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Review Article

Progress toward a comprehensive brain protein interactome

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Protein–protein interactions (PPIs) in the brain play critical roles across all aspects of the central nervous system, from synaptic transmission, glial development, myelination, to cell-to-cell communication, and more. Understanding these interactions is crucial for deciphering neurological mechanisms and the underlying biochemical machinery affected in neurological disorders. Recently, advances in proteomics techniques have significantly enhanced our ability to study interactions among the proteins expressed in the brain. Here, we review some of the high-throughput studies characterizing brain PPIs, using affinity purification, proximity labeling, co-fractionation, and chemical cross-linking mass spectrometry methods, as well as yeast two-hybrid assays. We present the current state of the field, discuss challenges, and highlight promising future directions.

Introduction

While proteins play important roles in the structure and function of every organ, they do not carry out these tasks alone, and the brain is no exception. In the central nervous system (CNS), an intricate network of protein–protein interactions (PPIs) underpins a myriad of cellular functions. Both stable and transient interactions are essential for maintaining the structural and functional integrity of neurons and other brain cell types, modulating signaling pathways, regulating neurotransmitter release, and ensuring proper neural connectivity.

For instance, neurofilaments are heteropolymers composed at their core of four protein subunits: neurofilament heavy, medium, and light chains, as well as α-internexin [1,2]. These subunits polymerize to form a robust and stable network of cytoskeletal filaments within the neurons, maintaining cell shape and integrity, particularly in the axon. In turn, neurofilaments are crucial for the radial growth of axons during development, for preserving axon size, and for transmitting electrical impulses along axons, which affects nerve conduction speed [3,4].

Disruption of PPIs has been linked to impairments of synaptic communication, protein aggregation, and dysregulation of cell death, contributing to varied neurological diseases (NDs) and neurodevelopmental disorders (NDDs) [5–7]. For example, deletions, duplications, and point mutations in SH3 and multiple ankyrin repeat domains 3 (SHANK3)—a gene encoding a major scaffolding protein —are strongly associated with autism spectrum disorder (ASD), as well as several other NDDs and neuropsychiatric disorders [8]. Located in the postsynaptic density, Shank3 interacts with many proteins and complexes downstream through its component domains, regulating the formation of dendritic spines and synapses [8,9]. Although not fully understood, it is likely that disrupting different specific Shank3 PPIs may contribute to phenotypic diversity across these disorders.

Despite significant challenges in studying the interactions among endogenous proteins in the brain, not least due to the high complexity in terms of cells, composition, and connectivity, analytical biochemical methods have begun demonstrating good progress at accurately mapping brain PPIs. The technologies available for examining PPIs in the context of both cell lines and native tissues are diverse and have evolved significantly in the last two decades, offering scientists a range of methods to study these interactions with varying levels of detail and throughput [10,11]. Here, we mainly discuss studies based on high-throughput mass spectrometry (MS)-based experiments, which have emerged as powerful tools for proteomics and protein research [11]. These large-scale studies provide systematic and reasonably unbiased views of the molecular organization of the highly specialized and unique neural architectures, from dendritic trees to synaptic structures, including the postsynaptic density. We discuss the advantages and limitations of individual assays applied to fruit fly, mouse, and human brains, to human and mouse neural cell lines, and

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to both diseased and healthy brain tissues. Additionally, we highlight important findings from these studies that are advancing our understanding of normal neuronal biology and disease mechanisms.

Affinity purification mass spectrometry

MS-based proteomic approaches to map PPI networks can be broadly classified into targeted and untargeted approaches. Affinity purification mass spectrometry (AP-MS) is one of the most common targeted MS approaches used to study PPIs sampled from their native cellular environment. In AP-MS, a protein of interest (referred to as the 'bait') is isolated from cells along with its interacting partners (called 'preys'). The bait protein is purified from the cell lysate using affinity reagents, such as antibodies or affinity resins, that bind to a molecular tag fused to the bait protein (e.g. FLAG, c-myc, HA, or GFP). After purification, prey proteins that co-purify with the bait protein are identified and quantified using MS (Figure 1).

A typical AP-MS workflow involves incubating the cell lysate with beads conjugated to antibodies specific to the bait's tag. This is followed by several stringent washes to reduce nonspecific binding, and then MS is used to identify the proteins that remain associated with the bait. These proteins are considered potential direct or indirect interactors. By repeating this process with different bait proteins, researchers can build a comprehensive interaction network by statistically inferring relationships between bait and prey pairs [12].

