Proteomics of the Synapse – A Quantitative Approach to Neuronal Plasticity*

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The advances in mass spectrometry based proteomics in the past 15 years have contributed to a deeper appreciation of protein networks and the composition of functional synaptic protein complexes. However, research on protein dynamics underlying core mechanisms of synaptic plasticity in brain lag far behind. In this review, we provide a synopsis on proteomic research addressing various aspects of synaptic function. We discuss the major topics in the study of protein dynamics of the chemical synapse and the limitations of current methodology. We highlight recent developments and the future importance of multidimensional proteomics and metabolic labeling. Finally, emphasis is given on the conceptual framework of modern proteomics and its current shortcomings in the quest to gain a deeper understanding of synaptic plasticity. *Molecular & Cellular Proteomics 15: 10.1074/mcp.R115.051482, 368–381, 2016.*

Chemical synapses of the brain configure the most complex cell–cell junction of the body (Fig. 1). Proteomic studies have revealed that more than 2000 different proteins are found in preparations of forebrain synapses following biochemical purification (1–12). This complex proteome creates a challenge for future research not only from a methodological point of view but also with respect to the molecular dynamics of protein exchange at this cell–cell junction. The activity-dependent association of proteins with synaptic junctions imposes fundamental questions about synaptic function that are the driving force for most of the proteomic research done so far. It is widely believed that synapses in the forebrain undergo structural and functional changes, a phenomenon called synaptic plasticity, that underlies learning and memory processes. The synaptic basis of memory formation is still far from being understood but compelling evidence suggests that activity-dependent alterations in the molecular composition of the synapse are a key mechanism for synaptic plasticity (13–17). However, to date the evidence for this notion is still largely based on the study of individual proteins and efforts to overcome these limitations with an unbiased large-scale proteomic approach have been facing several constraints that we will discuss in this review.

Neurons are highly polarized cells and the number of synapses is typically huge, their molecular makeup extraordinarily complex, and their distance from the cell body, where most protein synthesis occurs, can be enormous for both their dendritic and axonal processes. Neurons have therefore adopted a number of strategies to enable local and rapid changes in proteostasis and very recent research suggests that satellite synapto-dendritic organelles, that allow for protein synthesis, modification, degradation, and that give rise to highly specialized vesicles, are important for synaptic function (Fig. 2) (14, 18–20). The bewildering complexity in the interplay between local and somatic protein synthesis, mRNA and protein transport, protein modifications including, phosphorylation, acetylation, methylation, tyrosination, glutamylation, lipidation, and glycosylation as well as local protein degradation allows for a tightly controlled supply and removal of synaptic proteins. In principal, these interconnected machineries can give rise to a high molecular diversity in the synaptic protein make-up but the study of this question is still in its infancy (21, 22; see also below).

The Molecular Dynamics of Dendritic Spines—In the human forebrain dendritic spines harbor the most abundant synapse type (Figs. 1 and 2). They are found on excitatory principal neurons of the cortex and hippocampus as well as on GABAergic medium spiny neurons of the striatum, and a single pyramidal neuron can be equipped with up to 10,000 of these synaptic contacts (23, 24). Interestingly, up to 60% of hippocampal spine synapses are in tight contact with astrocytic endfeet (25) and engulfed with a complex extracellular matrix, which has led to the concept of the tri- and tetrartaplate synapse (16, 17). Spines are membranous protrusions from neuronal dendrites and it is widely believed that spines are semi-autonomous biochemical microcompartments separated from the dendritic shaft by the spine neck (Figs. 1 and 2). Spine synapses allow for compartmentalization of post-synaptic Ca\textsuperscript{2+} responses (26, 27) and they can continuously change their shape and volume and thus adapt to requirements of the synaptic contact (15, 17, 28–30). An important role for the topology of the spine synapse is played by an...

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**Electron micrograph of rat cortex showing multiple pre- and postsynaptic structures, as well as astrocytic endfeet (*) in close contact with synapses.** Note the presence of numerous synaptic vesicles in the presynaptic boutons. CAZ, cytomatrix at the active zone; PSD, postsynaptic density; SV, synaptic vesicles. Scale bar: 100 nm.

