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Proteomic Analysis of Postsynaptic Protein Complexes Underlying Neuronal Plasticity

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Abstract

Normal neuronal communication and synaptic plasticity at glutamatergic synapses requires dynamic regulation of postsynaptic molecules. Protein expression and protein post-translational modifications regulate protein interactions that underlie this organization. In this Review, we highlight data obtained over the last 20 years that have used qualitative and quantitative proteomics-based approaches to identify postsynaptic protein complexes. Herein, we describe how these proteomics studies have helped lay the foundation for understanding synaptic physiology and perturbations in synaptic signaling observed in different pathologies. We also describe emerging technologies that can be useful in these analyses. We focus on protein complexes associated with the highly abundant and functionally critical proteins: calcium/calmodulin-dependent protein kinase II, the N-methyl-D-aspartate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors, and postsynaptic density protein of 95 kDa.

Graphical abstract

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Notes

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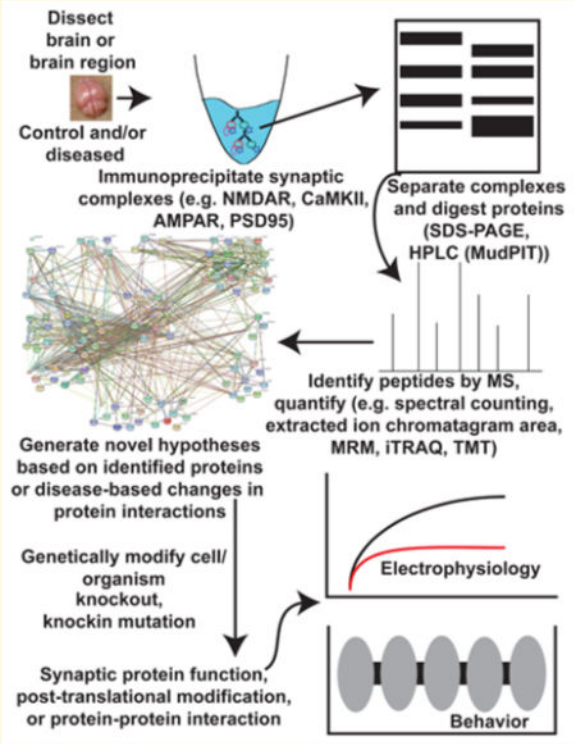

Keywords

Mass spectrometry; calcium/calmodulin-dependent protein kinase II; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; N-methyl-D-aspartate receptor; postsynaptic density protein 95; postsynaptic density; signaling

INTRODUCTION

While mass spectrometry based approaches have been used to quantify protein abundance changes that underlie long-term plasticity and learning and memory,[1] these approaches are also well-suited to understand postsynaptic protein post-translational modifications, interactions, and protein function that allow for a rapid response to alterations in neuronal excitability. Following presynaptic release, glutamate diffuses across the synaptic cleft to activate multiple classes of glutamate receptors, including N-methyl-D-aspartate receptor (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). Signaling molecules such as calcium/calmodulin-dependent protein kinase II (CaMKII) associate with glutamate receptors or voltage-gated calcium channels to respond to calcium that enters through these proteins. While CaMKII is one of the most abundant enzymes found in the postsynaptic density (PSD; Figure 1),[2,3] many other abundant proteins act as scaffolds and are required for localizing glutamate receptors and other machinery directly opposed to release sites (Figure 1).[4] While early studies employed electron microscopy and immunoblotting to isolated PSDs obtained via subcellular fractionation, shotgun-based proteomics approaches are exquisitely sensitive to identify and quantify components of the PSD. These approaches have utilized PSD enrichment strategies such as

subcellular fractionation to isolate PSDs followed by mass spectrometry to identify and quantify PSD proteomes.[1,5–18] It is important to note that different PSD preparations and mass spectrometry instrumentation may lead to distinct PSD proteomes.[19–21] However, there is overlap between many of these approaches. For instance, integrating multiple approaches and studies, Grant and colleagues defined what they called the consensus PSD, which contained 466 total proteins. They compared 8 different studies and found overlap between the PSD proteomes that ranged between 31% and 100%.[19] These approaches have identified highly abundant PSD proteins such as NMDARs, AMPARs, CaMKII, and PSD-95. Here we review how proteomics approaches have been used to interrogate postsynaptic protein complexes associated with the above proteins and discuss how this knowledge has increased our understanding of synaptic protein organization and function.

Understanding how synaptic proteins are organized is important in decoding physiological changes that underlie normal learning and memory. These learning and memory changes are encoded via a process termed synaptic plasticity. Long-term potentiation (LTP) and long-term depression (LTD) are two molecular correlates of synaptic plasticity. Reorganization of synaptic protein components occurs during LTP and LTD. This reorganization can be achieved by changes in the levels or activity of signaling molecules such as kinases and phosphatases as well as changes in synaptic ion channel number or conductance. Throughout this Review, we focus on how synaptic protein modifications and interactions convey proper postsynaptic organization and facilitate normal synaptic plasticity both acutely and long-term. Moreover, we describe how perturbations in synaptic protein modifications and interactions are associated with pathological conditions, such as stroke, autism spectrum disorders like Angelman syndrome, and Parkinson disease. Finally, we describe emerging technologies and approaches that can be used to enhance detection of low-abundance synaptic protein complexes and post-translational modifications.

