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Proteomic analysis of synaptic structures

Karl-Heinz Smalla[1], Ka wan Li[2], August B. Smit[2] and Eckart D. Gundelfinger[1]

[1]Leibniz Institute for Neurobiology, Brenneckestrasse 6, D-39118 Magdeburg, Germany;
[2]Vrije Universiteit, Department of Molecular and Cellular Neurobiology, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

Abstract
Proteomics is the large-scale analysis of protein expression in a biological system under defined conditions or factors which determine the expression level of proteins. This is complementary to DNA or RNA based genomic approaches and can lead to valuable information about the cellular function of proteins. The introduction of proteomics technologies into neuroscience research has now given new opportunities to study fundamental questions in neurotoxicology, neurometabolism and to investigate the molecular basis of brain function and diseases or mental disorders. Using proteomic protein profiling a more comprehensive view of functional diversity and specialisation of different brain regions, subregions, cell types or even organelles will broaden our understanding of brain physiology and pathology. The basic element to study mechanisms of neuronal plasticity is the synapse. Therefore this study focusses on proteomic approaches to different types of synaptic structures or biochemically defined synapse-enriched fractions.
Key words: synapse, synaptosome, postsynaptic density, proteomics, two-dimensional gel electrophoresis, mass spectrometry

Introduction

Proteomics as a global analysis of proteins has opened a wide range of new opportunities to study the protein composition in different brain regions, cell types or distinct subcellular structures by protein profiling. Moreover, proteomics provides excellent tools to investigate differentially regulated compositions of protein complexes thus determining functional implications of given proteins under defined experimental conditions. The technology basically consists of two-dimensional gel electrophoresis, in-gel digestion of protein spots and subsequent identification by mass-spectrometry. This allows not only to determine all proteins expressed but might disclose in addition posttranslational modifications of these proteins. The first part of this presentation addresses biochemical methods for enrichment or purification of synaptic structures. Then we discuss the proteomic work flow with emphasis on technical details of two-dimensional gel electrophoresis and mass spectrometry. Finally comments will be given on new strategies in proteomic analysis.

Biochemical preparation of synaptic structures

Chemical synapses are highly specialised asymmetric cell-cell contacts between neurons (Fig. 1). The presynaptic side contains a specialised secretion machinery for activity-dependent release of neurotransmitters into the synaptic cleft. At the postsynapse neurotransmitter receptors and the downstream signal transduction apparatus are organised mainly by the post-synaptic density (PSD) which is visible on electron micrographs as an electron-dense structure (Fig. 1B and C). Several protocols have been developed to purify postsynaptic densities, the most accepted procedure was first described by Carlin et al. 1980 and is still used to purify synaptosomes, synaptic membranes or postsynaptic densities with only slight modifications (Gundelfinger and tom Dieck 2000, Li et al. 2004). The most crucial step during this purification is the introduction of a sucrose step gradient to separate synaptosomes or synaptic membranes resp. from other subcellular organelles (Fig. 1A). Similar protocols have been used to prepare

