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Subcellular proteomics in neuroscience

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Sample preparation
3.1. Preparation of plasma membrane
3.1.1. Affinity two-phase partitioning of plasma membrane
3.1.2. Enrichment of plasma membrane without prior cell compartment isolation
3.2. Isolation of brain organelles
3.3. Isolation of protein complexes
3.4. Isolation of phosphopeptides
4. Identification of proteins
5. Quantitation of proteins in subcellular proteomes
5.1. Chemical tagging with stable isotope coded reagents
5.1.1. Quantitative proteomics with ICAT and related reagents
5.1.2. Quantitative proteomics with iTRAQ reagents
5.2. Quantitative comparison with a stable isotope coded internal standard
5.3. Quantitative analysis by metabolic labeling with stable isotope labeling
5.4. Label-Free quantitation
6. Perspective
7. References

1. ABSTRACT

The brain is the most complex and dynamically organized organ of the human body, with a high degree of computation capability enabling the execution of a wide spectrum of physiological processes and behaviors. In the past decades a large number of genomics studies have been undertaken to investigate brain function and brain disorders, but despite these efforts many of the underlying molecular mechanisms still remain largely unknown. The implementation of mass spectrometry based quantitative proteomics in recent years enabled to tap into condition-specific protein trafficking and protein interaction that are the key to organelle proteome (dys)function. The technology for neuroproteomics is still evolving; currently there are no standardized protocols. In this review we describe the most commonly used methods to prepare brain subcellular fractions suitable for proteomics analysis, and highlight the various approaches for quantitative neuroproteomics.

2. INTRODUCTION

The brain is the most complex and dynamically organized organ of the human body with a high degree of computation capability, which enables the execution of a wide spectrum of physiological processes and behaviors. For decades, studies from different disciplines have been performed aiming at the understanding of the biological basis of how we perceive, act, learn, think and experience emotion. These efforts aimed also to understand the disordered regulation of molecular processes in a variety of neurological and psychiatric diseases. These studies often focused on single to a few genes or proteins, which yielded insufficient insight into the mechanisms by which neural processes are accomplished.

In the current post-genome era it is possible to carry out functional genomics- or proteomics approaches to analyze the potential contribution of combinations of many gene products to behavior, mental processes and disorders

thereof. An integrative research strategy has been formulated that combines studies at different levels of molecular/cellular and network/system biology in order to explain aspects of behavior and investigating brain disorders. Within this strategy gene expression analysis has caught much attention due to the successful development of micro-array technologies enabling genome-wide assessment of gene expression. However, gene expression analysis of the brain has restricted value; it has no spatial resolution within the morphological polarized neuronal cells and leaves the role of proteins and protein complexes in subcellular compartments untouched. The dynamics of organelle-specific protein expression, protein posttranslational modifications and protein interactions, however, play major roles in nervous system function.

Proteomics technologies in conjunction with biochemical separation of proteins provide powerful tools to analyze the brain and can overcome the limitations of genomics tools. In this respect, a number of proteomics studies have been reported describing the proteomes of cellular organelles in the brains of model animals (1, 2) and human biopsies (3, 4).

Currently, thousands of proteins can be routinely separated and characterized in a single two dimensional (2D) Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) experiment. Chemical tagging reagents, especially the isotope-coded reagents, have been developed for the quantitative analysis of hundreds of proteins simultaneously. Several of these studies have been aimed at the demonstration of technical advancement (5-8), followed by studies focusing on the proteomics analyses of specific brain organelles or subcellular domains as an entry point to understand the molecular mechanisms underlying various aspects of brain physiology and brain disorders. In particular, protein machineries that drive synaptic neurotransmission have been examined extensively (1, 2, 9). Together, it is becoming apparent that quantitative proteomics is establishing as a powerful tool in brain research capable of generating unique insights into neuronal functions.

This review focuses primarily on the technical aspects of organelle neuroproteomics, in particular the quantitative LC-MS/MS analysis. Several recent reviews have summarized the biological significance of neuroproteomics (10-13).

