HHS Public Access

Author manuscript

Curr Opin Neurobiol. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

Curr Opin Neurobiol. 2017 February ; 42: 102–110. doi:10.1016/j.conb.2016.12.004.

Synapse biology in the 'circuit-age' - paths towards molecular connectomics

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Abstract

The neural connectome is a critical determinant of brain function. Circuits of precisely wired neurons, and the features of transmission at the synapses connecting them, are thought to dictate information processing in the brain. While recent technological advances now allow to define the anatomical and functional neural connectome at unprecedented resolution, the elucidation of the molecular mechanisms that establish the precise patterns of connectivity and the functional characteristics of synapses has remained challenging. Here, we describe the power and limitations of genetic approaches in the analysis of mechanisms that control synaptic connectivity and function, and discuss how recent methodological developments in proteomics might be used to elucidate the molecular synaptic connectome that is at the basis of the neural connectome.

Introduction

The proper function of the nervous system is defined by neuronal circuits, in which individual neurons are precisely connected via synapses with specific properties. Recent methodological developments, such as optogenetics, *in vivo* electrophysiology and imaging, virus-based tracing, tissue-clearing and light sheet microscopy, and three-dimensional reconstruction of circuits by serial electron microscopic imaging, have led to substantial progress in defining the core anatomical connectivity of the mammalian brain. However, it is

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Conflict of Interest Statement

The authors declare no conflict of interest.

unclear how this anatomical connectivity is established at the molecular and cellular level [1,2], and how the synapses within such circuits are endowed with synapse type-specific functional features.

For over 50 years, since Roger W. Sperry formulated the 'chemoaffinity hypothesis', posing that individual cells expose 'identification tags' by which they are distinguished to the level of single neurons and implying that such tags determine the specificity of synaptogenesis [3], it has been assumed that cell type-specific surface receptors and adhesion proteins determine the specificity of connectivity. Subsequently, many candidate proteins that might act as such identification tags have been identified, but the question as to whether and how these molecules define the connectivity and function of specific synapses has remained largely unresolved [4].

Two main problems account for this lack of progress. First, multiple synaptic adhesion proteins appear to operate in concert or even in parallel and redundantly, rather than individually, to control synapse formation and define synapse-specific functional features [5,6]. Accordingly, obtaining detailed insights into the mechanism of specific synapse and circuit formation from analyses of individual adhesion proteins or even of entire protein families has proven challenging. Second, it has long been impossible to systematically tackle the problem of synapse type-specific protein composition and function, beyond mere localization and characterization of individual protein species and their interactors.

From a biochemical point of view, synapses can be considered supramolecular protein machines that are assembled from the repertoire of synaptic adhesion proteins, scaffold proteins, receptors, ion channels, and the components of the synaptic signaling machinery available in the connected neurons [7]. The qualitative and quantitative features of the composition and stoichiometry of the synaptic protein machinery subsequently determine the specificity and unique properties of a given synaptic connection. In turn, the mutation of genes encoding synaptic protein components causes qualitative or quantitative changes in the composition of defined synapses, triggering perturbations in synapse and network function that are at the basis of multiple neurological and psychiatric diseases.

In view of the major role that the protein composition of synapses plays in brain function, the elucidation of the composition of synaptic protein complexes and an understanding of the molecular mechanisms that control synaptic specificity and function have immense importance. Here, we first describe the power and limitations of genetic approaches in the analysis of mechanisms that control the specific generation and composition of synapses. We then consider recent methodological developments in cell subcompartment-specific proteomics, and discuss how these approaches might be used to determine the molecular synaptic connectome that is at the basis of the neural connectome.