The robustness of AP-MS has enabled researchers to generate large-scale interaction networks in various model organisms, as well as healthy and diseased tissues. One notable example is the BioPlex 3.0 Interactome, derived from over 10,000 pulldown experiments performed in two human cell lines (HEK293T and HCT116), which targeted over half of all known human proteins [13]. This dataset has become a valuable resource for the systems biology community, advancing our understanding of underlying molecular mechanisms.

In the context of brain proteomics, AP-MS has been extensively used to investigate postsynaptic complexes, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptor subunits, and postsynaptic density 95 [14]. AP-MS has also been applied to study various NDs mechanisms, such as Alzheimer's Disease (ALZ), ASD, and neuronal ceroid lipofuscinoses (NCLs), a group of rare, fatal neurodegenerative diseases characterized by lipopigment inclusion in tissues. For example, mutations in palmitoyl protein thioesterase 1 (PPT1) are linked to infantile NCLs. Scifo et al. used AP-MS to identify 23 interacting partners of PPT1 in human neuroblastoma cells, implicating PPT1 in roles such as neuronal migration and dopamine receptor signaling [15]. Pires et al. used AP-MS to identify 125 significant interactors of phosphorylated tau (pTau) in fresh-frozen brain tissue from Alzheimer's disease (ALZ) donors [16].

Another study by Wang et al. [17] constructed a large-scale PPI network involving 100 high-confidence ASD (hcASD) risk genes using AP-MS in HEK293T cells. This network included over 1,800 PPIs, and AlphaFold2 structural predictions suggested that roughly 10% of these interactions were direct. The study also identified DDB1 and CUL4 Associated Factor 7 (DCAF7) as a potential scaffolding protein for multiple ASD-related proteins and showed that disrupting DCAF7 and its interactors via CRISPR-Cas9 in *Xenopus tropicalis* impaired neurogenesis and significantly reduced telencephalon size.

Despite its strengths, AP-MS has limitations. One potential drawback is the use of affinity tags, which can potentially alter the native structure, function, or interaction partners of the bait protein. Additionally, AP-MS may suffer from high background levels of nonspecific binding proteins, requiring careful optimization of washing procedures and, ideally, quantitative comparisons with negative-control pulldowns. Otherwise, these nonspecific interactions can lead to incorrect conclusions if mistaken for biologically relevant interactions. Researchers must balance preserving weaker or transient interactions with reducing nonspecific binding by selecting appropriate lysis and wash buffers. AP-MS typically cannot differentiate between direct PPIs and indirect associations that occur through intermediary proteins within a complex and, hence, identifies 'co-complex' interactions.

Overall, AP-MS remains a powerful method for elucidating protein interaction networks, particularly in the brain. When combined with other techniques, such as proximity labeling or co-fractionation MS, AP-MS can provide even deeper insights into the molecular mechanisms underlying neuronal function and disease.

