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TRENDS

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Proteomics of synapse

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Introduction

The brain is the most complex and dynamically organized organ with a high level of computational ability that enables the acquisition and integration of information in order to generate appropriate responses to environmental and physiological inputs. Pivotal to its function is its extensive neuronal connectivity, due in particular to the 10^{15} synaptic connections of the 100 billion central neurons of the human brain, via which chemical neurotransmission occurs. In brief, an action potential travels along the axon and reaches the presynaptic bouton, causing the neurotransmitter-containing synaptic vesicles to fuse with the membrane in the active zone. The released transmitter diffuses through the synaptic cleft, binds to the corresponding receptors and causes ion influx into the dendritic spine that ultimately drives neuronal activity. In nearly all neurons, both short- and long-term plastic changes can take place. Depending on the preceding neuronal activity, the synaptic strength can be enhanced or depressed; this is underpinned by molecular changes in the synapse, including alterations of protein expression pattern, post-translational modifications and protein interacting with partners.

With the advancement of proteomics technology, it is now possible to define the whole protein constituent of the synapse. Proteome analyses of synaptosome, synaptic membrane and pre- and post-synaptic compartments of the glutamatergic synapse revealed a surprisingly high complexity in the synapse, which possibly contains thousands of distinct proteins. Recent studies further revealed that protein phosphorylation is a common event in the synapse, which is consistent with the presence of diverse classes of kinases and phosphatases in the synapse. Quantitative synaptic proteomics is essential for deciphering the molecular changes in the synapse. Several laboratories are initiating this technically demanding task, and I anticipate that it will be a major research line in the coming years.

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Technologies for synaptic proteomics

Synaptic proteomics analysis requires, first of all, the purification of subcellular organelles from the brain regions of interest. The isolation of synaptosome, synaptic membrane and synaptic subdomains such as post-synaptic density (PSD) is well-established. Generally, multiple steps of discontinuous density gradient ultra-centrifugation are employed to enrich the distinct organelles. In special cases where high spatial resolution are needed—for example, in the proteomics analysis of neurofibrillary tangles in Alzheimer disease [1]—laser capture microdissection has been employed to collect the samples. To reveal the protein interaction networks in the synapse, a number of multi-protein complexes, including NMDA receptor and AMPA receptor protein complexes [2], have been isolated by means of peptide- and antibody-based affinity purification.

Contaminants are often present in the samples, and they need to be taken into account when interpreting the proteomics data.

All current proteomics methodologies have been used to analyze the proteomes of synapse and synaptic subdomains.

Gel-based proteomics

SDS polyacrylamide gel electrophoresis and mass spectrometry The first proteomics study used SDS gel electrophoresis to separate PSD proteins. Proteins were characterized based on the peptide mass fingerprint generated from the tryptic digests of each protein band. This approach is useful for the analysis of a single protein

or (at the most) a few proteins per protein band. In this early study about 30 proteins were characterized [3].

In order to address the limitations of peptide mass fingerprint analysis, recent studies have introduced one or two sequential liquid chromatography (LC) step(s) in order to separate the tryptic peptides, followed by tandem mass spectrometry (MS/MS) for structural characterization. This approach identified 10–20 times as many proteins [4, 5]. Isotope-coded reagents (ICAT) and AQUA peptides (see below for methodology) were used to quantify the samples [5].

Two-dimensional gel electrophoresis and mass spectrometry Two-dimensional gel electrophoresis has been used to separate and quantify proteins, including post-translational modified forms, from synaptic structures [6]. Proteins spots were characterized by (tandem) mass spectrometry. A disadvantage of 2-D gel is that it has only limited ability to fractionate hydrophobic proteins, proteins larger than 100 kDa, and very basic and acidic proteins. Consequently, many major players in synapse physiology, such as receptors and ion channels, which are generally large proteins containing many trans-membrane domains, cannot be analyzed.

Gel-free proteomics

The most commonly used gel-free proteomics consists of tryptic digest of the synaptic proteome, separation of the peptides by two or more LC steps, and characterization of the peptides with MS/MS. Using this approach, several hundreds to over a thousand synaptic proteins have been described [7, 8]. Post-translational modified peptides can also be characterized using this approach. Generally, an extra chromatography step is incorporated to isolate the post-translational modified peptides; for example, immobilized metal affinity chromatography (IMAC) is widely used for the enrichment of phosphopeptides [7, 9].