Genetic dissection of the mechanisms controlling synapse-specific organization and function

Synaptic adhesion molecules play an important role in synapse development, by mediating cell-cell recognition and by linking pre- and postsynaptic partners [8]. A subset of synaptic

adhesion molecules has the capacity to induce the differentiation of initial cell-cell contacts into pre- or postsynaptic specializations. This was first demonstrated for the postsynaptic adhesion molecules of the neuroligin family, which induce presynaptic differentiation when presented to contacting axons in cell culture [9]. Conversely, the presynaptic binding partners of neuroligins, the neurexin family of alternatively spliced adhesion molecules, induce postsynaptic differentiation [10]. The formation of a neurexin-neuroligin complex across the synaptic cleft induces pre- and postsynaptic differentiation by recruiting key components of the synaptic machinery, such as scaffolding proteins and neurotransmitter receptors [10–12] (Fig. 1A), thereby organizing synaptic protein composition [13]. A host of additional synaptic ligands for neurexins that expand or modulate the repertoire of neurexin interactions have since been identified. Among these are postsynaptic adhesion molecules of the leucine-rich repeat transmembrane neuronal protein (LRRTM) family, which also form trans-synaptic complexes with neurexins [14–16]. In addition, secreted proteins can bridge neurexins and postsynaptic receptors into tri-partite complexes. The astrocyte-derived secreted protein Hevin for example facilitates binding between neurexin and neuroligin splice variants that normally have weak affinity for each other [17], modulating the neurexin repertoire. Cerebellin-1 (Cbln1), a secreted glycoprotein of the C1q/tumor necrosis factor (TNF) superfamily, binds neurexins and the GluD2 glutamate receptor [18,19], whereas the related C1q-like proteins C1ql2 and C1ql3 couple neurexin-3 to postsynaptic kainate receptors [20], thus expanding the repertoire of neurexin interactions to directly organize postsynaptic neurotransmitter receptor composition at excitatory synapses. At inhibitory synapses, the extracellular matrix protein Punctin/MADD-4 was recently identified as a secreted synaptic organizer that binds both neurexin and neuroligin and regulates postsynaptic $GABA_A$ receptor clustering [21,22].

In addition to these neurexin-based adhesive complexes, other synaptic adhesion molecules capable of inducing synaptic differentiation have been identified. These include members of the LRR family of synaptic adhesion molecules [23], and the homophilic adhesion molecule SynCAM1 [24], which contributes to the patterning of the synaptic cleft into adhesive subcompartments, with different adhesion receptors occupying distinct regions of the cleft [25]. Of note, this summary is far from exhaustive, and is only meant to highlight the increasingly complex adhesive interactions that regulate synapse organization, formation and function. Other molecularly diverse families of adhesion molecules with important roles in regulating connectivity or synaptic function have been identified [4], and recent single-cell profiling studies are beginning to propose the functional relevance of cell type-specific repertoires of adhesion molecules [26,27]. Determining the role of cell type-specific signatures of adhesion molecules in synapse formation and the specification of synapse type-specific functional features constitutes a major challenge. Here we focus on the genetic dissection of adhesion molecules in the development of cerebellar Purkinje cell (PC) connectivity as an example of a well-characterized neural circuit. PC dendrites receive two main types of excitatory input: parallel fibers (PF) from cerebellar granule cells, which terminate on distal dendrites, and climbing fibers (CF) from the inferior olive, which terminate on proximal dendrites (Fig. 1B). In addition to these excitatory inputs, PCs also receive inhibitory input from various types of interneurons, stellate and basket cells (Fig. 1B).

A major synaptic organizer of excitatory PF-PC synapses is Cbln1, which is secreted from cerebellar granule cell axons and binds the postsynaptic GluD2 receptor on PC dendrites. Both are required for the formation and plasticity of this synapse [28]. Cbln1 also binds presynaptic neurexin, and the tri-partite neurexin-Cbln1-GluD2 complex is required for PF-PC synapse formation and plasticity [18,19] (Fig. 1B). C1ql1, which is secreted from climbing fibers, is a major organizer of excitatory CF-PC synapses. C1ql1 binds to the adhesion G protein-coupled receptor BAI3 on PC dendrites. Both C1ql1 and BAI3 are required for the formation and maintenance of the CF-PC synapse [29,30] (Fig. 1B). Whether C1ql1 also binds neurexins at CF-PC synapses to form a tri-partite complex, analogous to the interaction of C1ql2/3 with neurexin-3 at hippocampal mossy fiber synapses [20], or interacts with a different presynaptic receptor remains to be determined. Together, these studies show that secreted cues, released from distinct presynaptic inputs and through differential interactions with postsynaptic receptors, play a critical role in establishing specific excitatory synaptic connectivity in the cerebellum.

