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RECENT ADVANCES IN QUANTITATIVE NEUROPROTEOMICS

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Abstract

The field of proteomics is undergoing rapid development in a number of different areas including improvements in mass spectrometric platforms, peptide identification algorithms and bioinformatics. In particular, new and/or improved approaches have established robust methods that not only allow for in-depth and accurate peptide and protein identification and modification, but also allow for sensitive measurement of relative or absolute quantitation. These methods are beginning to be applied to the area of neuroproteomics, but the central nervous system poses many specific challenges in terms of quantitative proteomics, given the large number of different neuronal cell types that are intermixed and that exhibit distinct patterns of gene and protein expression. This review highlights the recent advances that have been made in quantitative neuroproteomics, with a focus on work published over the last five years that applies emerging methods to normal brain function as well as to various neuropsychiatric disorders including schizophrenia and drug addiction as well as of neurodegenerative diseases including Parkinson's disease and Alzheimer's disease. While older methods such as two-dimensional polyacrylamide electrophoresis continued to be used, a variety of more in-depth MS-based approaches including both label (ICAT, iTRAQ, TMT, SILAC, SILAM), label-free (label-free, MRM, SWATH) and absolute quantification methods, are rapidly being applied to neurobiological investigations of normal and diseased brain tissue as well as of cerebrospinal fluid (CSF). While the biological implications of many of these studies remain to be clearly established, that there is a clear need for standardization of experimental design and data analysis, and that the analysis of protein changes in specific neuronal cell types in the central nervous system remains a serious challenge, it appears that the quality and depth of the more recent quantitative proteomics studies is beginning to shed light on a number of aspects of neuroscience that relates to normal brain function as well as of the changes in protein expression and regulation that occurs in neuropsychiatric and neurodegenerative disorders.

Keywords

Two-dimensional gel electrophoresis; isobaric labeling; label-free quantitation; mass spectrometry; neuropeptidomics; proteomics; quantitative neuroproteomics

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INTRODUCTION

Challenges in proteomic studies of the central nervous system

The goal of this review is to discuss recent advances in quantitative proteomic methods and their application to studies of the central nervous system, i.e. so-called “neuroproteomics”. The mammalian brain presents unique challenges in terms of proteomic analysis due to its high degree of complexity that is found at a number of different levels. Firstly, there are specialized brain regions such as the cerebral cortex and its sub-regions that are involved in specific aspects of sensory perception and decision-making; the hippocampus that is involved in spatial learning; the striatum that is involved in the control of movement as well as of reward-based learning; or the amygdala that is involved in fear and emotional responses. In addition to the existence of many clearly anatomically-defined regions that are associated with specific behaviors, there is cellular heterogeneity within any given brain region. In some cases distinct cell types can be distinguished by their appearance revealed by Golgi or Nissl staining, or functional association with a specific protein marker, for example staining with a specific antibody to proteins such as parvalbumin, which is highly expressed in neurons that release the neurotransmitter GABA. However, it is now becoming clear from mRNA profiling and other genetic approaches that there is an even higher degree of heterogeneity of cell types throughout the brain resulting in distinct patterns of gene expression that can occur in intermixed neuronal and glial cell types, and which cannot be easily distinguished by morphology.

Large-scale initiatives to characterize mRNA expression patterns by *in situ* hybridization such as the Allen Brain Atlas (<http://www.brain-map.org>), analysis of patterns of gene promoter activity in transgenic mice carried out by GENSAT (<http://www.gensat.org>), and detailed transcriptional profiling of many sub-regions of the brain [1–3], (see also <http://www.brainspan.org>) have all highlighted large differences in gene expression between different brain regions, and in different neuronal sub-types. These distinct patterns of gene expression are predicted to lead to related patterns of protein expression but as shown in a number of studies there is at best only a limited level of correlation between mRNA expression and protein expression [4–6]. This discrepancy is likely due to the fine control of RNA and microRNA regulation, protein translation (see for example, [6]), as well as the complex control by protein degradation [7, 8]. Translational/ribosomal profiling of defined neuronal populations as done using so-called “TRAP” transgenic mice [9–11], and identification of cell type-specific patterns of microRNA expression [12], are likely to give a more precise picture of protein expression patterns in specific neurons. However, any direct study of protein expression will require quantitative proteomic methods that have the same cell-type specificity that is now becoming available for gene expression studies.

In addition to cell-type specificity, individual types of neurons have unique morphologies, with high levels of compartmentalization of neuronal cell bodies, axons, and dendrites, which can be spatially separated over large distances. Neurons communicate often through 1000s of synaptic contacts called synapses, which are specialized structures that allow chemical or electrical signals to be transferred from pre- to post-synaptic neurons. Thus both the pre-synaptic region that is involved in neurotransmitter release (exocytosis and endocytosis) and the post-synaptic region that contains receptors and signaling elements responsible for neurotransmission are highly specialized sub-compartmentalizations that need to be accessible to proteomic methods. In addition, proteins in the brain are highly regulated by many different types of post-translational modifications (PTMs) such as phosphorylation, ubiquitinylation, sumoylation, glycosylation, methylation, oxidation, and nitroxylation that are likely to be differentially regulated in the distinct types of neurons and cellular sub-compartmentalizations, e.g. excitatory versus inhibitory synapses. Ideally, quantitative proteomics of

the central nervous system would be required to assess protein expression and modification at both the cellular and sub-cellular level.

Why use proteomics to study neurological, neuropsychiatric and neurodegenerative disease?

Genome Wide Association Studies (GWAS), Copy Number Variations (CNVs), microarrays and next-generation sequencing (RNA-seq) have allowed researchers to investigate the contribution of rare genetic variation of low-frequency alleles that are associated with psychiatric diseases [13, 14]. CNVs are a form of structural variation of DNA (e.g. duplication or deletion) that results in the cell having an abnormal number of copies of one or more sections of the DNA [15]. CNVs can be inherited or caused by *de novo* mutation and are recognized to contribute both to normal genomic variability and to risk for human diseases such as autism-spectrum disorders (ASD) and schizophrenia, presumably through changes in protein expression levels. Changes in protein expression of single or multiple proteins at the synapse, or alteration of their properties by PTMs, may lead to functional modification of synapses and increasing evidence suggests that “synaptopathologies” underlie a range of neurological conditions including ASD and schizophrenia [16, 17].

ASD is defined by having deficits in social communication, impaired language development and stereotyped-repetitive behaviors. A number of the genes that are mutated in ASD are crucial for experience-dependent synaptic development and plasticity in the brain [18]. For example, mutations in synaptic adhesion proteins like neurexin, neuroligins and other components of the post-synaptic density (PSD) are associated with ASD. In addition to direct alterations in protein expression, altered protein expression may influence protein synthesis or degradation. Fragile X syndrome, a disease associated with mental retardation, is caused by mutations that result in decreased expression of the RNA-binding protein FMRP, a negative regulator of protein synthesis. In another example related to ASD, CNVs of the chromosomal region containing the HECT domain ubiquitin E3 ligase *Ube3a* gene have been implicated in Angelman’s syndrome likely through modulation of protein ubiquitinylation [8, 19].

Schizophrenia is a neurodevelopmental disorder with gene-environmental interactions contributing up to 80% of disease susceptibility [20, 21]. A number of CNVs have been identified in patients with schizophrenia, highlighting a strong genetic component as well as evidence for developmental and environmental influences (for example, on-set of disease after adolescence) [15]. In addition to altered protein expression and function being responsible for psychiatric disorders, the mechanism of action for many drugs used for treatment of schizophrenia, as well as bipolar disease and depression, while still not fully elucidated, are thought to act mainly via G protein-coupled receptors to control gene transcription and changes in protein expression in specific neural circuits [21–24].

Drug addiction is a psychiatric disorder characterized by a compulsion to seek and take the drug of choice, loss of control in limiting intake and the emergence of a negative emotional behavioral state (e.g. anxiety, irritability), that are thought to occur in response to repeated perturbation of signaling pathways that are coupled to altered gene and protein expression [25–28]. Drugs of abuse hijack normal learning and memory mechanisms resulting in long-lasting memories of the drug experience [29]. The effect of drugs of abuse (alcohol, cocaine, amphetamine, methamphetamine, marijuana, heroin/morphine and nicotine) in animal models as well as human post-mortem brain tissue from addicted individuals have been characterized using genomic and proteomics technologies in an attempt to elucidate the molecular and biochemical mechanisms underlying drug addiction.

As for neuropsychiatric disorders, neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) have genetic components that lead to changes in protein abundance, protein turnover, PTMs [30] and protein function that in turn can lead to local changes in neuronal function [31, 32]. Protein accumulation in the form of neurofibrillary tangles (hyper-phosphorylated tau protein) [33], Lewy bodies or amyloid plaques [31], are associated with the degeneration of neurons and pathology of disease. Notably, when proteins that are enriched in the human PSD (see further discussion below) were compared with an annotated database of human monogenic disease, Bayes *et al.* found that mutations in 133 of these genes were linked to 269 monogenic diseases of the nervous system [34]. Understanding neuropsychiatric and neurodegenerative diseases will therefore require quantitative methods to measure proteins and their modifications in the various defined neuronal sub-cellular compartments, as well as in specific brain regions and individual types of neurons.

Overview of quantitative proteomic methods

Quantitative proteomics in general, and more specifically quantitative neuroproteomics, aims to measure in a highly reproducible manner, relative or absolute protein abundance or PTMs that results from two or more experimental conditions (e.g. control versus drug treatment or normal versus disease state). Quantitative proteomics methods are based on a variety of mass spectrometric (MS) platforms (summarized in Figure 1, and discussed in detail below) that can measure relative protein abundance typically expressed as a fold change, or determine the absolute amount of the peptide/protein in the sample through the inclusion of internal stable-isotopic standards. While classical approaches such as two-dimensional gel electrophoresis (2-DE) and differential two-dimensional fluorescence gel electrophoresis (2D-DIGE), coupled with MS-based protein identification, have been widely used as a way to visualize and detect differential fold-changes of several hundred or so proteins on one gel, these electrophoresis methods are limited in terms of the types of proteins identified, and in their capability for comparing large numbers of samples (see further discussion below). Quantitative MS-based methods to analyze protein abundance and PTMs, have therefore evolved over the last 10–20 years from simple protein profiling experiments using mass spectrometry (typically referred to as MALDI-TOF MS and LC-MS/MS) to more complex analyses using quantitative LC-MS/MS approaches to detect changes in protein abundance across multiple biological and technical replicates. This has been made possible by technological advancements in mass spectrometer design combining electrospray ionization (ESI) or MALDI ion sources with ion traps, quadrupole and TOF mass analyzers, as well of the development of various label and label-free methods to allow for quantitative analysis (Figure 1).

Desiderio and colleagues conducted seminal studies in the field of quantitative MS-based proteomics using fast atom bombardment ionization-MS and multiple reaction monitoring (MRM) techniques and were the first to measure absolute levels of several neuropeptides in human brain tissue through the use of heavy labeled synthetic peptides [35–37]. However, it was not until the advent of widely available chemical labeling technologies such as isotope coded affinity tags (ICAT) and isobaric tags for relative and absolute quantitation (iTRAQ) that the field of quantitative proteomics has accelerated. In particular, the use of 4- or 8-plex iTRAQ labeling has allowed multiple biological samples or technical replicates to be compared in one LC-MS/MS. Such a multiplexed approach overcomes the inherent run to run differences observed in a single MS-based shotgun profiling experiment, and can generate information on the relative and absolute peptide abundance of hundreds of proteins at one time. More recently, Stable Isotope Labeling by Amino Acids In Cell Culture (SILAC) or Stable Isotope Labeling In Mammals (SILAM) is being increasingly used given its ability to quantitatively compare multiple samples while minimizing technical variation

between samples. Label-free quantitative techniques have also become more robust over the last few years as a result of the increased accuracy and sensitivity of MS instrumentation and improved data processing algorithms. Such label-free methods measure protein abundance based on the analysis of integrated chromatographic peak areas of peptides or the number of MS/MS spectra (spectral counting) in a LC-MS/MS run. SWATH-MS is a new data-independent label-free method that allows relative and absolute protein levels to be measured for hundreds of proteins in a single LC-MS/MS run. Finally, the highly quantitative method of MRM, also referred to as selective reaction monitoring (SRM), that has been used for many years to analyze levels of small molecules, has been optimized for peptides, and is increasingly being used as a targeted MS approach to obtain relative and absolute protein levels in a large number of samples.

Here, we will review recent neuroproteomic studies and highlight the continued development and implementation of MS-based quantitative methods that are being applied to various aspects of neurobiology. In particular, we will compare and contrast older methods using 2-DE and 2D-DIGE with newer quantitative techniques including SILAC, isotopic chemical labeling tags, MRM, and label-free methods. We will focus on the application of these various methods to neuroproteomic research, including highlighting the quantitative aspects of the methods with respect to neuropsychiatric and neurodegenerative disease. Only brief descriptions of the methods will be given since detailed discussion of specific quantitative proteomics methods are presented in this issue of *Methods*. We will limit most of our discussion to work published over the last five years (reviews of older work in the field of neuroproteomics can be found in a number of articles [38–41]. A number of other recent reviews also highlight the application of proteomic studies to studies of the nervous system [42–44].

GENERAL APPROACHES USED TO STUDY NEURONAL PROTEOMES AND SUB-PROTEOMES

General Approaches

While the whole brain or large regions of brain tissue can be used for proteomic analysis, the useful information that might be gathered is limited because of cellular and sub-cellular heterogeneity. Analysis of mixed populations of distinct cell types not only limits any understanding of where a particular protein expression change might have occurred, but minimizes the ability to detect meaningful changes in protein expression levels or modifications due to issues related to low signal to high noise. Such an approach has to be balanced with the ability to generate enough brain tissue that can be handled and processed effectively, especially if some sort of sample fractionation is employed. Minimally, however, any neurobiological profiling study should at least examine selected brain regions that have clear anatomical or functional distinction from one another (e.g. cerebral cortex, striatum, hippocampus, cerebellum etc.). Likewise, certain neurological diseases can preferentially affect particular brain regions, therefore, focus on specific brain regions associated with a particular disease might be expected to increase the likelihood of detecting meaningful changes in protein expression in studies of a particular disease or animal model of the disease.

Historically, a number of very useful sub-cellular fractionation methods have been developed to study neuronal preparations and these methods are also very useful in reducing the complexity of the brain proteome. Separating proteins by their cellular compartment, organelle type, or based on differential detergent solubility reduces the total number of proteins to be analyzed in one fraction, and significantly enriches for lower abundance proteins that may be located in one specific cellular compartment, e.g. neurotransmitter

receptors that are enriched in post-synaptic fractions [45]. Neuroproteomic studies attempting to detect protein expression changes at the synapse often begin with the preparation of synaptosomes, which are isolated and functionally intact “nerve endings” that are generated from neurons by mild liquid shear using homogenization in an isotonic sucrose solution [46, 47]. Through a series of centrifugation steps and finally using Percoll® or sucrose gradients, purified synaptosomes can be obtained and biochemically studied. Synaptosomes are sealed by a continuous plasma membrane and contain synaptic vesicles, mitochondria, cytoplasm as well as the attached post-synaptic density (PSD, see further discussion below). Further fractionation can be used to enrich for both pre- and post-synaptic elements of the synapse. Synaptosomal membranes can be obtained following differential detergent extraction while the PSD can be enriched using two TX-100 detergent extractions leaving a TX-100 detergent-insoluble protein matrix [48, 49].

It is important to consider that no fractionation procedure based on organelle separation or detergent solubility is completely effective. Proteins may be distributed across multiple fractions increasing the total number of samples to be analyzed and making analysis of protein expression levels challenging. In some cases, proteins identified in the “enriched or purified” fraction may be “contaminants” that may or may not have biological relevance and can complicate any subsequent analysis [45, 50]. As for classical biochemical fractionation, it can be instructive to measure the fold-enrichment of a protein or proteins through a series of enrichment steps, of for example, an organelle. Proteins that show the same fold-enrichment would be considered to be part of the purified fraction or organelle, while proteins that exhibited a fractional loss in fold-enrichment would be considered as likely contaminants. Proteins that show no increased or decreased enrichment might be more likely to be distributed in all cellular fractions. However, while quantitative approaches have the capacity to measure fold-enrichment, in our review of recent studies that employed subcellular fractionation, there were few examples, presumably because of the increased cost of processing additional samples.

Quantification of protein extracts prior to MS analysis

To identify actual biological changes in protein abundance between multiple samples, total protein needs to be accurately quantified before proteomic analyses. Colorimetric protein assays such as Bicinchoninic acid (BCA), Lowry and Bradford assays can be used to calculate the protein concentration in a sample but can be affected by contaminants (reducing agents, lipids), as well as pipette errors that can affect protein measurements. For accurate protein determination for downstream quantitative proteomic applications, Amino Acid Analysis (AAA) is the most reliable method. Hydrolysis of proteins and peptides results in the release of individual amino acids that can be separated by chromatography and absolute values determined by comparison to amino acid standards. Accurate knowledge of protein concentration also ensures that equal amounts of peptides are loaded on the liquid chromatography stage of LC-MS/MS which helps to ensure that peptide retention times are more consistent.

PSD Proteomics

The PSD contains a high level of glutamate receptors, signaling proteins and cytoskeletal proteins that are organized into a dense protein matrix by various accessory scaffold proteins (for a detailed review on the PSD architecture, see [51]). There is a growing appreciation that long-term changes in protein abundance (both up and down) at the post-synaptic membrane are responsible for processes linked to learning and memory. Post-synaptic signaling cascades regulate protein kinases and protein phosphatases that in turn can lead to changes in PSD composition by increasing or decreasing the number of receptors in the membrane, changes that are responsible for increased or decreased synaptic strength [52].

As mentioned above, a large number of PSD proteins have also been linked to human disease [34]. For example, a small number of rare genetic CNVs linked to schizophrenia have been found in proteins enriched at the PSD, such as PSD-95, ARC, and four members of the NMDAR complex [17], highlighting the likely importance of proteomic studies of synaptic proteins. A significant effort has therefore been placed on the proteomic analysis of the PSD with the goal of understanding the underlying mechanisms of altered synaptic signaling and synaptic plasticity.

A number of groups have focused their efforts on profiling the protein composition of the PSD-enriched fraction resulting in a core list of proteins that have been identified by the multiple studies [53–60]. The general approach in these various studies was to use standard peptide profiling methods and MALDI-TOF-MS or LC-MS/MS techniques. A meta-analysis by Collins *et al.* (2006) that examined six proteomic studies that profiled the PSD proteome concluded there were ~1,100 unique proteins in the PSD-enriched fraction [61]. However, the majority of proteins (58%) were only identified in a single study, while there were a total of 466 “consensus-PSD components” identified in two or more studies, yet only 72 (6%) proteins were found in 5 or more studies. Major biological differences between these studies can be attributed to the methods used for preparation of PSD samples as well as differences in PSD-associated accessory proteins and the probable variable association from non-PSD sources like mitochondrial proteins. It is notable that one of the earlier studies, from Smit and colleagues, used ICAT to measure enrichment of PSD proteins compared to relative levels in the cruder synaptic plasma membrane fraction and found only 60 proteins with an enrichment number greater than 1 [50], suggesting that the definition of a true consensus for the PSD proteome still requires further assessment, including analysis of more recent quantitative studies of the PSD (see further discussion below).

Quantitative proteomic approaches have been used to examine absolute levels of PSD proteins as well as changes in protein levels following an experimental procedure. These methods include ICAT, iTRAQ, label-free and MRM/AQUA quantitative techniques along with immunoblotting/ western blotting (IB) employed in some cases for secondary validation (see Table 1 for summary of PSD studies; specific discussion of individual quantitative methods is included below). An earlier, study using an absolute quantitation method (termed AQUA for Absolute Quantitation) looked at 32 “core” PSD proteins in rat forebrain, providing quantitative information about molar concentration and mass percentage of multiple proteins in the PSD [60]. iTRAQ reagents were used to analyze the relative levels of 2,159 “PSD proteins” as well as their phosphorylation in different brain regions (cortex, striatum, hippocampus and cerebellum) [55]. In this study, they observed brain region-specific protein expression, for example, protein expression of the NMDA receptor NR2B was higher in the hippocampus and cortex compared to low expression in the cerebellum, suggestive of different sorts of synaptic signaling in these brain regions. A recent review of quantitative methods to estimate glutamate receptors (AMPA and NMDAR) in the PSD expands on our discussion [62].

