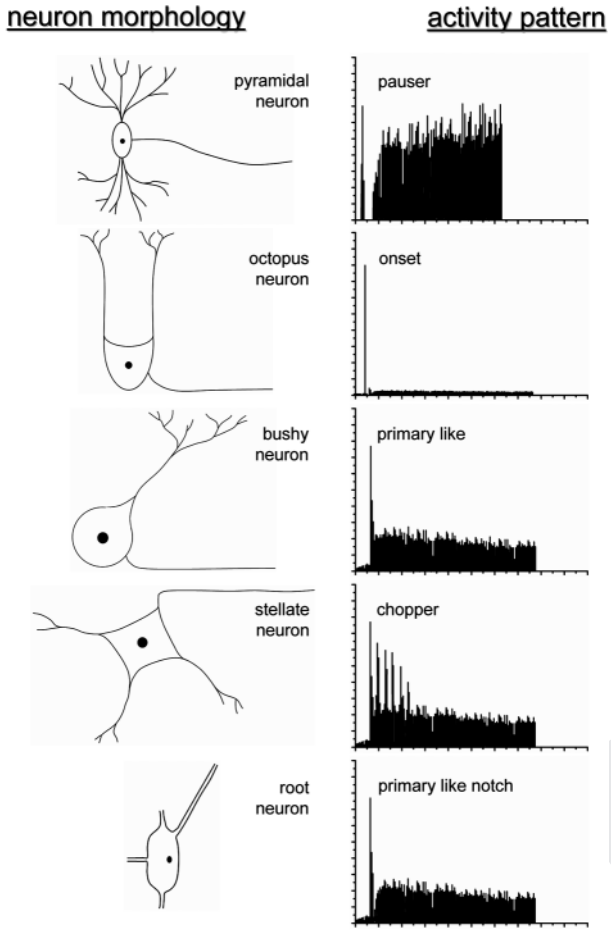

Figure 1. Morphology and electrical activity pattern of neuronal cell types. Morphology of five neuronal cell types in the cochlear nucleus (pyramidal neurons, octopus neurons, bushy neurons, stellate neurons, and root-neurons) and the corresponding electrical activity pattern, which reflects each cell's physiological function, are depicted [11].

Another element of complexity that has been seriously underestimated is generated by transcripts arising from intergenic and intronic regions of the genome. A combination of high-density tiling arrays for ten human chromosomes, rapid amplification of cDNA ends (RACE), and cloning/sequencing techniques revealed that approximately 10% of the genome corresponds to polyadenylated transcripts, of which more than half do not overlap with known gene locations. In addition, a large percentage of transcripts were not polyadenylated (43.7%) or were bimorphic, *i.e.*, present with and without polyA-tail (36.9%) [9]. The analysis of more than 50 000 000 oligonucleotides, positioned on average every 46 nucleotides along each strand of the human genome, also resulted in a higher number of transcribed sequences than predicted by previous analyses. Most transcripts arising from noncoding regions of the genome were previously dismissed as aberrant or at best judged as nonrelevant (but see [23]), yet their sheer number, as well as new insights concerning their impact on transcription and mRNA stability indicates a new level of transcriptional complexity [24]. Small RNAs, such as small nucleolar RNAs, microRNAs, short interfering RNAs, and small double-stranded RNAs, for instance, play important roles in many cellular processes such as chromosome architecture, stem cell maintenance, and differentiation [20–25].

Although the novel insights into transcriptome complexity were mostly gained from liver tissue [8] and non-neuronal or neural tumor cell lines [9], it is likely that they can be transferred to normal brain tissue. All previous analyses indicated that the nervous system contains the most complex repertoire of expressed genes when compared to other organs. A transcriptional study of human organs using serial analysis of gene expression (SAGE) detected more than 200 000 different transcripts in the brain, more than in any other organ [26]. Analysis of 160 genes located on chromosome 21 by RNA *in situ* hybridization and reverse transcription-PCR (RT-PCR) demonstrated that 65 to 80% of them are expressed in the mouse brain [27, 28], whereas only 21% were detected in muscle [27]. Taken together, these data indicate that likely more than 50% of all possible transcripts are present in the nervous system at a given time.