3. SAMPLE PREPARATION

To obtain physiological and behavioral relevant information it is important to focus on specific brain regions rather than the whole brain. For example, studies of learning and memory may focus on hippocampus, drug addiction research on nucleus accumbens and prefrontal cortex, and Parkinson's disease on dorsal striatum and substantia nigra. Furthermore, it is necessary to define which organelles should be studied. Proteomics studies that take whole brain regions for analysis (e.g. 14, 15) are somewhat limited in scope because (A) these studies display predominantly high abundant house-keeping proteins rather than the specific proteins underlying many of the specific features of the nervous system, e.g. proteins involved in synaptic transmission. (B) A proteomics tissue approach does not take into account that the physiological functions of the proteins are likely related to their specific subcellular localization. For example, the α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptors located in the post-synaptic density (PSD) are the ligand-gated ion channels that drive synapse activity in most glutamatergic synapses, whereas AMPA receptors in the dendritic compartment, endosome, or plasma membrane outside of the PSD, may represent the reserve pool of inactive receptors. (C) A proteomics tissue approach will not reveal the dynamically regulated local changes of protein constituents, notably protein trafficking, synaptic *de novo* protein synthesis and/or protein degradation, whereas these processes are important for neuroplasticity (16-18). The success of a quantitative neuroproteomics study therefore depends on the formulation of a working hypothesis with an experimental design that would be adequately handled by the present analytical techniques.

Current neuroproteomics experiments have been focused primarily on two main types of brain organelles, namely the plasma membrane and the synapse. Plasma membrane proteomics is pursued with the assumption that a number of transmembrane proteins with extracelllular domains, in particular receptors and ion channels, play central roles in the cause and treatment of many brain disorders. Synapse proteomics concerns the characterization of proteins or protein machineries of an average synapse. Quantitative proteomics analysis in this context is employed to reveal the global changes of synaptic proteins as a lead to understand the basis of neuroplasticity and brain disorders. As posttranslational modifications and dynamics of protein-protein interaction are known to be key elements in the regulation of neurotransmission, a number of studies have been designed to examine the synaptic phosphoproteome and to reveal synapse protein complexes.

3.1. Preparation of plasma membrane

3.1.1. Affinity two-phase partitioning of plasma membrane

Two-phase partitioning has been established decades ago. Generally, the two-phase system consists of two polymers, polyethylene glycol and dextran, dissolved in an aqueous solution such as Tris buffer. The two polymers do not mingle; polyethylene glycol is enriched in the top phase and dextran in the bottom phase. When the tissue extract is mixed with the two-phase system, plasma membrane is preferentially segregated into the polyethylene glycol phase. The enrichment of plasma membrane however is moderate. Recently, an affinity step is introduced to deplete contaminating membranes (19). The plasma membrane enriched in the polyethylene glycol phase is mixed with a new aqueous phase consisting of dextran conjugated with wheat germ agglutinin. Wheat germ agglutinin interacts with N-acetyl-D-glucosamine and sialic acid containing proteins. As many plasma membrane proteins are glycosylated containing N-acetyl-D-glucosamine and/or sialic acid the plasma membrane as a whole partitions into

