Published in final edited form as:
Curr Opin Mol Ther. 2007 June ; 9(3): 270–281.

Recent advances in neuroproteomics

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Abstract

The last few years have seen a rapid growth in the use of proteomic methods to study normal brain function as well as in the analysis of changes in protein expression that underlie the onset and progression of neuronal disease. However, the field of neuroproteomics faces special challenges given the complex cellular and sub-cellular architecture of the central nervous system. This article presents a review of recent progress in studies of neuroproteomics, and highlights the strengths and limitations of current proteomic profiling technologies used in studies of neuronal protein expression.

Keywords

Proteome; brain; gel electrophoresis; 2-DE; DIGE; LC-MS/MS; organelle; post-synaptic density; fractionation; phosphorylation; affinity chromatography

Introduction

Advances in instrumentation and bioinformatics have made it possible to analyze the entire genome and transcriptome. However, there often is not a direct relationship between the *in vivo* concentration of an mRNA and its encoded protein, and microarray technologies are unable to give information about post-translational modification of proteins. Identification of the proteome is therefore necessary to understand the normal and abnormal physiology of different types of cells and tissues. The central nervous system (CNS) poses particular challenges to proteomic studies. There is a huge level of cellular heterogeneity, with ~10^{11} neurons each with ~10^4 synapses, and a greater number of glial cells. Moreover, there is complex intermingling of cell bodies and neuronal processes with many genes expressed in only a small percentage of the ~10^3 different types of neurons [1]**. Moreover, neurons contain unique sub-cellular compartments, such as dendrites, spines, axons, and pre-synaptic terminals. Alternative splicing is a common feature in the CNS, and contributes to its complexity. Intracellular signal transduction is critical for CNS function and most protein kinases and phosphatases in the genome are expressed in the CNS. Any understanding of CNS function therefore requires identification and quantitation of the neuronal phosphoproteome, as well as of other post-translational modifications.

Since the first application of proteomics methods to studies of the brain in the late 1990s, there has been a substantial increase in publications on this topic with more than 250 in the last 2 years. Proteomic studies have analyzed the neuronal sub-compartments such as synaptic vesicles and the post-synaptic density. Other studies have analyzed differences in protein expression associated with CNS diseases such as depression, schizophrenia, drug addiction, Alzheimer's disease, Parkinson's disease, ALS, epilepsy and brain tumors. Along with this expansion of neuroproteomics, several large-scale initiatives have been organized to meet the challenges faced in studies of the brain. The Human Brain Proteome Project (http://www.hbpp.org) (see also below) aims to characterize all proteins in normal and

diseased human brain, as well as carry out analysis of aging. Other projects that focus on gene expression in the brain such as GENSAT (Gene Expression Nervous System Atlas, http://www.gensat.org) and the Allen Brain Atlas (http://www.brainatlas.org) [1] also provide a wealth of information that complements neuroproteomic research. In this review, we will briefly highlight advances made in the last 2 years in neuroproteomics, with a focus on studies related to psychiatric disease and the actions of drugs of abuse (see [2–4] for reviews of neurodegeneration and other neuroproteomic studies).

Neuroproteomic Techniques

2-DE and DIGE

The most commonly used neuroproteomic technique is 2-dimensional gel electrophoresis (2-DE) [for review of this and other neuroproteomic techniques, see [5]) (Table 1 and 2). With the standard method, samples are separated according to charge (isoelectric focusing) and molecular weight (SDS-PAGE). Proteins of interest can be identified using liquid chromatography tandem mass spectrometry (LC/MS/MS) or the higher throughput MALDI-Tof/Tof (MALDI-MS/MS). A major drawback to 2-DE is gel-to-gel variability, which can be overcome by the use of differential two-dimensional fluorescence gel electrophoresis (DIGE). In DIGE, typically, the control and experimental samples are labeled with the lysine- and protein (free) amino terminal-conjugating cyanine fluorophores (Cy3 or Cy5), while a mixture of equal amounts of the control and experimental samples is labeled with Cy2 and serves as an internal control. Dye-labeled samples are then mixed together and run on a single gel. The use of fluorescence tags enhances the sensitivity of detection. However, differences in dye-labeling of particular proteins can produce artifactual ratios; therefore, dye reversal is necessary to eliminate the possibility of dye bias. Disadvantages of 2-DE or DIGE include the fact that proteins that are large (>200 kDa), small (<10 kDa), or hydrophobic, are not resolved well. Another limitation is these methods tend to only identify abundant proteins, thus many neuroproteomic techniques that use these methods tend to identify the same proteins (see below).