**The tetrapartite synapse of principal neurons in the forebrain, consisting of the pre- and postsynaptic compartment, astrocytic endfeet, and the extracellular matrix has a tightly regulated protein composition.** A microsecretory system is present in synapses and dendrites that allows for translation of mRNA, local synthesis of, processing and insertion of transmembrane proteins. Hence the turnover of the synaptic protein machinery is controlled by local and somatic de novo protein synthesis, protein degradation by the ubiquitin proteasome system, lysosomes and autophagosomes. In addition, the association of proteins with pre- and postsynaptic compartments is highly dynamic. Molecular machineries and organelles for proteostasis are shared between synapses in dendritic segments. Proteins are transported in and out of the synapse as well as by diffusion of transmembrane proteins. These processes govern the activity-dependent assembly of the pre- and postsynaptic scaffold and the synaptic surface expression of receptors, calcium channels and cell adhesion molecules. Abbreviations: CAM, cell adhesion molecules; CAZ, cytomatrix at the active zone; COIN, Correlated optical and isotopic nanoscopy; ECM, extracellular matrix; FASS, fluorescence activated synaptosome sorting; FRAP, fluorescence recovery after photobleaching; FUNCAT, fluorescent noncanonical amino acid tagging; GABAC, cell adhesion molecules; GlyR, glycine receptor; HPG, homopropargylglycine; ICAT, isopeptide-coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantification; LTP, long-term potentiation; mGluR, metabotropic glutamate receptor mRNA, messenger ribonucleic acid; MS, mass spectrometry; NCAM, neural cell adhesion molecule; N-GlcNAc, N-linked-Acetylglucosamine NMDA, N-Methyl-D-aspartic acid; NMDAR, N-Methyl-D-aspartic acid receptor; O-GlcNAc, O-linked-Asparagine; polysia, polyasialylated; PTM, post-translational modification; SILAC, stable isotope labeling by amino acids in cell culture; SILAM, stable isotope labeling by amino acids in mammals; SIMS, Secondary ion mass spectrometry; SPILL, specific protein isotopic and fluorescence labeling; tRNA, transfer ribonucleic acid.
The presynaptic counterpart of the PSD is the cytomatrix of the active zone (CAZ) of neurotransmitter release (Figs. 1 and 2). The CAZ is like the PSD an electron dense area close to the membrane (Figs. 1 and 2). Protein components of the CAZ tether synaptic vesicles to the presynaptic membrane and mediate synaptic vesicle fusion, thereby allowing neurotransmitter to be released reliably and rapidly when an action potential arrives (38, 39). The protein composition of the CAZ has been elucidated in much detail and the topology of this structure is arguably better understood than those of the PSD. Protein components of the CAZ and the PSD cannot be separated by conventional proteomic approaches, which is because of the purification schemes employed in these studies (see below/Fig. 3) (Table I).

Experimental studies from the past 15 years have identified more than 2000 components of the synapse and this work has set ground to put the building blocks of synaptic neurotransmission together (15, 17, 32, 40–43). Our knowledge about the protein composition of the synapse is based to a large extent on this pioneering work. Converging targeted and shot-gun proteomic approaches have shed light on the protein composition of synaptic vesicles (44, 45), biochemically isolated fractions highly enriched in docked synaptic vesicles (46), single CNS synapse types (47), excitatory receptor complexes like the N-Methyl-D-Aspartate-Receptor (NMDAR)- (48), metabotropic glutamate receptor (mGlur5) (49), α-aminooxy-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (50–53), glycine receptor β subunit (GlyRβ)-interacting proteins (54), synaptic cell adhesion molecules (55, 56), and the major constituents of the pre- and postsynaptic scaffold (57–60). In case of the NMDAR initially a multiprotein complex with 77 proteins was identified that contained several kinases and phosphatases and other signaling components (48). This seminal study was followed by other approaches to identify AMPAR subcomplexes with interaction proteomics (50–53, 61). Important auxiliary subunits that regulate AMPAR function were identified by mass spectrometry (MS) analysis of purified complexes. In recent work, using an elegant combination of immunoprecipitation with a series of antibodies directed against AMPAR subunits, extensive validation and isolation of a AMPAR complex in native blue gel electrophoresis of roughly between 0.6 to 1 MDa, Schwenk et al. (52) could verify 34 proteins as part of distinct AMPAR complexes with different stability. This approach was then extended to different brain regions and developmental stages, where it was found that AMPAR complexes significantly differ in their protein constituents across postnatal development and between brain regions (53, 61). An ultimate question that arises is how a functional complex is defined and how large such a complex might be. Interestingly enough, using transgenic mice expressing TAP-tagged PSD-95, the probably most abundant postsynaptic scaffolding molecule (31, 32, 62), a complex of 118 proteins was identified (57). In an earlier study employing immunopurification of PSD-95 with a specific antibody 26 major components of the PSD-95 complex were identified with relatively little overlap apart from NMDAR and AMPAR subunits to the study of Fernandez et al. (58). This variability probably reflects the general problem of interaction proteomics from a complex matrix whose outcome depends on several variables and it is difficult to isolate one functional complex of the multidomain protein PSD-95 over a large number of synapses and with a different history of synaptic activation.