A systematic analysis of neuroligin function in cerebellar PCs revealed that all three neuroligins expressed in cerebellum, (Nlgn1, -2, and -3), are required for specifying the functional properties, but not the formation, of CF-PC synapses [31]. Combinatorial PC-specific loss of Nlgn1 and Nlgn3, which localize to excitatory synapses, decreases CF-PC synapse size and strength. A similar phenotype is found in *Nlgn1/2/3* knockout mice, but here the additional deletion of Nlgn2, which localizes to inhibitory synapses, also results in a loss of distal CF synapses, indicating that Nlgn2 indirectly contributes to CF-PC synapse development through a poorly understood mechanism [31] (Fig. 1B). The contributions of all three neuroligins at PF-PC synapses appeared dispensable in this study, but an independent study showed that Nlgn3 immunoreactivity mainly localizes to PF-PC synapses, and found that loss of Nlgn3 impairs long-term depression (LTD) at PF-PC synapses and causes an ectopic expansion of CF synapses onto distal PC dendrites [32].

The development of inhibitory synapses onto PCs is also regulated by several adhesion molecules. A subcellular gradient of Neurofascin-186 (NF186) on the PC soma and axon initial segment (AIS) directs the formation of pinceau synapses by basket cells (BCs) [33]. The secreted axon guidance cue Semaphorin 3A (SEMA3A), released by PCs, attracts BC axons via the SEMA3A receptor Neuropilin-1 expressed on BC axons. Neuropilin-1 then interacts in *trans* with NF186 on the AIS to form pinceau synapses [34], revealing an interplay between guidance and adhesive mechanisms to establish specific inhibitory synaptic connectivity (Fig. 1B). Neuroligins on the other hand contribute to specifying the functional properties of inhibitory synapses onto PCs, but are not required for the formation of inhibitory stellate/basket cell synapses. Loss of Nlgn2 impairs basket/stellate cell synaptic function, whereas loss of Nlgn3, which localizes to both excitatory and inhibitory synapses, has no effect. Combined loss of Nlgn2 and Nlgn3 however impairs inhibitory synaptic transmission more strongly than Nlgn2 deletion alone [31], indicating some redundancy between Nlgn isoforms at stellate/basket cell synapses, with Nlgn2 having a more important contribution (Fig. 1B).

Taken together, the emerging picture from this work is that many different cell-surface cues, axon guidance and adhesive, secreted and membrane-bound, act at specific types of inputs and subcellular compartments to construct precisely wired neural circuits.

Uncovering the molecular synaptic connectome: towards compartment-specific proteomics

The genetic dissection of the cerebellar PC circuit illustrates that different molecules act at distinct types of synapses. Systematically and comprehensively identifying synapse type-specific protein compositions has long been challenging. Novel proteomics-based approaches are rapidly changing this, enabling new insight into the mechanisms that define the connectivity and function of specific synaptic connections.

Most attempts to elucidate the composition of synaptic protein complexes have been based on the biochemical isolation of synaptic subfractions, such as synaptosomes, synaptic membranes, or postsynaptic densities. In combination with mass spectrometric analyses, over 2000 (potential) synaptic proteins were identified [35–42]. However, a key limitation in this context has been that whole brains or brain regions containing complex mixtures of different types of neurons, glial cells and synapses were typically used as input material.

Complementing classical subfractionation approaches, attempts were made to isolate more specific synaptic protein complexes, using purely biochemical techniques, antibody-based affinity purification of synaptic proteins, or mouse genetics to tag synaptic proteins in order to provide an additional level of specificity biochemical approaches alone could not afford. Examples include glutamatergic synaptosomes [43] (BOX 1), components of GABAergic or glutamatergic synapses [44,45], components of the pre- and postsynaptic constituents of synaptic protein complexes [46], or neurotransmitter receptor complexes [47–49]. To date, only a single study, employing transgenic cell type-specific expression of the GFP-tagged GluD2 receptor that localizes to parallel fiber inputs on Purkinje cells, isolated and characterized synaptic protein complexes originating from a defined population of neurons and synapses [50].