N-METHYL-D-ASPARTATE RECEPTORS (NMDARs)

The NMDARs are ionotropic glutamate receptors that allow for calcium and sodium influx into the postsynapse. The NMDAR is made up of an obligate GluN1 subunit and different GluN2 (or less commonly GluN3) subunits. In certain brain regions, there is a developmental shift from GluN2B to GluN2A such that GluN2B expression decreases and GluN2A expression increases.[22] In rats, GluN2B levels decreased in both Cornu Ammonis (CA)1 and CA3 regions of the hippocampus beginning 4 days after birth and reaching adult levels (~40% decrease) by postnatal day 21.[23] Similar results were observed in humans, where the GluN2A:GluN2B mRNA ratio increases in the CA1 and CA3 region, but not the dentate gyrus.[24] Despite age-dependent reductions in GluN2B levels, recent studies suggest that triheteromeric NMDARs containing GluN1/GluN2A/GluN2B may be the most abundant form in dissociated hippocampal neurons and adult hippocampal synapses.[25,26] Accordingly, global knockout (KO) of the GluN1 subunit in mice resulted in loss of NMDAR activity, reduction in expression of the GluN2B subunit of the NMDAR, and death of animals by 15 h after birth.[27] KO of the GluN2B subunit in CA3 pyramidal cells of the hippocampus abrogates NMDAR-dependent currents, decreases spine density, and attenuates hippocampal LTP.[28] In contrast to GluN2B KO animals, loss of GluN2A attenuated CA1 hippocampal LTP but had fewer additional effects.[29] The above data

demonstrate that NMDAR subunit composition is developmentally and spatially organized. Moreover, the NMDAR is critical in different forms of LTP and LTD. However, understanding the full complement of NMDAR interactions and how those differ under physiological and pathological changes is well-suited for proteomics-based studies.

NMDAR-dependent synaptic plasticity requires proper expression, localization, and function of the NMDAR. In one of the first neuroproteomics studies ever performed, multiple affinity isolation techniques, including immunoprecipitation and immunoaffinity purification of GluN1 complexes, and peptide isolation of GluN2B complexes, were used to identify 66 different proteins that coimmunoprecipitate with the NMDAR.[30] This approach allowed for identification of proteins that associate with both GluN1 and GluN2B or that may be enriched in one complex or another. For instance, SAP102 was weakly detected in the GluN1 immunoprecipitates and immunoaffinity purification, but was strongly detected in the GluN2B peptide isolation.[30] Using the string database (www.string-db.org)[31] to analyze a subset of these proteins, we highlight the interconnectivity of these molecules (Figure 2). We mapped those interactions that were experimentally validated and had medium to high confidence (0.4–0.9) to generate a connectivity map between the proteins that were detected in the GluN2B immunoprecipitates (Figure 2). NMDARs interact either directly or indirectly with kinases such as CaMKII, PKA, and PKC and phosphatases such as protein phosphatase 1, 2A, and 5, calcineurin, and tyrosine-protein phosphatase nonreceptor type 11 (Figure 2), signaling molecules that are known to regulate NMDAR function.[32] PKA interacts with scaffolding/regulatory subunits termed A-kinase anchoring proteins (AKAPs), whereas PKC interacts with regulatory subunits for C-kinase (RACKs) for PKC. These subunits help target them to the NMDAR.[33] CaMKII per se, AKAPs, or RACKs can target the kinase and/or interacting proteins to the NMDAR. In addition to kinases, the NMDAR was also found to associate with multiple scaffolding proteins including PSD-93, PSD-95, SAP-102, Homer 1, and Shank 1 and 2 (Figure 2), synaptic proteins that can modulate NMDAR targeting, localization at the PSD, and channel function.[34–41] The scaffolding and GTPase activating protein, SynGAP1, also associates with the NMDAR. The association between the GluN2B subunit of the NMDAR and SynGAP1 modulates synaptic transmission through structural roles and/or RAS-GTPase activity that, together with NMDAR function, modulate ERK/MAPK signaling.[42–45] Cytoskeletal proteins including actin, α-actinin, tubulin, and myosins also associate with the NMDAR. Interestingly, NMDAR activity regulates phosphorylation of cytoskeletal proteins such as MAP2 to modulate the synaptic cytoskeleton.[46] Moreover, cytoskeletal proteins such as actin and myosins regulate NMDAR properties.[47,48]
Proteomics studies to identify novel interacting proteins do not require a priori knowledge of those interactions and the data obtained in this initial proteomics study helped direct many subsequent studies probing the function of specific interactions. However, it is important to address some caveats of these interactome maps. Using the above criteria, only two proteins were not connected to any other protein. These include Ppp1ca (the catalytic subunit of PP1) and Hspa1a. However, PP1 is known to modulate NMDARs[49] and other synaptic proteins, suggesting a functional association with the NMDAR and additional proteins. Therefore, the fact that an interaction is not observed does not mean it does not exist. Moreover, while we restricted these maps to those associations for which experimental data is available, one can also utilize the string-db to glean information from other sources, such as data mining and

co-occurrence in the literature. However, while these approaches are useful to generate hypotheses, specific experimental approaches need to be performed to validate these large interactome maps as well as to test specific hypotheses.

This initial NMDAR proteomics study was performed under basal conditions. NMDAR activity and membrane localization is regulated by multiple factors, including phosphorylation. For example, CaMKII, PKC, and death-associated protein kinase 1 (DAPK1) phosphorylate GluN2B at Ser1303. Phosphorylation at Ser1303 and/or Ser1323 by PKC or DAPK1 can enhance channel conductance.[50] However, other studies have suggested that Ser1303 phosphorylation by CaMKII reduce channel conductance by enhancing desensitization in HEK293 cells.[51] Recent data suggest that the differential role of Ser1303 phosphorylation by different kinases is actually due to Cl^- levels in the cells, as Ser1303 phosphorylation enhances desensitization in the context of low intracellular Cl^-, but decreases desensitization in the context of high intracellular Cl^-.[52] In addition to Ser1303 phosphorylation, casein kinase 2 (CK2) phosphorylates GluN2B at Ser1480, which decreases GluN2B binding to synaptic scaffolding proteins such as PSD-95 and SAP-102 and attenuates surface expression of the receptor.[53] Increased CK2 activity during development decreased synaptic GluN2B expression.[54] Together, these data have identified multiple NMDAR-specific interacting proteins and have determined phosphorylation sites that modulate these interactions. However, many of these studies use phosphorylation-specific mutants such as alanine (to prevent phosphorylation) or aspartate/glutamate (to mimic phosphorylation). One of the benefits of proteomics approaches is that one can survey multiple phosphorylation sites on a protein of interest in an unbiased manner and without potentially perturbing additional sites. Interestingly, this type of approach uncovered multiple novel phosphorylation sites on GluN2A and GluN2B.[55] Moreover, coupling proteomics approaches to biochemical preparations that isolate different subcellular fractions can allow for tracking of specific phosphorylation sites in different parts of the neuron. This type of approach can also be used to determine how different pathological or developmental conditions impact phosphorylation at multiple sites at one time.