Several recent quantitative studies on the PSD have attempted to identify key regulators of synaptic plasticity or resulting changes in post-synaptic receptors after manipulation or administration of various types of drug exposure including the effects of drugs of abuse and antipsychotic drugs (see Table 1). For example, using 8-plex ITRAQ reagents Reissner *et al.* identified 42 proteins (from 337 proteins quantified) that were altered during cocaine seeking reinstatement in PSD preparations isolated from the nucleus accumbens (NAc) [63]. Secondary confirmation of these samples using immunoblotting confirmed three out of six changes, and follow-up functional studies identified a role for altered levels of the protein kinase A anchoring protein (AKAP79/150) in cocaine seeking reinstatement. Moron *et al.*

identified 23 proteins altered by morphine administration in mouse PSD fractions using ICAT labeling [58].

More recently, Trinidad and colleagues used 4-plex iTRAQ labeling to examine protein abundance changes after acute pharmacological stimulation of the brain using pilocarpine, a non-selective muscarinic receptor agonist that increases neuronal activity [53]. They were able to quantitatively examine 893 proteins in the PSD-enriched fraction, focusing attention on a group of 110 proteins they termed the “core-PSD complex”, which consisted of glutamate receptors and other membrane proteins, although several mitochondrial proteins and pre-synaptic proteins that may not be true PSD proteins were still included in the “core” list. Low variation in protein abundance was observed with 95% of proteins falling within $\pm 15\%$ range across four time points (0, 10, 20 and 60 min), supporting a high level of technical reproducibility at all stages of analysis, including brain dissection, PSD preparation, iTRAQ labeling and LC-MS/MS. Notably, the acute seizure induction using pilocarpine resulted in modest changes in protein expression at the time points examined. Subsequent bioinformatics analysis revealed two functional co-regulated clusters; one comprising mitochondrial and some synaptic proteins, and a second cluster consisting of glutamate receptors. The results showed co-regulation with a general increase within the first 20 minutes and a return to baseline levels for protein family members like Dlg, AMPA- and NMDA-receptors, septins, but an overall decrease in CaMKII protein levels after pilocarpine stimulation. Together, these more recent quantitative analyses are beginning to give clues to the role of altered PSD protein content in synaptic plasticity mechanisms.

QUANTITATIVE GEL ELECTROPHORESIS TECHNIQUES

Gel based quantitative methods: 2-DE and 2D-DIGE

Historically in neuroproteomics studies, 2-DE and 2D-DIGE have been the most commonly used proteomic techniques to compare protein profiles between control and experimental conditions. While there is growing shift towards MS-based studies utilizing stable isotope labels or more recently label-free approaches, these gel-based methods remain popular, likely as a result of their availability, and for limited sample numbers, their simplicity of preparation and processing. Proteins are initially separated by isoelectric focusing and then by their molecular weight (Mw) using SDS-PAGE. Protein changes are quantified using 2D-image analysis software. Replicate 2-DE gels are typically needed for comparison to align protein spots for analysis. To eliminate or minimize gel-to-gel differences observed in 2-DE, 2D-DIGE uses the fluorescent cyanine dyes Cy2, Cy3 and Cy5 to label different samples that are then combined and run in a single 2D-gel. The 2D-spots can be individually quantified on the same gel and multiple gels can be cross-referenced by an internal standard using the Cy2 label. In either case, quantitation occurs at the protein level in comparison to the peptide level in MS-based approaches. To identify proteins, the 2D-spots of interest are excised and enzymatically digested, usually with trypsin, prior to analysis using mass spectrometry (MALDI-TOF-MS and/or LC-MS/MS).

In addition to total protein levels, PTMs including protein phosphorylation, glycosylation, acetylation and methylation, can be observed by small shifts in the pI of the protein or Mw of the proteins visualized on the 2D-gel. The ability to visualize multiple PTMs using 2D-gels can increase the total number of related spots on the 2D-gel, making quantitation more difficult. Moreover, to identify the specific PTM observed on the 2D-gel spot requires immunoblotting (for example, through the use of phospho-specific antibodies) or MS-based methods to determine the specific-site of modification (phosphopeptide analysis to identify site of phosphorylation).

At their best, these gel-based methods are capable in allowing up to approximately 1,000 proteins to be separated and visualized on a single 2D-gel. However, a major disadvantage of any gel-based system is that the dynamic range is limited, and highly abundant proteins typically mask the identification of less abundant proteins that have similar pI/Mw's. Analysis by gel-based methods is also limited in terms of being able to identify easily high molecular weight proteins (Mw >100 kDa), acidic proteins (pI<3) or basic proteins (pI>9). The use of narrow-range, IPG strips (for example the pH 5.6–6.6 from Amersham Biosciences (GE Healthcare)) can provide higher resolution and increase the total number of proteins compared to broader range (pH 3–10 and pH 4–7, or pH 6–9) IPG strips more commonly used in proteomics studies. The use of overlapping narrow-range IPG strips does however increase the number of gels and reduces sample throughput. Analyses of membrane proteins are especially important in the neuroscience community; for example to detect and quantify glutamate receptors in total protein extract as well as PSD fractions. However, ionic detergents like SDS cannot be used during isoelectric focusing. The zwitterionic detergents CHAPS and ASB-14 have been used successfully to solubilize certain membrane proteins but membrane proteins are best analyzed using MS-based methods.

Despite these known limitations, 2D-gel based neuroproteomic studies continue to be used to compare control and experimental tissue/plasma from human post-mortem patients or from model organisms (see Table 2 for a selected neuroproteomic studies that used 2-DE or 2D-DIGE in the last 5 years). The majority of these studies have been focused on identifying differentially regulated proteins in studies of neuropsychiatric disorders, including schizophrenia/bipolar disease, and exposure to drugs of abuse, or in neurodegenerative disorders including PD, AD, and HD.

Gel-based methods - Neuropsychiatric disorders

In an attempt to identify biomarkers associated with schizophrenia, a number of proteomic studies have used 2-DE/2D-DIGE to analyze post-mortem human brain tissue from patients diagnosed with schizophrenia compared to normal controls (Table 2). In particular, neuroimaging has identified changes in grey matter areas (PFC, dorsal lateral PFC (dlPFC), anterior cingulate cortex (ACC)) as well as white matter in schizophrenic patients compared to control patients. These brain regions have therefore been the focus of several 2D-gel studies examining post-mortem human brains [64–70]. Two separate meta-analyses of 2D-gel based studies examining human schizophrenic brain tissue were by conducted by English *et al.* and Martins-De-Souza *et al.* [71, 72]. English *et al.* identified 145 proteins (8 studies) that were differentially expressed between control and schizophrenic human post-mortem tissue [72]. Martins-de-Souza *et al.* found 31 proteins (13 studies) that were consistently differentially expressed in schizophrenia brain tissue using 2D-gels [71]. Interestingly, 20 of these proteins had been previously implicated at the mRNA level in schizophrenia, while 9 proteins were implicated in bipolar disorder. Sixteen of the 145 proteins from English *et al.* were classed as candidate biomarkers that were differentially altered in the same direction in two or more investigations. A number of candidate genes that were identified with genomic CNV's in schizophrenia also showed altered protein expression in the human proteomic studies. The candidate genes/proteins identified included; AP3B2, CLTCL1, COG4, EXOC3, EXOC4, KCNQ5, MAP4, PRDX1, SNAP29 and SNAPIN. Future studies using MS-based quantitative proteomic methods will hopefully provide further insight and validate the candidate proteins as potential biomarkers for schizophrenia. Studies using rodent models have also examined the effects of anti-psychotic drugs used to treat human schizophrenic patients on protein expression [73–77].

Gel-based methods - Drugs of Abuse

The effect of drugs of abuse (alcohol, cocaine, amphetamine, methamphetamine, marijuana, heroin/morphine and nicotine) in animal models as well as human post-mortem brain tissue from addicted individuals have been characterized using genomic and proteomics technologies in an attempt to elucidate the molecular and biochemical mechanisms underlying drug addiction. Over the last 20 years, many proteomic studies using 2-DE/2D-DIGE techniques have examined the effect of several drugs of abuse (see Table 2 for a summary of work from the last five years; see also earlier review article –[41]). More recently, Wang *et al.* (2011) compared all published proteomic studies examining drugs of abuse (most studies used 2-DE, 2D-DIGE with a few using label-free LC-MS/MS) and compiled a list of 497 candidate proteins that were associated with exposure to one or more addictive drugs [78]. There were 160 proteins identified that were altered by at least two drugs, indicating possible overlap of biological pathway or mechanism of action. Functionally, 55 proteins were related to energy metabolism, 17 proteins were assigned to synaptic vesicle trafficking and 14 were oxidative stress response proteins.

Gel-based methods - Neurodegenerative Disorders

A number of studies of neurodegeneration associated with AD or PD have used 2-DE and 2D-DIGE to examine human post-mortem tissue [79, 80] (Table 2). Zellner and colleagues compiled a candidate list of proteins altered in AD compared to normal controls, that included 15 altered proteins from studies using post-mortem brain tissue and 19 using CSF [81]. Although likely to be very limited by variable protein dephosphorylation during the post-mortem interval, one study used 2-DE and attempted to identify phosphoproteins with Pro-Q diamond stain followed by Sypro Ruby total protein stain in hippocampal tissue from control and AD patients. They identified 17 altered phosphoproteins including ENOG, ENOA, GNAO, ALDOC, CRMP2, 6PGD, GFAP, GAPDH, MEK1 [82]. One other recent study using 2D-DIGE verified increased monoamine oxidase-B (Mao-B) protein and mRNA levels in blood platelet cells from AD patients but not in PD or normal controls [83]. A study by Licker *et al.* compared protein profiles from control and confirmed PD patients using 2D-DIGE and identified 32 proteins with altered protein levels in the substantia nigra (SN) [84]. They found increased CNDP2 protein levels in the SN of PD patients, that was confirmed by immunoblotting and immunohistochemistry (IHC).

The use of transgenic animal models of AD and PD can be useful to understand disease progression and was recently reviewed [85]. The mitochondrial proteome of triple transgenic (3xTg)-AD mice, that overexpress three AD human mutations in APP_{swe}, PS1_{M146V} and Tau_{p301L}, were compared at 6 months using 2D-DIGE [86]. This study identified altered levels in 23 mitochondrial proteins (both increases and decreases) that occurred before the development of amyloid plaques and neurofibrillary tangles, potentially giving insight into disease progression of AD.

Comparison of differentially expressed proteins identified in gel-based neuroproteomic studies

These various studies show that despite inherent technical limitations, 2D-gel-based methods do sometimes have the capability to identify protein abundance changes in human post-mortem brain tissue that may be related to the disease. Interestingly, when we compared the 145 candidate proteins that were altered in patients with schizophrenia/bipolar disease [72] to a list of proteins that were altered by at least two drugs of abuse (prepared by Wang *et al.*) [78], we found 14 of the proteins were common to both lists (ALDOC, CKB, DMN1, DPYSL2, ENO1, ENO2, GFAP, GLUL, GNB1, NSF, PGAM1, PRDX2, TP11 and UCHL1) while 2 proteins (INA and SEPT3) were not altered in the drugs of abuse list. Of the 8 white matter proteins altered in schizophrenia, IMPA1 was only altered in

schizophrenia, while 7 were altered in studies with drugs of abuse (DPYSL2, NEFL, PARK7, YWHAZ, LDHB, PRDX2, STMN1). A summary of candidate proteins that were altered in AD brain tissue also identified common protein or protein isoforms (YWHAZ, ENO1, ADOA, DPDX2, PRDX2, PGAM1) [81]. Several of the differentially expressed proteins identified using 2D-gel based techniques have been validated by immunoblotting as well as quantitative MS-based approaches that will be discussed in the next section, suggesting some common pathways between these diseases. However, it seems likely that the overlap in identified proteins reflects their abundance and ability to be readily visualized using the 2-DE/2D-DIGE methods. Thus there remains a need for further evaluation and confirmation using either more in-depth proteomic methods, or other methods of secondary confirmation. In addition, comparison between studies and reproducibility is not always possible due to the limited sample numbers used, especially for human post-mortem tissue due to the inherent biological variation between patients, and difficulty in normalizing for post-mortem interval.

QUANTITATIVE MASS SPECTROMETRY-BASED TECHNIQUES

Overview of MS-based methods

There has been a significant move from the use of 2D gel-based methods to MS-based neuroproteomics studies over the last few years. This is largely a result of improvements in MS instrument design, the introduction of more robust quantitative MS-based techniques, optimization for handling the relatively low amounts of protein available for neuroproteomic studies, and the interest in studying membrane proteins that are hard to analyze using the 2D gel methods. Larger scale, and more in-depth, MS-based experiments have benefited from the use of peptide fractionation methods such as multi-dimensional chromatography (MUDPIT) that separates complex peptide mixtures using strong-cation exchange chromatography (SCX). This dramatically increases the number of peptides sequenced by LC-MS/MS and can allow for the identification of less abundant peptides. All the quantitative techniques (see Figure 1) we will discuss have advantages/disadvantages depending on the type of sample and complexity (cell line compared to tissue), but we will highlight their usefulness in neuroproteomic studies published to date.

Chemical labeling techniques - Overview

Protein and peptide labeling using chemical isobaric tags (ICAT, cICAT, iTRAQ, mTRAQ, Tandem Mass Tags (TMTs)), anhydrides, or enzymatic labeling (^{18}O) during trypsin digestion, can all be used for quantitative purposes. ICAT, introduced by Aebersold and colleagues, was the first chemical labeling tag that gained widespread use as a quantitative technique [87]. ICAT uses a biotin tag and labels via cysteine residues. One disadvantage of ICAT is the low frequency of cysteine residues in typical proteins, which results in low sequence coverage. A cleavable form of the ICAT reagent (cICAT) allowed for affinity purification of ICAT-labeled peptides and the removal of the biotin affinity tag prior to LC-MS/MS [88]. The introduction of iTRAQ labeling by Ross and colleagues was a major breakthrough in quantitative proteomics as it allowed biological/technical replicates to be multiplexed (4-plex or 8-plex) and analyzed in one LC-MS/MS experiment [89, 90]. TMTs are another version of isobaric tags that use a set of structurally identical tags that label peptides at their free amino-termini and epsilon-amino groups of lysine residues [91, 92]. All these isobaric mass tags are specifically designed to contain a reactive group, a balancing group, a cleavable group and a reporter ion. The isobaric tags have identical mass in MS mode but differ in their fragmentation (MS/MS) pattern resulting in specific marker ions (e.g. 4-plex iTRAQ diagnostic ions are 114.1, 115.1, 116.1 and 117.1 Da). The introduction of isobaric tags into peptides that have differential mass typically occurs as a

late step in these analyses; therefore, there can be concern about introducing a greater level of technical variation resulting from the actual labeling steps.

In comparison to these isobaric approaches, isotope-coded protein labeling (ICPL) is a non-isobaric technique that labels free primary amines of intact proteins or peptides [93, 94]. Four different protein samples can be labeled using ICPL₀, ICPL₄, ICPL₆, and ICPL₁₀ reagents before [93, 95, 96] or after enzymatic digestion [97, 98]. This method is applicable to any protein sample, including tissue extracts or plasma, serum and CSF but has not been used as much as other quantitative techniques. mTRAQ is a non-isobaric version of iTRAQ that was developed specifically for MRM-based experiments that allowed absolute quantitation when used with heavy-labeled peptides [99].

Dephoure and Gygi recently introduced a multiplexed metabolic labeling and chemical labeling method using triplex metabolic labeling (SILAC) and 6-plex TMTs isobaric tags to quantitatively monitor 18 samples simultaneously [100]. This strategy was able to exploit the mass separation of intact peptides in the MS1 scan (light, medium and heavy metabolic labeling), peptide identification in the MS2 scan, as well as the relative quantitation using isobaric labels (6-plex) in an MS3 scan. They applied this hyperplex approach to monitor and quantify 2,666 yeast proteins involved in dynamic response to rapamycin, identifying cellular targets of the mTOR pathway, but it should be straightforward to apply the method to any cell-based system where SILAC methods are used (see discussion of SILAC below).

Isobaric chemical labeling techniques – Applications to neuroproteomic studies

Recent neuroproteomic studies that have used these isotopic chemical-labeling techniques are detailed in Table 3. As previously discussed, one notable study used iTRAQ labeling to identify and quantify activity dependent changes in 853 proteins in the PSD over a 60 minute period after acute brain stimulation with the pharmacological drug pilocarpine [53]. iTRAQ has also been used to analyze PSD fractions prepared from the NAc of cocaine-trained and yoked-saline controls rats [63]. Robison *et al.* used iTRAQ labeling to examine protein expression changes in the NAc (core versus shell) of rats either administered chronic cocaine or saline for 7 days [101]. They identified higher expression all four CaMKII isoforms in the NAc core compared to shell. Secondary validation by immunoblotting confirmed the protein expression increase in the CaMKII α isoform, as well as phosphorylation at specific sites. Moreover, they showed that the transcription factor Δ FosB is phosphorylated by CaMKII α at Ser-27; and that this is required for cocaine-mediated accumulation of Δ FosB and locomotor sensitization.

In another study of drugs of abuse, Prokai *et al.* used ICAT to identify 27 differentially altered proteins from synaptic plasma membranes in rats chronically administered with morphine for 7 days compared to saline-treated controls [102]. Moron *et al.* identified 23 proteins altered by morphine administration in mouse PSD fractions using ICAT labeling [58]. Abul-Husn *et al.* identified 30 presynaptic proteins altered by chronic morphine administration using iTRAQ, validating results using immunoblotting. Using protein-protein interaction network modeling, they identified clusters and used immunoblotting to confirm changes to several interacting proteins not observed using the iTRAQ analysis [103].

Other recent neuroproteomic studies using isobaric methods have been related to studies of neurodegeneration. Lehnert *et al.* identified 16 proteins altered in CSF of PD dementia patients [104]. This study is notable since they also used MRM to validate the iTRAQ results, finding however that only 2 of 14 tested proteins were significantly changed in PD patients - tyrosine-kinase non-receptor-type 13 and netrin G. In another study of PD, serum from PD patients ranging in disease progression, were labeled with iTRAQ reagents following the typical method employed to deplete highly abundant serum proteins [105].

They identified 26 differentially regulated proteins from a total of 258 proteins quantified. Eight proteins showed increased expression while 18 decreased including complement component 4B, apolipoprotein A-I, and alpha-2-antiplasmin. Mouse models of AD have also been examined using isobaric labels. The triple transgenic mice (3xTgAD), that express mutant presenilin-1, amyloid precursor protein and tau, exhibit AD-like amyloid and tau pathology [106]. Analysis of cortical and hippocampal tissue at 16 months of age identified a total of 142 and 91 proteins, respectively, that were significantly altered in expression. In another AD study, Fu *et al.* used cleavable ICAT to analyze enriched mitochondrial fractions from 3-, 6-, 12-month-old knock-in mice expressing the M146V presenilin-1 (PS-1) mutation. They identified 46 proteins with altered expression, 32 increased and 14 decreased, between young and old mice and confirmed 5 proteins using immunoblotting [107].

Végh and colleagues used iTRAQ 8-plex labeling to compare synaptic proteins in the hippocampus from wild-type and *Ercc1* mutant mice that show accelerated aging and progressive memory deficits at p28 and p112 of age [108]. They identified a progressive decrease in ionotropic glutamate receptors (AMPA receptors), changes in mitochondrial proteins and increased levels of G-proteins that can affect long-term changes in synaptic activity. A total of 288 proteins were identified at each time point, with 175 proteins significantly altered between wild-type controls at one of both time points. Secondary validation examining 8 proteins using immunoblotting confirmed a small subset of the iTRAQ results.