2.3 Complexity on the protein level

In addition to the complexity on the transcriptional level, proteome approaches have to deal with the considerable increase in protein isoforms due to multiple post-translational modifications (PTMs). Several hundred different types of PTMs exist, such as amino- and carboxy-terminal cleavage, phosphorylation, glycosylation, and myristoylation [29, 30]. The human proteome contains over 100 000 putative phosphorylation sites and about 50% of all proteins are supposedly phosphorylated by one of more than 500 known protein kinases [31]. A high degree of complexity is also generated by glycosylation. Today, more than 2700 unique glycan structures are known. They arise from variation in the type, number, and position of individual sugar residues, the degree of branching, and the level of acetylation, methylation, sialylation, phosphorylation, and sulfation [32]. It is thus estimated that a protein undergoes between 2 and 20 PTMs in average [33, 34]. Taking into account the existence of more than 56 000 protein-coding transcripts and the complexity of the brain transcriptome, several hundred thousand protein species are to be expected in the nervous system.

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Another important point in protein analysis is the broad range of protein abundance. In serum, the concentration of different proteins can vary by a factor of 10^{10}. Insulin was found in concentrations of 35–50 mg/mL, whereas interleukin 6 occurred at a concentration of 5 pg/mL or less [35]. These values represent an extreme case and the dynamic range of protein abundance in tissue is likely to be lower, but even more conservative estimations are in the order of 10^6 [36].

3 Genomics technologies

Modern genomics technologies come in many different flavors. Several powerful techniques are available which profile more than 10 000 transcripts in parallel. This includes hybridization-based high-density DNA microarrays [37] or sequence-based SAGE [38] and related techniques such as massively parallel signal sequencing (MPSS) [39] (for reviews see [40, 41]). Global gene expression analyses have procured insights into the molecular changes accompanying the differentiation of neural progenitors [42] and retina development [43], or have identified candidate genes underlying the left–right asymmetry of the human cerebral cortex [44]. In combination with RNA amplification [45–47], these profiling techniques have a very high anatomical resolution down to the single cell level. Single-cell microarray analysis of pyramidal neurons from the hippocampus CA1 region in combination with cluster analysis revealed two different neuronal populations, indicating that even a single morphologically defined cell type *in vivo* is not a homogeneous population of cells at the gene expression level [48].

Two other potent profiling techniques with cellular resolution are large-scale *in situ* hybridization [49, 50] and the use of bacterial artificial chromosome (BAC)-transgenic mice enabling expression of the reporter gene green fluorescent protein (GFP) under the regulatory elements of selected genes [51]. For both approaches, publicly available databases exist, providing insight into the developmental expression pattern of several hundreds (http://www.gensat.org) to thousands of genes (http://www.genepaint.org or http://geneatlas.org) in the brain on a cellular level.

In addition to the transcriptional profiling techniques, large-scale functional approaches based on gene-perturbing strategies are available, including gene knock-out by homologous recombination [52], random insertional mutagenesis, also called gene-trapping [53–55] or RNA interference [56] (summarized in [57]). For instance, a recent RNA interference screen in *Caenorhabditis elegans* identified 185 genes involved in acetylcholine-mediated synaptic transmission [58].

4 Proteomics technologies

Proteomics technologies have widely expanded in recent years. Similar to genomics technologies, they can be divided into expression profiling and functional approaches. Both major gateways to expression profiling employ MS for large-scale protein identification but differ in the protein separation techniques. Currently, the most widely used separation technique is 2-DE. The improvement of the originally reported 2-DE [59, 60] by the introduction of IPGs [61, 62] routinely allow the reproducible separation of several hundred to thousand protein spots. In addition, multidimensional LC was established to separate peptides from complex sample mixtures [63–65]. This approach resulted, amongst others, in the identification of more than 4500 different proteins from mouse cortex [66].

Powerful tools have also been developed concerning the important issue of quantitative proteomics [67, 68]. This includes 2-DE-based methods such as the 2-D difference gel electrophoresis (DIGE) [69] and the development of sensitive fluorescent dyes with an increased dynamic range [70, 71]. Likely more important are isotope coded protein-labeling techniques that allow MS-based strategies for protein quantification [72–74] (reviewed in [75]).