The Proteomic Toolbox to Analyze Neuronal Proteostasis in Plasticity—The early studies were based on biochemical purification schemes that impose limitations with regard to quantitative proteomics (i.e. shot-gun proteomics versus 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (63, 64), variability in the isolation of the structures of interest because of different density gradient centrifugation protocols, immunoprecipitation of protein complexes, Fig. 3). With the advent of quantitative proteomics using ICAT (65), iTRAQ (66), AQUA (67), SILAC (68), SILAM (69), and label-free quantification approaches (70) some of these issues have been resolved and differential proteomic profiling in combination with absolute and relative quantifications can address changes of the neuronal proteome associated with diseases (e.g. 71–79) or is able to tackle protein half-lives (80, 81). Crucial to all of these approaches are absolute high fidelity in the technical prerequisites of the analyses including accuracy of workflows, validity of data, dynamic range of protein assessment, or PTM levels. These important aspects have been recently covered in excellent technical reviews (82, 83).

However, the current proteomic toolbox still lacks the ability to grasp the full range of determinants of synapse proteostasis simultaneously because of special purification and separation requirements of the various protein modifications in combination with detection limitations of the mass spectrometers, i.e. we cannot go beyond the “mere” identification and quantification of specific proteomes such as the synaptic phosphoproteome (84–91) or the entity of palmitoylated proteins (92, 93) but rather have to address post-translational modifications (PTMs) one by one. In a seminal paper by the Burlingame lab, characterization of O-linked glycosylated and phosphorylated peptides from murine synaptosomes was performed in a sequential manner (90). A total of over 1750 O-GlcNAcylated and 16,500 phosphorylated sites were identified with heavily glycosylated proteins such as protein kinases always being extensively phosphorylated as well. This suggests toward a putative crosstalk of phosphorylation with glycosylation at catalytic grounds (90). However, identification of these PTM-subproteomes is not complete, particular PTM peptides originating from lower abundance proteins are missing because of the common, strong bias toward acquiring MS/MS data on higher abundance molecules. This type of issue also holds true for combining the different profiling approaches with metabolomics or neuropeptidomics. Moreover, recent evidence from several labs points to the pivotal...
importance of alternative splicing of mRNA for activity-dependent protein synthesis (95–97), the use of alternative translation initiation sites (98–100) and the involvement of point mutations to the genesis of several diseases (e.g., 101–103). These phenomena imply a severe problem for proteomics as full sequence coverage is a rather rare event during the

**Fig. 3.** Workflow for common brain and synapse proteomics approaches tackling proteins, glycans, lipids, and phosphorylation sites of synaptic proteins, as well as receptor complexes. Synaptic fractions such as synaptosomes, CAZ, and the detergent extracted PSD are prepared using sucrose or Percoll density centrifugations followed by subsequent specific MS analyses. Dashed lining indicates further necessary processing of fractions to analyze specific subproteomes such as the lipidome, the phosphoproteome, or the different glycoproteomes. In case of global brain lipidome analysis or interactome analyses of receptor complexes organic solvents or mild detergents, respectively, are used for extraction from homogenates prior to further processing and MS analysis. IP, immunoprecipitation; AC, affinity chromatography; CAZ, cytomatrix of the active zone; PSD, postsynaptic density.
identification process, and, therefore, missing domains encoded by differentially spliced internal exons or N-terminally truncated proteins in the case of alternative AUGs will be hardly identified in complex samples. Point mutations pose a significant problem especially in the case of human samples and yet unknown mutations as search engines attempt to match peptide sequences present in a given database. Here, customized databases (e.g., the SNAP database within the Global Proteome Machine (GPM), http://gpmdb.thegpm.org/snap/index.html, or PEPPPI, http://bioinformatics.iupui.edu/peppi (104)) and the combination with transcriptomics will help to resolve this issue. Finally, although a multitude of specifically neuronal proteins has been identified, synapses share identical proteins including receptors, channels, cell adhesion molecules, and regulatory proteins and all of their putative post-translational modifications, with intimately connected cell types, such as astrocytes, oligodendrocytes and microglia. Hence, without having the possibility of neuronal cell-selectivity one cannot be entirely sure about the true identity and dynamics of the current synaptic proteomes in