The TMT-6 isobaric labels and MRM were used to examine histone H3 site-specific K18/K23 acetylation in the temporal lobe from post-mortem tissue from control and AD patients [109]. The change in histone acetylation was comparable using TMT-6 labeling and MRM, but MRM gave higher sample-throughput and reproducibility. IPCL was used to identify protein changes in four different regions of the primary brain glioblastoma tumor in human biopsy samples [95].

This study identified 31 proteins with increased expression in the glioblastoma tumor region compared to the peritumoral brain tissue. Cellular prion protein (PrP^C) knockout mice were compared to wild-type controls at p1 and p67 using ICPL and 2D-DIGE techniques [96]. There were no significant protein changes that accompanied the loss of PrP^C in whole brain, total protein extracts or synaptosomal extracts using either technique, suggesting protein abundance changes may be localized to a specific brain region or sub-cellular fraction.

Metabolic labeling, SILAC, and SILAM techniques - Overview

SILAC was introduced as a quantitative proteomics technique in 2004 and has been increasingly favored as a robust method that is typically used in cell culture systems, but has the capacity to be adapted for analyzing animal tissue [110, 111]. The general principle of SILAC is to mix a protein sample containing natural-abundance isotopes with an identical protein sample containing heavy labeled isotopes (e.g., ²H, ¹³C, ¹⁵N or ¹⁸O). The introduction of stable isotopes into proteins alters their molecular weight by specific mass increases that can be detected using mass spectrometry. The relative protein ratio is then determined from ion chromatograms comparing unlabeled “light” to labeled “heavy” peptides. To simplify analyses, only lysine and/or arginine amino acids are usually labeled with heavy isotopes to maximize the number of unlabeled/heavy labeled pairs that are available for the quantitative analyses following tryptic digestion. Any biochemical fractionation or any further peptide fractionation steps (e.g. SCX, LC-MS) after mixing can then be carried out with low variation between samples. Moreover, any variation can be normalized to account for small differences in sample loading.

The strengths of SILAC include lower variability and relative ease of use compared to other quantitative proteomics methods available. SILAC has been very successful with experiments utilizing immortalized cell lines (e.g. HEK293), as the heavy label typically needs to be incorporated into 99% of the proteome for accurate quantitation and this can be easily achieved in mitotically dividing cells. However, neuroproteomic research typically uses rodent, non-human primate or human post-mortem tissue. It is possible in the case of rodents to feed animals a diet labeled with heavy isotopes (e.g. ^{13}C -lysine or ^{15}N). This may take several weeks to ensure all proteins are effectively labeled to >90% to ensure accurate quantitation. For example, in mammals; ^{15}N -labeled spirulina, ^{15}N -labeled bacteria or ^{13}C -lysine can be incorporated into the animal feed for 6 weeks at which point high protein turnover tissue like liver is sufficiently labeled. However, to label brain tissue in rodents, the dam and her offspring need to consume heavy labeled food for at least two generations [112–114]. An alternative approach is to prepare metabolically labeled proteins from mammalian cell lines that can then be used for relative quantification between experimental tissue and the labeled tissue. In this respect, a recent development by Mann and colleagues was the introduction of a super-SILAC mix (a combination of multiple cancer cell lines) that could be used as a standard when analyzing non-labeled cancerous tissue [115, 116]. The combination of multiple mammalian cell lines maximized the total number of labeled proteins identified, better representing proteins that are expressed in the different types of cancers found in human patients. Producing a super-SILAC mixture of brain-related cell lines may be a useful development for applications in neuroscience.

Metabolic labeling, SILAC, and SILAM techniques – Application to neuroproteomic studies

Quantitative metabolic labeling have been applied to several model organisms used in neuroscience [117] including *Drosophila melanogaster* “fruit-fly” [118, 119], *Danio rerio* “zebrafish” [120], *Caenorhabditis elegans* “nematodes” [121], *Mus musculus* “mouse” [122, 123] and *Rattus norvegicus* “rat” [124, 125] (Table 4). Although expensive to generate (depending on the cost of the diet and the time needed for incorporation), SILAM tissue can provide a solution for quantitation of brain tissue by spiking SILAM labeled tissue with unlabeled tissue before MS analysis [123–127]. Thus metabolically labeled mice can be generated and tissue extracts used as an internal standard for total extracts as well as subcellular fractions to improve quantitation across biological and technical replicates. This provides the benefit of SILAM but removes the necessity to label all animals in the study. For example, an application of the SILAM mouse brain as an internal standard was carried out McClatchy *et al.* to examine neurodevelopment in the rat brain. Quantitative proteomics was carried out using ^{15}N -labeled rodent brain (produced by SILAM) mixed in with rodent brains at each developmental time period to get a relative protein expression at each point. In one study, protein expression in cerebellum was examined at post-natal day p1, p10, p20 and p45, with 1,138 proteins identified and quantified resulting in insights into potential novel regulators of neurodevelopment [125]. In a more recent study, McClatchy and colleagues analyzed synaptosomal and mitochondrial fractions isolated from different brain areas at four post-natal times (p1, p10, p20 and p45) [128]. Sensory deprivation in the mouse has been widely used as a model for experience-dependent synaptic plasticity. Butko and colleagues used $^{14}\text{N}/^{15}\text{N}$ -labeled SILAM mice to identify proteins with altered expression in trimmed whiskers (sensory-deprived) compared to brushed whiskers [129]. They quantified 7,134 synaptic proteins in synaptosomes prepared from the barrel-cortex and found 161 proteins were significantly increased, whereas 89 were significantly decreased by at least 1.2-fold in sensory-deprived synapses. Secondary validation of 22 proteins with altered proteins expression was confirmed using immunoblotting.

Metabolic labeling methods have also been used in studies related to neuropsychiatric disorders. For example, there is an animal model of schizophrenia (G72Tg BAC transgenic

mice) that recapitulates several schizophrenia-like behaviors including impaired motor control and increased impulsivity [130]. Quantitative analysis of the cerebellar proteome from non-metabolically labeled G72Tg mice were compared to ^{15}N -labeled wild-type reference cerebellar tissue. A number of changes in myelination-related processes and oxidative-stress functions were identified in the G72Tg mice tissue. In another study, using metabolic ^{15}N labeling, protein expression changes were studied in a depression-like/anxiety mouse model [126, 131, 132].

Zhang *et al.* identified changes in expression of 312 cytosolic proteins and 206 microsomal proteins in hippocampal tissue between low and high anxiety mice [131]. In particular, alterations in expression were identified in proteins involved in metabolism, for example in the TCA cycle, oxidative phosphorylation and the glyoxylate and bicarboxylate pathways [132]. The effect of the ^{15}N isotope labeling on brain metabolism needs to be carefully considered, but it appears that protein samples from metabolically labeled mice can be used as spiked standards.

SILAC application for use with primary neuronal cultures

Dissociated neurons prepared from mouse or rat brain can be grown in culture, and have several advantages in terms of proteomic analysis. For example, primary neuronal cultures can be prepared from different brain regions such as striatum, hippocampus or cerebellum. Various types of glial cells such as astrocyte cultures can also be grown in culture. Neurons are grown for up to 28 days until axonal projections and dendritic arbors form thousands of synaptic connections. Relevant to proteomic studies, primary neuronal cultures are a simpler model compared to brain tissue since they have reduced number of neuronal sub-types, and are more practical for timed experiments using drugs to examine different signaling pathways [133, 134]. A disadvantage of primary cultured neurons is that while they are considered functionally mature, they represent model systems that do not necessarily represent the specific patterns of gene and protein expression that would be found in an intact brain.

An early assumption was the SILAC method would not be compatible with the use of post-mitotic neuronal cultures as there would be insufficient time for complete incorporation (>90%) of the heavy label into the proteome. However, recently SILAC has been used effectively to analyze changes in primary neuronal cultures following stimulation (Table 4). For example, SILAC studies have been carried out using primary neuronal cultures prepared from mice in which the fragile X protein, FMRP, was knocked out [135]. This study identified 132 proteins with altered protein expression in the absence of FMRP protein. Studies using neuronal cultures combined with SILAC have also examined protein phosphorylation. Two studies examined phospho-tyrosine signaling following stimulation with the neurotrophic factors, BDNF [136] and neurotrophin-3 (NT-3) [137] in primary neuronal cultures. Zheng *et al.* used the so-called “pulsed SILAC” method where two different sets of heavy labeled amino acids (Lys4/Arg6) and (Lys8/Arg10) are used to label the control and experimental samples. Using this approach they found SILAC ratios were quantifiable at >60% labeling after 2 days and greater than 90% after 10 days. Spellman and colleagues observed 80% labeling incorporation in all proteins after 14 days *in vitro*, indicating pulsed SILAC is suitable for use in neuronal cultures [136]. Both studies identified proteins phosphorylated on tyrosine residues such as TrkB, BDNF, and ERK following treatment with BDNF or NT-3 respectively. The phospho-tyrosine protein abundance levels were validated by immunoblotting confirming several of changes obtained by the SILAC procedure.

Label-free quantitative methods – Overview

To this point, we have highlighted several benefits of using different types of labeling techniques and their application to the field of neuroproteomics. There is however increasing interest in utilizing non-label MS-based techniques such as label-free-MS and MRM. Label-free approaches can be applied to any biological sample and has applicability to samples that cannot be labeled *in situ* (e.g. postmortem brain tissue, serum/plasma and CSF samples). Label-free quantitative methods are based on the correlation that peak area (area under the curve (AUC)) or the number of MS/MS spectra (spectral counting) for each peptide is linearly proportional to protein abundance in a single LC-MS run (for detailed reviews on label-free methods based on AUC and spectral counting, refer to [43, 44, 138–141]).

The general principle of label-free quantitation by AUC involves the measurement of ion abundances at specific retention times for a given peptide, first shown by Chelius and colleagues in 2002 [139, 140]. The integrated measurement of chromatographic peak areas for a given peptide in LC-MS runs is linearly proportional to the concentration of the peptide in a range from ~10 fmol to ~100 pmol [139, 140]. Label-free MS^E is a data-independent mode of acquisition that has been demonstrated to be the most-accurate label-free approach for estimating absolute protein abundance [142]. The mass spectrometer alternates between low energy (for MS precursor scans) and high-energy (fragmentation of product ions). Quantitation is based on the AUC from the extracted chromatogram.

Spectral counting is the second main approach used for label-free quantitation, first used by Liu and colleagues in 2004 [143]. It is based on the observation that the more abundant peptides in a complex sample will be selected for fragmentation and will produce more individual MS/MS spectra. Therefore spectral counting has been empirically found to be proportional to protein abundance. However, in many cases accurate spectral counting information may not be available as a result of the methods used to collect MS/MS data on more recently used MS platforms.

A number of studies have combined sub-cellular fractionation, MUDPIT and label-free quantitation to conduct a global survey of relative protein expression in multiple mouse organs [144–146]. Kislinger *et al.* used spectral counting to obtain relative protein expression levels of 4,768 proteins in 6 mouse organs and compared the expression levels of 1,758 proteins to mRNA expression [144]. They observed reasonable correlation of protein to mRNA using spectral counting, but observed a number of outliers due to blood-borne factors, low spectral counts or weak mRNA probes. Cutillas and colleagues (2007) used spectral counting to quantify the 1,000 most abundant proteins in mouse brain, heart, kidney, liver, and lung [146]. Similarly, Huttlin and colleagues (2010) used spectral counting to compare protein abundance and phosphorylation sites found in 9 mouse tissues including the brain [145]. They identified a total of 12,039 proteins of which 6,296 were phosphoproteins (36,000 phosphorylation sites). More recently, 31,480 phosphorylation sites (7,280 proteins) were quantified using spectral counting and compared across twelve mouse tissues [147]. These large quantitative data sets were able to compare protein and phosphorylation networks across multiple tissues and showed tissue-specific phosphorylation patterns that may be relevant to understanding tissue specific diseases.

Label-free quantitation approaches require high chromatographic separation, resolution and reproducibility across multiple samples to be analyzed by LC-MS. LC resolution, MS-intensity and biological variation can all effect the quality of the data, and need to be carefully controlled to ensure reproducible and accurate detection between samples over multiple LC-runs [44]. Raw LC-MS data is processed by software packages that normalize and correct for small differences in LC retention time, spectral intensity and other factors to minimize variation and enable comparison of multiple samples. To improve the accuracy of

label-free quantitation, at least triplicate LC-MS/MS analyses for each sample has been shown to be essential, but this can result in large numbers of LC-MS runs for fractionated or complex samples.

Label-free quantitative methods – Application to neuroproteomic studies

Recent neuroproteomic studies that have used label-free methods for quantitation are summarized in Table 5. These methods have been used for analysis of several disease-related studies. One study attempting to identify the effect of maternal binge alcohol consumption on the fetus looked at changes in caveolar proteins in the membrane fractions of uterine endothelial cultured cells that were treated with ethanol [148]. The study found dramatic decreases in 47 caveolar proteins, with only 5 increasing after chronic alcohol consumption compared to control. In another study, a systematic genomic and proteomic comparison of maternally separated (MSEW) B16 mice was carried out [149]. The label-free proteomics approach identified 9-up and 37-down regulated proteins from a total of 798 proteins quantified. Interestingly, the results indicated that a decrease in the myelin component MBP matched decreases in mRNA levels obtained by microarray and RNA-seq techniques. MRM was also used in this study to confirm several of the changes observed with the label-free approach.

Several label-free studies have recently been used in studies related to schizophrenia. Using the LC-MS^E approach, Chan and colleagues compared the protein profiles in dIPFC from human schizophrenic, bipolar and normal control patients [150]. They identified changes in protein expression between the control patients and schizophrenic patients with low, medium or high cumulative anti-psychotic medication throughout their lifetime. Examining cytosolic and membrane fractions, they found 30 differentially expressed proteins in bipolar disease and schizophrenic patients compared to normal controls, of which 17 of these proteins were previously described to change in schizophrenic and bipolar patients [150]. To identify binding partners for the schizophrenia susceptibility gene, dysbindin (DTNBP1), wild-type dysbindin or mutated dysbindin were expressed in SILAC labeled SH-SY5Y cells and used to immunoaffinity purify the BLOC-1 complex, a complex of proteins that include dysbindin, and other proteins like snapin and cappuccino, and which is involved in lysosome biogenesis. Immunoprecipitated samples, analyzed by GeLC-MS and the SILAC ratio as well as label-free spectral counting, identified 24 proteins that interacted with dysbindin [151]. Genomic CNVs in schizophrenic patients are known for 8 of these binding partners. The Golgi-tether COG complex and the antioxidant peroxiredoxins 1–2 were identified as new binding partners for dysbindin. Notably, using 2-DE, peroxiredoxin-2 protein levels were found to be decreased in both the grey and white matter of human schizophrenic patients compared to unaffected controls [152, 153]. To identify potential peripheral disease biomarkers of schizophrenia, the sweat proteomes from normal control and schizophrenic patients were compared using quantitative label-free LC-MS/MS (spectral counting) [154]. This study identified a total of 185 proteins in sweat, of which 30 proteins had altered protein levels in schizophrenic patients. Secondary validation using a multiplex MRM assay confirmed altered protein level changes in 17/19 of these proteins, including peroxiredoxin-1.

Several studies have used label-free methods to examine aspects of AD [79, 80, 155]. A longitudinal study examining protein expression changes in the Tg2576 amyloid mouse model at 12-, 15-, 18- and 22-months of age identified proteins that are altered during disease progression [156]. This study quantified 1,085 proteins at four stages of disease progression from hydrophilic and hydrophobic fractions. Changes were found in 57 mitochondrial proteins and 10 proteins previously shown to be up-regulated in amyloid pathology progression, providing clues to understand progressive neurodegeneration

disorders. A study by Donovan *et al.* (2012) using spectral counting identified 13 proteins that were significantly altered in membrane-enriched fractions from post-mortem brains of AD patients [155]. Gozal *et al.* compared human frontal cortex tissue (insoluble fraction) from normal controls, AD patients and frontotemporal lobar degeneration (FTLD) patients [157]. Using label free, they showed significant changes of 11 proteins (e.g. beta-amyloid, tau and apolipoprotein, serine protease 15, ankyrin B, and 14-3-3eta) in the detergent-insoluble fraction of AD patients compared to controls. Relevant to PD, studies looking at the effect of the neurotoxin MPTP in mice showed changes in 518 out of 4,895 proteins quantified [158]. Of these 518 altered proteins, 270 proteins changed selectively in the striatum or SN, regions of the brain specifically affected in PD. In another PD-related study, label-free (spectral counting) was used to identify 40 proteins enriched in Lewy bodies from human post-mortem brains, including previously identified components like α -synuclein [159].

Multiple reaction monitoring - Overview

MRM / SRM provides a targeted approach with high selectivity and sensitivity to detect relative and absolute peptide levels. (For detailed reviews on MRM, see [160–163]). MRM experiments are usually run on triple quadrupole (QQQ) mass spectrometers. The first (Q1) and the third quadrupole (Q3) are used to scan and filter ions while the second quadrupole (Q2) is used as a collision cell to fragment the peptide and to transmit ions to the third quadrupole. The selected “transition” is the combination of the precursor mass in Q1 and the fragment ion mass in Q3. Usually, two to three transitions per peptide and two to four peptides per protein are used for quantification. MRM has a large dynamic range as a result of the Q1/Q3 selection criteria and can therefore detect low levels of peptide in complex samples.

Peptides with missed cleavages, non-tryptic peptides; peptides containing cysteine or methionine residues are generally avoided when selecting peptides for quantitation by MRM, as they may be less reproducible between samples. Once identified, modification of peptides by PTMs (e.g. phosphorylation, acetylation, methylation, oxidation, ubiquitinylation [164, 165]) can be specifically targeted by MRM assays and compared to the non-modified peptides. However, variability in detection of a specific transition in an MRM assay may indicate the presence of a previously unknown PTM.

MRM is typically employed after the initial discovery phase used for profiling the proteome of the sample of interest. The Q1/Q3 transition(s) to be selected for MRM analysis are thus determined manually by analyzing shotgun proteomics data or using prediction algorithms that help determine Q1/Q3 transitions. However, some caution must be taken when comparing across different mass spectrometry platforms, as differences in peptide ionization may affect the relative intensities of Q1/Q3 transitions. Care must also be taken to correct for even small differences in chromatographic separation of targeted peptides when using different platforms.

To detect low abundance proteins in complex samples that may not be identified by shotgun discovery proteomics, a number of different strategies may be used. For example, specific sets of synthetic peptides can be prepared and analyzed to identify robust MRM transitions. Alternatively, proteins can be expressed in recombinant systems, enriched and analyzed following proteolysis by LC-MS/MS, and MRM transitions identified. An example of this approach focused on relatively low abundance human transcription factors that are not typically identified in profiling studies. Stergachis *et al.* expressed 96 human transcription factors *in vitro* and then empirically identified optimal Q1/Q3 transition pairs for each protein [166]. The relative abundances of four transcription factors were determined and the

values obtained were consistent with mRNA abundance showing this is an effective way to identify optimal Q1/Q3 transitions to identify these low-abundant proteins.

MRM is also often used as a secondary, quantitative, method that follows on from other quantitative, or semi-quantitative, MS-techniques. In this respect, it is an appropriate alternative to immunoblotting as a secondary validation method, and a number of examples of the MRM validation are included in the discussion above and below and in the various Tables. An additional advantage of MRM is the ability to determine the absolute peptide and/or protein levels in a biological sample, commonly referred to as absolute quantitation (AQUA). For example, Gygi and colleagues used heavy ($^{15}\text{N}^{13}\text{C}$)-labeled tryptic peptides and MRM/AQUA to measure absolute quantitation of peptides by comparing the ratio of light to heavy transitions [167] (see further discussion below).