In addition, platforms have been generated to complement MS-based proteomics by large-scale interaction studies using the yeast two-hybrid system [76], (tandem-) affinity-purification [77, 78], or protein-arrays [79] (for review see [80, 81]). The power of high-throughput yeast two-hybrid analysis has recently been exemplified by the identification of 3200 [82] and 2800 [83] human protein–protein interactions. Whereas these analyses focused on binary protein–protein interactions, a study on the *N*-methyl-D-aspartate (NMDA) receptor complex revealed the wealth of information that can be gained from tedious purification and analysis of protein complexes. This work identified 77 proteins, grouped into receptor, adaptor, signaling, cytoskeletal, and novel proteins, as part of the complex, illustrating the huge molecular network required for proper function of many proteins [84].

5 Challenges and future directions

As outlined, many potential applications for proteomics in neuroscience exist: Determination of the brain proteome, comparative protein expression profiling, PTM profiling, and mapping protein–protein interactions. All of them have their strengths and limitations, and one of the major challenges ahead is to determine the most appropriate application of proteomics technology to the system studied.

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5.1 Proteome mapping

Considering the figures cited above, the brain proteome likely consists of several hundred thousand proteins, and its actual composition depends on developmental stages, environmental conditions, and pathological states. Consequently, generating a complete inventory, or even covering a decent part of the protein repertoire in a global manner is not straight forward. 2-DE-based approaches, arguably the most established technique for proteome mapping to date, routinely identify several hundred to 1000 protein species [85–87]. Although the upper limit of 2-DE resolution might be close to 10 000 individual spots *per* gel [87, 88], annotation of such a high number of spots on a single gel has not yet been reported. In addition to this discrepancy between proteome size, resolving power, and identified proteins, 2-DE has several serious limitations for use in neuroproteomics. It requires large protein amounts and fails to separate the important class of hydrophobic membrane proteins [89]. Furthermore, high and low-molecular weight proteins are not displayed [87, 90]. Out of 4459 *Rattus norvegicus* proteins stored in the Swiss-Prot database (release 6.5), roughly 12.6% (563) would not appear on a gel separating from pH 3 to 10 in the first dimension and from 14 to 200 kDa in the second dimension, simply due to their M_r and p*I* (Fig. 2A). Some of these problems are partially alleviated by alternate gel-based methods such as different buffer systems [87, 89] or the use of alternative 2-DE systems such as the 16-BAC-SDS system [91, 92]. Another serious limitation in 2-DE is the low dynamic range of protein amount that can be displayed. Most often, a small and very similar subset of the proteome is detected by 2-DE. This corresponds to high- to medium-abundant proteins, whereas low-abundant proteins are not amenable to the analysis [93, 94]. To overcome this limitation, the use of narrow pH-gradient gels was suggested [95]. However, this technique requires huge amounts of sample and is hence rarely applied. Finally, an emerging critical issue is the presence of spot fusion and comigration of different protein species in the same gel spot [93, 96] (Fig. 2B and C).

To overcome these limitations of 2-DE, alternative methods have been developed. Chromatography-based approaches, such as SDS-PAGE coupled to LC-MS/MS or multidimensional LC-MS/MS, are rapidly gaining popularity as fast and reliable methods for high-throughput protein identification [97–100]. Although impervious to many of the 2-DE shortcomings, the number of proteins identified in LC-based studies is usually between several hundred and a few thousand [66, 98, 101].