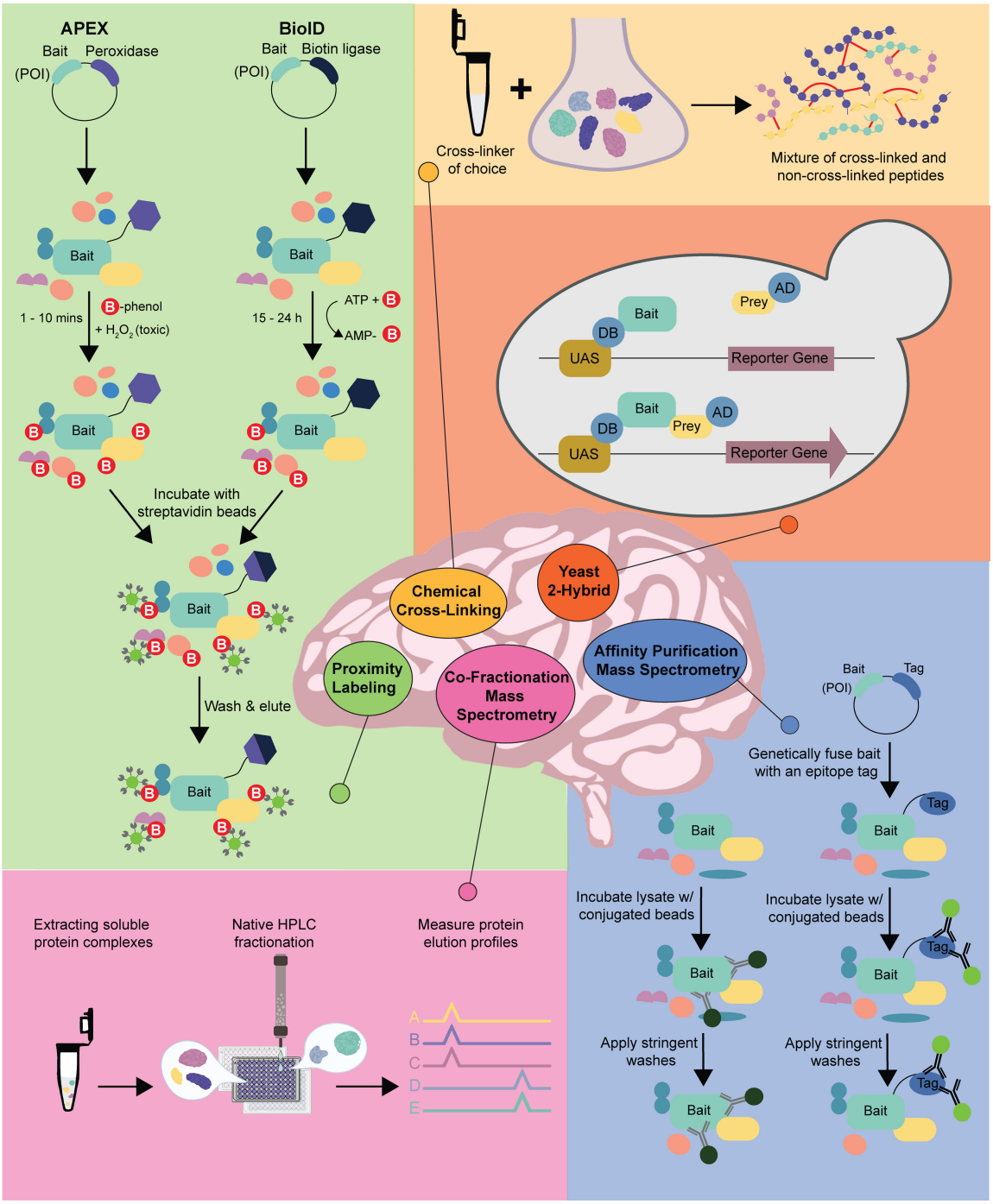

Figure 1: Overview of large-scale proteomics methods for mapping protein–protein interactions in the brain.

The figure summarizes the main experimental approaches used to identify and characterize interactions among brain-expressed proteins. The methods vary significantly in their ability to detect direct versus indirect interactions, identify multi-protein assemblies, use recombinant DNA or affinity reagents, and analyze endogenous proteins in their native cellular contexts.

Proximity labeling mass spectrometry

Enzyme-catalyzed proximity labeling mass spectrometry (PL-MS) is a related targeted MS approach that identifies PPIs by tagging proteins in close spatial proximity to a bait protein. In PL-MS, the bait protein is fused to an enzyme capable of biotinylating nearby proteins, such as a biotin ligase (e.g. BioID [18]) or

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its engineered variants such as TurboID [19] or a peroxidase (e.g. APEX [18]). When a biotin substrate is added, the enzyme catalyzes the addition of biotin to nearby proteins [20]. These labeled proteins are then purified using streptavidin beads and subsequently identified and quantified via MS (Figure 1).

Overall, this method allows for the detection of direct, indirect, stable, and transient interactors of the bait protein. As with AP-MS, PL-MS does not distinguish between direct and indirect interactions, as it labels all proteins in proximity to the bait, including those that may be part of larger protein complexes. Hence, proper controls are essential to distinguish specific interactions from spurious identifications. However, this aspect can provide interesting opportunities to identify proteins in general subcellular locations. With the addition of localization signals (e.g. nuclear localization) or the use of tissue- or developmental stage-specific promoters, this method can be modified to detect both spatial- and temporal-specific PPIs *in vivo*. The use of proximity labeling to identify spatial-specific proteomes and PPIs in the brain has been recently reviewed by Mathew et al. [20] Therefore, here, we will focus on key studies that have been published since then, as well as a few newly developed proximity labeling techniques that could have implications for the development of brain PPI networks.