Recent developments in the metabolic labeling of proteins (BOX 2) and the subsequent isolation of proteins from defined cell populations or subcellular compartments open new avenues for the analysis of specific synaptic proteomes. Bio-orthogonal non-canonical amino-acid-tagging (BONCAT), for instance, is based on the incorporation of non-canonical amino acids, such as azidonorleucine (ANL), instead of methionine into newly synthesized proteins [51] (Fig. 2A). In cells expressing an unnatural aminoacyl-tRNA-synthetase, translated proteins incorporate ANL and can subsequently be labeled with a functional group for affinity purification, for example by coupling the azide group of ANL to alkyne-bearing tags (e.g. biotin) using so-called 'click chemistry'. Several published studies utilized this methodology for the labeling and comparative proteome analysis of newly synthetized proteins in primary neuronal cultures or in brain slices after stimulation with neuronal receptor agonists or as response to neuronal activity [52–54]. However, initially developed and widely used for applications in cell culture, this methodology has recently been applied successfully for labeling of whole cell type-specific proteomes in *Drosophila* [55] and *C.*

elegans [56]. So far, BONCAT is mostly limited to applications in cell cultures and small animals. The delivery of non-canonical amino acids to the brain *in vivo* and possible side-effects caused by the expression of unnatural aminoacyl-tRNA-synthetases or the presence of ANL [55], still pose major barriers for applications in mammals.

Biotin proximity labeling by the promiscuous biotin ligase BioID [57] represents a second approach with potential suitability for cell-selective metabolic labeling of proteins with biotin (Fig. 2B). Compared to BONCAT, this approach allows not only for labeling of entire cellular proteomes, but also of subcellular nano-proteomes, by targeting BioID fusion proteins to defined subcellular compartments. Numerous studies have demonstrated the applicability of this method for the identification of components of different protein complexes, including protein complexes that mediate nonsense-mediated mRNA decay [58], control centrosome organization [59], or constitute the nuclear pore [60]. To date, the BioID methodology has only been applied in cell culture. Efficient biotinylation by BioID requires high, non-physiological biotin concentrations, and has relatively slow kinetics. A new and improved version of BioID requires lower biotin concentrations [61]. The application of this methodology in the brain would require an external supply of biotin by injections, which is feasible as biotin is efficiently transported across the blood-brain barrier [62,63]. Hence, BioID approaches have substantial potential for applications of selective biotin labeling of proteins in vivo, by expressing BioID in defined neuronal cell populations and/or by targeting BioID to defined subcellular compartments (e.g. postsynaptic spines or presynaptic terminals).

A BioID-related approach for proximity labeling employs ascorbate-peroxidase (APEX) [64]. This technique utilizes the enzymatic activation of tyramides, such as biotin-phenol, by ascorbate-peroxidase (APEX) in the presence of hydrogen peroxide [65,66] (Fig. 2C). Activated tyramides are highly reactive and conjugate rapidly with proteins in the proximity by covalent attachment to aromatic amino acids (preferentially tyrosines). Several recent publications demonstrated that the APEX approach is a powerful way to specifically isolate proteomes of selected cell types or subcellular compartments and organelles [64,67–69]. So far, the requirement of tyramides and hydrogen peroxide for protein labeling has limited the application of this technique to cell cultures or small animals. However, the fast kinetics of the reaction provides an avenue for the use of the APEX technique in situ, e.g. in acute brain slices from animals that express APEX in specific neuronal populations and/or subcellular compartments.

A recent publication demonstrated the feasibility of biotin labeling of synapses in vivo using split horseradish peroxidase [70]. Here, enzymatic activity of non-functional fragments of horseradish peroxidase fused to pre- and postsynaptic adhesion proteins, was restored upon formation of trans-synaptic adhesion complexes and association of the peroxidase fragments (Fig. 2D). This allowed for proximity biotin labeling and visualization of synapses in the intact mouse brain. While the initial use of this approach was limited to the visualization of synaptic contacts, it is also potentially applicable for the isolation and proteomic analysis of specific synaptic complexes in the intact brain.