MS-based methods

A feature of most protein profiling studies is their use of proteolytic digestion and mass spectrometry (MS), typically using tandem MS (MS/MS), to produce a mass fingerprint for any isolated peptide. Peptide masses and fragmentation patterns can then be used to search genomic and protein sequence databases to identify a particular peptide. Tandem MS/MS is often preceded by liquid chromatography (LC-MS/MS), which simplifies complex mixtures of peptides. For example, multi-dimensional chromatography (MudPIT) employing strong cation-exchange (SCX) and reverse-phase is often used. The complexity of peptide mixtures varies enormously from single proteins (2-DE), to many thousands from an unfractionated brain sample. Proteins may be identified by high quality MS/MS data from a single peptide, but identification of multiple peptides from a single protein adds confidence. Identification of multiple peptides from a single protein also helps assess the presence of protein isoforms. Recent studies also suggest that protein abundance is proportional to the numbers of peptides that are identified from any given protein [6].

A critical component of many neuroproteomic studies is the comparison of “control” and “experimental” samples. Differential isotope prelabeling methods like SILAC [7] are not easily applied to studies of intact brain. However, various methods can be used to label peptide mixtures with chemical tags prior to mixing the peptides and separation by LC-MS/MS [8;9]. A popular method is isotope-coded affinity tag (ICAT), where control and experimental protein samples are labeled on cysteinyl residues with a reagent containing biotin and either eight ^{1}H residues or eight ^{2}H residues [10]. The samples are mixed,

digested, and cysteine-containing peptides are purified using the biotin tag. The peptide pairs are then identified by LC/MS/MS and the relative ratio of the two peaks is calculated. The affinity step reduces peptide complexity, but comes at the cost of reducing the number of peptides (per protein) available for identification. Moreover, many proteins do not contain cysteine. A variant of ICAT is the use of isotopically labeled His6-tags (HysTag), a method used recently in studies of mouse brain membrane proteins [11]. Another attractive alternative that is beginning to be used in neuroproteomic studies [12–14]** is "isobaric tagging for relative and absolute quantitation" (iTRAQ) [15;16]. iTRAQ is a multiplex MS-based method in which control and experimental samples are labeled separately with one of four amine reactive tags, then pooled and subjected to LC-MS/MS. These tags contain isobaric balance and reporter groups that are cleaved during MS/MS and are used to quantitate the relative abundance of each protein in the control and experimental samples.

Characterization of the brain proteome

Global Expression Profiling

There has been significant progress in the last 2 years in the global characterization of the brain proteome (Table 1). Following on from studies by Yu et al who identified more than 4500 proteins in cultured cortical neurons [17], Wang et al used LC-MS/MS to identify ~7800 proteins from unfractionated brain [18]**. It is notable that a large number (>1400) of membrane proteins were identified.

Sub-cellular fractionation

Given the complex morphology of neurons, the use of sub-cellular fractionation is an obvious first step in any proteomic study. In addition, given their importance, considerable effort has been made to identify low abundance membrane proteins such as ion channels and neurotransmitter receptors. As part of a global protein expression survey of organelles, Kislinger et al analyzed cytosol, membrane, mitochondrial and nuclear fractions from brain and other tissues [19]. More than 2200 total brain proteins were identified, which was comparable with that in other organs. Greater than 1000 were membrane proteins, and >1000 were found in the mitochondrial fraction. A notable feature of this study was that multiple (7–9 repeats) LC-MS/MS analyses were needed to improve protein coverage in any individual fraction. Subcellular fractionation combined with 2-DE has been used to identify >1800 proteins from mouse brain that are the products of ~790 genes [20]. In this study, ~31% of the proteins identified were associated with cellular metabolism, likely reflecting the abundance of this class of proteins. Reflecting the use of 2-DE, few membrane proteins were documented. Subcellular fractionation and 1-DE has been used to study human brain (temporal lobe) resulting in identification of >1500 proteins, 24% of which were membrane proteins [21]. Other studies of rodent brain, using novel methods for isolation of plasma membrane fractions, have identified membrane proteins, including various ion channels and neurotransmitter receptors [22–24], while other studies have identified the protein content of myelin [25].

Synaptic proteins

A major focus of neuroscience research is to understand the mechanisms of synaptic transmission, as well as the processes involved in synaptic plasticity. Considerable effort has been made to identify the protein components of both pre- and post-synaptic compartments (see also [26]). Synaptosomes, pre-synaptic compartments that reseal after tissue homogenization, are isolated by differential and density-gradient centrifugation. They contain synaptic vesicles, usually 1 or 2 small mitochondria, remnants of the post-synaptic membrane and often the post-synaptic density (PSD). Using 2-DE and LC-MS/MS, Witzmann et al identified more than 240 proteins in synaptosomes [27], while Schrimpf et al

used ICAT and LC-MS/MS to identify more than 1130 proteins [28]. In the latter study, 608 proteins were identified in each of 2 experiments, while a substantial number of proteins (~200–300) were only found in one experiment or the other.