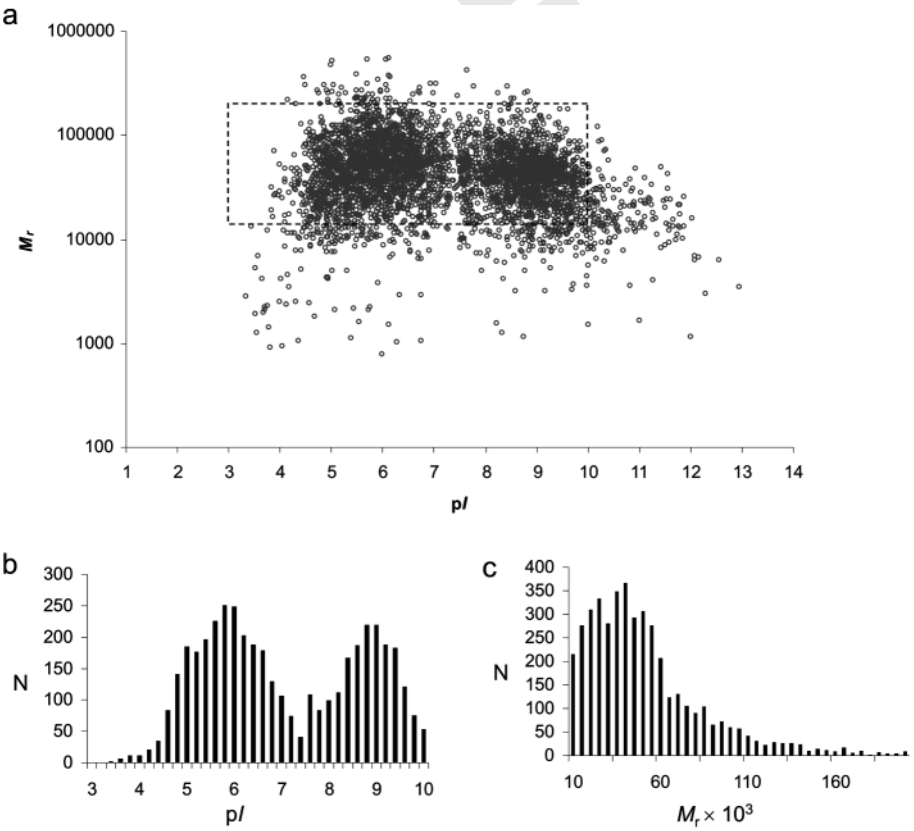

Figure 2. Limits of separation by conventional 2-DE. (A) Figure shows 4459■**check change**■ *R. norvegicus* protein entries of the Swiss-Prot database (release 6.5) plotted according to their p*I* and molecular weight (M_r). Values were calculated according to amino acid sequence using the ProtParam tool (www.expasy.org/tools/protparam.html). Dashed rectangle outlines the frequently used separation limits of conventional 2-DE from p*I* 3 to 10 and M_r 14 to 200 kDa. Five hundred sixty-three (12.6%) of the proteins in the database lie outside these limits and would not be detected by 2-DE. (B, C) Histograms displaying the distribution of the same protein set across the p*I* and M_r of a 2-DE. Distribution is not uniform in either dimension, which leads to spot clustering and reduction of the separation power. Bin size is 0.2 pH units (B) and 5 kDa (C). *N* = number of proteins *per* bin.

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A further caveat that must be considered is the performance of algorithms used to score mass spectra. LC-MS/MS experiments in particular generate an enormous number of peptide fragmentation spectra that can only be analyzed in a (semi) automated manner. Evaluation of different search algorithms is hindered by the fact that a precisely annotated reference dataset is necessary to reliably assess the sensitivity and false-positive rate for a particular algorithm. In a recent study, such a dataset was assembled and its analysis demonstrated that neither of the five algorithms evaluated (MASCOT, SEQUEST, Sonar, Spectrum Mill, and X!Tandem) returned a comprehensive list of true-positive hits [102]. Therefore, consensus scoring of two or more algorithms might be recommendable, but is not common practice right now. Also, false-positive rates of 1–5% must be expected using standard search parameters. By searching a dataset against a scrambled (*e.g.*, reversed) database, the number of false-positive hits can be estimated, allowing optimization of search parameters [103].

Regardless of the actual number of proteins identified in a single study to date and the accuracy of our estimate on the complexity of the brain proteome, it is apparent that complete coverage will require further technological improvements. These include further increases in sensitivity and sampling rate for mass spectrometers, and also improved prefractionation methods.