### Table I

| Synaptic subproteomes and their preparation | Description | Approx. number of unique proteins | Selected Literature |
|-------------------------------------------|-------------|----------------------------------|---------------------|
| Postsynaptic density (PSD) proteome       | The PSD is defined at the ultrastructural level as electron-dense material associated with the postsynaptic membrane. Core PSD proteins and their associated partners have been identified in synaptic junctional protein preparations (“PSD preparations” using detergents including Triton X-100, DOC and SDS. In addition to postsynaptic proteins, components of the CAZ, glial endfeet and ECM proteins are found in “PSD preparations” due to either similar biochemical features or general stickiness to membranous preparations. | Ca. 1,500 | (189–191) |
| Presynaptic cytomatrix at the active zone (CAZ) proteome | Electron-dense presynaptic counterpart of the PSD containing scaffolding molecules and the vesicle release machinery. Scaffolding components co-purify in PSD preparations. | Ca. 500 | (190, 192, 193, 43) |
| Synaptosomes | Also called synaptic membranes, collected after sucrose density centrifugation at the 1.0/1.2 M interface. Contain presynaptic, postsynaptic as well as astroglial and ECM components. Recent technical advancement: transmitter-specific synaptosomes can be isolated from knock-in mice carrying fluorescently labeled core components such as VGLUT1 for glutamatergic synapses (FASS methodology). | Ca. 6,620 in total: 163 enriched proteins via VGLUT1-FASS analysis | (90, 146) |
| Synaptoneurosomes | Crude synaptosomes including also dendritic components are prepared using either mesh filters right after homogenization of brain material to separate nuclei and large cellular debris, or brief sucrose density centrifugation. | uncertain | (e.g. 194–196) |
| Glial endfoot proteome | Astroglial endfeet are in close contact with spine synapses and components, therefore, copurify in PSD, and synaptosome as well as synaptoneurosome preparations. | unknown | (197, 198) |
| Extracellular matrix (ECM) proteome | ECM components located perisynaptically and within synaptic clefts are released by astrocytes and neurons and are tightly associated with the synapse. ECM components are found as co-purified proteins in “PSD preparations”. | unknown | (198- 200) |
| Synaptic phosphoproteome | Phosphoproteins or -peptides are enriched from PSD, synaptosome or synaptoneurosome preparations using affinity resins such as TiO₂ beads. | Ca. 16,500 phospho-sites | (90) |
| Synaptic brain lipidome | Isolated from synaptic membrane preparations followed by high Triton X-100 extraction. | Ca. 2,850 in human brain | (150) |
| Synaptic glycoproteomes | O- and N-glycoproteomes are enriched from detergent extracted synaptosomes using lectin affinity purifications. | Ca. 1,260 O-glycoproteins, ca., 1,300 N-glycoproteins | (90, 159) |
| Receptor complexes / interactomes | Receptor or scaffolding molecule specific antibodies are used to isolate respective complexes from brain extracts or synaptosome extracts. | NMDAR complex: 77 proteins; AMPAR complex: 34 proteins; PSD95 complex: 118 proteins | (48, 52) |
question. Novel approaches will circumvent this problem at least in part (see below).

The Conceptual Framework for Synaptoneuroproteomics—
The most critical aspect in the study of synapse proteostasis is to overcome the snap shot view imposed by a steady state analysis of the highly dynamic protein equilibrium in spine synapses. The exchange rate of proteins between spine synapses is quite high (105) despite the fact that protein turnover of key synaptic proteins is probably in the range of days (82, 83, 105) and that protein degradation and de novo synthesis alone can, therefore, probably not solely account for these high dynamics (14, 16). In addition the molecular composition of spine synapses is different depending upon their dendritic localization, size, and age (22, 107). Thus, although it has been proposed that the stoichiometry of the synaptic scaffold is not variable in mature spines (10, 62, 108, 109), a profound molecular diversity might exist between excitatory synapses and this heterogeneity is further increased by the existence of excitatory and inhibitory shaft synapses whose dynamics and molecular make up are not very well investigated. Thus, a number of biological constraints exist for a quantitative proteomic approach to synaptic function.

In consequence, one might ask which questions can be addressed with the currently available technologies and how future methodological developments might overcome these limitations. A central request for a molecular underpinning of activity-dependent changes in synaptic strength is the idea that the molecular make-up of a synapse changes for time periods of days if not weeks and months. An influential hypothesis in the field called “synaptic tagging” (110, 111) proposes that sustained synaptic activity like during the induction of long-term potentiation (LTP) initiates the creation of a short-lasting protein-synthesis-independent “synaptic tag” at the potentiated synapse. The nature of this tag is still unknown but it is supposed that it will sequester proteins to the activated synapse, which will subsequently help to maintain LTP (111). Despite more than a decade of research the acquisition of the corresponding molecular maintenance mechanisms by a synapse is also still elusive. The list of processes and mechanisms contributing to tagging is long: Various post-translational modifications (112), mRNA trafficking and local mRNA translation at potentiated synapses (113), incorporation of a pre-existing pool of plasticity-related proteins, protein degradation (114, 115), reconstruction of the postsynaptic cytoskeleton (116, 117), exchange of proteins between synapses (105), and finally weakening of neighboring inactive and nonpotentiated spine synapses (inverse tagging/118). Looking at this conundrum of different processes that are plausibly only relevant in a defined spatio-temporal context it is obvious to ask which answers can we expect from the current “static” synapse proteomics?