Proteomics experiments have revealed pathological perturbations in the NMDAR interactome. For instance, the GluN2B subunit of the NMDAR has enhanced association with synaptic proteins during aging.[56] In addition, withdrawal from chronic intermittent ethanol (CIE) treatment increased the association of GluN2B with activity-regulated cytoskeleton-associated protein (Arc) and Homer1,[57] two NMDAR interacting proteins that were detected in the initial NMDAR proteomics screen.[30] Translocation of Arc mRNA to synapses has been shown to require NMDAR activity.[58] Also, Arc mRNA synthesis is enhanced by epileptic seizures.[58–60] Interestingly, the observed CIE-dependent increased association of Arc and Homer with the NMDAR occurred in a synaptic subcellular fraction. Previous studies have found that synaptic and extrasynaptic NMDAR activation differentially modulate ischemia-induced injuries and neurodegenerative diseases.[61–63] Using proteomics to delineate unique subcellular differences in the NMDAR interactome in different disease states will greatly inform the function of different complexes and determine how alterations in these complexes may underlie specific pathologies.

CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II (CaMKII)

NMDARs allow for ions such as Ca^{2+} to enter the postsynaptic spine. Ca^{2+} entry via NMDARs and other sources activates molecules such as CaMKII. When the calcium signal is robust and persistent, the kinase autophosphorylates at Thr286 on CaMKIIα which allows the kinase to retain Ca^{2+}-independent activity and regulates CaMKII interactions and subcellular localization.[64–67] CaMKIIα expression and autophosphorylation are required for NMDAR-dependent synaptic plasticity. Both complete loss of CaMKIIα and mutant CaMKIIα mice that cannot autophosphorylate at Thr286 (T286A) have abrogated LTP and LTD and behavioral deficits.[68–72] It is important to note that there are four different isoforms of CaMKII: α, β, γ, and δ. While CaMKIIα is the most abundant CaMKII isoform in the forebrain, CaMKIIβ is the second most abundant CaMKII isoform in the forebrain with one CaMKIIβ molecule per three CaMKIIα molecules. In contrast to forebrain, CaMKIIβ is three times more abundant than CaMKIIα in the cerebellum[73] and CaMKIIβ KO mice have altered cerebellar plasticity that leads to deficits in motor coordination.[74] The above studies demonstrate that kinase expression and/or autophosphorylation are critical for normal synaptic function in different parts of the brain.

CaMKII is the most abundant protein in the PSD, with levels > 1.5 pmol/μg protein across CaMKIIα and CaMKIIβ isoforms.[4] This protein accounts for ~6% of the total protein in the PSD.[3,75] Biochemical estimates of the abundance of endogenous kinase suggest 80 dodecameric holoenzymes (~960 CaMKII molecules) per typically sized (~100 nm^2) PSD.[3,75] While this estimation may be conflated due to translocation and targeting postdecapitation during sample preparation,[76] it remains interesting that a kinase is so highly concentrated in this subcellular compartment. While estimation of CaMKII abundance in the PSD may be high, CaMKII actively translocates in slices and cultured neurons (within 30 min of stimulation),[77–80] indicating that activated CaMKII moves to preferentially associate with the PSD following glutamate signaling.

In contrast to Thr286 autophosphorylation enhancing CaMKII activity, Thr305/6 phosphorylation prevents Ca^{2+}/calmodulin-stimulation of CaMKII. Whereas T286 phosphorylation enhances CaMKII at the PSD, Thr305/6 phosphorylation diminishes CaMKII targeting to the PSD.[78,79] Functionally, Thr286 phosphorylation without Thr305/6 phosphor-ylation leads to Ca^{2+}/calmodulin-independent CaMKII activity along with Ca^{2+}/calmodulin binding. This form of the kinase favors enhanced synaptic strength.[81,82] However, T286D mutants in combination with TT305/6DD mutants decrease synaptic strength.[83] Proteomics-based approaches have recapitulated overexpression and immunoblotting data showing that CaMKIIα phosphorylation at Thr286 is greatest in the synaptic fraction whereas phosphorylation at Thr306 is greatest in a cytosolic fraction.[66]

As mentioned above, T286A mutant CaMKIIα had lower levels in a synaptic fraction; however, immunoprecipitation of the kinase followed by both immunoblotting and proteomics approaches revealed that the kinase that was detected in this fraction had a greater association with multiple synaptic scaffolding proteins and NMDARs.[66,84] Previous studies have suggested that the NMDAR is critical in targeting CaMKII to the PSD and that autophosphorylation of the kinase enhances the association of CaMKII with the GluN2B

subunit of the NMDAR.[85–87] This discrepancy may be due to differences in using the T286A mutant kinase, which can regulate additional phosphorylation sites on CaMKII as well as calmodulin binding affinity.[66,88] Together, the above data suggest that CaMKII binding to the NMDAR is critical in regulating autophosphorylation-dependent CaMKII targeting to the PSD; however, additional synaptic proteins may also play critical roles. For example, based on a recent proteomics study, the cytoskeletal protein, brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2, also known as IRSp53) had a decreased association with CaMKII in a synaptic fraction.[66] BAIAP2 is a highly expressed PSD protein with an abundance of 23.5 fmol/μg protein.[4] BAIAP2 KO mice have decreased PSD size, but increased insertion of NMDARs and enhanced hippocampal LTP.[89] Moreover, proteomics studies determined that BAIAP2 expression is decreased in human Alzheimer disease patients.[90] It is important to note that due to the multimeric structure of CaMKII, immunoprecipitation of CaMKII may isolate low levels of interacting proteins. If a single CaMKII molecule is bound to your protein of interest, immunoprecipitating that CaMKII molecule will copurify 11 other CaMKII molecules, but only one interacting protein. Therefore, low abundance CaMKII interacting proteins may be valid interactors and using technologies such as multidimensional protein identification technology (MudPIT) and other approaches to detect low abundance proteins will be useful in identifying novel interacting proteins (see Summary, Emerging Approaches, and Future Directions, below). Moreover, given the dodecameric structure and the high abundance of CaMKII in the PSD, as well as the panoply of CaMKII interacting proteins, CaMKII may also function as a structural or scaffolding protein in dendritic spines.[91]