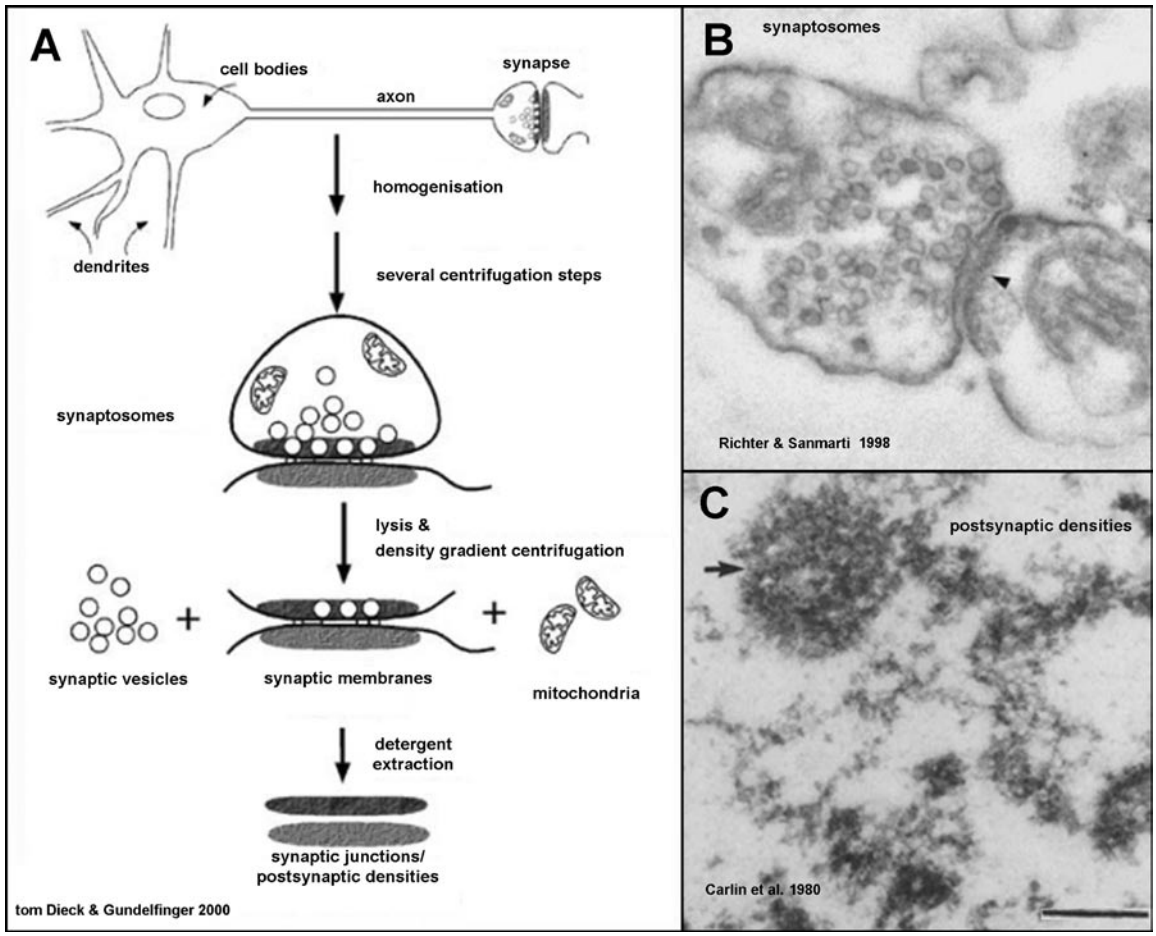

Fig. 1. Preparation of synaptic structures. (A) brain tissue is homogenised and after removal of cell debris, nuclei, cytosol and microsomes via differential centrifugation synaptosomes can be derived. Synaptosomes are lysed by hypoosmotic shock and after density gradient centrifugation synaptic membranes can be separated from synaptic vesicles and mitochondria. Synaptic membranes are delipidated by detergent extraction to yield the postsynaptic density (PSD) fraction. (B) Electron micrograph of a synaptosome with the same magnification as in (C) (Richter and Sanmarti 1998). The arrow points to the PSD. (C) Electron micrograph of a PSD fraction, scale bar 1 μm (from Carlin et al. 1980)

synaptodendrosomes which are analogue to synaptosomes but have membrane-enclosed compartments also at the postsynaptic part (Rao and Steward et al 1993). Since in several experimental approaches, e.g. experiments in cell cultures or with hippocampal slices, the amount of protein available for analysis is limited due to experimental design, other methods were introduced to obtain so-called "PSD enriched" fractions (e.g. Smalla et al. 2000).