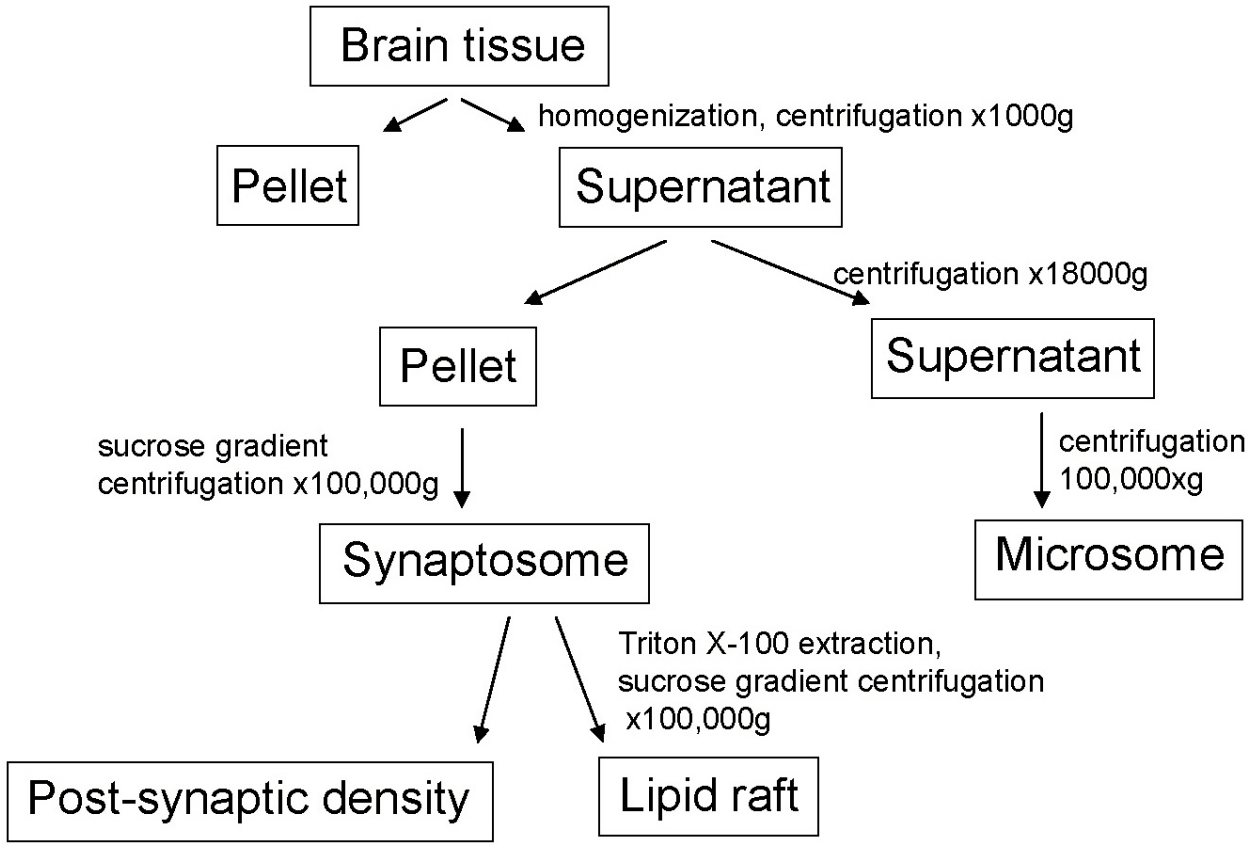

Figure 1. Schematic diagram showing the simplified procedures for the isolation of synaptic sub- domains.

the wheat germ agglutinin conjugated dextran bottom phase. All other membranes remain in the polyethylene glycol top phase. This extra step of purification enriches the plasma membrane content 12-fold, with a strong reduction of mitochondria and endoplasmic reticulum. More than 40% of the identified proteins in this preparation were plasma membrane proteins including numerous transporters, channels, and neurotransmitter receptors. An additional advantage of this approach is that it conserves membrane structure and protein interactions, and may be useful for the subsequent studies of membrane-associated protein complexes.

3.1.2. Enrichment of plasma membrane without prior cell compartment isolation

This method has been optimized to deplete non-plasma membrane proteins from the extract by means of sequential protein denaturing and stripping steps (20). The frozen brain tissue is broken with high speed shearing in high salt. After centrifugation the pellet is agitated at high pH, which disrupts protein-protein interaction and removes bulk of membrane-associated proteins. The solublized proteins are removed by centrifugation. The pellet is treated with chaotrophic reagent, urea, aiming at stripping off the remaining membrane-associated proteins. The plasma membrane is then fractionated by a Percoll-sucrose density gradient centrifugation. Low concentration of a detergent, digitonin, is also included that serves to improve separation of plasma membrane from other membranes. More than 60% of the proteins identified from the plasma membrane fraction were genuine plasma membrane proteins, with only a few proteins derived from mitochondria or endoplasmic membranes.

3.2. Isolation of brain organelles

Due to the central role that synapses play in neurotransmission, synaptic protein constituents have been examined extensively in the past decades. The protocol to isolate synapses and synaptic sub-domains has been well established (21). Recently, several studies have been reported to fine-tune the methods that mainly involve the affinity isolation of distinct synaptic sub-domains with specific antibodies (22, 23). Furthermore, the proteomics approach is increasingly used to identify synaptic protein complexes (9, 24, 25). The flow chart of the isolation procedure is shown in Figure 1.