A frequently employed means to address the problem of limited coverage is the dissection of the proteome into a number of subproteomes of reduced complexity. This dissection can take place on the anatomical level (by analyzing defined brain regions instead of whole brain samples), on the subcellular level (by fractionation according to protein localization within a cell), on the biochemical level (according to protein p*I*, M_r, solubility, hydrophobicity *etc.*), or by a combination of these methods [95, 100, 104–107]. In theory, information gained from screening the subproteomes can then be merged, eventually approximating complete coverage of the brain proteome. However, at the currently possible sampling depth, the overlap between the subproteomes is large. For example, a recent study identified 1000 proteins from cultured Neuro2A neuroblastoma cells. Proteins (97%) were also identified when using whole brain samples [108]. This shows that even when narrowing sample complexity to a single-neuronal cell type, novel identifications are scarce. When using subcellular fractionation methods, overlap is generated by contaminants as well as by so-called "moonlighting" proteins that fulfill different functions in more than one cellular compartment [109]. Even with no overlap, tiling the brain proteome out of subproteomes would require analysis of more than 100 independent fractions with 1000 annotations each. Considering current limitations of separation techniques, the required number is likely much higher. A comprehensive analysis of the entire brain proteome is therefore out of reach for the time being.

This fact also sounds a note of caution to differential proteome analysis which consists in the quantitative comparison of protein profiles with respect to different conditions such as developmental stages or normal *versus* disease. Due to the relatively small coverage, the analysis is most often restricted to highly abundant housekeeping proteins [34]. This will also hold true for techniques such as DIGE [110]. Furthermore, when compared to similar studies on the transcriptome level, the few differentially regulated proteins are either stand-alone candidates or loosely grouped according to general function. Full coverage of all members of a pathway is rare, and automatic clustering into functional groups, as customarily done with transcriptome data [111–113], is difficult. It seems therefore most appropriate to form a hypothesis prior to quantitative or qualitative expression profiling. For example, the experimental design will differ for studies of highly abundant proteins, such as cytoskeletal proteins, *versus* those aimed for low-abundant proteins, such as signal transduction molecules [114]. The focus on a single subcellular proteome might improve the chances of success. An attractive idea might therefore be the rapid and inexpensive prescreening of the sample(s) by microarray analysis in order to guide the researcher to the most interesting cellular subproteomes, which can then be analyzed by the most adequate proteomic technique. However, one should remember that any proteome approach will dismiss the emerging class of noncoding RNAs, which likely play an essential functional role [20].

5.2 Focused proteomic approaches

If both, mapping and differential proteome analyses of brain samples, are limited by coverage, what kind of studies hold the most promise? To answer this question, one must reconsider the main advantages of experiments at the protein level. First, it is possible to detect the existence of previously unknown proteins for which no ORFs have been described, and to confirm the actual existence of hypothetical proteins that have been predicted from the genome sequence or detected as transcripts [115]. Second, only proteomics can dissect the molecular composition of subcellular compartments and changes in the distribution of proteins between different compartments due to altered trafficking. Third, PTMs of proteins can be identified and characterized [116, 117]. Fourth, multiprotein complexes take center-stage concerning our understanding of physiological and pathophysiological processes [118, 119].

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Current high-throughput studies, however, often do not exploit these capabilities. Automatic and semiautomatic analyses of both peptide mass fingerprint and peptide sequence data are prone to dismiss unknown and modified peptides, or correct them to create a better fit with database entries. Consider, for example, a dataset generated from a protein not yet represented in a species-specific database. Automatic searches would generate low-scoring, nonsignificant hits, and cause the dataset to end up as unannotated. Even searches against a larger database containing homologous proteins are likely to return low-scoring hits if the peptides analyzed fall into nonconserved regions. As high-throughput data is primarily judged by search scores, some of the most interesting proteins may be lost this way.

One of the advantages of 2-DE separation is the ability to separate different isoforms of the same protein into distinct spots. Most proteins appear in two to five spots, and some may generate well above 100 spots [34]. At least several of these spots are due to biologically relevant modifications of the protein molecule that survive the rigors of sample preparation, while other spots might be artifacts generated during preparation. It is common practice to identify these spots, map them to the same, unmodified database entry and summarize them as a single annotation. This extends to LC-MS/MS data, where differently modified peptides are often subsumed under the same protein entry. However, the sheer number of datasets generated often prohibits a more extensive evaluation of each single one. One approach to ease this situation are databases, containing known PTMs for a given protein. This would allow rapid identification of PTMs without specifying the settings in the search algorithm.