Many of the studies that have delineated the function of CaMKII autophosphorylation at Thr286 and Thr305/6, have done so using phosphorylation mutants. These mutants do not fully recapitulate a nonphosphorylated residue (e.g., T286A) or a phosphorylated residue (e.g., T286D). Moreover, phosphorylation mutants at one site may influence the phosphorylation status of additional sites.[66] While Thr286 and Thr305/6 are the most well-studied sites of autophosphorylation, CaMKIIα is autophosphorylated at multiple other sites, including Thr253, Ser279, Thr305, Thr306, Ser314, Ser318, Ser331, Ser333, Thr334, Thr336, and Thr337 (Figure 3).[92–102] In addition to these sites on CaMKIIα, CaMKIIβ contains an actin-binding domain and can be phosphorylated at Ser315 and Thr320/1 nearby and within this domain. Phosphorylation at these CaMKIIβ sites is associated with the cytosolic fraction[66] suggesting phosphorylation at these sites may modulate CaMKIIβ interactions and synaptic targeting. While many of these sites have been identified as autophosphorylation sites, the interplay between them in vivo is not completely understood. Many of the above phosphorylation sites have been identified in vivo using proteomics approaches and future proteomics-based studies will be needed to track phosphorylation changes at multiple sites under different pathological conditions and/or subcellular fractions.

CaMKII phosphorylation is modulated in different disease states. For example, Thr286 phosphorylation is increased in animal models of Parkinson disease[103,104] and Thr305/6 phosphorylation is increased in an animal model of Angelman syndrome.[105] Rescue of changes in CaMKII autophosphorylation/activity normalizes behavioral deficits observed in these animals.[104,105] In addition to Parkinson disease and Angelman syndrome, CaMKII autophosphorylation and interaction with the NMDAR is increased in brain slices following

oxygen/glucose deprivation, a model of ischemia.[106] Furthermore, a recent phospho-proteomics study identified multiple phosphor-ylation sites on different synaptic proteins, including phosphorylation of Ser331 on CaMKIIα. Phosphorylation at this site was increased by cocaine-cue memory extinction, but decreased by cocaine-cue memory reconsolidation.[107] While individual phosphorylation sites have been interrogated on CaMKII, understanding the interdependencies of phosphorylation on the kinase would be very interesting. As an emerging field, top-down proteomics is useful in identifying not only individual phosphorylation sites, but the phosphorylation signal or code on an intact protein molecule.[108] For CaMKII, this approach would be useful in determining how a single kinase subunit within a holoenzyme is phosphorylated in a specific subcellular localization or in response to physiological or pathological changes in synaptic plasticity. Using proteomics to understand pathological changes in kinase autophosphorylation, protein interactions, and subcellular localization in different neurological diseases will increase understanding of the normal and pathological role of this multifunctional kinase and may uncover novel targets to treat multiple synaptopathies.

α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID RECEPTOR (AMPAR)

AMPARs are ionotropic glutamate receptors that allow for Na^+ (and in some cases Ca^{2+}) to enter the postsynapse. There are four subunits of the AMPAR, GluA1−4, with the GluA2 subunit being the most abundant, followed by GluA1, then GluA3, and then GluA4 (Figure 4). NMDAR-dependent synaptic plasticity requires CaMKII activation. Activation of CaMKII and the NMDAR modulate AMPAR localization at the synapse, causing long-term plasticity changes. Increased AMPAR levels in the membrane are essential for LTP, whereas decreased AMPARs in the membrane underlies LTD. Multiple, immunoprecipitation-based studies have identified novel proteins that interact with the AMPAR to modulate AMPAR insertion into membranes and/or AMPAR channel conductance.[109–111] Quantitative proteomics approaches have recently been used to determine absolute or relative abundance of AMPAR interacting proteins.[110–112] Based on these quantitative data, we have generated a schematic model depicting the ratiometric expression of AMPARs and their interacting proteins (Figure 4). The transmembrane-AMPAR regulatory proteins (TARPs) are localized to the membrane (shown as inner components in Figure 4). Multiple TARP isoforms include γ-8, γ-3, γ-2, γ-7, and γ-4. Knockout of TARPγ-2 (aka stargazin) leads to deficits in basal AMPAR synaptic transmission in hippocampal neurons and also AMPAR insertion in cerebellar granule cells.[113] Mice with a spontaneous mutation in what was later described as stargazin were found to have an ataxic gait and were prone to seizures.[114,115] Stargazin and TARPγ-7 are the main TARPs expressed in the cerebellum,[112] but other TARPs may compensate for stargazin activity at other synapses.[116] TARPγ-8 is one of the most abundant TARPs detected in the whole brain (Figure 4).[110] Recent studies have found that CaMKII phosphorylates TARPγ-8 at Ser277 and Ser281. Phosphorylation at these sites enhances AMPAR transmission and is required for LTP.[117] Conversely, mutating similar phosphorylation sites on stargazin in the context of knocking out TARPγ-3 and TARPγ-4 had no effect on LTP,[117] suggesting that other TARPs do not compensate for loss of TARPγ-8.