Activity-Dependent Regulation of the Synaptic Proteome—A recent survey on quantitative and qualitative proteomic studies of the synapse indicated that more than 2700 different proteins have been identified as integral components of excitatory synapses on post- and presynaptic sites (119). It should be noted, however, that physical constraints (size of the PSD, copy number of the most abundant proteins etc.) make it unlikely that all of these different proteins can fit into a single spine synapse. It is instead likely that sample contaminations, synaptic heterogeneity as well as protein exchange and mobility contribute to these findings. Several manually curated databases combine these data in online repositories including G2Cdb (http://www.genes2cognition.org/120), SynaptomeDB (http://metamoodics.org/SynaptomeDB/index.php, 121), SynProt (www.synprot.de, 116), or SynSysNet (http://bioinformatics.charite.de/synsys/122).

The majority of studies that compared the proteome of synapses were performed on human brain disease states (12, 40, 72, 73, 76, 78, 123–126) and despite significant methodological progress in recent years the resulting picture is still not representative. Apart from the problem of tissue preservation and preparation the limitations of conventional proteomic analysis lead to a situation where in several studies very different proteins where shown to be up- and down-regulated in the same brain region and disease state. Although usually more than one hundred proteins are regulated in disease states only cherry-picked candidate proteins that match to the disease process have been further investigated and up-or-down-regulation of very few other candidates were confirmed by other methods. Another problem of quantitative neuroproteomics is the stoichiometry of synaptic proteins that ranges from less than ten to more than several hundred (10, 31, 40, 62, 108, 109). Despite the fact that synapses are relatively stable imaging studies suggest that the turnover of proteins within a spine is fast on a time scale of minutes to hours and it is likely that both a relatively immobile and a rather dynamic pool for a given synaptic protein exist (127–130). The metabolic half-life of most synaptic proteins is usually in the range of several days (80, 81, 106) and several live-imaging studies revealed a prominent exchange of molecules between synapses (reviewed in 105). This points to another problem of a steady-state approach such as the current proteomic ones to a highly dynamic equilibrium: The high exchange rate of synaptic proteins requires a fast supply to prevent synaptic dysfunction and this in turn points to a readily available reserve pool of proteins in dendrites that might derive from a local protein synthesis machinery (14, 16). Knowledge about these pools is essential for the interpretation of quantitative proteomics. A central question therefore will be how important local protein translation is for synapse function and under which circumstances it is needed. However, the sensitivity of current MS-based techniques still requires sampling an entire heterogeneous brain region consisting of different subtypes of neurons and glial cells instead of a single cell or synapse and for the study of activity-dependent changes in the synaptic proteome usually larger tissue samples have to be manipulated.
The Synaptic Proteome in Learning and Memory—As a result of these difficulties considerably less work has been done on the dynamics of the synaptic proteome following the induction of synaptic plasticity. In consequence, very little is known to date whether patterns of protein expression are altered depending upon the synaptic input in vivo. Compelling evidence for experience-dependent changes in the synaptic proteome stem largely from studies where the sensory input was manipulated during development (131, 132). Pharmacological activation of synapses either in vivo (133) or in vitro (134, 135) with GABA-ergic antagonists to remove inhibition and thereby increase firing of excitatory synapses or after induction of LTP (136) results in profound changes in synaptic protein composition.

These reports prove the feasibility of proteomics to detect activity-dependent changes in synaptic protein content. Nonetheless very few studies were undertaken to learn about learning-induced proteomic changes (137–143). Interestingly, changes in the abundance of several hundred proteins were reported even in studies that used total protein homogenates. Moreover, very little overlap even in regulated protein networks is apparent from this work, which makes it difficult to provide a synopsis of the learning-regulated proteome. It is important to note that only a small percentage of synapses and cells usually are supposed to encode the memory and it is therefore surprising that with the relatively insensitive methodology employed so many proteins were detected that are up- and down-regulated. Cell-specific labeling and purification techniques as well as visualization of newly synthesized proteins might provide a technological advance to gain deeper insights (see below). Another important aspect is here clearly the time course and the need to differentiate between memory stages like acquisition, encoding and consolidation. Likewise synaptic activation as such does not necessarily lead to an engram that can be easily detected at the protein level. Even an enriched environment or simple physical exercise can lead to the expression of several plasticity-related genes in the hippocampus and dramatic changes in the synaptic proteome that can obscure the findings (144, 145). In other terms the number of control experiments to elucidate a learning-induced change in a small number of synapses is high and usually outside of the scope of a single study. It is therefore unlikely that this approach as it stands today will lead to major new insights into learning-induced changes of the synaptic proteome in the next coming years.