During the review of the manuscript two studies with a special importance for the discussed issues were published [71,72]. In the study published by Loh and colleagues, cultured neurons were infected with lentiviral constructs encoding for horseradish-peroxidase (HRP) targeted to the synaptic cleft of inhibitory or excitatory synapses, respectively, by fusion to the transmembrane and cytoplasmic part of synaptic adhesion proteins known to be localized at the respective type of synapses. Biotinylation of proteins localized to the synaptic cleft in the proximity of HRP was achieved by addition of biotin-phenol and hydrogen peroxide (see Fig. 2). Uezu and colleagues utilized the BioID approach to label components of excitatory and inhibitory postsynaptic densities (PSD) in vivo. BioID constructs targeted to inhibitory or excitatory PSDs by fusion to gephyrin or PSD95, respectively, were delivered by AAV injections into neonatal animals. To achieve efficient protein biotinylation by BioID, biotin was delivered exogenously by subcutaneous injections. In both studies specific biotinylation and subsequent isolation of proteins corresponding to inhibitory or excitatory synapses, respectively, could be achieved. The mass spectrometric analysis of isolated proteins identified known synaptic proteins as well as new components of inhibitory and excitatory synaptic complexes. Thus, both studies demonstrated the feasibility and power of the protein labeling technologies for synaptic proteome analysis. Further development of these approaches and their extension for the applications in defined neuronal populations will significantly contribute to our understanding of the molecular organization of neuronal synapses.

Conclusion

In summary, proximity-labeling methods represent fascinating new tools for the analysis of specific neuronal and synaptic proteomes (Fig. 2E). Depending on the specific targeting of the enzymatic agents, analyses can be very focused, even down to specific synapses. In combination with evolving mass spectrometric analysis, including conventional ‘shotgun’ proteomics as well as targeted and quantitative approaches, these techniques hold the promise to drive substantial progress in our understanding of the molecular determinants of synaptic specificity and function. In combination with sophisticated combinatorial genetic approaches, we can begin to understand how the molecular synaptic connectome controls the anatomical and functional connectivity of neural circuits.

Acknowledgments

We apologize to those authors whose work we could not include in this review, due to space limitations and the emphasis on recent publications in Current Opinion articles. Work in the authors’ laboratories is supported by NIH award R00DC013805-02 and Hartwell Individual Biomedical Research Award (to J.N.S.); the French Agence Nationale de la Recherche (ANR-12-JSV4-0005-01 VGLUTIQ, and ANR-10-LABX-43 BRAIN) and the French Fondation pour la Recherche Medicale (ING20150532192) (to E.H.); the Max Planck Society, the European Commission (EU-AIMS FP7-115300) and the German Research Foundation (CNMPB) (to N.B.); European Research Council (ERC) Starting Grant (#311083) and Research Organization Flanders (FWO) Odysseus Grant and Project Grant (#G094016N) (to J.d.W.).

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Highlights

- The CNS represents a precisely organized synaptic network formed by diverse neurons
- Neuronal adhesion proteins substantially contribute to synaptic complex organization
- Molecular compositions of synaptic complexes define their functional capabilities
- New techniques will help to gain insight in the molecular organization of synapses

BOX 1

Fluorescent sorting of specific synaptosomes

Synaptosomes are isolated, functional synaptic particles consisting of a resealed presynaptic compartment and partial postsynaptic element. A major limitation of conventional synaptosome preparations is that they contain a mixture of many synapse types together with neuronal and non-neuronal contaminants [73–75]. Fluorescence Activated Synaptosome Sorting (FASS) of a subset of glutamatergic synapses purified from a VGLUT1^{VENUS} (vesicular glutamate transporter 1) knock-in mouse allows to deplete most contaminants and to enrich for VGLUT1^{VENUS} synapses to near homogeneity [43,76]. Recent improvements on this technique include the fluorescent sorting of EGFP-labeled terminal fields of AAV transduced cortical mouse neurons (unpublished), enabling the analysis of synaptic proteomes of genetically identified afferents. Even though the amount of material recovered from these sorts is extremely low, current mass spectrometry technologies can confidently identify and quantify thousands of proteins from only a few micrograms of proteins and excel when analyzing samples with reduced protein complexity. FASS-based proteomics thus has the potential to contribute to unraveling the protein networks of distinct synapse populations.