The major advantage of PL-MS lies in its ability to study biological structures that are challenging to purify biochemically, such as synaptic junctions and multi-cell-type junctions in the brain. This powerful technique has been extensively applied to investigate protein interactions implicated in NDs. For instance, Bozic et al. [21] used PL-MS to study the interactions of C9orf72 dipeptide repeat proteins (DPRs), which are linked to frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). They found that one of the DPRs, poly-GA, sequesters vasolin-containing protein (VCP), impairing its function in autophagy. Interestingly, a recent preprint by Batra et al. [22] used a split-APEX approach and found that the tau protein associated with ALZ also interacts with VCP during its aggregation. Prikas et al. [23] focused on identifying tau interactors under physiological conditions by performing three PL-MS screens: in primary murine cortical neurons *in vitro*, as well as in mouse forebrain neurons in both wildtype and tau-mutant models *in vivo*. In doing so, they found that tau normally inhibits the N-ethylmaleimide-sensitive fusion ATPase, which regulates surface AMPA-type glutamate receptor (AMPAR) expression, ultimately affecting synaptic plasticity and learning.

Another study by Unda et al. [24] focused on OTUD7A, a deubiquitinase linked to 15q13.3 microdeletion NDD, ASD, and epilepsy. Using BioID2, they identified Ankyrin-G as a key interactor of OTUD7A, regulating its proteostasis and suggesting a role in neuronal development that is disrupted in disease.

PL-MS has also been used to investigate protein domain-specific interactors of neurological disorder-related proteins. Irala et al. [25] induced expression of a secreted version of TurboID conjugated to either the C-terminal or N-terminal peptide of neurocan (NCAN) in the cerebral cortices of wildtype mice. This protein is normally secreted by astrocytes into the extracellular matrix where it is proteolytically cleaved, and mutations in NCAN are associated with bipolar disorder, mania, and schizophrenia. Following *in vivo* biotinylation, these authors identified both overlapping and distinct interactomes for both NCAN peptides, and ultimately determined that NCAN-C-terminus facilitates inhibitory synaptogenesis. Bowen et al. [26] found N- and C-term-specific interactomes of the potassium channel Kv1.3 under both homeostatic and proinflammatory conditions of BV-2 microglia cultures *in vitro*. In addition, Xu et al. [27] identified interactors of the intracellular domain of Ten-m, which is important for synaptic matching in *Drosophila* and relevant for a variety of sensory and motor dysfunction disorders. The authors generated an endogenous knock-in of APEX2 in the Ten-m locus and found that it interacts with the RhoGAP Syd1 and modifies its signaling activity.

New methods to improve proximity ligation techniques have also been introduced in recent years that may prove useful for mapping brain PPI networks. In mammalian systems, the labeling enzyme must be genetically encoded and *in vivo* biotin administration can be challenging, as it requires long labeling periods that limit the ability to capture interactions within short temporal windows. To improve these aspects, *Takato* et al. [28] developed a photooxidation-driven proximity labeling strategy, PhoxID, to identify PPIs of endogenous receptors in the brain. In this method, they directly injected a ligand-tethered photosensitive probe into live mouse brain ventricles such that cerebrospinal fluid (CSF) flow distributed the probe throughout the brain, anchoring it to the endogenous receptors of interest. Upon optogenic light activation, the probe generates molecular oxygen that can oxidize proximal proteins in the presence of protein-labeling reagents. By injecting the labeling reagent into different brain regions and at different stages of development, these authors established the AMPR interactome across brain space and developmental time. Zhu et al. [29] developed a tyrosine-based strategy using *Bacillus*

megaterium tyrosinase (BmTyr), which they found exhibits low cytotoxicity, high labeling efficiency, and low background labeling. These authors used a similar ligand-tethered approach to anchor BmTyr onto endogenous brain receptors to identify Grm1- and Drd2-specific interactomes in mouse brain *in vivo*. Finally, as calcium signaling is vital to neuronal activity, Kim et al. [30] developed a calcium-gated proximity ligation strategy to study PPIs at calcium-signaling microdomains. Specifically, they conjugated RS20 and Calmodulin (Cam) to the N- and C-terminus of a split TurboID enzyme, with the addition of a pseudosubstrate to block the active site and reduce background biotinylation under normal physiological conditions. Upon increases in local calcium concentration, RS20 and Cam bind, allowing the enzyme to refold and label proximal proteins. Using this strategy, these authors identified calcium-signaling microdomain proteomes in primary mouse neurons *in vitro*, as well as in kainic acid-induced seizure mouse models *in vivo*.

One of the primary challenges in biotin-based PL-MS techniques, such as BioID or TurboID, is the interference from endogenously biotinylated proteins (e.g. carboxylases), which can generate high background signals and complicate data interpretation. Similarly, in horseradish peroxidase-based labeling approaches like APEX2, endogenous peroxidase activity in certain cell types or tissues can result in nonspecific labeling. This issue is particularly pronounced in complex samples such as brain tissue, where peroxidase-like activities contribute to unwanted background signals, reducing the specificity of the labeling [31].