Germ cell-specific gene 1-like protein (GSG1L) is a novel AMPAR interacting protein that was found using proteomics-based approaches and that uniquely modulates AMPAR function by altering desensitization kinetics.[110,111] GSG1L has also been shown to suppress the calcium permeability of AMPARs.[118] In addition to GSG1L, proteomics studies have uncovered other proteins such as Abhydrolase domain-containing 6 (ABHD6) and porcupine (PORCN), which modulate AMPAR trafficking to the membrane.[119] Unbiased, proteomics-based approaches are well-suited for identifying interacting proteins such as those where there is no a priori knowledge that the protein may interact with the receptor.

In addition to identification of AMPAR components, proteomics approaches have also been utilized to determine developmental and region specific differences in AMPAR levels and interactions.[112,120] The hippocampus, cortex, and cerebellum have the highest relative amounts of AMPAR levels.[112] While the quantity of the AMPARs are fairly stable during development, the associated proteins tend to vary. TARP γ-4 is highest at birth but decreases as animals age to adults. In contrast, TARP γ-8 increases during development to adult levels in rat brain.[112] Proline Rich Transmembrane Protein 1 (PRRT1) was expressed in, and interacts with, AMPARs most robustly in the hippocampus. GSG1L had robust interaction with AMPARs in the cortex and less association in the hippocampus. Ras-related protein Rap2B (RAP2B) and Abhydrolase domain-containing 12 (ABHD12) had robust interactions with AMPARs in the hippocampus. Characterization of AMPAR proteomes from different brain regions and/or subcellular fractions has greatly enhanced our knowledge of differential AMPAR complexes; however, it is currently unclear how different physiological (e.g., induction of LTP/LTD) or pathological perturbations may modulate these specific complexes. Recent work by Castillo, Tomita, and colleagues has shown that, in addition to its role in LTP in hippocampal slices (see above), phosphorylation of TARP γ-8 at Ser-277 and Ser-281, is critical for both cued and contextual fear memory.[117] While this is one change, it is likely that changes in the phosphorylation of, or association of AMPARs with, additional AMPAR interacting proteins also occurs in normal plasticity and under pathological conditions. Understanding pathologies associated with interactions in specific brain regions and/or cell types may reveal novel disease targets that have enhanced specificity for treating diseases associated with plasticity deficits.

POSTSYNAPTIC DENSITY PROTEIN OF 95 kDa (PSD-95)

PSD-95 is a highly enriched scaffolding protein that has multiple organization domains. PSD-95 and other scaffolding proteins interact with multiple synaptic proteins including NMDARs, AMPARs, and CaMKII (Figure 5) to properly localize them at the PSD. PSD-95 is expressed at a concentration of ~85 fmol/μg in forebrain PSDs, making it one of the most abundant adaptor/scaffold-type proteins.[4] Knockdown of PSD-95 decreases AMPAR levels in specific membrane patches.[121] KO of PSD-95 increases protein expression of a synaptic tyrosine phosphatase, striatal-enriched protein tyrosine phosphatase ($STEP_{61}$), causing a decrease in synaptic GluN2B protein at the membrane.[122] Moreover, PSD-95 KO animals have decreased spine density in the striatum and increased spine density in the hippocampus.[123] PSD-95 KO animals also have specific physiological and behavioral abnormalities,[124] including enhanced LTP, abrogated LTD, and impaired spatial memory deficits in aversive behaviors.[125,126]

While PSD-95 KO mice have many deficits, overall they are viable and do not have as severe of a neurological phenotype as may be thought given PSD-95 abundance and ability to recruit and stabilize proteins at the PSD. This minimal synaptic phenotype may be due to overlapping function of other adaptor/scaffolding proteins at the PSD. Specifically, 12 adaptor/regulatory proteins associated with PSD-95 in a proteomics screen, including: PSD-93, SAP-102, SAP-97, SAPAP1, SAPAP2, SAPAP3, and SAPAP4.[127] While these proteins may compensate for loss of PSD-95, they are normally expressed at 6–40-fold lower levels than PSD-95 (~2–15 fmol/μg).[4] Grant and colleagues identified 118 proteins in at least three biological replicates that coprecipitated with PSD-95 that they have defined as constituents of the "core" PSD. We have plotted these proteins using the String database and the protein function categories given by Fernandez et al. (Figure 5).[127] As expected there are multiple known nodes of association between the identified PSD proteins. To understand how the PSD-95 interactome compares to other synaptic protein interactomes, we can compare the NMDAR interactome (Figure 2)[30] with the PSD-95 interactome (Figure 5).[127] If we use the same parameters as Figure 2 and only plot associations that have been experimentally validated, a large majority of the proteins are not connected (data not shown). Therefore, we evaluated interactions that were not only experimentally validated, but also validated by textmining, databases, coexpression, neighborhood, gene fusion, and/or co-occurrence. Even adding other evidence of interactions, there were still 18 proteins that were unconnected. Given that the more recent studies use updated mass spectrometers, it is possible that these studies have probed deeper and have not been as well validated. Conversely, by detecting lower abundance interactors, it is possible that some of the interactions are nonspecific. Looking at specific proteins, one notices both overlap and unique proteins in each interactome. Examples of overlap include kinases, phosphatases, SynGAP, and cytoskeletal proteins. Conversely, some differences were observed. For instance, Shank and Homer were present in the NMDAR complex, whereas in the PSD-95 complex, Shank was only observed in a single biological replicate whereas Homer was not detected. Moreover, vesicular trafficking proteins and multiple ATPases were detected in the PSD-95 complexes, but not in the NMDAR complexes. These variances in data may be due to differences in the interactions between these molecules, but may also be due to technical advances in HPLC separations and parameters and mass spectrometers. As instrumentation and methodologies continue to improve, additional data will be generated, even from the same types of experiments. In addition to technical differences, isolation of NMDAR complexes would be predicted to include both synaptic and extrasynaptic complexes, which may have different interactions and organization. Comparing complexes from core PSD proteins such as PSD-95 with other synaptic protein complexes (like the NMDAR, which is localized both within and outside of the core PSD) will allow for the determination of the core components of the PSD as well as the identification of proteins that reside outside of the PSD that may have unique synaptic functions.