In general, a brain region of interest is homogenized in 0.32M sucrose that preserves the synapse structures. The sample is then subjected to ultracentrifugation in a discontinuous sucrose gradient to obtain a fraction enriched in synaptosomes. This synaptosome fraction contains the pre-synaptic bouton and the post-synaptic spine. Treatment of the synaptosome with 0.5-1% Triton-X 100 yields a soluble fraction and a fraction containing insoluble structures, the post-synaptic density and lipid raft. The post-synaptic density and lipid raft can be fractionated by another round of sucrose gradient ultracentrifugation (26).

The biochemically isolated synapse and synaptic subdomain preparations still contain contaminating substances derived from other organelles, notably proteins from mitochondria. An extra affinity isolation step may be used to reduce these contaminants (22).

In addition to the synapse, proteomics analyses of several organelles have been reported. This includes mitochondria and its electron transport chain enzyme complexes (27), and the neuromelanin granule (28).

3.3. Isolation of protein complexes

Immunoprecipitation using appropriate antibodies remains the method of choice to isolate protein complexes (24, 25). The synaptic organelle obtained by the procedure as shown in Figure 1 is usually extracted in 1% non-ionic detergent such as Triton X-100 or Nonidet P-40. After centrifugation, the supernatant is used for immunoprecipitation. The immunoprecipitated protein complex can be trypsin digested and analyzed by LC-MS/MS. Alternatively, the proteins are separated first by SDS-PAGE, stained with Coomassie Blue, and the stained protein bands digested and subjected to MS or MS/MS analysis.

The major functional domains of a synapse are the apposing pre-synaptic active zone and the post-synaptic density. The dynamics of post-synaptic density proteins and their specific interaction is thought to underlie synaptic plasticity. The characteristic feature of the post-synaptic density is that it is insoluble in 1% Triton X-100 (29) (Figure 1). Therefore, stronger detergent such as 1% deoxycholine and buffer with higher pH are usually employed to solubilize post-synaptic density proteins (24). This approach has been successfully applied to reveal the protein constituents contained in the large N-methyl d-aspartate (NMDA) glutamate receptor complex (24). The detergent, however, may disrupt protein-protein interactions. The reported disagreement on the nature of the synaptic protein complexes as revealed by immunoprecipitation experiments may be attributed, at least in part, to the use of different detergents for the solubilization of the proteins.

3.4. Isolation of phosphopeptides

Phosphorylation is frequently used by neuronal proteins to switch activity- or conformational states. Various studies have been carried out to identify the phosphorylation sites as the first step to elucidate their function. Recently, techniques have been developed for the global analysis of phosphopeptides. As phosphopeptides are the minor component of the tryptic peptides generated from the proteome of interest, their identification and quantitation are challenging. In general, the sample is digested and the peptides are partially separated by ion exchange chromatography. The phosphopeptides contained in each fraction are affinity isolated from the bulk of other peptides with an affinity matrix, for example by immobilized metal affinity chromatography. The phosphopeptides are selectively enriched via their higher affinity towards metal ions such as Fe3+ and Ga3+. More recently, titanium dioxide chromatography was developed as an althernative for enrichment of phosphopeptides. It is reported to have lower non-specific binding of acidic peptides than that of immobilized metal affinity chromatography.

After elution at high pH the phosphorylated peptides are fractionated with nano-reversed phase liquid chromatography and analyzed by mass spectrometry. Up to a thousand phosphopeptides from a post-synaptic density preparation have been identified in a single experiment (30). In addition to conventional collision induced dissociation MS/MS, multi-stage activation and MS^3 analysis, phosphopeptides can also be detected by electron-capture dissociation MS/MS.

A recent study has investigated the ability of three phosphopeptide isolation methods (phosphoramidate chemistry, immobilized metal affinity chromatography and titanium dioxide) to isolate phosphopeptides from complex mixtures (31). The methods differed in the set of phosphopeptides isolated. The results suggest that the three methods detect different, partially overlapping segments of the phosphoproteome. Thus, an interim conclusion is that multiple approaches for the analysis of a single sample should be followed in order to have a better coverage of the phosphoproteome of interest.