While PL-MS is a powerful tool for mapping proteins within a defined proximity to the labeling enzyme, it does not provide information about the exact site of labeling on a protein or its spatial orientation within a complex. This limitation is especially significant in densely packed cellular environments, such as synaptic junctions, where high protein density and structural complexity make it challenging to pinpoint specific PPIs or structural features being labeled.

Co-fractionation mass spectrometry

Co-fractionation mass spectrometry (CF-MS) is an untargeted MS approach used to study protein interactions in native cells and tissues without the need for genetic tagging or affinity reagents [32,33]. In CF-MS, protein complexes are separated by their biochemical properties through techniques such as high-performance liquid chromatography (HPLC), density centrifugation, or native gel electrophoresis, collecting biochemical fractions throughout the course of the separation. These fractionated protein complexes are then subjected to MS analysis, allowing researchers to identify co-eluting proteins that are likely to interact. This mapping strategy scales well to the complexity of endogenous brain protein assemblies, including hard-to-access cellular regions such as membrane, cytoskeleton, and extracellular matrix, and can be applied directly to brain tissue without recombinant DNA or antibodies.

A typical CF-MS workflow involves several steps, as described in Figure 1: (1) extract native protein complexes from cells or tissues using nondenaturing lysis buffer condition, (2) subject the protein lysate to separation, where HPLC size exclusion chromatography, ion-exchange, and hydrophobic interaction chromatography are commonly used to biochemically fractionate soluble protein assemblies based on their size, charges, and hydrophobicity, respectively, and (3) use MS to identify and quantify stably associated proteins that reproducibly co-elute. Co-elution signals are computationally evaluated using correlation coefficients or other statistical measures to assess the similarity between protein elution profiles. Most typically, a supervised machine learning framework is used to control and score false discovery rates, trained on known internal control complexes that are observed during the experiments. A review of CF-MS computational analyses was recently published by Goel et al. [34].

In neuroscience, CF-MS has been applied to systematically study endogenous protein interaction networks in mammalian brains by Pourhaghighi et al. [35] and Liebeskind et al. (2020) [36]. The reported protein interaction networks from both studies show an enrichment for associations with neuronal subcellular compartments, such as axons, dendritic spines, and synapses, as well as key neural pathways. While both studies used CF-MS to create fractionation profiles of hundreds of endogenous protein complexes from brain lysates, each study applied additional experimental strategies to improve their protein coverage. Pourhaghighi et al. [35] performed independent biochemical fractionations and MS profiling on ten different brain regions to examine the regional-specific protein assemblies. Liebeskind et al. [36] supplemented CF-MS with two methods employing chemical cross-linking to measure interactions among insoluble proteins. Both studies applied at least two different kinds of biochemical separations on mouse brain lysates, resulting in 8389 (Pourhaghighi et al. [35]) and 5799 (Liebeskind et al. [36])

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high-confidence protein interactions, respectively. These interactome maps reveal how disease-associated processes and genetic variants disrupt interactions between neuronal protein assemblies, which are essential for maintaining brain function. In particular, Pourhaghighi's [35] BraInMap highlighted the role of RNA-binding protein assemblies in the development of ALS and provided a valuable resource for linking molecular interactions to broader neurological studies. Liebeskind et al.'s [36] protein networks indicated that mutations in the guanine nucleotide-binding protein GNAO1 can lead to epilepsy by disrupting chemical signaling pathways.

Recently, in one of the first large-scale applications of CF-MS to human clinical samples, Shrestha and colleagues analyzed postmortem ALZ brain samples, identifying over 10,000 PPIs, with 2389 being novel, identifying 486 protein complexes [37]. Their findings shed light on how disease-related changes in protein interactions contribute to neurodegenerative processes, such as the partial disassociation of the mu-calpain complex, which is important for neuronal calcium regulation. The data showed partial disassociation of the mu-calpain heterodimer in the ALZ brain lysate, suggesting potential disruption of cellular homeostasis and negatively affecting neuronal structure and function.

Similar to AP-MS and PL-MS, CF-MS does not readily differentiate between direct and indirect interactions, serving to indicate proteins participating in the same complex. Data analysis requires computationally evaluating protein co-elution to detect interactions. Current tools, such as PrInCE [38], PCprophet [39], and cfmsflow [40], employ machine learning frameworks to enhance the accuracy of interaction identifications, but the analysis remains computationally intensive. Finally, CF-MS requires proteins to be well observed in order to detect their interactions, and thus tends to be biased toward higher abundance proteins and longer-lived interactions. Nonetheless, its generality and lack of dependence on genetic tools or affinity reagents allow it to be widely applied to diverse tissues and species.