The MRM approach has generally been used to assay a limited number of proteins at the same time since a typical workflow would include designing up to 5 transitions per peptide, and 2–3 peptides per protein, although there is as yet no clearly defined standardization in terms of the minimal transitions needed for accurate quantitation. Scheduling the MRM method clearly becomes more complex as the number of proteins/peptides/transitions to be analyzed increases [168]. However, a number of targeted MRM assays have begun to be developed that can analyze up to a few hundred proteins in one LC-MS run. For example, a MRM-assay to monitor 67 putative cardiovascular disease biomarkers in human was effective in the dynamic range of 41 mg/ml down to 100 ng/mL [169] (see also Table 6 for other examples). This type of assay has the potential to be an important biological and screening assay that could replace or complement ELISA type assays for clinical analyses to detect biomarkers in human patients. Another example of the large-scale functionality of MRM assays examined the global activity of caspases-3-7-8 and 9 in the apoptotic Jurkat cell line after treatment with staurosporine [170]. This study analyzed 676 caspase-derived peptides and was able to assign 180, 58 and 66 peptides to caspase-3, -7, -8, -9, respectively. The analysis of protein-protein complexes in signaling networks is another application of MRM. For example, the protein stoichiometry and dynamic reorganization of the cullin-ring ubiquitin ligase complex was determined by analyzing quantitative changes in 23 target proteins of the ligase complex by MRM/AQUA [171]. These results suggested that the abundance of adaptor molecules drives network organization rather than cycles of the PTM-neddylation. In another study using MRM/AQUA, the protein stoichiometry of five proteins in the human spliceosomal hPrp19/CDC5L complex was also determined [172]. It is worth noting similar protein stoichiometry for the CRL complex was obtained by multiple MS-based quantitative methods used in this study; MRM/AQUA, LC-ESI (MS1 extracted ion chromatograms), and MALDI-TOF/TOF-MS (based on peak area). However, MRM was found to be more consistent and reproducible, whereas peptide quantitation using the two other MS methods were affected by co-eluting “contaminating” peptides that caused peak overlaps in chromatograms and MS spectra.

Multiple reaction monitoring – Application to neuroproteomic studies

A MRM assay was developed to examine the levels of 7 glycolytic enzymes (HK1, ALDOC, TPI1, GAPDH, PGAM1, PGK1 and ENO2) that were previously shown to be affected in post-mortem brain tissue from schizophrenic patients using 2D-gel and MS-based quantitative methods [173] (Table 6). They used a model of psychosis, in which rats were treated with the NMDA antagonist phencyclidine (PCP). Protein levels of TPI1 and PGAM1 decreased in the rat frontal cortex consistent with the decreases observed in human post-mortem tissue. The levels of HK1, ALDOC, GAPDH and ENO2 were not significantly altered in the rat frontal cortex compared to control tissue. This result may be explained simply by differences in human to rodent frontal cortex specialization. Alternatively, the

model of psychosis used in rat may not reproduce protein changes observed in human schizophrenic patients.

In the area of neurodegeneration, Danielson and colleagues quantified the reversible oxidation of 34 distinct cysteine residues in the murine mitochondrial Complex I. They found six cysteine residues were increased in an *in vivo* oxidative stress model of PD [174]. Using TMT-6 isobaric labeling and an SRM/MRM assay, Zhang *et al.* showed significantly lower histone acetylation on Histone3-K18/K23 in post-mortem tissue from AD patients compared to aged controls in the temporal lobe [109]. The higher throughput of the SRM/MRM assay compared to TMT-labeling and immunoblotting techniques was suggested by these authors to be more suitable to clinical applications to diagnose patient samples [109].

SWATH-MS

An alternative to MRM includes SWATH-MS recently developed by Aebersold and coworkers in collaboration with AB Sciex [175, 176]. SWATH-MS allows both relative and absolute protein levels to be measured in a biological sample. SWATH-MS is a data-independent MS/MS acquisition method that can systematically generate in a single measurement, complete recording of all fragment ion spectra of which the precursor ions are within a pre-determined m/z vs. retention time window. This data is obtained by repeatedly cycling through “sequential isolation windows” (SWATHs) over the entire chromatographic elution range. The high-specificity fragment ion maps can then be queried for the presence and quantity of any protein of interest using a targeted data analysis strategy. SWATH-MS relies on the high resolution, mass accuracy and high spectral acquisition rates of the AB5600 TripleTOF mass spectrometer to generate peptide identification and quantitation. The SWATH method appears to be as accurate as MRM but can be possibly applied up to a thousand proteins in one LC-MS/MS run. In a recent study by Li *et al.* comparing protein quantitation using MRM and SWATH measurements, SWATH was found to be 2–3 times less sensitive than MRM but showed similar accuracy and variability, and dynamic range compared to MRM [177]. A recent study has also compared SWATH and MRM in the analysis of PSD proteins and found excellent agreement between the two methods (Colangelo, C., ASMS conference 2012, abstract).

DIRECT COMPARISON OF QUANTITATIVE TECHNIQUES AND ABSOLUTE QUANTITATION

Overview

As discussed above, while relative quantitation is often sufficient to obtain useful information from most proteomic or neuroproteomic investigations, the ultimate goal of quantitative proteomics will be to obtain measures of absolute peptide or protein abundance. Knowledge concerning the specific copy number of a specific protein and its protein-interacting partners will provide meaningful insights into cellular biology, as will knowledge of the absolute stoichiometry of PTMs [178, 179]. In principle, the different quantitative LC-MS/MS methods discussed above, including label-free analysis, metabolic labeling, or isobaric chemical labeling methods, would be expected to identify the same peptides and to report equivalent relative quantitative differences in peptide/protein levels between control and experimental samples. While a number of the examples we discussed use a second MS-based technique (e.g. MRM) to confirm an initial MS-based technique (e.g. label-free), these are a small minority and are not often carried out in a systematic and comprehensive way. Here we will briefly review some examples where direct comparisons of quantitative methods have been made and also discuss a number of emerging approaches that are being developed for systematic absolute quantitation.

Direct Comparison of quantitative methods

While some of the general advantages and disadvantages of the various quantitative methods available have been discussed, it is important to compare these methods directly with each other using the same protein sample and where feasible using the same mass spectrometer and data analysis method. While not specifically using protein samples related to neuroproteomics, the information gathered from such comparisons would be expected to have general relevance. An earlier study by Wu *et al.* (2006) using 2D-DIGE, cICAT and iTRAQ showed the three methods may be used in a complementary manner to increase the number of identified proteins in a sample [180]. Reflecting the inherent limitations of DIGE and ICAT, of 32 proteins identified by the three methods to be proteins differentially expressed between HCT-116 and HCT-116 p53 knockout cell lysates, 10 proteins had less than 5 cysteines and were not identified using cICAT, and 8 proteins were not identified using DIGE since the pI/Mw was outside the range of resolution. A comparison of isobaric reagents performed by Pichler *et al.* (2010) showed the 4-plex method produced the highest number of peptide matches, and unique peptide and proteins identified, compared to TMT or iTRAQ 8-plex reagents [181]. At the protein level, iTRAQ 8-plex labeling resulted in 60% fewer proteins identified compared to iTRAQ 4-plex labeling.

Recently, a systematic study by Li *et al.* (2012) compared label-free (spectral counting), $^{14}\text{N}/^{15}\text{N}$ metabolic labeling, iTRAQ and TMT using a bacterial sample and a LTQ Orbitrap Velos mass spectrometer [182]. They found that the label-free method generated the largest number of unique protein identifications (1,980), compared to metabolic labeling (1,606), iTRAQ (1,473) and TMT (1,404). However, fewer proteins were accurately quantified using the label-free method, especially those of lower abundance. Interestingly, they also concluded that the chemical isobaric tagging methods had the highest level of quantitative precision.

What is the best quantitative proteomic technique to use?

The experimental design of any proteomic study should always consider multiple approaches (e.g. 2D-gels, different MS-based proteomics techniques, immunoblotting). Obtaining statistically significant, reproducible quantitative protein abundance changes using multiple proteomic approaches can be used as validation that the results are technically reproducible and of biological relevance. We have provided numerous examples of 2D- and MS-based studies that validate the results of a subset of candidate proteins using immunoblotting or MRM-based assays (Tables 1–8). However, the success rate for secondary validation is typically low, indicating that one or more secondary validation method is critical (see further discussion below).

The combined use of 2D-gels, MS-based proteomics techniques along with quantitative immunoblotting has been used successfully to identify protein expression. For example, Zhang and colleagues successfully used 2D-DIGE, iTRAQ labeling and immunoblotting to identify putative cysteine string proteins alpha (CSPa) clients in synaptosomal fractions [183]. Recently, Thiede *et al.* combined SILAC labeling with the resolving power of 2-DE to quantify proteins levels in control and apoptotic HeLa cells [184]. Combining the techniques, they were able to show quantitation based on 2-DE staining intensities were imprecise as the majority of 2-DE spots consisted of 1–5 proteins. However, in more than 30% of 2-DE spots, the dominant protein accounted for less than 70% of spot intensity. Quantitation based on the SILAC ratios from 2-DE spots revealed significant changes in 434 proteins species derived from 27 unique proteins. Multiplexing of MS-based TMT isobaric labeling with SILAC labeling allowed 18 samples to be analyzed simultaneously to obtain quantitative data on 2,981 proteins with a 1% false discovery rate [100]. The number of studies using utilizing SILAM to metabolically label whole organisms has allowed ~4,000

proteins to be accurately quantified and compared between biological comparisons [185]. A similar study using 2D-gels reached a similar conclusion in that they only found small changes in levels of ~200 out of ~4000 protein spots [186]. Therefore, combination of different proteomics methods can take advantage of each method to increase the number of proteins quantified and inclusion of both biological and technical replicates to increase statistical significance of the results.

Absolute Quantitation methods

Many of the previous quantitative approaches we have discussed give relative quantification between proteins from control and an experimental (disease) state expressed as a percentage or fold change. Absolute quantitation methods aim to provide meaningful, accurate and precise information that expresses protein levels as number of protein copies per cell or moles of peptide/protein per weight tissue. This information can be used in systems biology research to model protein-protein networks and measure potential protein biomarkers in tissues or body fluids. (For more detailed reviews on absolute quantitation methods, see [179, 187]). The early quantitative neuroproteomics studies by Desiderio and colleagues mentioned earlier measured absolute levels of a number of neuropeptides in human brain tissue through the addition of stable isotopic peptides labeled with deuterium to human brain tissue. The absolute levels of the neuropeptides leucine-enkephalin and methionine-enkephalin in thalamus tissue were quantified by comparing MS signal intensities of the endogenous to heavy labeled peptides [37]. They followed up this study by looking at larger β -endorphin neuropeptide, but used an enzymatic digestion prior to MS analysis to optimize peptides for MS analysis [35]. These techniques allowed picomolar levels of endogenous methionine-enkephalin to be quantified in human pituitary tissue [36].

Several methods for absolute quantitation have been developed that use synthetic peptides, recombinant expressed protein fragments, or recombinantly expressed full-length proteins (Figure 2). These include: AQUA [167], QCAT [188] and later modified as QConCAT [189], Protein Standard Absolute Quantification (PSAQ), Full-Length Expressed Stable Isotope-labeled Proteins for Quantification (FLEXIQuant) [190], Absolute SILAC [191], SILAC combined with Protein Epitope Signature Tags (PrESTs) [192], super-SILAC [115, 116], and SWATH-MS by addition of heavy-labeled synthetic peptides to the peptide mixture [177]. The addition of the internal standards in the sample preparation depends on the characteristics of the standard, for example synthetic peptides need to be added at a late stage whereas full-length proteins can be added prior to protein fractionation and enzymatic digestion (Figure 2).

As discussed above, the AQUA method has been used for quantitation of both protein levels as well as peptides containing site-specific PTMs including phosphorylation or ubiquitinylation [164, 167]. The AQUA method requires the chemical synthesis of a heavy labeled peptide (or matching heavy labeled peptide with the PTM) for each peptide to be analyzed. Although costs for synthetic peptides have become cheaper, synthesis of large numbers of heavy labeled peptides remains expensive and can limit the number of peptides per protein analyzed. The AQUA heavy labeled peptide is typically added (“spiked”) at a late stage of the analytical process, usually after tryptic digestion and before LC-MS analysis. Therefore, the AQUA strategy cannot be used when any pre-fractionation is used in the analysis. One earlier study of the PSD proteome also included AQUA to measure the absolute amounts of a 32 important PSD proteins [57]. In another comprehensive study, Bennett *et al.* used a multiplex AQUA to elucidate the architecture of the cullin-RING ubiquitin ligase (CRL) network by determining absolute levels of each component in the complex [171].

A number of isotope labeling strategies have been developed to take advantage of recombinant DNA techniques in order to simplify production of peptides or proteins used for quantitation. Bayon *et al.* used synthesis and metabolic labeling of artificial peptide concatemers called QCAT [188] or later QConCATs [189]. Originally the individual Q-peptides in a QconCAT protein were adjacent to each other, but more recently additional native flanking regions of each peptide have been included for each Q-peptide/polypeptide. The 6xHIS-tagged QconCAT proteins can be produced in cell-free systems or in bacteria and are a cost effective means to produce up to 50 tryptic peptides in one QConCAT construct. By adjusting the number of copies of each Q-peptide in the construct, the resulting isotope labeled peptides can be used to match the expected concentration range in the sample (i.e. high vs low abundant proteins in complex mixtures). A disadvantage of QconCAT proteins is they usually behave poorly when combined with any pre-fractionation and are therefore added prior to trypsin digestion. A recent study by Chen *et al.* used QconCAT to measure absolute protein levels of the glycoprotein clusterin in human post-mortem frontal and temporal cortex brain tissue of control and AD patients [193]. Quantification of clusterin levels was found to be elevated in the frontal cortex of AD patients (39.1 ± 9.1 pmol/mg tissue) compared to control group (24.4 ± 4.4 pmol/mg tissue). Levels in temporal cortex were not significantly altered in control (29.0 ± 7.9 pmol/mg tissue) and severe AD patients (28.0 ± 8.4 pmol/mg tissue).

Due to the complexity of brain tissue, sub-cellular fractionation is often used to simplify and enrich for lower abundance proteins or proteins at the synapse. Therefore, the addition of defined amounts of *in vitro*-synthesized heavy isotope-labeled full-length target proteins at the beginning of the fractionation process can correct for sample loss during protein fractionation and differences in enzymatic digestion efficiency. Brun and colleagues described the PSAQ strategy that is based on full-length isotopically labeled proteins [179]. PSAQ standards are usually produced in cell-free systems or using bacterial expression. A limitation of PSAQ is the requirement to express, purify, determine purity and the concentration for each PSAQ standard to be analyzed [194]. In one study, Kaiser *et al.* used PSAQ's to measure and compare absolute cellular concentrations of ubiquitin species in mammalian cell lines, mouse brain and human frontal cortex [195].

FLEXIQuants can determine absolute quantity of a protein as well as track changes in relative peptide abundances as a function of PTMs [190]. Each full-length protein is recombinantly expressed in a cell free system produced with heavy labeled isotopes, with the FLEXIQuant design being used to quantify multiple peptides in a single protein. In addition, through a lack of detection of one or more heavy peptide, this method can potentially detect specific-sites of PTMs. FLEXIQuant's were used to measure changes in the differential regulation of two subunits of the anaphase-promoting complex CDC27 and APC5 in HeLa H3 cells. After mitotic arrest using the microtubule-disturbing drug nocodazole, dynamic changes in APC5 PTMs were found to occur faster than in CDC27 suggesting differential regulation of the two subunits.

Absolute SILAC utilizes full-length target proteins that are expressed in bacteria autotrophic for arginine and lysine, with minimal media including heavy labeled arginine and lysine (or alternatively by protein synthesis in a cell-free system). The full-length labeled protein is then spiked into a tissue or cell extracts in defined amounts. Recently, an advance to the absolute SILAC method uses fragments of proteins (approximately 50–150 amino acids) that are expressed in auxotrophic bacteria with heavy labeled arginine and lysine [192]. A “master mix” of different PrESTs were used to determine the copy number of proteins in HeLa cell lines. Firstly, the labeled PrESTs were quantified so each PrEST had a SILAC ratio of one to one compared to unlabeled HeLa cell lysates. To simplify the analysis, 43 unlabeled PrESTs were spiked into heavy labeled HeLa cell lysates and the SILAC ratios of

PrEST peptides to endogenous target proteins yielded their cellular quantities. The absolute amounts obtained in the HeLa cells were then used to calculate corresponding copy numbers in cells. For example, the transcription factor FOS was present at 6,000 copies per cell compared to the cytoskeletal protein vimentin that has 20 million copies per cell.

SWATH-MS

SWATH-MS allows both relative and absolute protein levels to be measured in a biological sample. To calculate absolute levels of proteins using SWATH-MS, heavy isotope labeled peptides for each peptide/proteins are spiked into the peptide mixture. SWATH was found to be 2–3 times less sensitive than MRM but showed similar accuracy and variability, and dynamic range than MRM [177]. MRM methods detected plasma glycoproteins down to 5 ng/mL whereas SWATH detected glycoprotein levels at 50 ng/mL. The SWATH method has high potential for use in the neuroproteomics community to obtain accurate, reproducible relative and absolute quantitation of multiple types of samples.

FUTURE DIRECTIONS IN QUANTITATIVE NEUROPROTEOMICS

General Overview

As described in the discussion above, there have been significant advances in several aspects of quantitative proteomics over the last 5 years, especially in the expanding application of quantitative methods to neuroproteomics. It seems likely that general advances in quantitative proteomics will continue, with improvements in instrument sensitivity and precision, and in methods of data analysis. However, there remain a number of important general challenges in quantitative proteomics as well as specific challenges that are related to proteomic studies of the central nervous system.

It is clear that through the use of the data-dependent approaches that are typically used in most peptide identification workflows, a lot of information in the mass spectra obtained is not actually analyzed. There is also the intrinsic limitation of typical protein profiling studies of complex samples that each experiment essentially “rediscovers” the same proteome or sub-proteome.

There is therefore a need for more standardized, targeted methods that systematically interrogate proteomes in a large-scale but still quantitative manner. As with analysis on any complex tissue that contains proteins expressed at widely different levels, there is also the challenge of interrogating lower abundance proteins and peptides. A variety of approaches can be used to enrich for particular types of protein or PTMs. Complex peptide mixtures can also be subjected to different types of fractionation. However, each of these procedures can introduce variables that affect technical reproducibility in terms of peptide identifications. More importantly, additional fractionation can lead to problems with precise quantitation when comparing different samples since any fractionation is usually incomplete and proteins or peptides distribute across multiple fractions. Using some of the absolute quantitation methods discussed where labeled proteins are spiked into a sample early on in the processing steps may be able to overcome issues related to fractionation.

With respect to neuroproteomics, as was discussed in the introduction, a major challenge is the development of methods that will allow for interrogation of the proteomes of specific populations of the many different types of neurons and glia that are present in the mammalian nervous system. This challenge extends to analysis of sub-compartments of these individual cell types, and the associated problem that as more specific cell types and cellular compartments are assayed, the amounts of protein become less and less. Additional challenges that are relevant to neuroproteomics are the development of robust methods to analyze neuropeptides involved inter-cellular signaling, which requires a distinctly different

approach from the sorts of LC-MS/MS “bottom-up” techniques described above. Finally, improved methods are needed to interrogate protein and peptide expression in cerebrospinal fluid (CSF), since it seems likely that this bodily fluid might contain important biomarkers that can be used to assess human neurological disease, and help in the development of drugs to treat such diseases. These topics will be briefly discussed.

Technical and biological reproducibility of proteomics data

Though not discussed here in detail, areas of proteomics research that can improve significantly include experimental design, data analysis and bioinformatics. The challenges of technical and biological reproducibility remain a problem for the proteomics community, in general. Attempts to define standards to facilitate data comparison, exchange and verification, have been proposed by a number of groups. The ABRF Proteomics Standards Research Group (ABRF-sPRG) (<http://www.abrf.org/index.cfm/group.show/proteomicsstandardsresearchgroup.47.htm>) and the HUPO Proteomics Standards Initiative (HUPO-PSI) (<http://www.psidev.info>) have raised attention to the need to develop technical standards for design of proteomics experiments, data analysis and reporting of data to the scientific community. A positive step is that proteomics journals now require stringent reporting of MS data and 2-DE/DIGE data, producing guidelines for the documentation and quantitation of peptides, proteins and PTMs, and public access to the raw data. For large-scale discovery protein identification, a false-discovery rate is typically required that is assessed on the methods of analysis and statistical threshold required. However, clear-cut standards remain to be established for the numbers of biological and technical replicates needed, and the statistical analysis methods used.