4. IDENTIFICATION OF PROTEINS

Today, mass spectrometry is the method of choice for the structural characterization of proteins. However, the number of distinct proteins even in the purified plasma membrane or in synaptic subdomains is high; several hundreds to thousands of different proteins may be present. A two-dimensional separation procedure is needed to reduce the sample complexity to a level that it can be handled by mass spectrometry. The most commonly used techniques are 2-D gel electrophoresis and 2-D LC. While 2-D gel electrophoresis is excellent in the separation of most cytosolic proteins, the membrane proteins and basic proteins are poorly resolved (29). On the other hand, the 2-D LC approach fractionates tryptic peptides digested from the protein pool of the sample. As digestion of a single protein yields multiple distinct peptides, it is reasonable to expect that a number of these peptides are amendable for LC-MS/MS analysis. Indeed, membrane proteins, high MW proteins and proteins with extreme PI values have all been characterized by the 2-D LC MS/MS approach (1, 9, 29, 32). In most cases, the first liquid chromatography step involves separation of peptides with a strong cation exchange column. This column generally has good binding capacity but moderate separation efficiency. In the second dimension a high resolution nano-reversed phase liquid column is used. The small internal diameter of the reversed phase column, typical ranged 75-100 μm, concentrates peptides into a small volume, which facilitates their detection in the mass spectrometer.

There are several popular search engines for the identification of peptides based on the tandem mass spectra, for example, Mascot and SEQUEST. The SEQUEST search engine compares the experimental

MS/MS spectrum with a theoretical mass spectrum, from which a (confidence) score is calculated for the assignment of the protein identity. Mascot on the other hand uses a probabilistic algorithm. From a data set, the use of different search engines identify an overlapping set of peptides, and peptides that are scored uniquely by the individual search engines (33). The search results from several search engines may be combined to increase the confidence of the protein hit.

The identification of the peptides critically depends on the quality of the database. The NCBI database is comprehensive but contains considerable redundancy. Swissprot has no redundancy; however, it is a smaller database. We routinely use Swissprot as a first-pass data analysis tool. Mass spectra that are not identified are subjected to a second round of analysis with the NCBI database, yielding an extra 10% protein identity hits. The European Bioinformatics Institute International Protein Sequence database (IPI) is another widely used database in proteomics. Recently, a new MSIPI database has been developed (34; ftp://ftp.ebi.ac.uk/pub/databases/IPI/msipi). The C-termini of the protein sequences from the IPI database are appended with new sets of tryptic peptides that include the N-terminal peptide after removal of the signal peptide. It also contains the peptides corresponding to the precursor regions that carry single nucleotide polymorphisms or conflicts in the reported protein sequences that are not included in the original protein sequences in the IPI database. The use of this extended MSIPI database allows identification of a larger number of peptides.

5. QUANTITATION OF PROTEINS IN THE SUBCELLULAR PROTEOMES

In recent years a number of strategies for quantitative (subcellular) proteomics have been reported. In particular, stable isotope labeling in conjunction with (multidimensional) LC-MS/MS has been successfully implemented in molecular brain research. With current technology, a chemical stable isotope tagging strategy may be considered as the method of choice to examine the changes of protein constituents in brain organelle extracts. Quantitative comparison of samples with a stable isotope coded internal standard is another approach. There are several variants of internal standards of which proof of principle of the approach for neuroscience studies has been demonstrated. Some of these approaches seem to hold great promise for large scale relative and absolute quantitation of organelles. Finally, label-free quantitation has been applied in several occasions.

5.1. Chemical tagging with stable isotope coded reagents

There are several classes of stable isotope coded reagents for chemical tagging of proteins and peptides. The Isotope Coded Affinity Tags (ICAT) was the first introduced stable isotope chemical tagging technique for quantitative proteomics. Recently, Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) is gaining popularity. The uses of several alternative reagents are also reported, including ExacTag, Isotope coded protein labeling (ICPL) and HysTag.

5.1.1. Quantitative proteomics with ICAT and related reagents

ICAT reagent consists of a biotin moiety and a thiol reactive moiety that is separated by an ethylene glycol linker (35, 36). The substitution with stable isotopes ^{13}C in the linker region creates the heavy form, which is 9 Da heavier than the isotopically light form. The two ICAT forms can be used to label proteins from two samples at the side chain of reduced cysteinyl residues. The samples are then mixed and digested with trypsin. The ICAT tagged cysteine-containing peptides are affinity isolated with an avidin column, which retains the biotin moiety of the ICAT reagent. After elution from the column peptides are fractionated by liquid chromatography and then subjected to mass spectrometry. The same peptides from the two samples will be identified as doublets in the mass spectra and they differ by 9 Da. The relative quantities of the proteins are inferred from the ratio of the peak intensities of the peptide doublets. The sequence of the peptide is determined from its tandem mass spectrum. As only cysteine-containing peptides are examined, this approach greatly reduces sample complexity. However, proteins with no or low number of cysteine residues will be missed. Peptides with posttranslational modifications are generally not detected, unless the peptides also contain cysteine residues.