In the light of current technical limitations, it is also worthwhile to narrow the focus of brain proteome studies to a very limited subproteome. Ideally, the complexity of this subproteome would be well-below sampling limits (*e.g.*, a few hundred proteins), thus enabling nearly complete coverage. As a side effect, with a small dataset, thorough analysis of each single spectrum is feasible without becoming bogged down in detail. This can be achieved by various approaches, but unquestionably the most valuable and meaningful are preparing very well-defined subcellular structures or the isolation of protein complexes. The additional effort involved is likely to pay off well.

To neurobiologists, the most interesting subcellular structure is certainly the plasma membrane, the primary site of chemical and electrical signal transmission. Proteins, in the form of neurotransmitter receptors, ion channels, and transporters, are the major players in this process. As such, the interplay of neurobiology and proteomics holds much promise. In addition, electrophysiology, in combination with pharmacology, represents a powerful tool in the neurobiologist's arsenal, suitable for the functional assessment of identified plasma membrane molecules, their PTMs and associated proteins. The plasma membrane of neurons is further subdivided into different functional areas, including the axonal area with the presynaptic terminal, or the dendrites with postsynaptic membranes. Pre- or postsynaptic localization of a protein can have significant impact on its function. For instance, the glycine receptor is a classical, neurotransmitter-gated Cl–channel in the postsynaptic membrane [120, 121]. In mature neurons, its activation causes hyperpolarization due to Cl–influx [122, 123]. Surprisingly, at the Calyx of Held, a giant synapse in the auditory brainstem, the glycine receptor is also found presynaptically, where its activation depolarizes the nerve terminal, leading to an increase in intraterminal Ca^{2+}, and synaptic efficacy [124].

Lipid rafts are another important domain of the neuronal plasma membrane. These cholesterol and sphingolipid enriched microdomains [125] play important roles during neuritogenesis and proper neuronal function. For instance, the association of the neural cell adhesion molecule (NCAM) with lipid rafts is essential for the induction of neurite outgrowth [126]. In mouse brain plasma membrane vesicles, depletion of cholesterol perturbed the uptake of the neurotransmitter glutamate. This data implicate a role of lipid rafts in homeostatic maintenance of synaptic function [127]. Finally, association of proteins with lipid rafts might also be relevant to pathological processes. α-Synuclein, a presynaptic protein involved in Parkinson's disease, is found in lipid rafts, and a mutation, associated with the disease, disrupts its interaction with lipid rafts [128]. Differential proteome analysis of lipid rafts during development, in diseased state, or after induction of long-term potentiation and similar phenomenons could provide new insights into the cell biology of neurons. As lipid rafts frequently contain modified proteins such as GPI-anchored proteins, doubly acylated proteins, and palmitoylated proteins [129, 130], their analysis represents a worthwhile challenge for proteomic PTM analyses in particular.

Closely associated with the plasma membrane and of similar importance to neurobiologists are synaptic vesicles. These neuronal organelles still await their final proteome characterization, which might help to dissect the molecular machinery underlying the essential steps of vesicle docking, priming, fusion, and endocytosis from the plasma membrane during synaptic transmission [131]. Even less is known about developmental changes in the protein composition of synaptic vesicles or differences between brain regions.

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So far, only few studies have been carried out toward detailed analysis of these subcellular compartments. Novel, highly efficient fractionation protocols for the purification of these neuronal subproteomes are required. Despite recent progress, such as the enrichment of total plasma membrane from rat cerebellar tissue by affinity partitioning [100], further refinements are required to isolate and analyze the various plasma membrane microdomains in sufficient purity and yield. A recent proteomic analysis of synaptosomes isolated by differential and density-gradient centrifugation from mouse brain, resulted in more than 1100 protein identifications [132]. However, only 50% overlap was observed between two independent experiments and known synaptosomal proteins, including the inhibitory neurotransmitter receptors and vesicular glutamate transporters were not detected at all. This illustrates that, even for a tightly restricted subproteome, current technology falls short of achieving sufficient coverage and underlines the need for improvement of purification protocols and analytical methods.