Two-dimensional gel electrophoresis (2DE)

The most mature platform for large-scale analysis of proteins is still 2DE due to the unique resolving power arising from the combination of two high-resolution electrophoretic techniques, isoelectric focussing (IEF) and sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the first dimension proteins are resolved according to their isoelectric point. General problems of IEF have been overcome since focussing gels with immobilised pH gradients became available (IPG-strips). Nevertheless, removal of perturbing substances (salt, DNA, lipids, etc.) from samples is critical for good results because IEF is very sensitive to contaminants. To start the second dimension IPG-strips with focussed proteins have to be equilibrated to SDS-PAGE running conditions by incubation in SDS loading buffer. Afterwards IPG-strips are placed on top of an SDS gel and proteins are separated according to their apparent molecular weight. The protein staining method should be very sensitive and compatible with

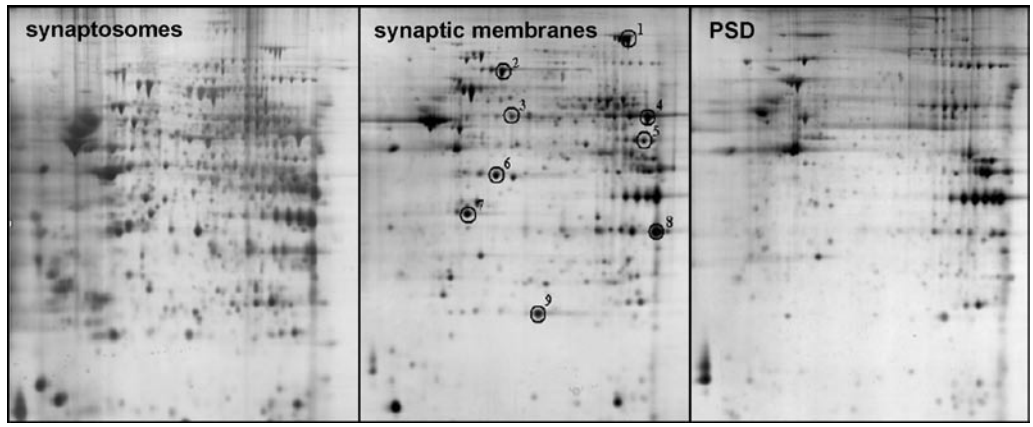

Fig. 2. Comparison of 2DE separations of different fractions containing synaptic structures with increasing content of "true" synaptic proteins from synaptosomes via synaptic membranes to PSDs. Encircled protein spots in the gel from synaptic membranes are enriched compared to synaptosomes but are almost not detectable on the gel from PSDs. Therefore these proteins are rather "contaminants" from other subcellular components than directly associated to synaptic structures (From Li et al. 2004).

MS. Therefore, several staining protocols have been improved, especially silver staining or detection with flourescent dyes like Sypro Ruby.

An important limiting factor for the application of 2DE is related to the problem that usually proteins larger than 100 kDa frequently form aggregates before leaving the IPG strip or on top of the SDS gel. Especially integral membrane proteins have a strong tendency to aggregation and are therefore particularly difficult to analyse. A comparison of 2D gels from synaptosomes, synaptic membranes and postsynaptic densities is shown in Fig. 2, which clearly demonstrates the high resolution and reproducibility of the technique. Furthermore, there is an obvious reduction in the number of different proteins the more defined the subcellular compartment under investigation is.

An image analysis software is essential for the detection of spots and the verification of differentially expressed proteins when comparing different samples obtained under different conditions/treatments. Automated spot detection, matching, normalization and statistical evaluation are the most important features to increase the reliability and reproducibility of results obtained by 2DE.

Although the high resolution and improved reproducibility of 2DE makes the application of this technique very attractive there are also several limitations. Thus, the resolution of high molecular weight proteins, hydrophobic proteins and extremely basic or acidic proteins is still a challenge. In these cases one-dimensional high-resolution SDS-PAGE can be used for separation alternatively.