In addition to traditional isolation of PSDs using immuno or peptide isolation strategies, recent studies overexpressed a PSD-95 protein fused to an E. coli. biotinylating enzyme, BirA.[128] This fusion protein allows for biotinylation of proteins near the fused PSD-95 protein and streptavidin pull-down to identify proteins in proximity to PSD-95. Following streptavidin pulldown and mass spectrometry, multiple known PSD-95 interacting proteins

were identified, including NMDARs, AMPARs, and scaffolding proteins (e.g., Shank, PSD93, SAP97, SAP102).

Robust characterization of the PSD-95 interactome has identified multiple synaptic proteins and interactions, many of which have been implicated in neurological diseases, including autism spectrum disorder and psychiatric diseases.[129–131] Future studies will need to identify cell-specific changes in the PSD-95 interactome under different conditions and/or disease states.

SUMMARY, EMERGING APPROACHES, AND FUTURE DIRECTIONS

Proteomics approaches have identified and characterized multiple synaptic protein complexes. These approaches are well-suited to understanding how proteins interact and how those interactions are regulated in different cell types and under pathological conditions. Moving forward, technological advances will allow for a deeper understanding of the synaptic interactome. This deeper understanding will aid in identification of novel therapeutic targets.

As mentioned above, proximity labeling approaches were used to identify proteins in proximity to PSD-95. One potential drawback to this approach is that it may not detect tertiary interacting proteins, depending upon the distance of the tertiary interacting protein from the biotinylating enzyme. Also, addition of the biotinylating enzyme may induce ectopic changes to the interaction network. Furthermore, appropriate controls must be performed to account for proteins that nonspecifcally bind to streptavidin.[132] However, proximity labeling approaches like these[133] allow for detection of both direct and indirect interactions, as well as interactions that tend to be more transient or that are difficult to biochemically isolate.[134,135] Moreover, this approach allows for temporal and cell specific isolation of protein complexes. This proximity approach was used to biotinylate proteins that are near gephryin,[128] a protein enriched at inhibitory postsynaptic densities, an area that is difficult to biochemically isolate. This approach can enhance immunoprecipitation approaches that have been used to immuno-isolate inhibitory and purkinje synapses.[136,137]

Another proximity labeling approach uses horseradish peroxidase (HRP) or an engineered peroxidase called APEX (or APEX2 depending on iteration) that uses hydrogen peroxide and biotin-phenol substrate to label nearby proteins.[138,139] Specifically, neuronal cultures expressing HRP-tagged proteins were incubated with hydrogen peroxide and a membrane impermeable biotin phenol. This allowed for biotinylation of proteins in close proximity to the HRP-conjugated protein. Both excitatory and inhibitory synaptic cleft proteins were identified using mass spectrometry on streptavidin-isolated proteins.[132] Further refinement of this approach allows the APEX2 protein to be split into two parts, allowing for identification of proteomes only where both parts of the protein are expressed. Fusing one-half of APEX2 to neurexin and one-half to neuroligin allowed for identification of specific synapse proteomes in the visual system.[140] One of the drawbacks to these approaches may be toxicity of using hydrogen peroxide. However, this is not an issue with the BirA (BioID) approaches. Moving forward, one could envision using these types of approaches to further restrict expression of tagged proteins to specific cell types in addition to specific regions.

While proximity labeling approaches will allow for the identification of proteins in specific compartments such as the PSD, newer technological advances will improve identification of synaptic proteins as well. A standard workflow (see Abstract graphic) for isolation of synaptic protein complexes includes immunoprecipitation of the complex, followed by separation of the complex and identification of the complex components using mass spectrometry. Standard separation techniques include gel-based separation. However, advances in separation technologies, such as MudPIT, will allow for identification of lower-abundance proteins. This approach couples strong cation exchange followed by reversed phase HPLC to enhance the separation of peptides.[141] In addition to detecting more proteins by increasing detection of lower-abundance proteins and peptides, we will be able to identify post-translational modifications (PTMs) that occur at low levels. Coupling MudPIT with other technological advances such as improvements in mass spectrometers as well as in ionization approaches (e.g., electron transfer dissociation (ETD)) will be useful in detecting more PTMs by enhancing coverage and more faithfully preserving PTMs such as phosphorylation.[142] Moreover, top-down mass spectrometry approaches to analyze intact proteins will be useful in identifying specific “proteoforms” of proteins.[108,143] Specifically, this approach could map all of the PTMS on a single protein molecule. Furthermore, using multiple enzymes to digest complex mixtures along with the above newer technologies and chromatagrophy improvements has allowed for near complete examination of the yeast proteome.[144–146] One would predict that utilizing these approaches would allow for identification of a near-complete or complete interactome. These approaches will be critical for identification of low abundance, endogenous synaptic interactomes. However, with improved detection capabilities and the high abundance of certain synaptic proteins, especially in fractionated samples, appropriate controls including KO-based strategies are important in validating specific interactions.[147]

Moving forward, improvements in mass spectrometry instrumentation and data analysis tools will allow us to further identify protein interactions that underlie normal and pathological synaptic communication. In addition to improvements in instrumentation, label-based approaches are being developed to allow for relative or absolute quantification of proteins using stable-isotope labeling of amino acids in culture (SILAC) or isobaric mass tags (e.g., iTRAQ and TMT).[148] These approaches have been used to quantify tens to hundreds of proteins concurrently[149] and have been used to analyze relative PSD protein abundance and abundance of phosphorylation sites.[150] Targeted, label-free quantitative techniques such as selected reaction monitoring (SRM) and Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS) can be used to analyze tens to hundreds (SRM) or tens of thousands (SWATH-MS) of proteins at one time.[151] Specifically, SRM methods have been designed to quantify ~100 rat PSD proteins.[152] In addition, newer label free quantitation approaches such as isoQuant[20] or MaxQuant software and delayed normalization and maximal peptide ratio extraction[153] are useful. MaxQuant has been utilized to quantify levels of over 13 000 protein groups isolated from different structures and different cell types in the brain.[154]

Taken together, advanced methodologies along with improved hardware will enhance our ability to quantitatively probe the depths of global synaptic protein abundance, PTMs, and protein interaction networks under normal and pathological conditions in specific brain

regions and cell types. In addition to novel approaches and methodologies, performing multiple biological and technical replicates is also important in enhancing the full complement of detected proteins. Obtaining a complete and specific synaptic interactome and understanding physiological and pathological changes that regulate this interactome will be critical for identifying novel druggable pathways to treat myriad different neurological disorders.