Chemical cross-linking mass spectrometry

One challenge in isolating intact native protein complexes from cells or tissue is that only PPIs that withstand the lysis and purification conditions can be detected by MS. Chemical cross-linking mass spectrometry (XL-MS) is a powerful proteomic technique used to study PPIs by stabilizing interacting partners through covalent bonds prior to their subsequent identification. Hence, XL-MS can capture transient and weaker protein interactions that would not otherwise survive cell lysis. In XL-MS, cross-linking reagents, which are typically small organic molecules (cross-linkers) containing two reactive groups separated by a spacer arm, are used to covalently link amino acid residues that are in proximity (usually between 5 and 30 Å) within or between interacting proteins [41,42], as demonstrated in Figure 1. After cell lysis, protein isolation, and proteolytic digestion, the resulting covalently linked peptides are then identified using MS, providing insights into the spatial relationships and interactions between proteins. Depending on the length of the cross-linkers employed, XL-MS can reveal both direct and co-complex binding relationships [42,43].

XL-MS is a well-established tool in structural biology to investigate interactions and spatial relationships between proteins or within different regions of a single protein, but it is only recently that high-throughput XL-MS analysis of *in situ* protein complexes and structures in brain cells or tissues has become feasible. Gonzalez-Lozano and colleagues performed the first large-scale XL-MS experiments on mouse synaptosomes using the MS-cleavable cross-linker disuccinimidyl sulfoxide, generating a detailed interaction map of synaptic proteins [44]. This study identified over 1500 intermolecular cross-links from 1036 different protein pairs and 5583 intramolecular cross-links within 1472 proteins. The authors identified novel PPIs and provided insights into the structural dynamics of important synaptic proteins, such as CaMKII, a kinase involved in synaptic plasticity. By combining structural modeling and dynamic simulations approaches with their cross-link data, the authors proposed that the kinase domains of Camk2 are more flexible than previously thought, which could be important for binding multiple partners.

In 2021, Wittig et al. [45] used XL-MS to investigate the protein composition of purified synaptic vesicles from rat brains. Using the cross-linker bis(sulfosuccinimidyl) suberate, they identified 407 cross-links corresponding to 260 intramolecular and 147 intermolecular cross-links. Among their results, the authors could differentiate two functional states of the V-ATPase: an open conformation representing the active, fully assembled V_1V_0-ATPase and a closed conformation locking the V_0 domain. They also identified cross-links between VAMP2, which aids synaptic vesicle fusion with the presynaptic membrane, and proteins within the synaptic vesicle complex, finding that VAMP2 plays an important role in complex formation in purified, unstimulated synaptic vesicles.

While XL-MS has many advantages in being able to sample native protein interactions *in situ* without the need for affinity reagents or antibodies, it also has several shortcomings, including a bias toward more abundant proteins. Moreover, analyzing XL-MS data can present challenges, as successfully cross-linked peptides are typically present in lower abundance than accompanying un-cross-linked and mono-linked peptides, in which the cross-linker is attached only to one residue. The method relies critically on disentangling the sequence information from the cross-linked pairs of peptides, either by separating each cross-linked peptide pair during the course of the MS analysis so that peptides can be analyzed independently (e.g. using MS [3] tandem MS) or by computationally deconvoluting their overlapping spectra. State-of-the-art XL-MS software, such as xiSEARCH/xiFDR [46], XlinkX (Proteome Discoverer 3.2) [47], and Scout [48], have all introduced methods to control false identification rates. With these improved analyses, XL-MS is proving a valuable approach for probing PPIs of brain proteins directly in their native cellular contexts.

Yeast two-hybrid

Finally, we consider the dominant non-MS approach for mapping brain protein interactomes. Originally developed 35 years ago [49], the yeast two-hybrid (Y2H) assay remains one of the most widely used techniques to study and detect PPIs. The Y2H assay is based on the idea that a transcription factor can be split into two separate domains: a DNA-binding domain (DB) and a transcriptional activation domain (AD). If a protein bearing an AD interacts with or comes near a protein bearing a DB, the AD and DB reconstitute a functional transcription factor, driving the expression of a reporter gene [49] (Figure 1).

Several variations of the Y2H system exist, but in general, it has been instrumental in mapping disease-related interactomes in the brain. Although many studies have applied Y2H for singular bait brain proteins [50–57], to name a few, we focus here on high-throughput studies using Y2H to map PPIs among brain disorder-related proteins. Here, as well, studies using Y2H have identified PPIs related to Huntington's disease (HD), spinocerebellar ataxias (SCAs), and a variety of neurodegenerative disorders such as ALZ, Parkinson's disease, ALS, and FTD [58–60].