We have presented findings from a number of neuroproteomic studies that utilized many of the quantitative proteomic techniques currently available. With respect to gel-based methods, issues of 2D-gel spot detection, resolution and reproducibility, are important. For MS-based methods, dynamic range, peptide selection, technical replicates and reproducibility are concerns for the proteomics community. In a number of the neuroproteomic studies we examined, proteins with altered protein expression “candidate protein lists” were not validated in the same study through the use of a second method (such as immunoblotting or MRM). In other cases, only a selected number of proteins from the initial discovery stage targets were ever validated using secondary confirmation, and the success rate for such secondary validation is often low or not made clear. A number of neuroproteomic studies utilize immunoblotting or a second MS-based approach (most commonly MRM assays), to validate protein expression changes obtained using 2-DE/DIGE or MS-based quantitative results. The validation of quantitative data should incorporate both technical and separate biological replicates as well as quality control samples in the proteomic study.

Many proteomics experiments likely use insufficient technical or biological replicates and use the data to state conclusions of biological significance beyond the scope of the study. Indeed, a number of recent in-depth profiling studies have shown that biological variability may be a much larger problem than technical variability. For example, a study of the effect of aging on mouse brain identified and quantified ~4000 proteins using SILAM. They found excellent agreement in any pair of technical replicates but larger differences between biological replicates that potentially obscured any ability to see age-dependent effects on protein expression [185]. Interestingly, a 2-DE analysis of the effect of aging in mouse brain protein expression reached somewhat similar conclusions in that they only found small changes in levels of ~200 out of ~4000 protein spots, that was accompanied by markedly increased variability in biological replicates with advancing age [186]. Another study that used SILAC labeling in drosophila observed large individual biological variation that could be reduced by pooling at least 3 drosophila per biological sample [196]. Reproducibility in

the mammalian neuroproteomics field is therefore likely to be inherently more difficult as a direct consequence of the high level of protein expression variation in different brain regions, and the individual variation of thousands of neuronal cell types and synapses in the brain. Careful experimental design is therefore especially important in neuroproteomic studies in order to minimize variation at the biological and technical level to hopefully reveal biological insight into the neurological phenotype or disease being examined.

Proteomic studies of neuropeptides

Bioactive peptides play important and diverse roles in inter-cellular signaling that occurs within the central nervous system and also connects the brain with peripheral organs and vice versa [197–201]. Historically, the use of heavy labeled peptides by Desiderio and colleagues was an important advance in terms of the use of MS-based methods to measure neuropeptide concentrations in brain tissue. Moreover, LC-MS/MS and other mass spectrometric methods have been used increasingly to identify and quantitate these neuropeptides in a variety of brain regions and under a variety of experimental conditions (see Table 7 for some recent examples). However, the proteomics of naturally-occurring peptides, termed peptidomics and the associated field of neuropeptidomics, still face several unique challenges that are distinct from those of the typical methods of bottom-up peptide discovery used in the LC-MS/MS approaches described above. Peptides and neuropeptides are a challenge for proteomic analysis because they are typically the products of complex patterns of processing from pro-peptides, and can also be subject to specific types of PTMs. This may make it very hard to identify specific peptides using the typical types of approach that includes proteolytic digestion with proteases like trypsin followed by LC-MS/MS and the use of standardized software methods and databases. Post-mortem protein degradation can increase the total number of peptides recovered from brain tissue due to protein proteolysis. Techniques such as fixation, focused microwave irradiation (FMI) have also been shown to be beneficial by preserving brain tissue and PTMs [202–205]. In many cases matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is used in the absence of any proteolysis. Specific databases dedicated to mass spectrometric information obtained empirically for neuropeptides, like NeuroPedia [206] (<http://proteomics.ucsd.edu/Software/NeuroPedia>) will hopefully help in efforts to more systematically analyze these important signaling factors. An interesting aspect of the use of MALDI-based methods to analyze peptides is the ability to combine this method with tissue imaging which has the potential capacity to reach the level of individual cells [207, 208].

Proteomic studies of CSF

Proteomic analysis of protein and peptide expression in bodily fluids including serum and CSF is likely to provide important information about how the brain is functioning both in normal conditions and in neurological disorders [209–212]. In particular, because CSF is in direct contact with the brain tissue, it may be a useful source of biological material in humans to identify “biomarkers” that would be diagnostic of a specific neurological or neuropsychiatric disease – such as AD or other neurodegenerative diseases – or be able to measure changes in response to drug intervention that would be diagnostic of improved brain function. A few quantitative proteomic studies have analyzed CSF in the hope to identify disease biomarkers and selected studies are summarized in Table 8 and recently reviewed by Kroksveen and colleagues [210].

CSF has many of the same challenges as serum in terms of the high dynamic range of protein/peptide abundance that spans different and broad orders of magnitude. Typically, commercial kits are used to affinity deplete approximately the 14 highest abundant proteins from CSF enabling a deeper analysis of the proteome to identify changes in lower-abundant proteins, for example neurotrophic factors. However, it is likely that depletion of high

abundance proteins may lead to depletion of other lower abundance proteins through meaningful protein-protein interactions. A series of guidelines have been proposed by Teunissen and colleagues for the collection, handling and storage of CSF for biomarker studies [213]. Contamination of CSF with blood complicates the proteomic analysis since CSF has a lower protein concentration (~1/200) compared to blood. To remove blood contamination, CSF should be centrifuged immediately after collection to remove white blood cells then snap-frozen and stored at -80°C [210, 214]. CSF is a clear and transparent fluid that is produced in the choroid plexus but proteins from neurons and glia contribute to the total protein amount (~20%) via the drainage of the interstitial fluid [210]. The protein profile of CSF is similar to serum containing a high amount of albumin and other abundant serum proteins. Protein profiling experiments have identified 1,000–3,000 proteins in CSF with a high overlap with the serum proteome (~60–80%). For example, Schutzer *et al.* (2010) have successfully identified 2,630 proteins in CSF from normal subjects after depletion of the 14 high-abundance proteins [215].

While the number of high quality proteomic studies of CSF is low, recent work is beginning to utilize some of the quantitative methods described above, to the study of CSF in disease. For example, Kroksvenn *et al.* summarized potential protein biomarkers identified in CSF proteomics studies from a number of neurological diseases, multiple sclerosis (Msc), AD and PD [210]. Another recent study by Kroksvenn and colleagues used iTRAQ labeling to obtain quantitative data on ~600 proteins, identifying 5 proteins (>2-fold change) that reached statistical significant difference (p value <0.05) in Msc patients (n=5) compared to unaffected controls [216]. To validate the iTRAQ results, they designed a MRM assay examining 20 differentially abundant proteins using a larger cohort of Msc patients and controls (n=132). A significant difference was found between Msc and controls for 11 proteins using this MRM assay.

In other recent studies, Lehnert *et al.* used iTRAQ to compare CSF from human PD patients and healthy controls [104]. They identified 16 differentially regulated proteins in PD patients compared to controls; but only 2 proteins (2/14 analyzed) showed a statistical difference between PD/controls using a MRM-based assay (based on 1 peptide/protein). Ringman *et al.* quantified approximately 600 proteins using label-free LC-MSⁿ and identified 56 differentially abundant proteins in the CSF of patients with familial AD with PSEN1 and APP mutations compared to healthy control patients [79]. Mouton-Barbosa *et al.* used a combinatorial peptide library to reduce the dynamic range of protein concentrations in CSF [217]. They identified 1,212 proteins in total, with 745 low-abundance proteins in CSF detected only after the peptide library treatment. Giron *et al.* combined cysteine-containing peptide enrichment with TMT isobaric labeling of human CSF [218]. Stoop *et al.* used label-free quantitation of health CSF samples identifying sets of CSF proteins with low or high variation that can be used for the wider community when selecting potential biomarkers [219]. Steinacker *et al.* used SELD-TOF MS to quantify and detect elevated levels of Tau, 14-3-3-protein, and ubiquitin in the CSF of patients affected by Creutzfeldt-Jakob disease [220].

These more systematic and quantitative studies indicate that interrogation of the human CSF proteome is an area for growth in the future of quantitative neuroproteomics, with the goal of revealing potential protein biomarkers of neurological diseases. However, the individual variability of CSF between patients, and sample preparation and potential for blood contamination will need to be carefully accounted for [214].

Approaches to studying single neuronal populations in the brain

Probably the single biggest challenge faced in most neuroproteomic studies is related to the fact that the brain is comprised of many different types of neurons and several different

types of glial cells that are intermixed. Moreover, there is a large variety of cell morphology with individual neurons typically being in very close synaptic contact with several different types in neuron along extensively arborized dendrites. Axonal projections from an individual neuron can also project to other brain regions, often over long distances. Given that it is now clear that individual types of nerve cell exhibit distinct patterns of gene expression, it is imperative to have access to these neuronal sub-types to allow for proteomic studies to identify protein expression and functional changes with high signal to noise.

One approach is to use micro-dissection to collect homogenous cell populations from heterogeneous brain tissue sections under the microscope, using morphology for guidance or following antibody staining of a marker protein [197]. Thus the technique of laser (capture) micro-dissection can allow for preparation of hundreds to a few thousand cells, which can be isolated with reasonable effort and can provide enough material after amplification for gene expression studies [221]. While the amount of material is limited, it is possible to carry out proteomic analysis on these samples. For example, micro-dissection of specific neuronal and vascular cell populations from healthy control and infarcted brain tissue revealed an up-regulation of the matrix metalloproteinases (MMPs) MMP-9 and TIMP-2 in brain microvessels, while MMP-10 was notably increased in neurons of the ischemic brain [222]. Micro-dissection has also been used to capture Lewy body inclusions from PD human-post mortem tissue [223], as well as neurofibrillary tangles (NFTs) from hippocampal CA1 pyramidal neurons in patients with AD [224], and identify associated proteins. However, laser micro-dissection is labor intensive and the typical yields of protein are much less than are currently needed for quantitative proteomic analysis.

A second approach would be to take advantage of the growing resources of transgenic mice that express a specific protein (for example, GFP) in one or other neuronal cell type under the control of a specific gene promoter. In this respect, the Allen Brain Atlas and GENSAT programs (using Bacterial Artificial Chromosome (BAC)-transgenic mice) have been influential in the identification of neuronal and glial genes that display cell and/or brain region-specific gene expression.

One example, which made use of BAC transgenic mice, was aimed at analyzing dopamine signaling in striatal neurons. At the cellular level, the striatum contains a fairly homogenous neuronal population of approximately 90–95% medium spiny neurons (MSN's) and approximately 2–3% interneurons [93, 225]. However, there are two different classes of intermingled MSN's in the striatum which though morphologically similar, exhibit marked differences in gene expression [10]. Importantly, the so-called striatonigral neurons selectively express the dopamine type-1 receptor (D1R-MSNs), while the striatopallidal neurons express selectively the D2 receptor (D2R-MSNs). (For a detailed review on cell-specific technologies used to distinguish D1/D2-type molecular and functional effects related to drug addiction, see [226]). Based on the distinct patterns of D1R and D2R G protein coupled signaling, stimulation by dopamine leads to opposing cellular signaling by either increasing (D1R) or inhibiting (D2R) cAMP production and activation of protein kinase A (PKA) [227]. To assess the cell type-specific regulation of DARPP-32, transgenic mice were generated that expressed FLAG-tagged or Myc-tagged DARPP-32 selectively in either the D1R-MSNs or the D2R-MSNs, under the control of the gene promoters for either the D1R or D2R, respectively [228]. The transgenic mice, which expressed both tagged DARPP-32 variants in the same line, were then treated with various drugs, the differentially tagged DARPP-32 proteins isolated by immunoprecipitation, and the status of phosphorylation probed by immunoblotting with specific phosphorylation site-selective antibodies. Notably, the phosphorylation of DARPP-32 at Thr-34 increased in the D1R-MSNs in response to cocaine but decreased in the D2R containing MSN's. When the total striatum was examined the value was the average of that observed in the individual D1R and

D2R MSN populations. While focused on one protein, this study nicely illustrates the necessity for cell type-specific proteomics in studies of the nervous system, especially in terms of analysis of post-translational changes.

In other brain regions, the cellular complexity is even greater than that of the striatum, often containing multiple neuronal cell types, glia, astrocytes, interneurons and inhibitory neurons. Attempting to identify quantitative changes in protein expression levels or their PTMs in such a complex tissue would clearly be very difficult without being able to selectively interrogate these changes with cell type-specificity. Hopefully, future neuroproteomics studies can take advantage of transgenic mice that selectively express marker proteins in a cell type-specific fashion. There are several potential directions that might be developed. One approach would be to use the growing number of mouse lines that express GFP in specific cell types combined with FACS sorting [229–231]. While this approach can be useful in some areas of biology such as studies of the immune system, using FACS to sort neurons is limited by the fact that axonal and dendritic processes are usually lost during this procedure. An alternative approach would be to utilize marker proteins, or tagged proteins that could be made specific for subcellular organelles or compartments of selected neuronal cell types. One recent example of this was the purification of nuclei from Purkinje cells in the cerebellum from transgenic mice that selectively express a tagged ribosomal protein in these cells [232]. These mice, which were developed to be used for TRAP ribosomal profiling have the added feature that since ribosomes are assembled in the nucleolus, the nuclei of any of the TRAP lines available can be isolated by FACS sorting. While the Purkinje cell study focused on DNA modification, it seems likely that the nuclear proteome from specific cell types could be interrogated using this FACS-based approach.

Related strategies should also be feasible to analyze subcellular compartments of neurons, including synapses. Two recent studies have shown that different types of synapses can be studied at the proteomic level following selective expression of tagged neurotransmitter receptors in transgenic mice, followed by immunoprecipitation and mass spectrometric analysis [233, 234]. In one study, the parallel fiber/Purkinje cell synapse in cerebellum was analyzed through the use of a specific type of glutamate receptor [234]. Proteins from several functional classes were identified in these synapses including a novel kinase that is likely to be involved in dendritic spine development. In the other study, synapses of inhibitory neurons in cortex were targeted using a tagged GABA receptor [233]. While neurotransmitter receptors and adhesion proteins were found in this inhibitory receptor complex, unexpectedly there were none of the sorts of signaling proteins that are typically associated with PSD protein complexes in excitatory glutamatergic synapses (see above and Table 1). Using a similar approach, a Tap/TAG strategy has been used in transgenic mice to purify proteins associated with PSD-95, a major component of the PSD [235]. Targeted expression of tagged PSD-95 or of neurotransmitter receptors associated with different types of synapses in defined neuronal populations is clearly a strategy that should be taken advantage of in future quantitative neuroproteomics studies.

Convergent development of MS-based methods to analyze limited amounts of protein generated from neuroproteomic studies

While it is clear that the field of neuroproteomics will have to develop methods that will allow for analysis of single types of neurons, and their subcellular organelles and compartments, a resulting consequence will be the reduction in the amount of tissue that is available for proteomic analyses. Similar challenges will exist in analyzing the proteins and peptides of high functional relevance that are expressed only at relatively low levels, such as is the case with CSF and plasma. This limitation is much less of an issue for mRNA profiling studies using microarrays, RNA-seq, TRAP or qPCR techniques, since one or more amplification step typically can be used. However, amplification methods are not available

at the protein or peptide level. This highlights the need for parallel development of highly sensitive quantitative techniques that can take advantage of the limited protein amounts produced by focused approaches. Ultra-sensitive MS-based proteomics that can be applied to single adult stem cells and small amounts of homogenous cell sorted diseased tissue is a likely approach (see [236]). Other approaches include the development of targeted highly sensitive MRM assays, that will reproducibly be able to analyze perhaps up to 100s of proteins from different functional categories, or MRM assays that are designed to examine the proteomes of specific organelles, sub-cellular fractions or partially enriched samples. Advances in MS approaches like SWATH have the potential to increase the speed, accuracy and reproducibility of protein quantitation for use in the neuroproteomics community. However, quantitative neuroproteomics studies will require complimentary approaches to be used to increase the total number of proteins identified and subsequently quantified, gaining a greater understanding of the relative and absolute protein changes between biological samples.

One other challenge in the proteomics field is managing the high dynamic-range of protein abundances. One recent finding by Fonslow and colleagues exploited the digestion kinetics of trypsin to selectively digest and deplete high abundant proteins, a method they called DigDeAPr [237]. They found trypsin usually digests $85\% \pm 10\%$ of proteins under normal conditions. The remaining $\sim 15\%$ of the proteome was then re-digested with trypsin and analyzed by LC-MS/MS. DigDeAPr changed the protein-abundance profile; after the first digestion high abundant proteins are digested and analyzed. During a second digestion, the lower-abundance proteins were 10-fold more abundant within this sample. Strategies such as these will hopefully improve identification and quantification of lower-abundant proteins in the proteome.

Concluding remarks

In this review, we have introduced a wide variety of quantitative MS-based quantitative proteomics techniques and attempted to show how these approaches have been applied to neurobiological questions. Obviously all -omics studies, including the many neuroproteomic studies discussed here, begin with lists of protein or peptide targets that go up or down or do not change, and the biological insight gained depends largely on the quality of the follow-up studies. While we have pointed out that the more limited 2D gel approaches appear to have provided more limited biological information, it seems clear that the frequency of publications that include quantitative neuroproteomics has increased dramatically over the last two years. These studies both report more in-depth proteomic results and more clearly address important biological questions. While many challenges remain, taken together with other likely advances in MS instrumentation, software development, the availability of heavy isotope peptide libraries and SILAM tissue for absolute quantitation, the future looks bright for rapid contributions of quantitative neuroproteomics to studies of both normal brain function as well as of the contributions of altered protein expression and regulation in various neuropsychiatric and neurodegenerative diseases.

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Abbreviations

AD	Alzheimer's disease
CSF	Cerebrospinal fluid

ELISA	enzyme-linked immunosorbent assay
HD	Huntington's disease
IHC	immunohistochemistry
IP	immunoprecipitation
NAc	Nucleus accumbens
Msc	Multiple sclerosis
PD	Parkinson's disease
PSD	post synaptic density
TMAB	trimethylammoniumbutyryl-N-hydroxysuccinimide
IB	immunoblotting

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Highlight

- Review article examining quantitative neuroproteomics studies (2008–2013)
- We discuss 2D-gel and MS based methods for relative and absolute quantitation
- We discuss proteomic studies of psychiatric diseases and neurodegenerative diseases
- We list neuroproteomic studies in table format according to quantitative method utilized
- We discuss future directions and challenges for neuroproteomics studies

Figure 1.
Quantitative proteomics methods used for relative and absolute protein and peptide quantitation in neuroproteomic studies.

Figure 2. Absolute quantitation of peptides and proteins using heavy isotopic labeling strategies

A. Production of heavy-labeled protein standards for absolute quantitation. Tryptic peptides identified with reliable MS/MS transitions are selected from experimental proteomic data for two proteins of interest, protein A (6 peptides) and protein B (8 peptides). (1) The cDNA for each protein is cloned into protein expression vectors either for cell-free or bacterial protein expression. Full-length proteins are used for absolute SILAC, Flexi-Quant and PSAQ. A protein fragment (e.g. the N-terminus for protein A and the C-terminal fragment for protein B) is cloned into expression vectors. To produce QCAT/QconCAT concatemers, multiple tryptic peptides are cloned in series into an expression vector. (2) Protein is expressed in cell-free system or using bacteria. (3) Proteins are purified by affinity purification (e.g. metal ion affinity to bind 6xHIS tag). (4) Proteins are accurately quantified using amino acid analysis to determine concentration. (5) Precise amounts of heavy labeled protein standard are then added to the protein extract depending on characteristics of the standard.