BOX 2

Quantitative proteomic analysis of mammalian synapses

Metabolic labeling of rodents with a modified diet highly enriched in stable, heavy isotopes (such as nitrogen-15) facilitates the relative quantitation of thousands of synaptic proteins. The benefit of this strategy compared to label-free proteomic analysis is that mixing “light” and “heavy” brain extracts prior to biochemical enrichment of synaptic compartments provides experimental control over the inherent variability of these procedures [77]. Isotopic labeling in combination with gene knockout allows the analysis of the effect of loss of a single protein on synaptic protein composition [78]. Isotopic labeling in combination with modulation of sensory stimulation (e.g. whisker trimming, noise exposure, or eye lid suture) allows in-depth proteomic characterization of the barrel, auditory, or visual cortex [79]. Further, pulse-chase labeling of rodent brains with stable isotopes enables the analysis of synaptic proteome dynamics and identified an extremely long-lived neuronal adhesion protein [80,81]. While isotope-based strategies ensure accurate protein quantitation, they still lack the spatial resolution needed to resolve changes in protein levels of specific synapses. Isotopic labeling in combination with synapse-specific proximity-labeling has the potential to resolve this issue.

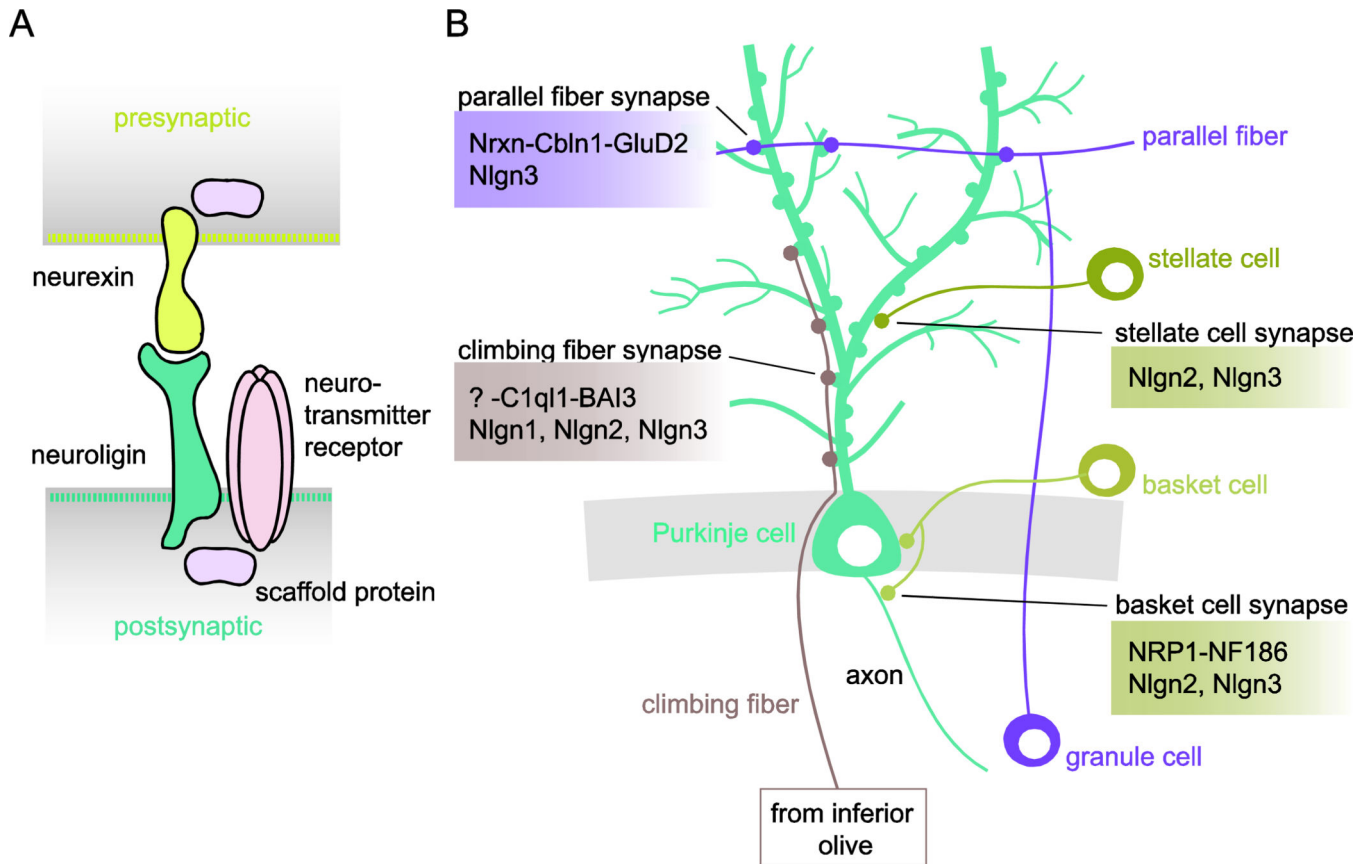

Figure 1. Genetic dissection of Purkinje cell connectivity in the cerebellum