Synaptic vesicles contain neurotransmitters and fuse with the synaptic plasma membrane in response to neuronal activity. Proteomic methods using 1-DE, 2-DE, and LC/MS/MS have identified more than 400 proteins in various studies of synaptic vesicles [29–32]. These include proteins known to be involved in synaptic vesicle function, including neurotransmitter transporters and proteins involved in vesicular trafficking. Of note is the comprehensive study by Takamori et al., who combined quantitative proteomics, lipidomics and modeling to generate a detailed molecular rendering of a synaptic vesicle [32]**. Following exocytosis, synaptic vesicles are retrieved as clathrin-coated vesicles and are locally recycled. Proteomic studies of clathrin-coated vesicles identified greater than 200 proteins, many of which were known to be associated with synaptic vesicles [33;34]. These studies also identified ~90 new proteins and follow-up studies characterized several of these, identifying a novel AP-2-binding motif.

A number of high quality studies have focused on characterization of the PSD, the electron-dense, post-synaptic element of glutamatergic excitatory synapses [35]. 1-DE, 2-DE, ICAT and MudPIT, as well as immuno-affinity purification, have identified more than 1200 proteins in the PSD [36–39] (see also [40–42]). In one of these studies, Phillips et al derived two fractions from a synaptosome preparation by differential detergent extraction – the insoluble PSD and the soluble presynaptic specialization – and analyzed both using MudPIT [36]. More than 340 proteins were identified, 60 of which were found in both fractions, 50 in the presynaptic specialization, and 231 in the PSD fraction. In addition to their experimental approaches, Collins et al also performed a bioinformatics analysis of most of the available proteomics studies [38]**. Notably, the majority (58%) of proteins were identified only in one study. Based on their identification in two or more studies, Collins et al found 466 proteins that they considered as the “core” PSD proteins, with many of these being confirmed to be present in PSD fractions by immunoblotting. Certain methods were more successful in identifying membrane proteins [38], while others identified a greater number of low molecular weight proteins [41]. Repeated analysis of the PSD fraction by the various investigators may have resulted in greater overlap in protein identification, as has been seen in other studies of organelles [19].

Typically total brain was used in these studies, and the results reflect the mixing of PSDs from many different brain regions and types of neurons. Recent studies have begun to analyze PSD fractions from specific brain regions. Cheng et al found that out of 296 proteins, 43 differences were found in PSDs from forebrain and cerebellum [43]. Dosemeci et al carried out small-scale purifications from hippocampal slices and have identified more than 100 proteins from ~10 μg of PSD protein [44]. McNair et al dissected the CA1 region of hippocampal slices and used DIGE to examine the effect of high frequency stimulation on protein expression, finding changes in 79 proteins potentially involved in synaptic plasticity [45]. Olsen et al have used an isotope-tagging method to quantitatively compare membrane proteins in mouse cortex, hippocampus and cerebellum, and measured differences in ion channels, ion transporters, and receptors [11]*. It is also likely that a number of proteins that have been identified in the PSD are not truly enriched in this synaptic fraction. Both ICAT and iTRAQ have been used to compare enrichment of PSD proteins relative to protein levels in other synaptic fractions [14;46**]. Studies have also begun to provide quantitation of protein amounts in the PSD fraction [14;43*]. These quantitative approaches will be important in future characterization of the PSD or other neuronal fractions and organelles.

Affinity Chromatography

A large variety of affinity methods are currently used in proteomics studies [47] and many of these approaches have been applied to studies of the CNS, for example antibody-based enrichment of glutamate receptors [38] or metal-based affinity for phosphopeptides [48]. To enrich for plasma membrane proteins, Chen et al used a surface biotinylation method to label the extracellular regions of membrane proteins prior to avidin-affinity chromatography [24]. Berggard et al were able to identify 140 proteins in mouse brain that interacted with the Ca^{2+}-binding protein, calmodulin [49]. Lectin affinity chromatography was used to enrich for PSD proteins modified by O-linked N-Acetylglucosamine [50]. Affinity chromatography was also used in studies that identified proteins in embryonic mouse brain that interacted with 14-3-3ε [51]. This study found >160 proteins, many of them novel, and also identified 85 phosphorylation sites in the proteins, consistent with 14-3-3 proteins interacting with conserved phospho-motifs. Olsen et al used immunoprecipitation to enrich for proteins that bind to the MALS/CASK/Mint complex, a presynaptic scaffold and found that it interacted with cytoskeletal and adhesion proteins [52]. Subsequent work established a role for the MALS protein in synaptic vesicle exocytosis. Immunoprecipitation was also used in a study of microtubule-associated protein light chain 3 (LC-3) in autophagy, a mechanism of protein degradation [53]. A feature of this study was the use of a transgenic mouse line that expressed GFP-LC3 in brain and immunoprecipitation using anti-GFP antibody. This type of approach, of expressing tagged proteins in specific neuronal populations in vivo, is likely to be useful for future studies of protein:protein interactions [54]. Local protein synthesis is known to take place in neuronal sub-compartments, a process that is likely to contribute to the complexity of the neuroproteome [55]. A recent notable study has developed a method to selectively incorporate azidohomoalanine into newly synthesized proteins, followed by their affinity purification and identification by tandem MS [56]*. Proteomic studies of ribonucleoprotein complexes from rat cortex may also help in analysis of mechanisms of regulation of local protein synthesis in neurons [57].