ICAT reagents have been used to distinguish contamination in a synaptic organelle preparation, the post-synaptic density (32). It is well known that the biochemically isolated post-synaptic density preparation is not free from contaminants; some of the identified proteins from the preparation may be derived from other organelles. A correlation profiling experiment on post-synaptic density proteins and synaptic membrane proteins has been performed in order to identify contaminants. The post-synaptic density and the synaptic membrane were labeled with ICAT heavy and light reagents, respectively. Comparison of the ratio of the ICAT doublets indicated that glutamate receptors and scaffolding proteins that are core post-synaptic desnity proteins were enriched in the PSD preparation. Mitochondrial proteins and transporters were generally strongly depleted, indicating that they were likely contaminants of the post-synaptic density preparation.

Another ICAT-like reagent is HysTag. This reagent is used for stable isotope protein labeling at thiol moieties and used for peptide complexity reduction. It is a 10-mer derivatized peptide consisting of a His-tag for affinity isolation, a tryptic cleavage site, an Ala residue of which the heavy form contains 4 deuterium atoms, and a thiol-reactive group. This approach has been applied for the relative quantitation of membrane proteins in the mouse brain cortex, hippocampus and cerebellum (37). The study demonstrated good correlation between the proteomics data and the mRNA expression levels or intensity of immunostaining.

Alternatively, ICPL may be used (38). The reagents also contain the heavy and light forms that differ by 6 Da. Instead of a cysteine residue, the reactive group of ICPL forms covalent bonding with lysine. It can be used for the interrogation of nearly all the peptides in a sample including those containing posttranslational modifications.

5.1.2. Quantitative proteomics with iTRAQ reagents

iTRAQ is one of the widely used chemical tagging reagents for quantitative proteomics analysis. The iTRAQ reagents are the multiplexed, amine-specific, stable isotope reagents that can label all peptides in different biological samples enabling simultaneous identification and quantitation (39). In addition, peptides with posttranslational modifications are not lost. The original iTRAQ reagents are 4-plex. Recently, the 8-plex iTRAQ reagents have been developed, which can label total peptides in up to eight different biological samples. In a typical experiment that compares the proteomes from two experimental groups, 4 independent biological replicates per group can be used. This provides a reasonable number of replicates for statistical analysis within a single set of experiments.

iTRAQ reagents are isobaric tagging reagents consisting of a reporter group, a balancer group, and a peptide reactive group. The peptide reactive group of the iTRAQ reagents forms a covalent bond with lysine or the N-terminal group of a peptide. All the peptides in a given protein sample digest are tagged with one of the iTRAQ isobaric tags. After tagging, peptides from all the samples are pooled, separated by (2-D) LC and individual peptides subjected to MS/MS analysis. During MS/MS, the isobaric tag is fragmented that generates the signature ions from the reporter groups of the 4-plex iTRAQ reagents at the low mass region of 114 to 117 Da. The intensities of the signature ions are directly correlated to the amount of peptide. The y- and b-ion series of peptide fragments are used for protein identification.

Recently, we have optimized iTRAQ methodology for comparative synaptic proteome analysis. In this study proteins of the synaptic membrane fraction isolated from the hippocampus of wild type mice and mutant mice (3'UTR-calcium/calmodulin-dependent kinase II α mutant in which CaMKIIα can not be locally synthesized in the synapse (40)) were examined (1). Synaptic proteins were solubilized in 0.85% RapiGest, digested with trypsin without prior dilution of the detergent, and the peptides from two groups of wild type mice and two groups of CaMKIIα 3'UTR mutants were tagged with iTRAQ reagents 114, 115, 116 and 117, respectively. The experiment was repeated once with independent biological replicates to obtain a sample number of n=4, which is necessary for statistical analysis of the difference of protein expression between the two groups. Peptides were fractionated with 2D LC, and collected off-line onto MALDI metal plates and analyzed on an ABI 4800 MALDI tof/tof mass spectrometer. There was a 3 fold decrease of CaMKIIα in the synaptic membrane fraction of the 3'UTR mutant mice. No other major changes were observed suggesting that the protein constituents of the synapse of the mutant mice were not substantially altered as a result from the deficient local translation of CaMKIIα.