An Integrative View of the Molecular Dynamics of the Synapse—Perspectives for the Integration of New Technologies—Dendritic spines can be isolated in so called synaptosomal preparations and kept for cellular in vitro assays for several hours (Fig. 3, Table I). Different purification schemes have been described to increase sample and, thus, synapse specificity. A major advance might be a recently published Fluorescence Activated Synaptosome Sorting (FASS) method that is based on VGLUT1-Venus knock-in mice and that allows an enrichment of glutamatergic synaptosomes of the forebrain to near homogeneity (146). By focusing on more homogeneous samples differential profiling with high protein identification and quantification completeness among several time points in development or during plasticity events might be feasible.

Lipidomics—A future development that holds a lot of promise for a deeper appreciation of signaling pathways lies in the combination of proteomics, lipidomics, glycoproteomics, and metabolomics of synapses. Lipidomics of brain tissue is still in its infancy (147, 148). Several lines of evidence suggest that the lipid composition of synapses is highly dynamic (149). The existence of more than 40 different lipids known to modulate signaling and/or to influence membrane geometry in neurons, synapses, and synaptic vesicles demands for a systematic large scale study of lipid abundance and functional regulation in neuronal subcompartments. A very recent large-scale MS-based analysis of the lipid composition of the human brain shows a bewildering complexity of lipid composition: From 5713 lipid compounds analyzed in the study 76% were either enriched or depleted in brain (150). Currently >600 lipids can be quantitatively accessed, whereas many lipid classes cannot be analyzed owing to impaired ionization and solubility, as well as low abundance (151). Modern lipidomic tools can provide access to understand the complexity of lipids, their homeostatic regulation, and their role in neuronal plasticity and in synaptic diseases (for a recent review see 148). Studies are ultimately warranted that break ground and provide a lipid inventory of synapses and that address whether corresponding lipid alterations occur in paradigms of synaptic plasticity and efforts to develop novel lipid quantification techniques tailored for the analysis of the synaptoneurilipidome will most likely open up new avenues in synapse biology (152).

Lipids impact on cellular functions and it is therefore of fundamental importance to correlate lipid dynamics with proteins that are essential for the synthesis, modification, and turnover of lipids and vice versa. A limitation that has to be overcome is that so far different biological building blocks, i.e. proteins, lipids and metabolites have largely been investigated independently. Direct correlations between metabolic and signaling events frequently remain concealed and studies targeting different molecular classes at once are cumbersome, rare and unique. Systems scale integration of lipidomics data within data sets derived from proteomics or metabolomics experiments is conceivable by concentration change coupling analysis to investigate the interconnectivity between the different molecular layers at different condition (see for instance 153). Thus, workflows to study the complexity and dynamics of lipids and their roles within synaptic membranes and synapto-dendritic organelles under physiological and pathological conditions are in principle established.