Posttranslational modifications

An important component of proteomic approaches is their ability to identify post-translational modifications. Protein phosphorylation and dephosphorylation plays a critical role in intracellular signal transduction in the brain. Other important modifications of proteins such as glycosylation, methylation, acetylation, myristoylation, palmitoylation, prenylation, nitrosylation, oxidation and ubiquitination also play important roles in the functions, cellular targeting and degradation of proteins in the CNS. In the case of phosphorylation, peptides that contain a phosphorylated tyrosine, threonine, or serine, are typically present in low abundance. However, there are several methods of enrichment, such as immunoprecipitation using phospho-tyrosine and phospho-serine/phospho-threonine antibodies, affinity chromatography with immobilized metal-ion affinity chromatography (IMAC) and metal oxides, SCX, as well as chemical modification of the phosphate combined with affinity purification [58]. While not as extensive as studies in cells in culture (see [7]), there have been several phosphoproteomic studies of neuronal preparations [39;48;59–63]. Of note are three comprehensive studies of synaptic proteins [14;39;48**]. In each study, a large number of novel phosphorylation sites were identified (80–90%) but there was little identification of phospho-tyrosine. Individual proteins were often phosphorylated on multiple sites, and based on bioinformatic analysis it appeared likely that multiple proteins are phosphorylated by individual protein kinases.

The Human Proteome Organization – Brain Proteome Project (HUPO BPP)

With the growth in neuroproteomics, the use of standardized protocols and analysis has become imperative to allow for appropriate peer review, comparison between datasets and

potential for re-analysis by others (for review, see [64]. The Human Brain Proteome Project (HUPO BPP) has been established to carry out neuroproteomic studies of human and animal model brain tissue, specifically in relation to neurodegenerative diseases. HUPO BPP, in conjunction with the HUPO Proteomics Standards Initiative (HUPO PSI) also will determine the best ways to collect and disseminate neuroproteomic data [65;66]. The results and analysis of the first stage of this initiative has recently been published in the September 2006 issue of Proteomics [67]**. To determine the feasibility of reanalyzing datasets from a number of different labs and to examine heterogeneity in sample analysis, HUPO BPP invited labs from around the world to characterize two sets of samples: biopsy material containing parts of the hippocampus and temporal lobe from an epileptic patient and postmortem tissue from the corresponding regions of another patient as control; and brain tissue from C57BL/6 mice at three different developmental stages [68]. Participating labs were asked not to pool samples and to use a common sample extraction method. However, a variety of 2-DE, DIGE and LC-MS/MS methods using different instrumentation and database mining were used. The most notable outcome was the high degree of variability. Despite large differences in the number of proteins identified, almost one third of any dataset were unique. Moreover, in attempts to identify differentially expressed proteins in the mouse samples, only 1 differentially expressed protein was found by all 5 laboratories involved, while 436 differentially expressed proteins were identified by a single laboratory. The reasons for these differences appear to be intrinsic to the specific methods used for protein and peptide isolation, peptide identification, and data analysis.

Proteomic studies of psychiatric disease and the actions of drugs of abuse

Psychiatric disorders remain one of the largest medical problems in developed countries. While there are treatments for illnesses like schizophrenia, bipolar disorder, and major depression, the underlying mechanisms of these disorders remain largely unknown. This is also true for drug addiction, where the effects of short- or long-term use is not well understood. Within the last two years, there has been a significant increase in proteomic studies of the actions of drugs of abuse and various psychiatric diseases. As discussed above, a critical component of these types of studies is accurate comparative quantitation of proteins in control and experimental samples and to date the majority of studies have used 2-DE (Table 2).