One example is the work by Goehler et al. [58] who used Y2H to develop an HD interactome, identifying 186 PPIs between 35 bait proteins and 51 prey proteins. Lim et al. [59] expanded on this by screening 54 SCA-related proteins, ultimately discovering 752 PPIs involving 36 SCA-related baits and 541 prey proteins. Haenig et al. [60] considerably scaled up their assay by using a systematic mating approach to screen their ND-related protein set against the human ORFeome. Ultimately, these authors identified 18,663 PPIs between 471 neurodegenerative disease-associated proteins and 3482 prey proteins. These studies underscore the scalability of the Y2H method for studying brain-related interactomes and have contributed large interactome networks specifically relevant for multiple neurological disorders.

Despite its utility, the Y2H system has certain limitations, such as high rates of false positives and false negatives unless appropriate controls are included [61]. Such errors can arise due to recombinantly expressing animal proteins as fusion constructs in a yeast cell's nucleus, out of the proteins' normal brain and organismal contexts. Additionally, Y2H experiments primarily detect direct-binding interactions, as mediating subunits in larger multi-protein complexes are not co-expressed with the assayed subunits in these assays. Nevertheless, these recent high-throughput adaptations of the Y2H system have allowed for large-scale screenings, providing valuable insights into protein networks relevant to brain function and NDs. Notably, the three brain PPI studies discussed above experimentally validated their interactome maps using pairwise co-immunoprecipitation methods, and both Lim et al. and Haenig et al. [60] also bioinformatically supported their results via Gene Ontology-enrichment analysis, with the idea that true protein interactors should be annotated with similar cellular compartments and/or biological and molecular functions. Haenig et al. [60] also found high concordance of their Y2H interactome with previously published protein complex datasets, such as the MS-based CORUM dataset [62–64]. As with any interactome, functional analysis is required to understand the biological relevance of the PPIs identified by Y2H. All three studies performed some level of functional analysis, with Haenig et al. [60] notably performing extensive *in vitro* cell culture, *in vivo* animal disease models, and human *ex vivo* functional analysis of PPIs within their ND interactome, providing evidence that genetic modifiers of NDs are likely present in the interactome. Clearly, Y2H remains a powerful tool to describe disease-related hubs of protein interactions, although this method has yet to be used to establish brain-wide interactome maps.

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Table 1: Overview of proteomics approaches used to survey brain PPIs, with strengths and weaknesses

Approach	Studies	Strengths	Weaknesses
Affinity purification mass spectrometry	Scifo et al. [15] 2015: PPT1 interactome Pires et al. [16] 2023: pTau interactome Wang et al. [17] 2024: hcASD	High specificity High throughput	Laborious sample preparation and purification Requires either recombinant expression or bait-specific antibodies Unable to distinguish direct vs. indirect interactors Potentially losing weaker/transient PPIs from washing steps High background rates if not properly controlled
Proximity labeling	Bozic et al. [21], 2021: *c9orf72* dipeptide repeat protein interactomes (FTD and ALS) Unda et al. [24], 2022: OTUD7A interactome (15q13.3 microdeletion syndrome, ASD, and epilepsy) Prikas et al. [23], 2022: tau interactome (ALZ) Irala et al. [25], 2024: NCAN C-term and N-term interactomes (Bipolar disorder, mania, and schizophrenia) Bowen et al. [26], 2024: Kv1.3 C-term and N-term interactomes (chronic neuroinflammatory and neurodegenerative disorders) Xu et al. [27], 2024 preprint: Ten-m intracellular domain interactome (sensory and motor dysfunction disorders) Batra et al. [22], 2024 preprint: aggregating tau interactome (ALZ) Takato et al. [28], 2024: Photoreactive PhoxID Zhu et al. [29], 2024: Tyrosine-based BmTyr-ID Kim et al. [30], 2024: Calcium-gated Cal-ID	*In situ* labeling, can capture PPIs in their native environment Can capture indirect PPIs in addition to direct-binding partners Can capture cell type- and/or compartment-specific PPIs	Must use appropriate controls to limit identification of nonspecific PPIs *In vivo* administration of biotin substrate can be challenging in mice The use of peroxidases can create cytotoxic products Unable to distinguish direct vs. indirect interactors
Co-fractionation mass spectrometry	Pourhaghigh et al. [35], 2018 Liebeskind et al. [36] 2020 Shrestha et al. [37] 2024	High throughput Can be applied to a wide variety of samples without the need for genetics or affinity reagents Detects PPIs among endogenous proteins	Labor intensive Unable to distinguish direct vs. indirect interactors Data analysis is computationally intense and complex Tends to be biased toward more abundant proteins
Chemical cross-linking mass spectrometry	Gonzalez-Lozano et al. [44], 2020 Wittig et al. [45] 2021	High throughput Detects direct binders Provides amino acid resolution structural information on the interactions	Low abundances of cross-linked peptides, benefits from enrichment Limited cross-linkers and chemistries to choose from
Yeast two-hybrid	Goehler et al. [58], 2004: Huntington's disease (HD) interactome Lim et al. [59], 2006: Spinocerebellar Ataxia (SCA) interactome Haenig et al. [60], 2020: Neurodegenerative disease interactome	Detects direct binders Can be performed by most labs without expensive equipment Can clone entire cDNA libraries into Y2H-compatible expression vectors to test large gene sets Can be automated to scale up the number of interactions that can be tested	High false-positive and false-negative discovery rates unless properly controlled Requires the use of engineered fusion proteins that may not fold properly Yeast cells may not mimic the natural environment of the PPI of interest (*i.e.* differences in PTMs, codon usage, etc.) Proteins are typically expressed out of context, e.g. in the nucleus, and lack other binding partners