Three approaches have been used for quantitative synaptic proteomics: the isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), and absolute quantification (AQUA) strategies.

Protein AQUA A known amount of a set of isotope-labeled synthetic peptides that correspond exactly to the selected tryptic peptides of the proteins contained in a sample will be mixed with the sample, separated and mass spectrometrically analyzed together. The synthetic peptides serve as an internal standard. The quantity of native peptides can be calculated using mass spectrometric peak ratios. A very large number of isotope labeled peptides will have to be synthesized to quantify the synapse proteome.

ICAT The cysteine residues of the proteins are tagged with ICAT reagents. There are two ICAT reagents, the heavy and light versions that differ in mass by 9 Da. One sample is labeled with ICAT heavy reagent, and the other sample with ICAT light reagent. The two samples are then mixed together, digested, and the ICAT-tagged peptides affinity-isolated by an avidin column. The mass spectrometric peak ratio of a peptide tagged with light and heavy ICAT reagents from the two samples, respectively, reveals the relative expression level of the corresponding protein. Obviously, proteins that do not contain cysteine residue will not be detected.

iTRAQ iTRAQ reagents are a multiplexed set of four isobaric reagents that are identical in mass. Tryptic peptides from up to four samples can be tagged with the four isobaric reagents respectively in a single experiment. Upon the collision-induced dissociation of a tagged peptide, signature ions will be produced for each iTRAQ reagent. The peak ratios of the four iTRAQ signature ions represent the relative quantities of this peptide contained in the four samples. As quantitation is coupled to MS/MS analysis of the peptides, the analysis of a sample of high protein complexity may be difficult due to the high demands placed on precursor ion selection in order to avoid the overlapping of the same signature ions produced from distinct peptides of similar masses eluting in the same LC fractions. Furthermore, a large number of MS/MS analyses will have to be performed. In a typical experiment, the abundant proteins are preferentially selected for analysis. Therefore, proteins at lower expression levels may be missed.

The proteomes of the synapse and synaptic subdomains

Post-synaptic density

In the past three years, many publications have reported the characterization of the proteome of PSD [2, 4–8, 10, 11]. The number of proteins identified have ranged from around a hundred to over a thousand. In general, proteins identified from PSD preparation can be classified into a number of functional groups: groups including ion channels and adhesion molecules, the scaffold and signaling protein complexes and the receptors, proteins of the cytoskeleton and their interacting partners for the maintenance and modulation of synaptic architecture, proteins involved in sorting and trafficking of membrane proteins, components for energy supply, the proteasome system for specific synaptic protein degradation, the chaperone system for correct protein folding and the local protein synthetic machinery. This is consistent with our knowledge that PSD is one of the most complicated subcellular structures in the cell and furthermore appears to have the capacity to function in a (semi-)autonomous manner.

Although a large number of proteins have been identified from PSD fractions, it should be realized that not all of these proteins are contained in each PSD. First, excitatory synapses are extremely heterogeneous in nature. The proteins identified may represent the sum of the proteins present in PSD from all synapses in the brain region under study. Second, the biochemical isolation procedure merely enriches the organelle of interest, i.e. the PSD preparation may contain a considerable amount of

contaminants. To address this problem, ICAT-based correlation profiling has been carried out on PSD proteins and synaptic membrane proteins [12]. As expected, core PSD proteins such as glutamate receptors and scaffolding proteins were enriched in the PSD preparation. Other groups of proteins with various functions, such as cytoskeleton-associated proteins, protein kinases and phosphatases, components and regulators of signaling pathways, and proteins involved in energy production may be associated with multiple organelles and multiprotein complexes, and these were consequently, as groups, enriched in the PSD fraction to a lesser extent. Mitochondrial proteins and transporters were generally strongly depleted, indicating that they were likely contaminants of the PSD preparation.