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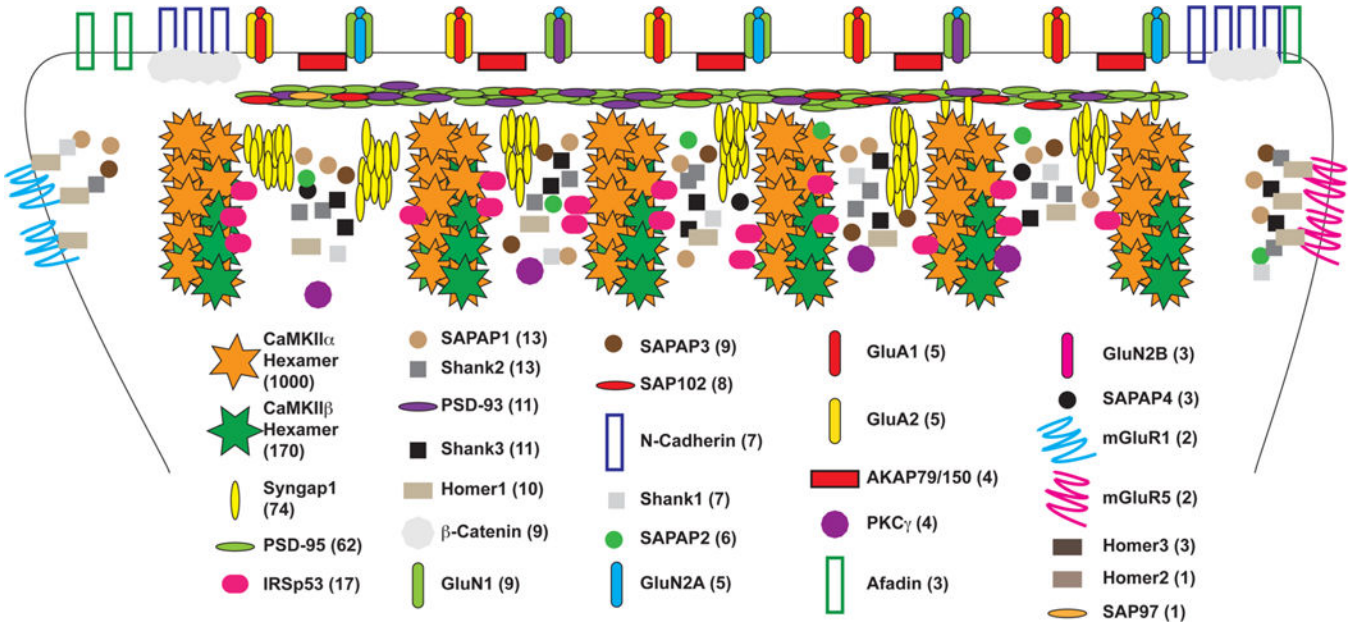

Figure 1.

Relative expression of PSD proteins. Schematic representation of multiple PSD-enriched proteins shown at relative abundance based on previous quantitative data.[4] Values in parentheses represent approximate arbitrary expression of PSD molecules with CaMKII, the most abundant PSD protein, set to 1000 individual subunits. The number of molecules shown is representative of this arbitrary expression.