5.2. Quantitative comparison with a stable isotope coded internal standard

In this approach, relative or absolute quantitation of peptides is achieved by the comparison of the MS ion intensities of the peptides of interest with chemically identical internal peptide standards that contain stable heavy isotopes. Several strategies have been developed, including the Absolute quantification of protein (AQUA), Culture-derived isotope tags (CDITs), Protein standard absolute quantification (PSAQ) and Concatemer of standard peptides for absolute quantification (QconCAT).

AQUA uses a stable isotope coded internal peptide standard that is synthesized by chemical methods (41). A pre-determined amount of the peptide standard is spiked into the sample, followed by LC-MS analysis. Because the concentration of the peptide standard is known, the comparison of the MS peak intensities of the isotope coded peptide standard to the native peptide yields the absolute quantity of the native peptide/protein. This method has been applied to examine the molar abundance of 32 key post-synaptic density proteins in forebrain and cerebellum, including receptors, scaffold proteins and signaling molecules (42). One example is the relative stoichiometry of AMPA receptor subunits in the post-synaptic density, from which the ratio among AMPA receptor subunits 1/2/3 (94:100:19) as measured by AQUA is found to be similar to that measured from the purified AMPA receptor complex (43). One limitation of the AQUA approach, however, is that it does not take into account the actual digestion yield of different peptides from the native protein. Furthermore, for global analysis of a tissue proteome it requires the synthesis of large number of expensive stable isotope coded peptide internal standards. CDITs, PSAQ and QconCAT, on the other hand, can produce large number of isotope coded peptide standards, and may be more appropriate for quantitative proteomics.

The QconCAT approach requires the production of a protein consisting of an artificial concatamer of standard peptides corresponding to the native peptides of interest predicted from different proteins (44, 45). Distinct peptides derived from more than 40 proteins have been synthesized within a single artificial QconCAT protein. The gene encoding the QconCAT protein is inserted into a high-level expression vector, expressed in *Escherichia coli*, and cultured in medium containing selected stable isotope amino acids. The QconCAT protein is isolated, mixed with the sample, and digested to release the isotope coded heavy peptides from the QconCAT protein and the native peptides from the native proteins. Quantitation is made by comparison of the MS peak intensities between the heavy and light forms of the same peptide. One concern of this approach is the potential differences in (trypsin)

digestion efficiency between the QconCAT protein and the native protein. This factor may yield over- or under-estimation of the absolute protein quantity in the sample, but it should have no negative effect on relative protein quantitation across multiple samples using the QconCAT peptides as internal standard.

The PSAQ strategy is based on the use of synthesized isotope labeled full-length proteins as standards for quantification. This solves the potential problem of differences in digestion efficiency (46). For the analysis of the proteome, the synthesis of large number of full-length proteins will be needed. In a earlier study (47), six isotopically labeled recombinant proteins corresponding to the full-length or partial sequence of the native synapse proteins were mixed with proteins of the post-synaptic density preparation and separated on a short SDS gel. All proteins were digested. Peptides were quantified and characterized by LC-MS and MS/MS analysis. PSD-95 is 5-fold, 4-fold and 7-fold more abundant than NMDA receptor 1, Guanylate kinase-associated protein and Shank1, respectively. This study highlights the fact that the PSD-95 family of proteins probably function as post-synaptic scaffolds for many other proteins in addition to the NMDA receptors.