B. Typical flow of a typical MS-based absolute quantitation experiment. Flow-diagram of a typical MS-based experiment from sample to LC-MS/MS analysis. The heavy-labeled full-length protein standards (e.g. Absolute SILAC, Flexi-Quant and PSAQ protein standards) can be added prior to fractionation of the protein extract, as they should behave the same as endogenous proteins. Protein fragments (PSAQs) or QCAT/QconCAT concatemers are added after any protein fractionation and prior to trypsin digestion. Proteins are then digested with trypsin. After trypsin digestion, heavy-labeled peptides will be present in defined amounts. Synthetic AQUA heavy labeled peptides can only be added to the peptide mixture. mTRAQ reagents can be used to chemically label tryptic peptides after digestion and prior to any peptide fractionation. The resulting heavy-labeled tryptic peptides

are used to determine absolute levels of peptides/proteins in the biological sample of interest using LC-MS/MS instrumentation and software.

Table 1
Neuroproteomic studies on protein profiling and quantitation of the Post Synaptic Density (PSD)

Tissue Source (Animal, brain regions)	Fractionation method	Quantitative method	Proteins identified/quantified/altered proteins	Year	Ref.
General neuroproteomic studies					
Mouse forebrain after pilocarpine induced brain stimulation	PSD preparation by sucrose gradient, SCX,	iTRAQ (4-plex)-time course	Defined core list of PSD proteins (PSD ₁₁₀). Quantified protein abundance changes in 893 proteins over different time points	2013	[53]
Mouse forebrain Tagged-PSD-95 ^{TAP} /TAP mice compared to <i>wt</i> mice	Total protein extract, FLAG IP followed by Ni ²⁺ -NTA resin	Number of peptides/protein <i>IB</i> (13)	Identified 118 PSD-95 interacting proteins (confirmation of PSD consensus proteins)	2009	[235]
Mouse cortex, midbrain, cerebellum and hippocampus	PSD preparation by sucrose gradient, SCX, TiO ₂	iTRAQ (4-plex) <i>IB</i> (5)	Relative quantitation of 2,159 proteins and 1,564 phosphorylation sites in different brain regions	2008	[55]
Rat forebrain and cerebellum	PSD preparation by sucrose gradient	cIICAT, <i>IB</i> (12); AQUA (32)	296 proteins identified, 43 proteins showing difference between forebrain and cerebellum	2006	[60]
Rat forebrain	Synaptic membranes, PSD, sucrose gradient, SCX, LC-MS/MS	ICAT (129 pairs) <i>IEM</i> (2)	Comparison of synaptic membranes and PSD fraction. 60 proteins enriched in PSD (i.e. ICAT pairs had ratio >1)	2005	[50]
Rat forebrain	PSD preparation by sucrose gradient, 1D-SDSPAGE	Number of peptides/protein AQUA (6)	374 proteins identified, calculated pmol/20 µg of protein in PSD for 6 proteins by AQUA	2004	[57]
Psychiatric disease					
Rat hippocampus Treatment with lithium or valproate	PSD preparation by mannitol buffer and Ficol [®] gradient SCX, LC-MSMS	Label free –Peak intensities <i>IB</i> (3)	Identified 605 proteins; 21 and 43 proteins altered with lithium and valproate respectively in PSD (7 proteins in both) Identified 424 of the consensus PSD proteins	2011	[238]
Drugs of abuse					
Rat NAc after 2 week cocaine self-administration with 2 week extinction)	Crude PSD, SCX, LC-MS/MS	iTRAQ (8-plex) <i>IB</i>	337 proteins identified 42 proteins altered with cocaine self-administration and extinction	2011	[63]
Mouse hippocampus, (2 days of morphine administration)	PSD preparation by sucrose gradient, SCX	ICAT, <i>IB</i> (5), 2-DE, <i>IP</i> (2), <i>IEM</i> (1)	102 proteins identified, 10 proteins altered after morphine treatment. Dynamin and AP-2 increased in PSD	2007	[58]
Neurodegeneration –Alzheimer's disease					
Human frontal cortical synaptosomes and PSD from AD patients and control	Synaptosomes, PSD preps, sucrose gradient, SCX, lectin weak affinity	iTRAQ (2-plex) Pulsed Q dissociation (PQD)	21 proteins altered in synaptic fractions of AD patients.	2011	[239]

The following tables (tables 2–8) are organized by (1) **Tissue Source (Number of sample)**: aims to give a brief description of the disease, number of control and patients analyzed e.g. patients (11) represents 11 individually patients were used in the study. (2) **Methods**: the basic methods used to prepare samples for analysis such as fractionation, Immunodepletion or SDS-PAGE. (3) **Quantitative methods**: the type of quantitative method(s) used. We have added secondary confirmation using immunoblotting (*IB*) and immunohistochemistry if applicable. (4) **Proteins identified/quantified/ altered proteins**: number of proteins identified and/or quantified, number of differentially regulated between the samples analyzed. For example, *IB* (2) indicates that two unique proteins were analyzed by immunoblotting techniques.

Table 2

Recent neuroproteomic studies that used 2-DE and 2D-DIGE

Tissue Source (Number of samples)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
General studies					
Mouse whole brain from wild-type and glutaminase deficient (Gls ^{+/−} and Gls ^{−/−})	Total protein extract	2-DE IB (16)	32 proteins altered across genotypes	2013	[240]
Identify clients of the cysteine string protein alpha (CSPα) using knockout mouse at p10 and p28	Synaptosomes	2D-DIGE, iTRAQ, IB (15)	22 proteins decreased in CSPα-null mouse. Identified SNAP-25 and dynamin-1 as clients	2012	[183]
Mouse hippocampus, extinction of contextual fear conditioning	Total protein extract	2-DE IB (4)	24 altered proteins between good and poor extinguished mice	2011	[241]
Mouse, whole brain from E10 to 100 weeks of age	Total protein extract	2-DE	3,990 2-DE spots examined at different time points, 217 proteins showed >1.2 fold change. High heterogeneity after 42 weeks of age	2010	[186]
Neuropsychiatric disorders					
Mouse frontal cortex from <i>wt</i> and interferon-γ knock-out mice. (<i>Ifng</i> is down-regulated in human schizophrenic brains)	Total protein extract	2-DE, IB (3) RT-PCR (4) IHC	10 proteins altered in <i>Ifng</i> knockout	2012	[242]
Rat neural stem cell culture treated with antipsychotic drugs haloperidol and risperidone (96 h)	Total cell extract using urea buffer	2-DE	12 proteins altered with haloperidol 14 proteins altered with risperidone treatment (compared to controls)	2012	[77]
Human post-mortem hippocampal tissue (DG, CA4, CA2/3 and CA1) from schizophrenic (20), bipolar disorder (20), normal controls (20). Mouse hippocampus from haloperidol treated mice for 28 days	Laser-assisted micro-dissection of DG, CA4, CA2/3 and CA1 regions	2D-DIGE ELISA (1), IB (4) Dot blot (1)	108 proteins altered in schizophrenic patients, 165 proteins altered in bipolar disorder. 61 proteins altered in both disorders compared to normal controls	2011	[65]
Human post-mortem anterior cingulate cortex tissue from schizophrenia patients (11) and normal controls (8). Male and female comparison		2-DE IB (2)	30 proteins altered in schizophrenic patients compared to controls. 11 proteins altered in schizophrenic males / 7 in females	2010	[69]
Human lymphoblastoid cell line from patients with schizophrenia and normal controls – 24 h clozapine treatment		2-DE	Increased oxidation on 7 proteins after clozapine treatment	2010	[76]
Human post-mortem DLPFC white matter from schizophrenic (35), bipolar disorder (35) and normal controls (35). Mouse cortex from haloperidol treatment (28 days)	Total cell extract using urea buffer	2D-DIGE IB (4) ELISA (3)	32 proteins altered in schizophrenia; 19 proteins altered in bipolar disorder compared to normal controls. 4 proteins altered by haloperidol in mice	2009	[68]
Human post-mortem DPLC grey matter from schizophrenic (10), bipolar disorder (10) and normal controls (10)	Membrane microdomains (2% triton X-100 insoluble), 1D-SDS-PAGE	2D-DIGE, GeLC-MS/MS Label-free (spectral counts), IB (3)	101 proteins altered in the three groups. 16 proteins altered in schizophrenia or bipolar (using both proteomic methods).	2009	[64]
Rat cerebral cortex and hippocampus. Rats treated with antipsychotic drugs for 34 days (either chlorpromazine, clozapine, quetiapine) or vehicle-control	Mitochondrial enrichment, Ficoll [®] , sucrose gradient	2-DE qRT-PCR IB (6)	14 mitochondrial proteins altered with antipsychotic drugs compared to control	2009	[75]

Tissue Source (Number of samples)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
Rat cerebral cortex treated with different antipsychotic drugs for 34 days	Synaptosomes	2-DE	Metabolic and mitochondrial proteins altered with drug treatment	2009	[74]
Human post-mortem DLPFC from schizophrenic (10) and controls (15). Rat cerebral cortex with sub-chronic dose (30 mg/kg) of ketamine or vehicle-control for 5 days. Rats treated for 14 days with clozapine (5 mg/kg)	Synaptic membranes. lipid removal	IB (1) IHC (3)	41 proteins altered in schizophrenia patients compared to controls 12 altered 2D-spots after ketamine treatment in rats	2008	[70]
Human post-mortem (insular cortex) from schizophrenic patients (12) and normal controls (13). Micro-dissected layer 2 insular cortex tissue	Total protein extract	2D-DIGE IB (2)	104 proteins altered in schizophrenia patients compared to controls. 41 proteins identified	2008	[67]
Human post-mortem tissue DLPFC grey matter from schizophrenia (35), bipolar disorder (35) and normal controls (35).	Total protein extract	2-DE IB (1)	11 proteins altered in schizophrenia, 48 proteins altered in bipolar patients. 7 proteins altered in both diseases	2008	[66]
Drugs of Abuse – Alcohol					
Uterine artery endothelial cells exposed to alcohol for 3h per day, 3 days per week for 2 weeks to model fetal alcohol disorders	FAC sorted cells, alcohol exposure	2D-DIGE IB (2)	49 proteins altered after chronic binge-like alcohol exposure	2011	[243]
Non-human primates (10) after chronic alcohol administration. Human plasma from alcohol dependent (8) and controls (7)	H14 high-abundant protein depletion	2D-DIGE IB (6)	190 proteins altered after binge drinking monkeys (46 proteins identified)	2011	[244]
Human post-mortem tissue, superior frontal gyrus from cirrhotic alcoholics (6), alcoholics without comorbid disease (6) and non-alcoholic non-cirrhotic controls (6)	Synaptosomes	2D-DIGE	99 proteins altered in cirrhotic alcoholics compared to controls and non-comorbid alcoholics	2011	[245]
Human post-mortem brain tissue – corpus callosum from uncomplicated alcoholics (7), cirrhotic alcoholics (6) and controls (9)	Total protein extract	2-DE IB (4)	99 proteins altered in alcoholics. 39 proteins altered in uncomplicated/ 60 in complicated alcoholics (25 in both)	2009	[246]
Rats chronically consuming ethanol for 12 months (5) or with nutrient-matched controls (5). Brain and liver tissue collected after 24 h withdrawal	Total protein extract	2-DE, Microarray, RT-PCR (23)	1 protein altered in brain, 4 proteins altered in brain tissue and 10 proteins altered in liver DNA Microarray - 45 altered genes in brain	2009	[247]
Human post-mortem brain tissue-corpora callosum from uncomplicated alcoholics (7), complicated alcoholics (6) and controls (12)	Total protein extract	2-DE	35 proteins altered in the uncomplicated alcoholic and 56 in the complicated group. 25 altered in both	2008	[248]
Drugs of Abuse (cocaine, amphetamine, methamphetamine, morphine)					
Mouse NAc tissue from 7 days of cocaine-paired and cocaine-unpaired training	Total protein extract	2-DE, IB (2), IHC (1)	11 proteins altered in cocaine paired compared to unpaired control	2011	[249]
Human monocyte-derived mature dendritic cell culture, incubated for 48 h with cocaine or methamphetamine or vehicle	Total protein extract	2D-DIGE, IB (6), RT-PCR (14)	26 proteins altered with cocaine, 28 proteins altered with methamphetamine compared to vehicle	2009	[250]
Rat NAc tissue. Separated cohort by extinguished (E) or did not extinguish (NE) cocaine-seeking behavior. Compared single saline/cocaine injection	Total protein extract	2-DE	5 proteins altered between E SAL and NE-SAL. 6 proteins altered in E-COC. 9 proteins altered in NE-COC	2009	[251]

Tissue Source (Number of samples)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
Non-human primate (Rhesus monkey). NAC from cocaine self-administered (4) and control animals	Cytosolic protein extract	2D-DIGE Pro-Q diamond phosphoprotein	22 proteins altered in cocaine-exposed monkeys. 15 cocaine-altered phosphoproteins	2008	[252]
Rat striatum from cocaine treated rats with or without Isoliqurigenin administration	Total protein extract	2-DE, IB (8), RT-PCR (6)	56 proteins altered in response to isoliqurigenin	2008	[253]
Human post-mortem NAC tissue from control and cocaine overdose victims	Cytosolic protein extract	2D-DIGE	18 proteins altered after chronic exposure to cocaine	2007	[254]
Neurodegeneration – Parkinson's disease					
Human post-mortem tissue, substantia nigra pars compacta tissue from PD patients (3) and controls (3). CSF from PD patients (7) and controls (7)	Total protein extract	2-DE IB (1) IHC (1)	32 proteins altered in PD patients	2012	[84]
MN9D cell line (model of dopamine neurons). DJ-1 knockdown and treatment with 6-hydroxydopamine (6-OHDA).	Total protein extract	2-DE IB (3) RT-PCR (3)	27 proteins altered in DJ-1 knockdown DJ-1 knockdown accelerated 6-OHDA MN9D cell death	2012	[255]
Human plasma from PD patients (36) and controls (16)	Albumin and IgG affinity deletion	2-DE ELISA (1)	2 proteins altered in PD patients	2011	[256]
Human serum from PD patients (18) and controls (7)	Albumin deletion	2-DE IB (3)	13 proteins altered in PD patients	2010	[257]
Human CSF from first-episode schizophrenic patients (17) compared to healthy controls (10)	No depletion	2-DE	6 proteins altered in schizophrenia patients.	2010	[258]
Rat striatum from hemiparkinsonian animal model. Rats were unilaterally lesioned with neurotoxic 6-OHDA in the substantia nigra (6) and controls (6) at 6 months of age	Total protein extract	2-DE IHC (1)	144 proteins were altered in lesioned side of striatum compared to controls (101 proteins identified)	2010	[259]
Rat striatum from striatum and substantia nigra from hemiparkinsonian animal model. Unilateral lesion with 6-OHDA in the substantia nigra (6) or vehicle control (6). Sacrificed after 5, 10 and 20 days	Total protein extract	2-DE IB (2) IHC (3)	70 proteins altered between the hemiparkinsonian animal model and controls. Observed time-dependent protein alterations in specific proteins	2010	[260]
Neurodegeneration – Alzheimer's disease					
Human post-mortem tissue - Hippocampus from AD patients (5) and control (5) Pro-Q	Cytosolic extract	2-DE, IHC (1) Pro-Q diamond	Phosphoprotein 2D-map, 17 phosphoproteins altered in AD patients	2011	[82]
Mouse cerebral cortex from 3xTg AD mouse model (4) and non-Tg controls (4) Sacrificed at 6 months of age	Mitochondria, Percoll gradient	2D-DIGE Blue native-SDS-PAGE	23 proteins altered in 3xTg AD mouse	2011	[86]
Mouse – Whole brain from AD hAPP-mutant Tg-mouse model (5) and control (5)	Cytosolic protein extract	2-DE, IB (2) IP (1)	6 proteins altered in the Tg-AD mouse	2011	[261]
Human – post-ventricular CSF from AD patients (47), non-demented controls (NDC) (43) and non-AD dementias controls (17)	Albumin and IgG affinity deletion	2D-DIGE	21 proteins altered in NDC/AD 19 of these altered comparing NDC/C 14 proteins altered comparing NDC/AD	2009	[262]

Tissue Source (Number of samples)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
Human post-mortem tissue –hippocampal and inferior parietal lobule from AD patients (6), amnesic mild cognitive impairment (MCI) patients (6) and controls (6)	Lectin affinity (N-linked glycoproteins)	2-DE IB (2)	N-linked glycoprotein 2D-map, 6 proteins altered in AD tissue and 4 proteins altered in MCI compared to controls	2009	[263]
Human post mortem tissue - Inferior parietal lobule from early AD patients (4) and controls (4)	Total protein extract	2-DE IB (1)	8 nitrated proteins increased in AD (3-Nitrotyrosine)	2009	[264]
Neurodegeneration – Huntington's disease					
Human CSF and serum samples from HD patients (6) and controls (6)		2-DE, IB (2) ELISA (1)	3 proteins altered in HD patients	2011	[265]
Mouse – Whole brain from HD Tg-mouse model (5–8) and control (5–8). Analyzed brains at 2-, 4-, 6-, 8- and 12-weeks-old	Total protein extract	2-DE	Longitudinal time course-protein changes after 2 weeks HD-Tg. 226 proteins altered in HD mouse	2009	[266]
Human post-mortem tissue - striatum and cortex from HD patients (8) and controls (8)		2-DE IB (8) Enzyme (3)	18 proteins altered in HD patients	2008	[267]
Human CSF from HD early (10) and HD-late mid-stage patients (10) and normal control (10). Multi-lab comparison of 2D-DIGE, label-free (spectral counts), d ₀ /d ₃ acetylation, AMT and iTRAQ	No. Immuno-depletions for other methods	2D-DIGE, label free, d0/d3 acetylation, AMT, iTRAQ	44 proteins altered in HD patients. Brain proteins are 1.8X more likely to be found in CSF compared to liver proteins	2009	[268]

Table 3

Neuroproteomic studies using isotope chemical labeling techniques

Tissue Source (Number of sample)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
<i>General neuroproteomic studies</i>					
PC12 cell line deficient of neurofibromin using NF1 siRNA and stimulated with NGF.	Fractionation, LC-MS/MS, microarray	iTRAQ (8-plex), 2D-DIGE, Pro-Q Diamond, IB (7)	Quantified 123 proteins (2D-DIGE); 3,239 (iTRAQ)	2013	[269]
Rat medulla oblongata from spontaneously 474hypertensive rats		iTRAQ IB (5)	Quantified 474 proteins, over 3 runs (218 in all 3), 17 altered proteins.	2012	[270]
Rat sensory and motor nerve comparison	Dorsal root and anterior root of spinal cord, T-PER tissue extraction, SCX, LC-MS/MS	iTRAQ (4-plex) IB (8), IHC (8)	Quantified 1,472 proteins, 100 proteins differential between neurons	2012	[271]
Rat frontal cortex from unaffected and brain tissue infected with tuberculosis meningitis	Total protein extract, SCX, LC-MS/MS	iTRAQ (4-plex)	Quantified 434 proteins, 134 altered proteins	2012	[272]
Mouse cortical lesion / axonal degeneration	Synaptosomes	iTRAQ (8-plex) IB (16)	Quantified 178 proteins, 47 proteins altered after 48 h of the lesion	2012	[273]
Mouse hippocampus from wt and Ercc1 mutant mice at p28 and p112	Synaptosomes, 2D-LC MS/MS	iTRAQ (8-plex) IB (4)	Quantified 288 proteins, 175 altered proteins	2012	[108]
Mouse cortices from 13-month-old HIV/gp120-Tg mice and wild type controls. Human patients with HIV-associated neurocognitive disorder (HAND)	Synaptosomes, MUDPIT (SCX, LC-MS/MS)	TMT (6-plex) IB (3)	107 proteins altered in HIV/gp120-Tg mice	2012	[274]
Mouse hippocampus from wt and FMRP knockout.	Synaptosomes, 2D-LC MS/MS	iTRAQ (8-plex) IB (9)	Quantified 205 proteins, 23 altered proteins	2011	[275]
Mouse visual cortex development at p30 and p46 with ocular deprivation (4 conditions)	Synaptosomes, sucrose gradient	iTRAQ (4-plex) IB (14)	Quantified 467 proteins, 35–84 altered proteins between conditions	2011	[276]
Human post-mortem brain tissue from MSc patients (3) and controls (4)	Formalin fixed tissue, Laser micro-dissection of Msc lesions, SCX, 2-D LC MS/MS	iTRAQ (8-plex), spectral counting	82 proteins altered in MSc patients	2011	[277]
Rat cerebral brain tissue, post-ischemia (0, 4 and 24 h)	SCX, LC-MS/MS	iTRAQ (4-plex) IB (9)	Identified 2,242 proteins, 61 altered proteins	2011	[278]
Mouse cerebral cortices D-galactose-induced mice and vehicle control mice	Synaptosomes, Blue native-SDS-PAGE	iTRAQ IB (7)	84 protein altered in D-galactose mouse from glycan-end product accumulation	2011	[279]
Rat ventral midbrain at embryonic days (E11–14)	SCX, LC-MS/MS	iTRAQ (8-plex) IB (4), IHC (1)	Quantified 3,000 proteins	2010	[280]
Mouse cortical tissue from mice with mutated Ca(V)2.1 calcium channels and wild-type control	Total protein extract, SCX, 2D LC-MALDI-TOF-MS/MS	iTRAQ (8-plex) IB (3)	19 proteins altered in mouse with mutated channel	2010	[281]
Mouse brain, post-ischemia (3, 6 and 20 h)	Synaptosomes, LC-MS/MS	clicCAT	62 proteins altered	2010	[282]
Neuroblastoma cells treatment with S- and R- enantiomers of ibuprofen	Total protein extract, 2D-SCX-LC-MS/MS	iTRAQ (4-plex) RT-PCR (6)	13 proteins altered in ibuprofen treated cells	2008	[283]