The characterization of PTMs occurring in defined subcellular localizations represents a further important step. For instance, phosphorylation of α-amino-3 hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptors affects not only their biophysical properties but also receptor trafficking and thereby synaptic plasticity [133–135]. Comparative studies on the phosphorylation status of receptors, ion channels, and transporters in different intracellular compartments and the plasma membrane could provide whole new insights into such regulatory mechanisms. An analysis of NMDA receptors in transient global ischemia observed an increased amount of receptor phosphorylation in synaptic lipid rafts compared to postsynaptic densities. In parallel, a redistribution of NMDA receptors from synaptic lipid rafts to the postsynaptic density occurred, suggesting a modulation of NMDA receptor distribution by phosphorylation [136]. Comprehensive analysis of such relations between phosphorylation and subcellular localization might open up new avenues in our understanding of how molecular switches such as phosphorylation execute their function besides inducing conformational changes.

The systematic analysis of PTMs will also provide a better understanding of the molecular consequences of many mutations. A recent analysis of 10 047 mutations in 577 genes encoding proteins trafficked through the secretory pathway, identified 142 potential gain-of-*N*-glycosylation missense mutations in 77 genes [137]. Thus, an unexpectedly high proportion of mutations that cause human genetic diseases might lead to the creation of new *N*-glycosylation sites. This might hold true for other PTMs as well.

Equally important as subcellular approaches are proteome analysis of defined protein complexes [119, 138–140]. Their importance for proper function of the nervous system was neglected for some time due to the fact that expression of the widely studied pore-forming ion channel proteins in heterologous expression system resulted in functional channels. However, most often, these lonely channel proteins do not recapitulate all the biophysical and physiological properties of the native ion channel. This is mainly due to the missing signaling complexes closely associated with ion channels and transporters. K^+-channel interacting proteins (KChIPs) for example, specifically modulate the density and gating kinetics of a subfamily of voltage-gated K^+-channels [141]. A further role of large protein complexes is the organized transfer of information to precise locations within a neuron. A striking example was recently provided by characterization of the NMDA interacting protein Tiam1. This guanine nucleotide exchange factor couples NMDA receptor activation to changes in dendritic morphology [142]. The comprehensive identification and characterization of protein complexes is hence a prerequisite for improved understanding of regulatory and signaling mechanisms in neurotransmission. Furthermore, this analysis will break new grounds for polygenic disease traits, by identifying sets of genes with a functional link [118]. An in-depth investigation of the NMDA receptor complex identified 185 proteins. Forty-seven of the corresponding genes were associated with nervous system disorders such as autism and schizophrenia [84, 118, 143]. However, the actual composition of the NMDA receptor in different neuron types is unknown and likely subject to changes. Finally, the characterization of proteins associated with the small conductance Ca^{2+}-activated K^+-channels by affinity chromatography using His-tagged fusion proteins, 2-DE and MS identified the protein kinase CK2 as a regulator of channel gating [139]. As this study exemplifies, the use of current proteomics technologies can lead to the identification of a single protein of important physiological function.

It might be also very instructive to purify a protein complex from different species to search for evolutionary differences in protein composition related to species-specific functions such as cognition [144].

6 Concluding remarks

In conclusion, we believe that an inventory of the complete brain proteome will provide new routes to understanding brain function in a bottom-up manner. Considering the current technological standards, major efforts in protein expression profiling should focus toward meth-

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odological refinements, as major technical hurdles need to be overcome before most of the currently used proteome approaches can be meaningfully applied to the nervous system. Existing proteomics technologies, because of limited coverage, allow comprehensive analysis of only narrowly defined subproteomes. To neurobiologists, synaptic vesicles, the plasma membrane, and its subdomains such as the postsynaptic density or lipid rafts are of the highest interest. Protein complexes, especially those associated with receptors and other membrane proteins are likely the most limited subproteomes. Although they represent only a fraction of the brain proteome *per se*, analysis of such subsets can provide significant insights into neurophysiological processes, as pinpointed by several remarkable studies.

7 References

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