Pre-synaptic compartment

A proteomic analysis of the synaptic vesicle identified 36 proteins, including seven integral membrane proteins and vesicle regulatory proteins [13]. Unlike synaptic vesicle, it is technically difficult to obtain a pre-synaptic membrane preparation. In one study, monoclonal antibodies were used to purify two synaptic vesicle-containing fractions from a synaptosome preparation, and 72 proteins were identified from the free vesicle and 81 proteins in the plasma membrane-containing fraction [14]. It was concluded that the latter fraction contained synaptic vesicle proteins, components of the pre-synaptic fusion and retrieval machinery and other proteins potentially involved in regulating the functional and structural dynamics of the nerve terminal. In another study, a presynaptic particle fraction and PSD was obtained by sequential extraction of synaptosome in Triton X-100 firstly at pH 6 and then pH 8 [11]. The presynaptic particle fraction contained a high representation of proteins that reside in, or mediate the trafficking of, the intracellular compartment as well as signaling molecules.

Synaptic proteome

Several hundred to a thousand proteins have been identified from synaptosome preparations [15, 16]. Curiously, the number of proteins characterized from PSD [7] is larger than that from synaptosome. As PSD is a subdomain of synaptosome, it must be concluded that the lower number of proteins identified from the synaptosome proteomics studies is most likely due to methodological differences. The use of more starting material and a more extensive separation of tryptic peptides before MS/MS will increase the number of synaptic proteins identified.

Synaptic phosphoproteome

Large-scale phosphoproteome analyses have been carried out on synaptosome and PSD preparation. In 2005, a study used IMAC for the enrichment of both phosphoproteins and phosphopeptides from a synaptosome extract, followed by LC–MS/MS analysis. This study reported the identification of 288 phosphorylation sites, representing 79 synaptic proteins [9].

Recently, nearly 1000 phosphorylated peptides from 287 proteins were identified from PSD preparation [7]. Notably, the phosphorylation sites of glutamate receptor subunits were also detected. Interestingly, this study followed a conventional approach for the analysis of phosphopeptides; i.e. sequential separation of peptides with ion exchange chromatography, IMAC and reversed-phase liquid chromatography, followed by MS/MS analysis. The success of this study relied on the maximization of the separation of the peptides through the use of a large number of fractions collected from cation exchange chromatography and the detailed analysis of each fraction by LC–MS/MS.

Quantitative synaptic proteomics

Performing quantitative proteomics is a challenging task, and only a few studies have been reported. In one study, ICAT reagents were used to study the alteration of synaptic membrane proteins after chronic morphine administration, and this revealed changes in several synaptic plasma membrane proteins [17]. In another study, both ICAT reagents and AQUA peptides were used for the comparative analysis of PSD isolated from forebrain and cerebellum [5].

Currently, we are using iTRAQ reagents to examine the differences in the expression patterns of synaptic membrane proteins in the hippocampi of wild-type and various mutant mice that have well-defined behavioral abnormalities [18]. In a typical study it is possible to assign 1000–1500 protein clusters (non-redundant protein sequences). For quantitative purposes we only consider proteins that are matched with at least three peptides with the highest CI % of a peptide (>95%). Generally, 300–600 proteins can be quantified. In a mutant mouse for which the mRNA of calcium/calmodulin-dependent protein kinase II α cannot be targeted to the synapse and therefore cannot be synthesized de novo locally, we detected a 70% decrease in this kinase in the synaptic membrane of hippocampus. Finally, a first draft of a synaptic protein interaction network has been constructed using commercial available software—Ingenuity Pathways Analysis—and the synaptic proteins have been organized into 13 (interconnecting) functional groups belonging to the pre- and post-synaptic compartments, including receptors and ion channels, scaffolding proteins, cytoskeletal proteins, signaling proteins, adhesion molecules, and proteins of synaptic vesicles as well as those involved in membrane recycling.

Outlook

Synapse proteome studies have produced a wealth of qualitative data so far. In the near future, quantitative

models will need to be constructed in order to explain how a complex protein network drives synaptic functions in the brain. In order to generate experimental data and to construct models in a concerted manner, it is important to iterate between system analysis, model construction and system prediction. Quantitative synaptic proteomics describing the changes in synapse proteome in response to environmental, genetic and/or pharmacological perturbations will be fed into the models for adjustment purposes, and to identify new elements to test during a new round of experiments. I foresee that bioinformatics will play a major role when analyzing the vast number of datasets involved, and when constructing and testing the models.

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