Glycoproteomics—Attachment of carbohydrates to proteins is as lipidation vital for a large number of cellular processes. Especially synapses are enriched in N- and O-linked
glycoproteins and glycosylated proteins—among them many receptors, channels, cell adhesion molecules, extracellular matrix (ECM) proteins, and regulatory proteins—fulfill crucial functions in cell differentiation, neuronal growth, signal transduction, cell–cell recognition, LTP (154), and memory formation (155). Complex O- and N-linked glycosylation are heterogeneous in their nature, more or less permanent PTMs, and, therefore, glycosylated peptides are rarely found in their unmodified form in contrast to phosphorylated or acetylated peptides. In contrast, simple O-linked glycosylation with β-N-acetyl-α-glucosamine (O-GlcNAc) is a very dynamic PTM resembling similar features as protein phosphorylation (156, 157). Despite the importance of glycosylation for neuronal and synaptic function, very little is still known on the exact composition and regulation of glycosylation of synaptic proteins because of the heterogeneity and complexity of the static glycosylation patterns as well as the dynamics of simple O-GlcNAc glycosylation. The lab of Alma Burlingame recently presented the combined analyses of the phospho- and O-glycoproteome as well as the O- and N-glycoproteomes of murine synaptosomes (90, 158) using a sequential purification strategy for glycosylated, phosphorylated, and unmodified peptides. As mentioned above, over 1750 O-GlcNAcylated and 16,500 phosphorylated sites were identified in the first study (90) and a total of over 2500 unique N- and O-linked glycopeptides with remarkable microheterogeneity of attached oligosaccharides on 453 proteins was discovered in the second study (158). Considering the issue of missing PTM-peptides of lower abundance proteins, the authors estimate the content of O-glycosylated proteins to be 19% and the phosphorylated proteins at 63% of the total synaptosome proteome (90). Combination of filter-aided lectin affinity purification and high-accuracy MS revealed 3162 N-glycosylation sites in mouse brain, with 1140 being unique to the brain and allows the simultaneous quantification of the N-glycoproteome via SILAC during age (159). However, also this seminal study falls short to reflect the wide diversity and likely dynamics of oligosaccharide structures and subtle glycosylation changes could not be detected. Especially the very terminal ends consisting of sialic acids and to a lesser extend L-Fucose of glycans are of critical importance for glycoprotein function and specificity. NCAM is a polysialylated (polySia) glycoprotein and the polySia serves as a negative regulator of cell–cell apposition, interferes with cis- and trans-interactions of NCAM. Moreover, it modulates as a scavenger of soluble factors receptor activation (reviewed in 160). Fucosylated carbohydrate structures in the brain have been implicated in molecular mechanisms that underlie neuronal development, learning, and memory. Plasticity phenomena including hippocampal LTP and memory formation, for instance, are accompanied by a transient increase in fucose incorporation into membrane glycoproteins (161–163). Most notably, inhibition of protein fucosylation does not interfere with LTP induction or memory acquisition, but prevents specifically the maintenance of LTP and long-term memory (162, 164).

Likewise to the lectin-based approaches for the characterization of the O- and N-linked glycoproteomes, Murrey et al. (165) used a more L-fucose-specific lectin from Ulex europaeus to enrich for fucosylated protein from different brain regions of adult and P3 mice. The 32 identified candidates belong to the classes of cell adhesion molecules, ion channels and solute carriers/transporters, ATP-binding proteins, synaptic-vesicle associated proteins and mitochondrial proteins, and most of them are predominantly expressed and developmentally regulated in the olfactory bulb. It is highly likely that with more specific enrichment approaches such as other lectins and in combination with bio-orthogonal labeling strategies (see below) and improved sensitivity of the mass spectrometers the repertoire of fucosylated proteins and the understanding of fucosylation dynamics will expand dramatically in the near future.

Metabolic Labeling—To tackle dynamics of post-translational modifications or alterations in protein expression patterns and to enrich for these subproteomes bioorthogonal labeling approaches have emerged during the last 15 years that use the cell’s own biosynthetic machinery. In these approaches that mainly use azide or alkynes as chemical handles proteins are endowed with this novel azide or alkyne functionality that serves to distinguish them from the pool of pre-existing or recently unmodified proteins. Employing either copper-catalyzed azide-alkyne ligation (commonly referred to as “click chemistry”) or strain-promoted cycloaddition, the reactive azide or alkynes groups can be covalently coupled to respective alkyne- or azide bearing tags in the second step enabling subsequent imaging, affinity purification, and MS identification procedures of tagged proteins (166). As shown for the de novo synthesized proteome using the noncanonical amino acids azidohomoalanine (AHA) or homopropargylglycine (HPG) in combination with BONCAT (bioorthogonal amino acid tagging) or FUNCAT (fluorescent noncanonical amino acid tagging) the presence and incorporation of AHA and HPG are nontoxic and do not affect global rates of protein synthesis or degradation. Moreover, a broad range of functional and biochemically diverse proteins have been identified and temporally and spatially visualized by these techniques (167–169). With BONCAT the dopaminergic subproteome in rat hippocampal neuropil was assessed (169). Many of the candidate proteins identified in the dopamine agonist-treated sample belonged to Gene Ontology (GO) categories specific for protein synthesis and synaptic function. The introduction of another noncanonical amino acid azidonorleucine (ANL) for labeling newly synthesized proteins might pave the way for an advancement of the NCAT technologies. Link et al. modified the methionine binding pocket of E. coli Methionyl-t-RNA synthetase to allow binding of ANL that otherwise can not be processed by the endogenous synthesis machinery because of a large side chain (170). The mutants E. coli MetRS 1305 and
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_E. coli_ MetRS<sup>NL</sup> were found to effectively activate ANL and cell specific expression of these mutants made cell-specific labeling of protein synthesis possible (171, 172). In very recent work, two groups presented with such an genetically introduced amino acid tagging cell-specific metabolic labeling with spatiotemporal resolution in living _Drosophila melanogaster_ and _C. elegans_ using ANL and Azidophenylalanine, respectively, in combination with genetically engineered amnonacetyl tRNA synthetases (173, 174). The future implementation of this cell-selective metabolic labeling approach into a mammalian context in combination with live-tagging methods (strain-promoted cycloaddition, see below), promises to track proteome dynamics of distinct neurons or astrocytes in coculture systems or even in living rodents. This is of special importance in particular for understanding the reciprocal interplay between neurons and astrocytes. Both cell-types share despite their functional differences in large parts identical proteins including neurotransmitter receptors, cell adhesion molecules, and signal transduction proteins. Therefore, it is not surprising that in recent years a tight reciprocal relation between neurons and astrocytes has been disclosed covering general aspects of cellular activity but also the astroglial secretion of synaptogenic factors (175, 176). This now well recognized concept of the “tripartite synapse” points to the importance of glia cells for neuronal function and development. A large body of literature shows that astrocytes participate in all essential brain functions, for example they are important for the formation and maintenance of synaptic contacts, sense neuronal activity, and actively participate in homeostatic scaling. However, it is still unclear what proteins are indeed unique to astrocytes and neurons, if the astroglial proteome is as dynamic as it has been shown for the proteomes of different neuronal subtypes, and what the common and unique modes of regulation of the neuronal and astroglial proteome indeed are. Answering these questions will unarguably deepen our understanding of synapse biology and will pave the way to ultimately understand the complex and heterogeneous nature of the brain itself.