(A) Presynaptic neurexin molecules bind to postsynaptic neuroligin molecules to form a trans-synaptic adhesive complex that organizes the pre- and postsynaptic machinery by recruiting scaffolding proteins and neurotransmitter receptors. (B) Purkinje cells (PCs) receive excitatory input from climbing fibers (CFs) originating from the inferior olivary nucleus and from parallel fibers (PFs) originating from cerebellar granule cells. Local interneurons, stellate and basket cells, provide inhibitory input. At excitatory PF-PC synapses, Cbln1 is secreted from PFs and forms a tri-partite complex with presynaptic neurexin (Nrxn) isoforms and the postsynaptic GluD2 neurotransmitter receptor to regulate synaptogenesis. Neuroligin-3 (Nlgn3) is not required for synapse formation, but is required for long-term depression of PF-PC synapses, although this is under debate. At excitatory CF-PC synapses, C1ql1 is secreted from CFs and binds the postsynaptic adhesion GPCR BAI3 to regulate CF synapse formation. Whether C1ql1 also binds a presynaptic receptor at CF-PC synapses to form a tri-partite complex remains to be determined. Nlgn1, Nlgn2, and Nlgn3 all contribute to the specification of CF-PC synapse functional properties. At inhibitory basket cell-PC synapses, Semaphorin 3A is secreted from PCs to attract basket cell axons via its receptor Neuropilin-1 (NRP1) on these axons. NRP1 then binds in trans to Neurofascin 186 (NF186), which is expressed in a gradient on the PC soma and the axon initial segment. Both Nlgn2 and Nlgn3 contribute to stellate/basket cell inhibitory synapse function.