Human and animal studies related to schizophrenia

In a study of the anterior cingulate cortex of patients with schizophrenia, bipolar disorder, or major depressive disorder, Beasley et al found 35 proteins with altered expression in two or all of the disorders [69]. Nineteen of these were identified and found to be from mitochondria or the cytoskeleton. Differential expression of 3 proteins was confirmed by immunoblotting (DRP-2, a guanine nucleotide binding protein, and NP25). Another study of the anterior cingulate of schizophrenic patients also found altered expression of many of the same proteins involved in metabolism and cytoskeletal function, as well as additional proteins involved in signaling and trafficking [70]. No secondary analysis was done in this study. These two studies, as well as an earlier study [71], highlight altered brain metabolism in schizophrenia. In an attempt to clarify the mechanisms of action of antisychotic drugs, La et al identified alterations in protein expression in hippocampus following administration of either clozapine, an atypical antipsychotic, or chlorpromazine to rats [72]. In another study from the same group, serum apolipoprotein-AI was found to be significantly increased in chlorpromazine-treated rats but decreased in patients with treatment-resistant schizophrenia [73].

Human and animal studies related to depression and anxiety

Schlicht et al found that 3 proteins associated with gliosis, GFAP, SOD2, and CRYAB, were increased in the prefrontal cortex of suicide victims when compared to controls [74]. Major depression may be linked to hyperactivity of the hypothalamic-pituitary-adrenal axis, and increased levels of cortisol. Skynner et al have examined the effects of chronic corticosterone treatment on protein expression in several brain regions [75]**. Using a rat model of psychosocial stress, Carboni et al examined changes in protein expression in hippocampus [76]. Alterations in 69 proteins were detected, 21 of which were identified and found to be involved in regulation of a variety of cellular processes. In a separate study, Carboni et al also examined the effects of fluoxetine and other antidepressants on protein expression in hippocampus and frontal cortex and found alterations in proteins that regulate synaptic plasticity [77]. Some of the proteins altered by fluoxetine treatment were also found in a previous study [78].

In a study of anxiety, Kromer et al outbred mice to have high or low anxiety-related behavior [79]**. Increased expression of the metabolic enzyme, glyoxylase, was found in amygdala, hippocampus and cortex from animals with low anxiety. The benefits of exercise on mental health as well as cognition are well established. Ding et al examined the effects of voluntary exercise on protein expression in the hippocampus [80]. Increased expression of proteins involved in energy metabolism were identified, one of which was confirmed by immunoblotting (ubiquitous mitochondrial creatine kinase, uMtCK). Using ProQ Diamond stain, they also identified changes in phosphorylation states of beta tubulin, neurofilament, GFAP, heat shock protein 8, and TAPP.

Neuroprotomics and drug addiction

A central feature of drug abuse research is the hypothesis that addictive drugs lead to long-term adaptive changes in gene and protein expression. Gene microarray studies have elucidated transcriptional changes that occur in various animal models of drug abuse [81]. However, only in the last year have studies begun to appear documenting effects of drugs of abuse on the neuronal proteome (see also [26;81]).

Human studies of addiction

Tannu et al measured protein expression in nucleus accumbens from cocaine overdose victims compared to controls [82]. Eighteen proteins were found to be differentially expressed, with increases in levels of β-tubulin, liprin-α3, and neuronal enolase, and decreases in parvalbumin, ATP synthase β-chain and peroxidoxin 2, being found in the cocaine overdose tissue. Alexander-Kaufman et al showed differences in uncomplicated alcoholics (69 regulated proteins) and alcoholics complicated with hepatic cirrhosis (81 regulated proteins) [83]. Of these, 30 were changed in both groups when compared to control. Most of these proteins were related to metabolic function, consistent with another recent study [84]. There have also been a number of studies focusing on utilizing various proteomic techniques in determining alcohol dependence and susceptibility [85].

Animal models of addiction – studies of nicotine

Two studies of chronic nicotine exposure in rats has been carried out [86;87]. Altered expression of several proteins was found in striatal tissue, consistent with similar changes in mRNA expression for these proteins (DNase 1/3, THIK-2, and BDNF) [86]. Altered expression of 41, 49, 46, 36, and 28 proteins in the amygdala, nucleus accumbens, prefrontal cortex, striatum, and ventral tegmental area, respectively, was also found [87]. Seven spots that were confirmed to change in multiple brain regions include proteins involved in energy metabolism (aldolase A, enolase 1α, and laminin receptor 1), synaptic function (dynamin 1,

N-ethylmaleimide-sensitive fusion protein, and *N*-ethylmaleimide-sensitive factor attachment protein), and protein folding (Hsc70).