The CDITs approach can produce a much larger number of stable isotope coded peptide standards than QconCAT. To obtain isotope labeled peptide standards that are useful for neuroscience studies, Neuro2A cells are cultured in a stable isotope enriched medium (48). Brain tissues from different samples are then mixed with a fixed amount of stable isotope tagged Neuro2A cells, and then subjected to protein extraction, digestion and LC-MS analysis. The ratio of a doublet corresponding to the isotopic tagged heavy form and the isotopic normal light form of a peptide is derived from their MS peak intensities. Changes of protein levels in two tissue samples can be determined by calculating the ratio of the two ratios, a procedure that cancels out the internal standards. Using CDITs about 1000 proteins have been quantified, 97-98% of which were expressed in both mouse whole brain and Neuro2A cells. This approach, however, increases the overall sample complexity by about two-fold because each peptides is represented by doublets. Therefore, the approach is more suitable for the analysis of samples with lower complexity, in particular subcellular proteomics. However, the organelles isolated from Neuro2A and the brain regions of interest may not match completely. Alternative neuronal cell lines that are a better match of the brain region of interest should then be explored.

5.3. Quantitative analysis by metabolic labeling with stable isotope labeling

Stable isotope labeling by amino acids in cell culture is one of the most widely used methods for MS-based quantitative proteomics analysis of cells culture *in vitro*. However, this method is generally not applicable for the analysis of a tissue proteome. Recently, metabolic ^{15}N labeling of rat brain for quantitative proteomics has been reported (49). A female rat was fed a ^{15}N diet after weaning, remaining on the ^{15}N diet through its pregnancy, and while weaning its pups. With this feeding regime the ^{15}N enrichment in the synapse of the pup was determined to be 94%. To examine the developmental changes of the synaptosome proteome, rat brain synaptosomes at four postnatal development time points were prepared and mixed with the ^{15}N labeled synaptosomes. The $^{14}N/^{15}N$ ratios of the peptides yielded quantitative data of the corresponding proteins; 324 proteins were quantified from which 82 proteins were up-regulated and 67 proteins were down-regulated during development.

5.4. Label-Free quantitation

This approach is based on the observation that peptide ion intensities generated from electrospray ionization mass spectrometry correlate with the concentration of the peptides, and may be used for comparative analysis of the same peptides from different samples that are separately analyzed by LC-MS and LC-MS/MS. Label-free approaches place a high demand on the robustness and reproducible of the LC-MS/MS system, and sophisticated data analysis programs. For complex samples containing several hundreds to thousand proteins, proteins are separated first by SDS-PAGE. The gel is cut into several pieces, digested, and peptides from each of the gel pieces are subjected to label-free LC-MS/MS quantitation. 2-D LC MS/MS label-free quantitation has also been applied to examine the differences of protein expression between the membrane preparations of mouse fore- and hindbrain (50). About 1000 proteins were quantified. Proteins that show large differences between the samples were discerned. For example, AMPA receptors 1-3 were more abundant in forebrain, whereas AMPA receptor 4 was more abundant in hindbrain. The transmembrane AMPA receptor regulating protein isoform, voltage-dependent Ca2+ channel ç-8 subunit, was more abundant in forebrain, whereas voltage-dependent Ca2+ channel ç-2 subunit was abundant in hindbrain. These results are generally in agreement with the known mRNA expression levels of the proteins, and comparable to that obtained with the HysTag approach.

6. PERSPECTIVE

The advancement in methodology and upgrading of instruments in the years to come will no doubt allow a further more detailed analysis of brain sub-proteomes. Currently, multiplexed stable isotope reagents enable quantitative analysis of up to eight samples. There is no major technical hurdle to develop reagents for quantitation of tens of samples simultaneously. Multiplexed sample analysis in conjunction with the increase in mass spectrometer sensitivity and dynamic range will contribute significantly to the establishment of large-scale quantitative neuroproteomics. It is expected that techniques for organelle isolation will be refined further. Together, global subcellular proteomics will shed light on the dynamics of protein expression and posttranslational modifications, protein trafficking in and to cellular sub-domains, and protein interactions. No doubt this will lead to novel insights into the relationship of processes at the protein

level with specific cellular functions of the brain and their dysregulation in brain disorders.

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Abbreviations: 2D: two dimensional; AMPA: α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid; AQUA: Absolute quantification of protein; CDITs: Culture-derived isotope tags; ICAT: Isotope Coded Affinity Tags; iTRAQ: Isobaric Tag for Relative and Absolute Quantitation; ICPL: Isotope coded protein labeling; LC: liquid chromatography; MS/MS: tandem mass spectrometry; NMDA: N-methyl d-aspartate; PSAQ: Protein standard absolute quantification; QconCAT: Concatemer of standard peptides for absolute quantification

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