PPIs, protein–protein interactions. Y2H, yeast two-hybrid. hcASD, high-confidence autism spectrum disorder. pTau, phosphorylated tau. CF-MS, Co-fractionation Mass Spectrometry.

Conclusions

Advances in technology are enabling increasingly detailed studies of neural connections, yet the global mapping of the molecular equivalent—the PPIs and protein functional networks in the brain—remains challenging. Recently, proteomics strategies have begun illuminating the extensive networks of protein interactions that underpin both healthy and diseased states in the CNS. In this review, we highlighted four MS-based techniques and a non-MS method that have been instrumental in mapping brain interactomes (Figure 1 and Table 1). These approaches have revealed the critical roles of both stable and transient PPIs in neural function. Ongoing innovations in PPI discovery techniques are poised to enhance sensitivity and throughput, paving the way to uncover functions for many uncharacterized brain proteins.

Complementing these experimental advancements, artificial intelligence (AI)-based tools such as AlphaFold [65] and AlphaFold-Multimer [66] are revolutionizing the field of protein structure prediction and PPI research. Traditional methods for studying PPIs and protein structures, including X-ray crystallography and cryo-electron microscopy, are limited in scalability, while AI-driven approaches offer unprecedented opportunities to model protein complexes with high accuracy on a large scale. In the context of brain research, where the proteome is exceptionally complex, AI predictions can aid in mapping synaptic protein networks, exploring neurodevelopmental pathways, and understanding disease mechanisms. Integrating AI-generated predictions with experimental data from proteomics approaches, such as CF-MS, PL-MS, and structural insights from XL-MS, offers the potential to significantly enhance the accuracy and depth of interactome maps. As AI algorithms continue to evolve and integrate multi-modal datasets, they hold the potential to accelerate discoveries in understanding the molecular basis of neural function and dysfunction.

Perspectives

- The rapid growth of technologies for identifying and characterizing protein–protein interactions (PPIs) has transformed our ability to study brain proteins. A comprehensive map of PPIs in the brain is crucial for understanding the mechanisms underlying normal cognitive processes and the pathophysiology of neurological disorders.
- Recent advances in PPI mapping provide a holistic view of neuronal protein function and dysfunction. Each method has unique strengths and limitations (Table 1), but integrating these approaches offers unparalleled potential to uncover functional protein complexes in the brain. Greater adoption of PPI techniques in neuroscience research will be key to 455 unlocking their full utility.
- Brain proteomics is advancing rapidly, driven by cutting-edge technologies and interdisciplinary efforts. Continued development of innovative PPI discovery tools will enhance spatial resolution, sensitivity, and throughput, enabling deeper insights into brain function and disease.

Conflicts of Interest

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ALZ, Alzheimer's Disease; AP-MS, affinity purification mass spectrometry; ASD, autism spectrum disorder; CF-MS, co-fraction mass spectrometry; CF-MS, Co-fractionation mass spectrometry; CNS, central nervous system; NDDs, neurodevelopmental disorders; NDs, neurological diseases; PL-MS, proximity labeling mass spectrometry; PPIs, protein–protein interactions; XL-MS, chemical cross-linking mass spectrometry; Y2H, yeast two-hybrid.

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