Mass spectrometric analysis

Nowadays the most commonly used method for protein identification is mass spectrometry (MS), under certain circumstances microsequencing and immunoblotting can also be used. MS is based on the separation of ions in a strong electric field according to their specific charge. For MS analysis single protein spots are excised from 2D gels and proteolytically cleaved by a protease with well described specificity (in most cases trypsin). After cleavage the resulting peptides are subjected to MS analysis and yield a "peptide finger print" which is then searched against a computer-generated list formed from a simulated digestion of a protein database using the same protease. If the protein is in the database it could be identified this way, otherwise the sequence has to be determined by additional MS of single peptides. There are two ionisation methods commonly used for MS of proteins and derived peptides: Matrix-assisted laser-desorption-ionisation-time-of-flight (MALDI-TOF) and electrospray ionisation (ESI). Both are useful for high throughput analysis and are used in combinations with distinct methods for fractionation of complex protein mixtures. A typical MS spectrum is shown in Fig. 3.

New strategies for proteomic approaches

As mentioned before, gel-based proteomics has several limitations in particular for large proteins or for proteins which are highly hydrophobic or have extreme isoelectric points. Therefore many attempts have been

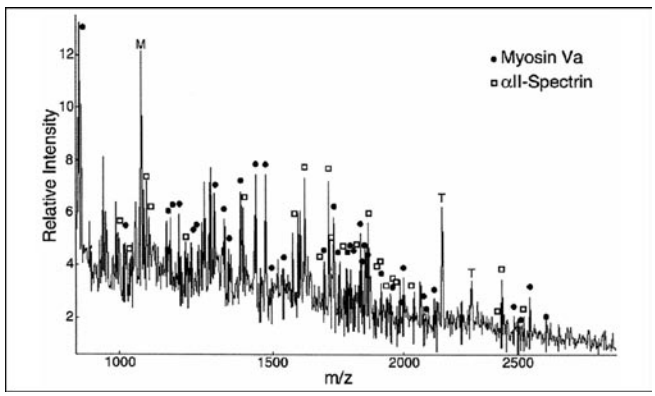

Fig. 3. Typical mass spectrum obtained from the tryptic digest from a protein band cutted from an one-dimensional SDS-PAGE gel. Peptide peaks derived from Myosin Va and αII Spectrin are indicated as well as peaks arising from trypsin fragments. (From Wallikonis et al. 2000)

made to search for alternative separation methods. One successful concept is to digest the whole protein mixture with trypsin or Lys-C and to separate the derived complex peptide mixture by multidimensional high performance liquid chromatography (nanoHPLC). In most cases two-dimensional HPLC is applied using strong ion exchangers in the first and reversed-phase chromatography in the second dimension. When combined with ESI-MS this approach has a high potential for full automation and high throughput. Advances in HPLC equipment, separation media and sensitivity of mass spectrometers bring the theoretical resolution of this approach close to that achieved with 2DE, but quantitation of proteins with nanoHPLC is still not possible.

Quantitative evaluation became possible when isotope-coded affinity tag (ICAT) technology has been introduced. The ICAT consists of a cysteine-reactive group (e.g. iodoacetamide) connected to an affinity tag (e.g. biotin) *via* a linker, which containes either eight ^{1}H residues or eight ^{2}H residues for differentiation between two samples to be compared. After cysteine-derivatisation of each single sample both samples are pooled and subjected to trypsin digestion followed by both, cation exchange and avidin chromatography. Reversed-phase HPLC and tandem MS is then used to identify ICAT peptide pairs and to quantify the relative heavy/light ratios.

In summary, neuroproteomic protein profiling is currently a strongly growing field with powerful traditional and emerging technologies. Recently the proteome of the postsynaptic density has been analysed by different labs independently with a remarkable overlap of proteins particularly attributed to the PSD although different techniques have been used (Li et al. 2004, Yoshimura et al. 2004, Peng et al. 2004, Jordan et al. 2004). Based on these data sets proteomic studies of synaptic structures will be very useful to study questions in fields ranging from fundamental mechanisms of neuronal plasticity to detailed molecular analysis of the action of drugs or molecular changes accompanying brain dysfunction.

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