Studies of opiates

Morphological changes at excitatory synapses following addictive drug administration is a well established phenomenon for a number of different drugs of abuse [88]. Using ICAT, Prokai et al characterized the synaptic proteome following chronic morphine exposure [89]. Twenty-seven proteins were regulated, including those involved in cell adhesion and synaptic vesicle exocytosis and endocytosis. No secondary confirmation was carried out in this study. A similar study also using ICAT was carried out by Moron et al [90]**. In this case the PSD fraction from hippocampus was enriched using the method of Philips et al described above [36]. Changes following chronic morphine exposure in various brain regions (without any subcellular fractionation) has also been studied using 2-DE [91–93]. Proteins involved in protein turnover, metabolism, and synaptic function were identified as being differentially expressed, but no secondary analysis was done in these studies. Studies of the phosphotyrosyl proteome in the frontal cortex in rats has also been examined following chronic analgesic exposure [94;95]. Proteins were separated by 2-DE and then immunoblotted with an anti-phosphotyrosine antibody. Altered tyrosine phosphorylation of several classes of protein were found in response to morphine or butorphanol.

Studies of psychostimulants

Freeman et al have studied the consequences of self-administration of amphetamine, and subsequent abstinence and relapse, on protein expression in hippocampus [96]. They found that of a large number of alterations, the expression of 22 proteins in the abstinent group, was the most significant. The proteins identified are involved in metabolic function and protein turnover. Iwazaki et al measured altered protein expression in striatum following acute methamphetamine exposure [97]. Thirty-six proteins were differentially regulated by drug treatment, of which 26 were identified, including proteins involved in metabolism, the cytoskeleton and protein turnover. Another similar study identified 5 proteins that were regulated by methamphetamine, 2 of which were confirmed by immunoblotting (α-synuclein and ubiquitin carboxy-terminal hydrolase)[98].

Studies of alcohol

Studies of alcohol addiction in animal models has included the generation of inbred alcohol-preferring rats. Protein expression studies have recently shown that alcohol intake is associated with altered protein expression in the amygdala and nucleus accumbens [99]. Using DIGE, Damodaran et al treated zebra fish with alcohol via their tank water for up to 10 weeks and showed altered protein expression in 8 proteins, including apolipoprotein A1 and voltage-dependent anion channel 1 and 2 [100].

Studies of reward

Drug addiction appears to result from abnormal control of basic reward mechanisms. Protein expression in rat prefrontal cortex has been analyzed following sucrose self-administration [101]. Altered expression of 28 proteins was found, including those involved in the cytoskeleton, energy metabolism, and synaptic function. Quantitative RT-PCR found that mRNA for 5 of the proteins identified (SNAP25, Ddah1, Ddah2, Grp58, and Crym) were also regulated by sucrose self-administration.

Conclusions and future directions

The last two years has seen significant progress in the use of proteomics techniques to study brain function. Global shotgun methods can now identify many thousands of proteins from

relatively small amounts of tissue. It is likely that greater than 22,000 genes are expressed in mouse brain [1]. Thus, while additional work is needed to identify greater numbers of the remaining, presumably lower abundance proteins, these recent studies provide a basis for future analysis of specific brain regions, and for quantitative global neuroproteomic studies. There has also been significant progress in characterization of the proteome of subcellular fractions such as the PSD and organelles such as synaptic vesicles. Recent studies have also begun to characterize post-translational modifications on a large scale, and methods for comparative and absolute quantitation of protein levels are now being used more frequently.

While advances in neuroproteomics have been made, there are still important challenges for the future (see also [26;68;102;103]). The complexity and heterogeneity of cell types in the brain, each with their own distinct protein expression profiles, poses a serious challenge. Changes in a specific subset of neurons are easily diluted out in the face of unchanging expression levels in surrounding cells. The dissection of discrete brain nuclei and the use of techniques such as laser-capture microscopy may help in this regard. In any respect, identification of the cellular and subcellular location of proteins identified in proteomics studies is a necessary goal. However, the lack of availability of high quality antibodies is a serious limitation. Indeed only a few of the studies discussed above utilize antibodies even for secondary confirmation by immunoblotting. Efforts to generate antibodies relevant to neuroproteomics are likely to intensify in the future [102], and it is hoped that these will be made available at low cost given the large numbers needed. The availability of antibody arrays relevant to CNS research will also be a useful complement other neuroproteomic techniques.