Tissue Source (Number of sample)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
Neuropsychiatric disorders-Schizophrenia					
Human post-mortem mediodorsal thalamus from schizophrenic (11) and controls (8); and CSF from first episode schizophrenic patients (17) and control (10)	Isoelectric focusing (IEF), 47 fractions, LC-MS/MS	iTRAQ (8-plex) 2-DE, IB (7)	Identified 551 proteins, 50 altered proteins	2010	[284]
Human post-mortem DLPFC tissue from schizophrenia patients (9) and control (7)	Total protein extract, Isoelectric focusing	ICPL-protein level	84 proteins altered in schizophrenic patients	2009	[285]
Drugs of Abuse					
Rat NAc (core versus shell) comparison after 7 days of cocaine administration	Total protein extract	iTRAQ IB (10)	>1,700 proteins quantified in core and shell. CaMKII signaling altered	2013	[101]
Rat striatum (5 days of morphine administration)	Synaptosomes, TX-100 soluble fraction	Succinic acid anhydride isotopes	30 proteins altered by morphine	2011	[103]
Rat NAc after 2 weeks of cocaine self-administration and 2 weeks extinction)	Crude PSD, SCX, LC-MS/MS	iTRAQ (8-plex) IB	337 proteins identified 42 proteins altered	2011	[63]
Rat mPFC, after 16 days of heroin self-administration, sacrificed after 21 days withdrawal.	Synaptic membranes, 2D LC-MS/MS	iTRAQ (4-plex), qPCR, IB (2)	486 proteins identified. 27 proteins altered	2010	[286]
Rat mPFC during adolescent development (at days P34, P44 and P78)	Synaptic membranes, sucrose gradient,	iTRAQ IB (14)	Identified 1,816 proteins, 78 altered proteins between age groups	2010	[287]
Rat liver from chronic ethanol feeding and control animals	Enriched proteasome fraction	Cleavable ICAT, Label-free (spectral counting), IB (11)	51 proteasomal proteins altered in chronic alcohol group	2009	[288]
Rat brain synaptic membranes from chronic morphine exposure and saline controls	Synaptosomes, Sucrose gradient	ICAT-protein labeling	27 proteins altered with morphine treatment	2005	[102]
Neurodegeneration –Parkinson's disease					
Human CSF from patients with PD (12), PD dementia (PDD) (12) and non-demented controls (NDC) (12)	Albumin and IgG depletion. MUDPIT (2D-LC-MS/MS)	iTRAQ (4-plex) MRM assay (14)	16 proteins altered in PD. Only 2 proteins validated in MRM assay	2012	[104]
Human serum from slightly affected PD (8), middle to seriously affected PD (8) and control (6) patients	MARS high abundant proteins depletion 2D-LC-MS/MS	iTRAQ (4-plex) IB (3)	26 proteins altered in PD	2012	[105]
Neurodegeneration –Alzheimer's disease					
Human frontal cortical synaptosome and PSD from AD patients and control	Synaptosomes, PSD preps, sucrose gradient, SCX, lectin weak affinity	iTRAQ (2-plex) Pulsed Q dissociation (PQD)	21 proteins altered in synaptic fractions of AD O-GlcNAc site map of synaptic proteins	2011	[239]
Human frontal cortex from control and AD patients	TiO2 phosphopeptide enrichment, LC-MS/MS	iTRAQ (4-plex) IB IHC	Increased levels of neurofilament phosphorylation in AD	2010	[289]

Tissue Source (Number of sample)	Methods	Quantitative methods	Proteins identified/quantified/ altered proteins	Year	Ref.
Mouse cortex from pre-senilin knockin (PS-1) Tg-AD mouse (3) and controls (3). Profiled at 3-, 6- and 12-months of age	Percoll gradient, mitochondrial fraction, 2D-HPLC	ICAT IB (4)	46 proteins altered in PS1-Tg mouse	2009	[107]
Mouse hippocampal and cortical brain tissue from <i>w^t</i> (4) and 3xTg-AD (4) mice at 16 months old	NP-40 based lysis buffer, SCX, LC-MS/MS	iTRAQ (4-plex) IB (16)	142 and 91 proteins altered in cortex and hippocampus respectively	2008	[106]
Neurodegeneration – Huntington's disease					
Human CSF from HD early (10) and HD-late mid-stage patients (10) and normal control (10) (<i>findings previously described in table 2</i>)	Multi-lab comparison of d ₀ /d ₃ acetylation, AMT and iTRAQ Immunodepletion of high abundant proteins	d ₀ /d ₃ acetylation, acrylamide labeling and label-free (AMT), iTRAQ	44 proteins altered in HD patients. Proteins from brain 1.8X more likely to be found in CSF compared to liver proteins	2009	[268]

Table 4

Neuroproteomic studies using SILAC, SILAM and metabolic labeling

Sample (cells, tissue)	Fractionation method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
SILAC labeling in mammalian cell lines					
Human HCT116 cells, Parkin dependent ubiquitination after mitochondrial depolarization	diGly ubiquitin antibody affinity purification	SILAC heavy:light ratio Lys0:Lys8	Quantified 4,772 ubiquitinated sites in 1,654 proteins. Identified 582 Parkin-dependent diGly peptides (303 proteins)	2013	[290]
Mouse primary culture of cerebellar granule neurons (CGNs). Comparison of secretome from apoptotic and surviving CGNs	SDS-PAGE gel; LC-MS/MS	SILAC heavy:light ratio Heavy: Arg10/Lys8, Light Arg6/Lys4, Arg10 IB (3)	47 proteins altered in the supernatants of apoptotic and surviving CGNs. A total of 1375 proteins were identified in CGN-conditioned media	2012	[291]
SH-SY5Y cell line SILAC labeling and MG132-treatment of heavy cells	RIPA buffer, High density sucrose gradient	SILAC: Heavy: $^{13}\text{C}_6$ -Arg/D $_2$ -Lys IB (4)	644 insoluble proteins after 8 h or 12 h of proteasomal inhibition using MG132	2011	[292]
Human HEK293 cells-overexpression of TDP-43 and TDP-S6 to model FTLTD	Sarkosyl-insoluble fraction from mock, HA-TBP-43 or HA-TBP-S6 transfected cells	Multiplex SILAC: Light/Med/heavy ratio; -Light (Arg0/Lys); -Medium (Arg6/Lys4); -Heavy (Arg10/Lys8); Label free (Spectral counts)	Identified 1,265 proteins with 267 proteins altered. Quantitation of 2,570 proteins. 6 proteins altered with overexpression including ubiquitin, SUMO2/3, ARP1 and dynamin 3	2010	[293]
Neuropsychiatric disorders					
SH-SY5Y and HEK 293 cell lines transfected with 3xFLAG-Dysbindin or 3xFLAG-mutant	Cross-linked proteins in cells, FLAG IP, 1D-SDS-PAGE	SILAC: Light/heavy ratio Heavy: $^{13}\text{C}^{15}\text{N}$ Arg/Lys Light: $^{12}\text{C}^{14}\text{N}$ Arg/Lys, Label free (Spectral counts)	Identified 24 proteins that associate with the BLOC-1 complex Identified subunits of BLOC-1 and AP3 complex	2012	[151]
Drugs of abuse					
Rat HAPI microglial cell line after ethanol exposure and control	SCX, LC-MS/MS	SILAC: light/heavy ratio $^{13}\text{C}_6$ -Arg/ $^{13}\text{C}_6$ -Lys	Identified more than 1,000 proteins	2012	[294]
Metabolic labeling using model organisms (SILAM)					
Mouse tissue (28 individual tissues), protein expression	Total protein extract,	SILAC: light/heavy ratio $^{13}\text{C}_6$ -Lys "Spike in" internal standard	Identified 7,349, quantified 6,974 proteins, atlas of protein expression across tissues	2013	[295]
Drosophila melanogaster (SILAC fly), fmr1 null fragile X mutant		SILAC: $^{13}\text{C}_6$ / $^{15}\text{N}_2$ -Lys (1 generation), internal standard (IS), MRM (1)	Observed high individual variation. Quantified 1,902 in wt, 1,617 proteins in FMRP mutant with internal standard	2012	[196]
Mouse barrel cortex from mice sensory-deprived (trimmed versus bushed whisker)	Synaptosomes from barrel cortex, MUDPIT, LC-MS/MS	SILAM, ^{15}N -labeled mice, ($^{14}\text{N}/^{15}\text{N}$ ratio) IB (22), IHC (2)	Quantified 7,000 synaptic proteins. 89 decreased and 161 elevated with sensory deprivation	2012	[129]

Sample (cells, tissue)	Fractionation method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
Rat brain and liver tissue examined at 6 weeks, 6 months and 12 months, global protein turnover	¹⁵ N-labeled diet for 6 weeks then swapped to ¹⁴ N diet, MUDPIT.	Pulse chase metabolic labeling, ¹⁵ N spectral counts (¹⁵ N/ ¹⁴ N ratio)	At t=0, 3,400 ¹⁵ N proteins compared to 9 ¹⁴ N-labeled proteins. Rapid turnover in liver, slower turnover in brain	2012	[114]
Mouse model of low and high anxiety	Synaptosomes LC-MS/MS	¹⁵ N/ ¹⁴ N ratio	1,214 proteins identified, 264 altered proteins in high anxiety mouse model	2011	[296]
Age related proteome of mouse hippocampus, frontal cortex, heart and kidney at 5 or 26 months of age	SCX, LC-MS/MS	SILAC mouse, ¹³ C ₆ -lysine diet as internal standard	Quantified 4,200 proteins between age groups. 5 proteins significantly altered by age, high biological variation noted	2011	[185]
Hippocampal proteins from a mouse model of depression and anxiety	Fractionation, 1D-SDS-PAGE	¹⁵ N/ ¹⁴ N ratio of peak area of peptide ion chromatogram	Quantified 1,576 proteins. 312 proteins altered in cytosolic fraction. Quantified 1,349 proteins in microsome, 206 altered in expression	2011	[131]
Rat brain and liver tissue at p45, comparing ¹⁵ N-labeled brain compared to ¹⁴ N-unlabeled brain	Nuclei purification. Phosphopeptidase enrichment IMAC, MUDPIT	SILAM (¹⁵ N), ¹⁴ N/ ¹⁵ N ratio	Quantified 3,472 phosphopeptides in nuclear extracts from brain and liver (1014 brain/ 849 liver). A total of 439 phosphoproteins and 654 phosphopeptides identified in both	2011	[127]
Zebrafish - examine protein turnover and tissue regeneration (brain, blood, fin, heart, intestines, liver, muscle, spleen)	GeLC-MS/MS	SILAC: light/heavy ratio ¹³ C ₆ -Lys/ ¹² C-Lys	Highest labeling in tissues with high regenerative capacity (e.g. intestine, liver, and fin), lowest (brain and heart). Proteins with high labeling (catalytic or transport activity pathways)	2011	[120]
Mouse brain, liver and blood at nine time points	1D-SDS-PAGE, In gel digestion, MUDPIT	SILAM (¹⁵ N), ¹⁴ N/ ¹⁵ N ratio	Analyzed ~2,500 proteins in multiple organs to look at protein turnover Protein turnover rates differ (brain ~9 d, liver ~3 d and blood ~3.5 d)	2010	[123]
Mouse cerebellum tissue at p5, p14, p28 and p56 in mouse model for trait anxiety	Cytosolic protein extract. LC-MS/MS	SILAM, ¹⁵ N/ ¹⁴ N ratio RT-PCR (1), Behavioral Tests (2)	Determined 50%, 70%, 80% and 90% ¹⁵ N incorporation at different times points. 2 proteins were verified by behavioral (anxiety) tests	2009	[126]
Drosophila melanogaster, sexual specific proteins, male versus female comparison	Whole fly, RIPA buffer extract, 1D-SDS-PAGE, LC-MS/MS	SILAC: light/heavy ratio Heavy: Lys 8 ¹³ C ₆ ¹⁵ N ₂ Light: Lys0 ¹² C ₆ ¹⁴ N ₂ Label-free	1,913 proteins identified and quantified. Identified sex-specific proteins, 1,578 proteins quantified by label-free	2010	[119]
Drosophila melanogaster (looking at embryonic development)	In-solution digested, SCX, LC-MS/MS	SILAM, ¹⁵ N/ ¹⁴ N ratio	2,232 proteins quantified, approximately half change during development. 500 proteins increased	2009	[117]
Drosophila melanogaster (Phosphatase inhibitor treatment or phosphatase knock down by RNAi)	GeLC-MS/MS SCX, LC-MS/MS, TiO ₂ enrichment	SILAC: light; heavy ratio ArgO/ LysO: Arg10/Lys8 RT-PCR	10,000 phosphorylation sites; 288 of 6,478 changed after phosphatase Ptp61F knockdown	2009	[118]
Primary cultured neuronal and astrocyte cultures					
Primary cultured neurons, neurotrophin-3 (NT-3) signaling	IP: Phosphotyrosine (pY), 1D-SDSPAGE, LC-MS/MS	Multiplex SILAC using Heavy: medium ratio Medium: Lys4Arg6 Heavy: Lys8Arg10	Incorporation checked at 2, 6, or 10 days <i>in vitro</i> . 60% incorporation after 2 days, 90%	2011	[137]

Sample (cells, tissue)	Fractionation method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
		IB (3)	after 10 days. 15 min treatment of NT-3 resulted in 27 proteins altered in pY abundance		
Primary astrocyte culture, Brefeldin A (BFA)-induced protein changes	1D-SDS-PAGE, LC-MS/MS	SILAC, light: heavy ratio IB (7) MRM (6)	21 proteins altered after BFA treatment. 92 proteins enriched in astrocyte-conditioned media compared to cell lysate	2010	[297]
Primary cultured cortical and hippocampal neurons. BDNF treatment (15 min)	RIPA buffer, IP:pY, 1D-SDS-PAGE, LC-MS/MS	SILAC light:heavy ratio Heavy: ^{13}C -Arg/Lys Light: ^{12}C -Arg/Lys; IB	13 proteins protein altered after BDNF treatment	2008	[136]
Primary cultured neurons from FMRP-KO mice (Fragile X syndrome) and wild-types	Synaptosomes, MUDPIT	SILAC light:heavy ratio Heavy: ^{13}C ^{15}N Arg/Lys Light: ^{12}C ^{14}N Arg/Lys IB (10)	132 proteins altered in FMRP-KO. Also compared proteomic /microarray data	2008	[135]

Table 5

Neuroproteomic studies using label-free quantitative techniques

Tissue Source (Number of sample)	Fractional method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
General neuroproteomic studies					
Zebrafish wild-type and BACE1 knockout brain proteomes		Label-free (peak area) iBAQ (intensity peptide / total peptides)	Quantified 4,770 proteins in both wild-type and BACE1 brains. Determined protein abundance using iBAQ algorithm. 24 membrane proteins altered in BACE1 knockout brains	2013	[298]
Human frontal cortex from control (11) and FTLD (12) patients.	Detergent-insoluble protein extraction, 1D-SDS-PAGE, LC-MS/MS	Label-free (spectral counting), SILAC-labeled HEK293 internal standard IB (9);	Pairwise comparison brain/SILAC labeled HEK293 cells, 18 proteins enriched in FTLD by label-free, 21 proteins enriched by SILAC internal standard method	2012	[299]
Chicken vesicular hair bundle	1D-SDS PAGE, LC-MS/MS	Label-free (peak area), iBAQ (intensity peptide/total peptides)	Quantified >1,100 proteins, 336 enriched in bundles	2012	[300]
Human dense core secretory vesicles (DCSV)	Soluble and membrane protein extracts	Label-free (spectra counts)	Quantified 300 proteins and peptides	2012	[301]
Global examination of protein phosphorylation in 14 different rat tissues	Protein activity inactivation using Stabilizer T.1. Total protein extract. TiO ₂ enrichment.	Label-free (LC-MS ^E)	Quantified 31,480 phosphorylation sites from 7,280 proteins in the different tissues	2012	[147]
Human post-mortem tissue. Occipital lobe (primary visual cortex) and cerebellum	Total protein extract	Label-free (LC-MS ^E)	Comparison of proteins in two brain regions (391 proteins in occipital lobe/330 cerebellum). 95 proteins unique in occipital lobe, 34 unique to cerebellum	2012	[302]
Human CSF from obese women with hypertension compared to non-obese women	Ultrafiltration	ICPL proteins > 10 kDa. Label-free (spectral counting) < 10 kDa. IB (1), ELISA	18 proteins altered in obese women	2012	[303]
Primary cerebellar neuronal culture (unexposed and exposed to food-contaminant non-dioxin like polychlorinated biphenyls (PCB138, PCB180)	Total protein extract. 1D-SDS-PAGE, LC-MS/MS	Label free (spectral counting) IB (2), IHC, RT-PCR	41 and 25 proteins altered with PCB180 or PCB138 respectively. Also compared proteomic /microarray data	2012	[304]
Mouse sciatic nerve myelin proteome,	1D-SDS-PAGE, LC-MS/MS, myelin purification, 2D-IEF/SDS-PAGE	Label-free (LC-MS ^E) IB (13)	Identified 545 proteins associated with myelin	2011	[305]
Medial prefrontal cortex from mice from MSEA mice (model of early life neglect)	Total protein extract, RNA, DNA, LC-MS/MS	Label-free, MRM (6) RT-PCR, microarray, RNA-seq	46 proteins altered in MSEA mice compared to controls. Used MRM, RNA-seq and microarray data on the same brain tissue. MRM confirmed 4 changes	2011	[149]
Glioblastoma (GBM) cancer stem cells line (CSCs) (from primary brain tumor) treated with gamma-secretase inhibitor	Total protein extract LC-MS/MS	Label free (spectral counts), MRM (1), IB (5)	117, 187 and 213 proteins altered by 2-, 10- and 50µm inhibitor	2011	[306]