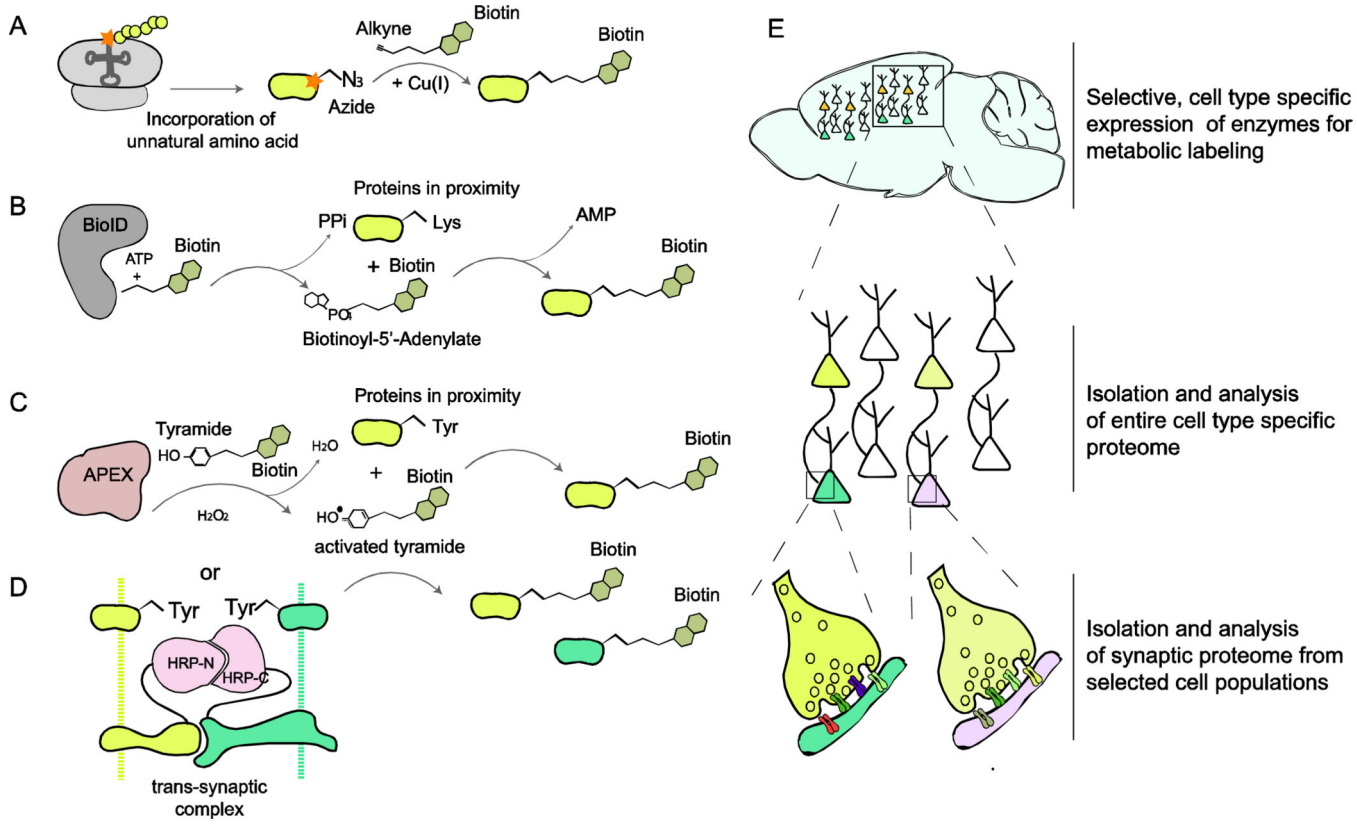

Figure 2. Overview of chemical labeling techniques and their application for targeted proteome analysis

(A) Bioorthogonal noncanonical amino acid tagging (BONCAT): the approach is based on the in vivo incorporation of non-canonical, azide containing amino acids such as L-azidohomoalanine (AHA) into newly synthesized proteins. These proteins can be subsequently labeled with biotin by 'click-chemistry' and isolated for further analysis. (B) In vivo proximity protein biotinylation by promiscuous biotin-ligase (BioID): biotinylation occurs through Biotinoyl-5'-Adenylate which is released by a mutated variant of E.coli biotin ligase BirA. Biotinoyl-5'-Adenylate is a highly reactive compound that quickly reacts with lysines of proximal proteins. (C) Biotin-labeling by ascorbate-peroxidase (APEX) and (D) by split-horseradish-peroxidase: both techniques utilize the ability of peroxidase enzymes to generate highly reactive species from tyramide derived compounds such as biotin-phenol. These react quickly with aromatic groups (usually tyrosine and tryptophan, but also histidine and cytosine) of proteins in close proximity. (E) Application of metabolic labeling for analysis of cell-type/compartment-specific proteomes: Selective expression of biotin-labeling enzymes in cells of interest (e.g. utilizing the Cre-Lox-system) allows isolation of proteins expressed in identified cells. Additionally, targeting these enzymes to synaptic compartments provides an opportunity for the labeling and subsequent determination of synapse-type specific protein composition in desired cell populations.