The magnitude of changes induced by brain disorders or experimental manipulations is also an important issue. The CNS, with its 10^{15} synaptic connections, forms an exquisitely tuned network, and even small changes in protein expression are likely to produce noticeable effects on network activity and hence behavior. Thus accurate methods are needed to quantitate small changes in protein levels, and appropriate procedures and standards need to be developed for statistical analysis. In this respect the comprehensive pilot projects carried out by the HUPO BPP sets the stage for greater standardization in future large-scale neuroproteomic projects. Standardization will be important in virtually all areas of experimental design, from choice of species and strain, gender, and drug treatment paradigm through tissue dissection and fractionation methods, precise use of proteomic technique and methods and databases used for peptide identification.

A notable feature of many of the shotgun protein profiling studies is the lack of overlap of protein identities found by different investigators ostensibly studying the same proteome or sub-proteome. A likely explanation is the necessity to perform repeated analyses on the same samples to provide saturation of protein identifications. However, these results also highlight the contributions that individual studies can make using complementary approaches. In contrast, studies related to different psychiatric diseases or to drugs of abuse often identify the same subset of proteins. This is likely largely a reflection of the general use of 2-DE methods that only allow analysis of highly and moderately abundant proteins, many of which are involved in metabolism or control of the cytoskeleton. These latter studies will need to reach deeper into the proteome by using more sensitive methods, defined brain regions, subcellular fractionation and other methods of protein enrichment. However, the results obtained to date are still likely to be important. For example, general changes in metabolism may be linked to altered neuronal function that is common to a variety of CNS disorders. Finally, it is important to emphasize that any list of candidate proteins generated by a proteomics study is the beginning, rather than the endpoint, of any particular investigation. Given the recent advances, future neuroproteomic research is likely

to identify novel candidate proteins and open up new avenues of research in many areas of neuroscience.

Acknowledgments

Supported by NIH grants DA018343 and DA10044. We thank Dr. Ken Williams for helpful comments.

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Table 1

Characterization of the brain proteome.

Tissue Source (#samples)	Fractionation/enrichment	Technique	# Proteins Identified	Secondary Analysis	Ref.
Brain proteome					
Mouse whole brain	No	With/without cysteinyl peptide enrichment	7792	No	[18]
Fractionation					
Rat whole brain	Synaptic vesicle fraction	16-BAC/SDS-PAGE and 1D SDS-PAGE	410	Immunoblot (85)	[32]
Rat cerebral cortex	Synaptosome	2-DE and shotgun LC-MS/MS	246	No	[27]
Mouse - Whole brain (7–9)	Total, cytosol, microsome, mitochondria, nuclei	MudPIT	2243, 1366, 1040, 1075	Microarray	[19]
Rat - Whole brain (3)	Synaptic vesicle fraction with SV2 immunoprecipitation	16-BAC/SDS-PAGE	127	Immunoblot (17)	[30]
Mouse (WT versus MALS triple knockout) - Whole brain	MALS immunoprecipitation	1D SDS-PAGE	6	Immunoblot (4)	[52]
Mouse - Whole brain	Synaptosome	ICAT	1131	No	[28]
Rat - Forebrain	PSD	1D SDS-PAGE	~300	Immunoblot, Immunocyto. (1)	[37]
Rat – Cerebral Cortex	Presynaptic fraction, PSD	MudPIT	110, 291	Immunocyto. (2)	[36]
Rat - Forebrain and cerebellum	PSD	cleavable ICAT and AQUA	296	No	[43]
Rat – Whole brain	Synaptic vesicle	1D SDS-PAGE	159	Immunoblot, Immunocyto. (6)	[31]
Mouse whole brain (4)	Cytoplasm	Affinity chromatography followed by 2-DE	1841	No	[20]
Human temporal lobe (1)	Soluble (cytoplasm and nucleoplasm), Membrane, DNA-binding proteins	1D SDS-PAGE	792, 1335, 343	No	[21]
Mouse - cerebral cortex (1) and hippocampus (1)	Plasma membrane	Affinity chromatography	862, 724	No	[22]
Rat - Cerebellum	Plasma membrane	Affinity chromatography followed by 1D SDS-PAGE	506	No	[23]
Rat - Hippocampus tissue and embryonic hippocampal cultures	Plasma membrane	1D SDS-PAGE, affinity chromatography	345, 336	No	[24]
Mouse - Forebrain	PSD	Affinity chromatography	244	No	[63]
Mouse - Whole brain	PSD and PSD with NR1, NR2B, GluR2 IP	1D SDS-PAGE	698	Immunoblot (21)	[38]
Mouse - Embryonic forebrain and midbrain (57 pooled)	GST-14-3-3ε pulldown - Phosphoprotein binding cleft mutant versus wildtype	1D SDS-PAGE	163	Immunocyto. (1)	[51]