Tissue Source (Number of sample)	Fractional method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
Mouse brain, kidney spleen, lung, testis, brown fat, heart, liver and pancreas at p21 days	RIPA buffer. SCX followed by IMAC. GeLC-MS/MS	Label-free, (spectral counts)	Identified 12,039 proteins, 6,296 phosphoproteins, 36,000 phosphorylation sites Compared protein abundance and phosphorylation levels across tissues	2010	[145]
Mouse frontal cortex and uterus	Total protein extract.	Label free-spectral counting	28 proteins altered in mouse uterus. A total of 118 proteins identified	2009	[307]
Mouse brain, heart, kidney, liver, and lung.	1% TX-100 buffer extract. 1D-SDS-PAGE. LC-MS/MS	Label-free (sum of all peptides per protein) Absolute quantities (ion intensity). IB (2)	Profiled ~1,000 most abundant protein in mouse tissue	2007	[146]
Astrocyte (AC) or astrocytoma (CT-2A) cell lines	Membrane fraction, 1D-SDS-PAGE	Label free, (spectral counting) RT-PCR (4)	25 plasma membrane proteins altered in CT-2A. A total of 599 and 587 proteins identified in AC and CT-2A respectively	2008	[308]
Psychiatric diseases					
Human serum, from antipsychotic-naïve schizophrenia patients (20) and controls (20)	Depletion of high abundant proteins (MARS 14 kit). IMAC-Fe3+- phosphopeptide enrichment	Label-free (LC-MS ^E) ELISA (2)	35 proteins altered in schizophrenia patients. 72 altered phosphoproteins. ELISA results were consistent with label-free results for 2 proteins	2012	[309]
Human DLPFC post-mortem tissue from Major depression disorder MDD (24) and healthy controls (12)	Total protein extracts 1D-GeLC-MS/MS	Label-free (LC-MS ^E) SRM (2), IB (2)	39 proteins altered in MDD patients	2012	[310]
Human DLPFC post-mortem tissue from schizophrenic patients (10), bipolar disorder (10) and normal controls (10). Separated low / medium antipsychotic medication	Sub-cellular fractionation, (cytosol and enriched membrane fractions)	Label-free (LC-MS ^E) Metabolomic profiling IB	30 proteins altered in low-SCZ and bipolar disorder patients compared to controls. No changes in medium-SCZ compared to controls	2011	[150]
Human serum from first-onset paranoid schizophrenic patients – (DSM-IV) (22) and 33 healthy controls	High abundant depletion (20 proteins)	Label-free (LC-MS ^E) ELISA (4)	10 proteins altered in schizophrenia patients	2010	[311]
Rat frontal cortex from rats administered antipsychotic drugs for 21 days (haloperidol and olanzapine)	Sub-cellular fractionation (cytosol and membranes)	Label-free (LC-MS ^E) IB (6)	59 proteins altered by haloperidol. 74 by olanzapine and 21 common proteins altered by both antipsychotic drugs	2009	[312]
Neurodegeneration – Parkinson's disease					
Mouse cortex, cerebellum and striatum and "rest of the brain" from MPTP-induced parkinsonian (4) and control (4)	Total protein extraction. SCX	Label free (spectral counting) IB (1)	518 proteins altered in MPTP model. 270 of these changes occurred in striatum or "rest of the brain" regions containing substantia nigra	2010	[158]
Human post-mortem cortical tissue from patient with Lewy bodies pathology or controls	Total protein extract, sucrose gradient	Label-free (Spectral counting)	~700 proteins identified in Lewy bodies compared to controls. 40 proteins co-purified with Lewy bodies	2008	[159]
Neurodegeneration-Alzheimer's disease					

Tissue Source (Number of sample)	Fractional method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
Neuroblastoma N2a cells treated with Huperzine A and A β (1–42) oligomers	Total protein extract, SCX enrichment	Label-free	2,800 proteins quantified, 198 proteins altered with Huperzine A and A β (1–42) oligomers	2013	[313]
Tg2576 amyloid mice model of plaque formation. Longitudinal study (12,15,18, and 22 months of age).	Triton X-114 protein extraction. Hydrophilic water-soluble and hydrophobic membrane proteins	Label-free (peak intensities)	1,085 proteins quantified at four stages of disease progression. 57 mitochondrial proteins altered	2012	[156]
Human CSF from familial AD patients with PSEN1 and APP mutations (14) and control (5)		Label-free (peak intensities)	56 proteins altered between mutation carriers and controls. 14 protein previously reported by proteomic studies	2012	[79]
Human post-mortem frontal cortex tissue from AD patients (5) and control (5)	Membrane-enriched fraction	Label-free (spectral counting) IB (2)	13 proteins altered in AD patients, a total of 1,709 proteins identified	2012	[155]
Human post-mortem temporal cortical tissue from AD patients (10) and controls (10)	Total protein extract	Label-free IB (3)	197 proteins are more abundant in AD. (21 proteins previously reported increased in AD)	2012	[314]
Human post mortem (insoluble frontal cortex) from FTLD patients and controls	Inclusion bodies, IMAC, LC-MS/MS	Label-free (spectral counting)	6 phosphoproteins altered in FTLD	2010	[315]
Human post mortem from AD patients (10), FTLD patients (10) and controls (10)	Insoluble frontal cortex	Label-free (Peak intensities) IB (6)	81 proteins altered in AD patients (512 proteins quantified)	2009	[157]
Mouse-whole brain and human cortical tissue from normal-control brains	Synaptosomes, phosphorylated and non-phosphorylated α -synuclein bait	Label-free (spectral counts) IB (12)	85 proteins enriched with phosphorylated α -synuclein bait. Repeated using human brain protein extracts by IB and found similar results	2008	[80]
Neurodegeneration-Huntington's disease					
Mouse – Brains from FLAG-tagged HD mouse mode (140Q) (2) and controls (7Q) (2)	Cytosol and synaptic membrane extracts. IP: FLAG to purify Htt complexes. MUDPIT	Label-free (spectral counting) IB (4)	Identification of Htt associated proteins. 236 and 129 proteins identified in 7Q and 140Q respectively in cytosol. 127 and 226 proteins identified in 7Q and 140Q in synaptosomal membrane fraction	2012	[316]

Table 6

Neuroproteomic studies that used targeted MRM quantitative techniques

Tissue Source (Number of sample)	Fractional method	Quantitative method	Proteins identified/quantified/ altered proteins	Year	Ref.
Cell lines					
Glioblastoma (GBM) cancer stem cells line from primary brain tumor) treated with gamma-secretase inhibitor	Total protein extract. LC-MS/MS	Label free (spectral counts), MRM (1), IB (5)	117, 187 and 213 proteins altered a 2-, 10- and 50µm of secretase inhibitor	2011	[306]
Brain tissue					
Mouse medial prefrontal cortex from mice from MSEW mice (model of early life neglect)	Total protein extract, RNA, DNA. LC-MSMS	Label-free, MRM (6) RT-PCR, microarray, RNA-seq	46 proteins altered in MSEW mice compared to controls. Used MRM, RNA-seq and microarray data on the same brain tissue. MRM confirmed 4 changes	2011	[149]
Psychiatric disease					
Rat frontal cortex. Rats were administered phencyclidine (PCP) at 1 mg/kg (9), 2.5 mg/kg (7) or saline for controls (5)	Total protein extract	MRM (6)	Levels of TP11 and PGAM1 were decreased in frontal cortex. The levels of HK1, ALDOC, GAPDH, ENO2 were no significantly altered	2012	[173]
Neurodegeneration –Parkinson's disease					
Mouse whole brain from mice treated with a glutathione synthesis inhibitor). A mouse stress model of PD and controls.	Mitochondria immuno-affinity purification of Complex I. 1D-SDSPAGE	MRM (269 precursors), D ₅ -NEM and d ₀ -NEM labeling.	Reversible cysteine oxidation of 34 distinct cysteine residues in murine Complex I. 6 cysteine residues increased with GSH depletion	2011	[174]
Neurodegeneration –Alzheimer's disease					
Absolute protein and PTM levels of clusterin in control and AD patients	Total protein extract	QconCATs	Determined absolute protein levels of clusterin in brain tissue	2012	[193]
Human post-mortem temporal lobe tissue from AD patients (11) and controls (4)	Histone purification	TMT (6-plex) labeling, SRM, IB (1)	Specific site of histone acetylation lower in AD patients shown by SRM, IB and TMT techniques	2012	[109]

Table 7

Summary of mass spectrometry-based neuropeptidomics studies

Tissue Source	Methods	Quantitative method	Neuropeptides identified/quantified/ altered proteins	Year	Ref.
Circadian-related peptides in rat suprachiasmatic nucleus	Brain punch, boiled 10 min in water	Label-free (peak intensities)	10 endogenous peptides showed differences between night and day	2013	[317]
Rat striatum after treatment with cyanobacterial toxin	Enzymatic inactivation of brain tissue using Stabilizer	Label-free	25 neuropeptides from 13 precursors were altered in the striatum	2013	[318]
Insect neurons, paraformaldehyde-fixed and immune labeled	Enzyme or heat-induced treatment. MALDI-TOF-MS	None	Profile neuropeptides from single cells (neurons) stored for up to 2 or 42 weeks	2012	[207]
Human pheochromocytoma tissue. Isolated dense core secretory vesicles (DCSV)	Sucrose gradient, 1D-SDS-PAGE gel, LC-MS/MS	Label free (Spectral counts)	Quantified profiles based on soluble and membrane proteins. 600 neuropeptides were identified	2012	[301]
Human pheochromocytoma from adrenal medullary. Isolated SV's from the sympathetic nervous system	Sucrose gradient, 1D-SDS-PAGE gel, LC-MS/MS	None	Identified novel neuropeptides derived from proenkephalin, pro-NPY, proSAAS, CgA, CgB, and SCG2 prohormones	2010	[319]
Human pheochromocytoma tissue. Isolated dense core secretory vesicles (DCSV)	Sucrose gradient, Soluble/membrane fractions, 1D-SDS-PAGE gel, LC-MS/MS	Label-free (normalized spectral abundance factor)	Identified over 600 proteins from DCSV's. Quantified 8 neuropeptides/neurohumoral factors in the soluble and membrane fractions	2010	[320]
Bovine Adrenal medulla. Isolated dense core secretory vesicles (DCSV)	Sucrose gradient, Soluble/membrane fractions, 1D-SDS-PAGE gel, LC-MS/MS	None	371 and 384 proteins identified in soluble and membrane fractions respectively. 69 common in both. Identified 18 neuropeptides in soluble, 19 in neuropeptides in membrane fraction	2010	[321]
Mouse hypothalamus, amygdala, and striatum from prohormone convertase 1/3 null (PC1/3) and WT mice	Microwave-irradiation, Heat extraction, LC-MS/MS	TMAB-labeling (D ₀ , D ₃ -D ₆ -and D ₉)	92 peptides identified, 35 known neuropeptides. Approximately 30% increased, 33% decreased and 33% did not change in PC1/3 null mice	2010	[322]
Mouse brain from prohormone convertase-2 (PC2) knockout and WT mice	Microwave-irradiation, Heat extraction, LC-MS/MS	Stable isotopic tags: H ₉ -TMAB and D ₉ -TMAB	115 peptides quantified. 41 peptides were not detected in PC2 knockout. 24 peptides were decreased by 20–80% in PC2 knockout mice	2010	[323]
Honey bee brain peptides	Acidified acetone extraction, Succinic anhydride isotopic labeling	Isotopic labeling, peak intensities	Quantified 51 brain peptides from 18 precursors	2009	[324]
Mouse hypothalamus and spinal cord, treatment with prolylendopeptidase (PREP) inhibitor or vehicle-control)	Heat extraction and acetic acid extraction, LC-MS/MS	Label-free (Peak areas)	23 peptides altered after 1 h inhibitor treatment	2009	[325]
Mouse pituitary from PREP null mice, heterozygous and homozygous WT controls)	MRM	MRM assay (5 and 7 transitions for MSH (1–13) and MSH (1–12) peptides. IB (1)	Monitored melanocyte stimulating hormone (MSH) levels by MRM Acetylated alpha-MSH (1–13) and alpha-MSH (1–12) is increased in PREP null mice	2009	[326]
Rat striatum in basal or after neuronal KCl depolarization conditions.	<i>In vivo</i> micro-dialysis to collect secretome, LC-MS/MS	Label-free (Spectral counting)	Identified 100 processed peptides and 88 proteins from 29 precursors in the secretome	2009	[327]

Tissue Source	Methods	Quantitative method	Neuropeptides identified/quantified/ altered proteins	Year	Ref.
Mouse amygdala, hippocampus, hypothalamus, PFC, striatum, and thalamus in Cpe (<i>fat/fat</i>) and WT mice	Heat extraction LC-MS/MS	Stable isotopic tags: H ₂ -TMAB and D ₂ -TMAB	111 neuropeptides/peptide identified in WT mouse. Most secretory pathway peptides were lower in Cpe (<i>fat/fat</i>) mouse brain. Other peptides were higher in Cpe mice	2008	[328]
Drugs of abuse					
Rat, (ventral mPFC and core /shell regions of the NAc and dSTR) from amphetamine-sensitized (8) and controls (4)	DHB matrix extraction. MALDI-MS/MS; LC-MS/MS	Label-free (Peak Intensity)	11 peptides altered after sensitization	2012	[329]
Mouse, hypothalamus, treatment with imipramine	LC-MS/MS	Label-free (Peak intensity)	14 peptides altered with imipramine	2012	[330]
Rat, ventral mPFC and the core and shell regions of the NAc and dSTR (cocaine-sensitized rats and control)	MALDI-MS/MS; LC-MS/MS	Label-free (Peak Intensity)	7 peptides altered	2010	[331]
Rat NAc (core and shell) after 14 days of morphine treatment, naloxone-induced morphine withdrawal	Stabilizer-heat inactivation, internal standards added	Label-free	Compared expression 27 neuropeptides	2009	[332]
Neurodegeneration – Parkinson's disease					
Mouse striatum, (unilateral 6-OHDA-lesioned mice with or without sub-chronic L-DOPA administration)	Microwave-irradiation. Acetic acid, heat extraction. LC-MS/MS	Label-free (Peak intensity) IB (1)	15 peptides altered in between treatment groups	2009	[333]

Table 8

Neuroproteomic and neuropeptidomic studies of CSF

CSF source	Methods	Quantitative method	Proteins or peptides identified/ altered proteins	Year	Ref.
Human CSF from MS patients treated with natalizumab		Label-free MRM (4) absolute quantitation	Quantified 301 proteins, 5 altered after natalizumab treatment	2013	[334]
Human CSF from control (n=5) and MS patients (n=5), MRM validation (n=132)	MARS Hu-14 affinity depletion, SCX LC-MS/MS	iTRAQ (4-plex) MRM (20)	11 proteins altered, 600 proteins quantified	2012	[216]
CSF from human preterm infants with post-hemorrhagic hydrocephalus	IgY-14 affinity depletion	Label-free ELISA (1) IB (5)	438 altered proteins compared to controls	2012	[335]
Human CSF peptideome from control subjects with no neurological disorder	Ultrafiltration (30 kDa MWCO) LC-MS/MS	None	730 peptides identified in CSF (originating from 104 proteins)	2012	[336]
Rat CSF from control or experimentally induced autoimmune encephalomyelitis (EAE) by minocycline treatment	LC-MS/MS	MALDI-MS/MS (Peak areas) in SRM mode. SRM (4)	20 proteins altered	2012	[337]
Human CSF from control (4) and patients with diffuse intrinsic pontine glioma (DIPG) (8)	Ultrafiltration (3 kDa MWCO), 1D-SDS-PAGE. LC-MS/MS	Label-free (spectral counting) IB (3) IHC (2)	334 proteins increase in DIPG	2012	[338]
Human CSF were pooled and either incubated with 15 mM glucose or no treatment	Glucose incubation. Glycan enrichment. LC-MS/MS	Stable isotope labeling ratio light:heavy $^{12}\text{C}_6/^{13}\text{C}_6$ glucose	Identified 111-glycation sites/48 glycosylated proteins in untreated CSF. Identified 265-glycation sites/101 glycosylated proteins using 15 mM glucose	2012	[339]
Human CSF from obese women with hypertension compared to non-obese women	Ultrafiltration	ICPL proteins > 10 kDa. Label-free (spectral counting) < 10 kDa. IB (1), ELISA	18 proteins altered in obese women	2012	[303]
Rat CSF from Msc model (autoimmune encephalomyelitis (EAE))	LC-MS/MS	Peak areas SRM (2)	44 proteins altered in EAE animals compared to both control groups	2012	[340]
Non human primate model of anemia	No depletion	iTRAQ (8-plex)	5 proteins altered during anemia, low number of proteins quantified	2012	[341]
Rat CSF after moderate or severe spinal cord injury compared to normal control	SCX, LC-MS/MS	Stable isotope labeling: light (CH_2O), medium (CD_2O) and heavy ($^{13}\text{CD}_2\text{O}$), IB (4)	Identified 42 putative biomarkers of spinal cord injury	2011	[342]
Human CSF individual variation between day and night	Cysteine-reactive covalent capture tag	TMT (6-plex)	Quantified 55 proteins, 11 proteins identified using cysteine affinity	2011	[218]
Human CSF from Neurologic post treatment Lyme disease (nPTLS) (25), chronic fatigue (CFS) (43) and healthy controls	Immunoaffinity depletion of 14 high abundance proteins, SCX, LC-MS/MS	Label-free Peak intensity. Accurate mass and time (AMT) tag	738 and 692 proteins only identified in CFS or nPTLS, (305 in both) but not in healthy controls	2011	[343]
Human CSF from control and patients with stage 2 Human African Trypanosomiasis	Immunoaffinity depletion of 14 high abundance proteins	2-DE and TMT (6-plex) ELISA, IB	73 proteins overexpressed in stage 2 of disease. 2 proteins confirmed by ELISA and IB	2010	[344]

CSF source	Methods	Quantitative method	Proteins or peptides identified/ quantified/ altered proteins	Year	Ref.
Human CSF from healthy controls and subjects	Immunodepletion of 14 high abundance proteins, SCX fractionation, LC-MS/MS	Label-free Peak intensity, Accurate mass and time (AMT) tag	22 proteins altered differences between neurologic patients and healthy controls. 2630 proteins in CSF from normal subjects were identified (56% were CSF-specific)	2010	[215]
Human CSF from healthy individuals	No depletion	Label-free (average peak heights)	126 proteins analyzed for biological variation in CSF	2010	[219]
Human CSF depleted with peptide ligand library	Combinatorial peptide ligand library affinity purification	Label-free (peak areas)	Identified 1,212 proteins, 745 proteins only detected after depletion	2010	[217]
Human CSF from patients with Creutzfeldt-Jakob disease (CJD)		SELDI IB (2)	3 peaks altered in CJD from 72 mass peaks detected	2010	[220]
Human CSF from control and MS patients	No depletion	2D-DIGE, ELISA	910 protein spots altered in MS patients.	2009	[345]
Human CSF from control and patients with CJD	Albumin and IgG depletion	2D-DIGE	5 altered proteins	2008	[346]
Drugs of abuse					
Nonhuman primate CSF, plasma, colon and lymph nodes, 20 day morphine administration	8M urea	Label-free (Accurate mass and time (AMT) tag)	Quantified 253 protein in CSF, 113 proteins altered by morphine treatment	2012	[347]
Psychiatric diseases					
Human CSF from first-episode schizophrenic patients (17) compared to healthy controls (10)	No depletion	2-DE	6 proteins altered in schizophrenia patients	2010	[258]
Neurodegeneration – Parkinson's disease					
Human CSF from patients with PD (12), PD dementia (PDD) (12) and non-demented controls (NDC) (12)	Albumin and IgG depletion. MUDPIT (2D-LC-MS/MS)	ITRAQ (4-plex) MRM assay (14)	16 proteins altered in PD. Only 2 proteins validated in MRM assay	2012	[104]
Neurodegeneration – Alzheimer's disease					
Human CSF from familial AD patients with PSEN1 and APP mutations (14) and control healthy-controls (17)	Immunodepletion of high abundant proteins	Label-free (peak intensities)	56 proteins altered between mutation carriers and controls. 14 protein were previously reported by proteomic studies	2012	[79]
Human CSF from AD patients (159) and healthy-controls (17)	No depletion. CE-MS/MS	Peak Area (AUC) IHC	5 potential AD-diagnosis biomarkers identified	2011	[348]
Human CSF from control and AD patients (n=47 in total).	Immunodepletion of 6 high abundance proteins,	2-DE Validated by ELISA	Identified YKL-40 from 2-DE study. Validated results in new cohort and patients with FTLID using ELISA assay	2010	[349]
Human CSF from AD patients and controls, post-mortem	Albumin and IgG depletion	2D-DIGE	22 altered proteins	2009	[262]
Neurodegeneration – Huntington's disease					
Human CSF and serum samples from HD patients (6) and controls (6)		2-DE, IB (2) ELISA (1)	3 proteins altered in HD patients	2011	[265]