Critically, metabolic labeling of proteins using bioorthogonal chemical reporters is not restricted to monitor global _de novo_ protein synthesis as other classes of biomolecules such as glycans and lipids can be targeted with the same chemistry as well. Carolyn Bertozzi and colleagues applied the metabolic labeling strategy to monitor glycoproteins with different noncanonical monosaccharides in cells, tissues and in zebrafish (177–181). Moreover, lipid-containing molecules can be tracked via azidolipid precursors tackling N-myristoylation, S-palmitoylation, or farnesylating in living systems (182, 183).

**Imaging Mass Spectrometry**—In the last decade, Imaging MS (IMS) opened a new avenue of bioanalytical research by matching histological features of a tissue sample to molecular localization patterns (recently reviewed in 184). Although, there are still major technical challenges to be resolved including sample preparation and throughput as well as comprehensive protein identification itself, a few studies using MALDI IMS have already shown its potential for the investigation of spatiotemporal neuropeptide and protein regulation in brain tissues, which is complementary and in some cases even superior to conventional approaches using antibody-based imaging and proteomics techniques. For instance, Hanrieder et al. (185) detected elevated levels of the two neuropeptides dynorphin B and alpha neoendorphin specifically in the striatum of mice suffering from _L_-DOPA induced dyskinesia. For both neuropeptides no specific antibodies exist. Secondary-Ion MS (SIMS) detecting the isotopic composition of the sample or material with its high spatial resolution of about 50 nm in the lateral and 10 nm in the z direction, here then referred to as Nano-SIMS, has been recently used in combination with STED super resolution microscopy to quantify protein and organelle turnover in organelles including synaptic vesicles, mitochondria, or Golgi compartments of hippocampal neurons (COIN, 186, 187). Recently, Nano-SIMS was coupled with click-chemistry based labeling of individual proteins (SPIII, 188) allowing their precise visualization and cellular protein turnover. This clearly opens new possibilities for not only the identification and quantification of a particular cellular proteome but also to address its dynamics _in situ_.

**Conclusions and Future Perspectives**—Despite 15 years of progress proteomics of the synapse is still an evolving field. The technological advances described above and the combination of different omics will allow for much deeper insights into signaling networks and the topology of signaling pathways in the near future. To this end the full gamut of approaches will also include systems biology. It is possible that exchange rates at synapses, _de novo_ protein synthesis and degradation are overrated as the key determinants of plasticity in synaptic function and that post-translational modifications well beyond phosphoproteomics have their share in altering synaptic strength even at time scales of hours and longer. The appealing concept of the tri- and tetrapartite synapse has gained considerable interest in recent years and some of the technological advances that will contribute to a deeper appreciation how different cellular membranes interact with matrix components to establish changes in neuronal connectivity. Several lines of evidence suggest that mainly mRNA splicing and much less gene transcription is altered in response to enhanced synaptic activity. Modern proteomic approaches with a much better peptide coverage will help to determine the role of alternative splicing or the usage of alternative start codons for activity-dependent protein expression in synaptic function.

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