Tissue Source (#samples)	Fractionation/enrichment	Technique	# Proteins Identified	Secondary Analysis	Ref.
Mouse - Whole brain from GFP-microtubule-associated protein light chain 3 (LC3)	GFP immunoprecipitation	1D SDS-PAGE	4	Immunocyto. Co-IP (1)	[53]
Rat - Cerebral cortex	Ribonucleoprotein complexes	Affinity chromatography	76	Immunoblot (8)	[57]
Post-translational modification					
Rat - Cerebral cortex: phosphatase-treated vs. control	No	DIGE	42	No	[61]
Human cerebral cortex (3)	Synaptosome	IMAC	26	No	[60]
Mouse - Forebrain	Synaptosome	Double IMAC protein and peptide enrichment	339 phosphorylation sites from 79 proteins	Bioinformatic analysis	[48]
Mouse - Forebrain	PSD	SCX and IMAC	723 phosphorylation sites from in 1264 proteins	No	[39]
Mouse - cortex	PSD, synaptic vesicles	SCX and IMAC	974 phosphorylation sites from 499 proteins	iTRAQ used for quantitation	[14]

Table 2

Neuroproteomic studies related to psychiatric disease and drug addiction.

Tissue Source (#samples)	Tissue	Technique	# Altered Proteins	Protein categories	Secondary Analysis	Ref #
Human - suicide victims (17) and controls (9)	Prefrontal cortex	2-DE	3	Protein folding, glial activity, oxidative stress	No	[74]
Human - schizophrenic (15), bipolar (15), major depressive (15) patients and controls (15)	Anterior cingulate cortex	2-DE	26	Energy metabolism, synaptic plasticity	Immunoblott (3)	[69]
Human – schizophrenic patients (10) and controls (10)	Anterior cingulate cortex	2-DE	39	Energy metabolism, synaptic plasticity	No	[70]
Rat - Clozapine-treated(12), chlorpromazine-treated (14), and control (12)	Hippocampus	2-DE	4	Energy metabolism, oxidative stress, signaling	No	[72]
Mouse - Bred for high (8) versus low (8) anxiety-related behavior	Hypothalamus, amygdala, and motor cortex	2-DE	1, 1, 1	Energy metabolism	Microarray and Immunoblott (1)	[79]
Rat - With voluntary exercise (5) and sedentary animals (5)	Hippocampus	2-DE	80	Energy metabolism, synaptic plasticity	Immunoblott (2)	[80]
Mouse - Chronic corticosterone treament (8) and control (8)	Hypothalamus, hippocampus, and cerebral cortex	DIGE	25, 33, 22	Energy metabolism, synaptic plasticity, cell death	Immunoblott (4)	[75]
Human - Complicated (6), uncomplicated alcoholics (8) and controls (10)	dorsolateral prefrontal cortex	2-DE	18	Energy metabolism, synaptic plasticity	No	[83]
Rat - Nicotine treated (10) and control (10)	Striatum	2-DE	7	Cell death, signaling	RT-PCR (3)	[86]
Rat - Nicotine treated (6) and control (6)	Amygdala, N. accumbens, prefrontal cortex, striatum, VTA	2-DE	14, 11, 19, 13, 19	Energy metabolism, synaptic function, oxidative stress	No	[87]
Rat - Sucrose self-administration (6) and control (5)	Medial prefrontal cortex	2-DE	28	Synaptic plasticity, energy metabolism, synaptic function	RTqPCR (5)	[101]
Rat - Chronic morphine exposure (3) and control (3)	Forebrain synaptosomes	ICAT	27	Synaptic plasticity, energy metabolism, synaptic function	No	[89]
Mouse - Morphine exposure (5 pooled) and control (5 pooled)	Hippocampal PSDs	ICAT	23	Signaling, synaptic function	Immunoblot (5) EM (1)	[90]
Rat - Chronic morphine exposure (5) and control (5)	Nucleus accumbens	2-DE	15	Energy metabolism, synaptic function, synaptic plasticity	No	[93]
Rat - Morphine exposure (3) and control (3)	Frontal cortex	2-DE + pTyr blotting	46	Energy metabolism, synaptic plasticity, signaling	No	[95]
Rat - Amphetamine self-administration: abstinent (3) versus binge (3), relapse (3), and control (3)	Hippocampus	DIGE	22	Synaptic plasticity, oxidative stress, energy metabolism	No	[96]
Mouse - Methamphetamine exposure (12) and control (12)	Striatum	2-DE	5	Synaptic function, synaptic plasticity, oxidative stresss	Immunoblot (2)	[98]
Rat - Exposed to repeated social defeat (6) and control (6), single social defeat (5) and control (5)	Hippocampus	2-DE	21, 8	Energy metabolism, synaptic plasticity, protein folding	No	[76]