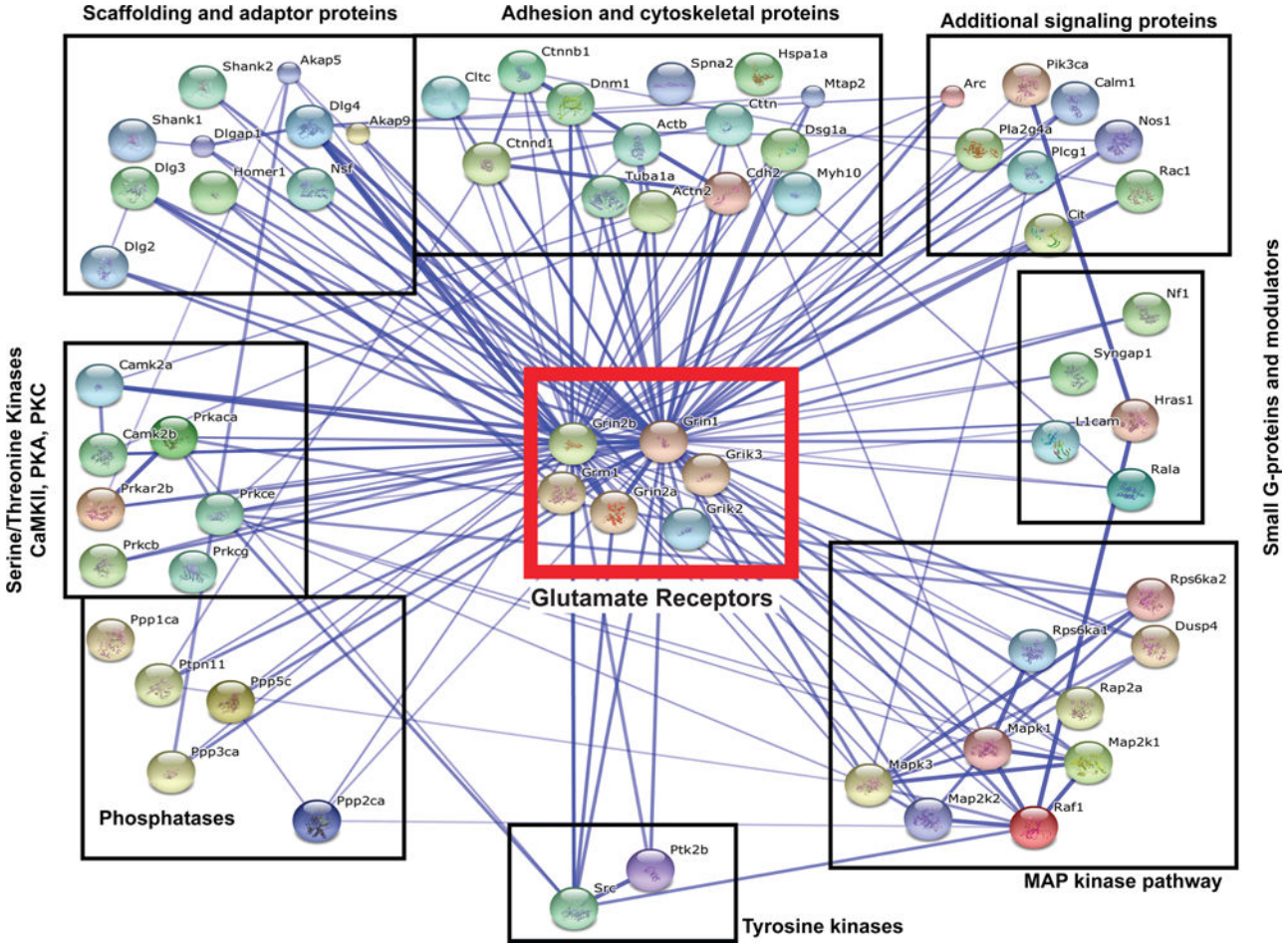

Figure 2.

Interconnectivity of the NMDAR interactome. The NMDAR interactome[30] was input into the string database (http://string-db.org/) to visualize interactions between the identified components. 66 proteins were evaluated. We set parameters to only detect interactions that were validated experimentally. The thickness of the line corresponds to the confidence of interaction (thin lines, >0.4; medium lines, >0.7; thick lines, >0.9).[31]

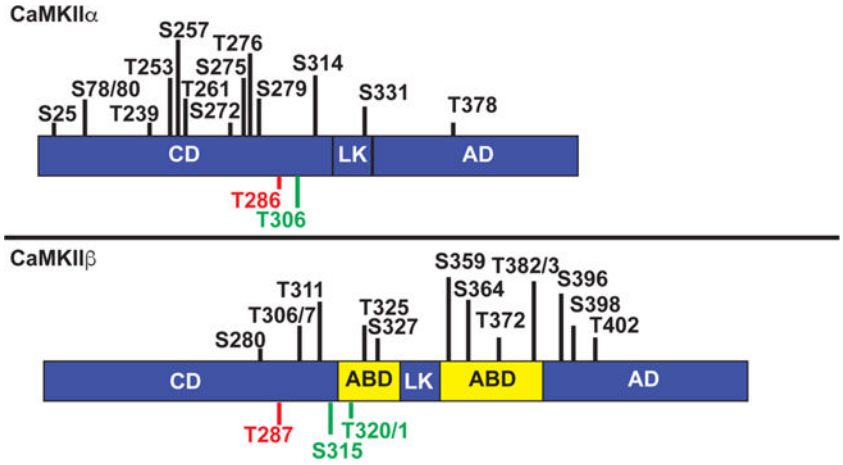

Figure 3.

CaMKII autophosphorylation sites.[102] Those autophosphorylation sites that are enriched in nonsynaptic (green) or synaptic (red) locations are labeled. CD, catalytic domain; AD, association domain; LK, linker region; ABD, actin-binding domain.

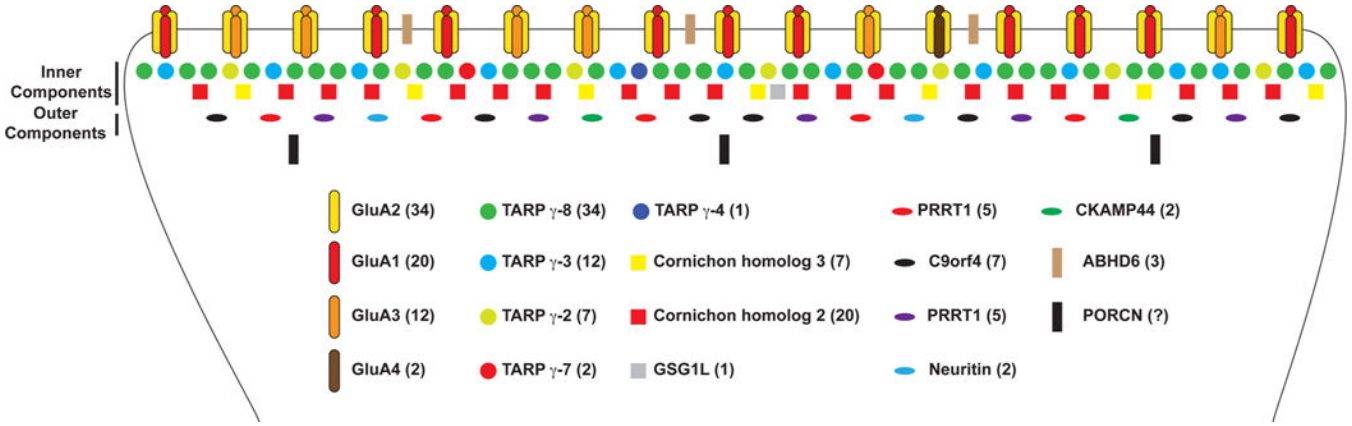

Figure 4.

Relative expression of AMPAR interacting proteins. AMPAR interacting protein abundance[110,111] was used to generate a schematic showing the relative abundance of AMPAR and proteomically quantified interacting proteins. Interactions are split into integral membrane proteins, inner components, and outer components. Ratiometric expression is normalized to the highest abundance protein, GluA2, which is set at an arbitrary value of 34.

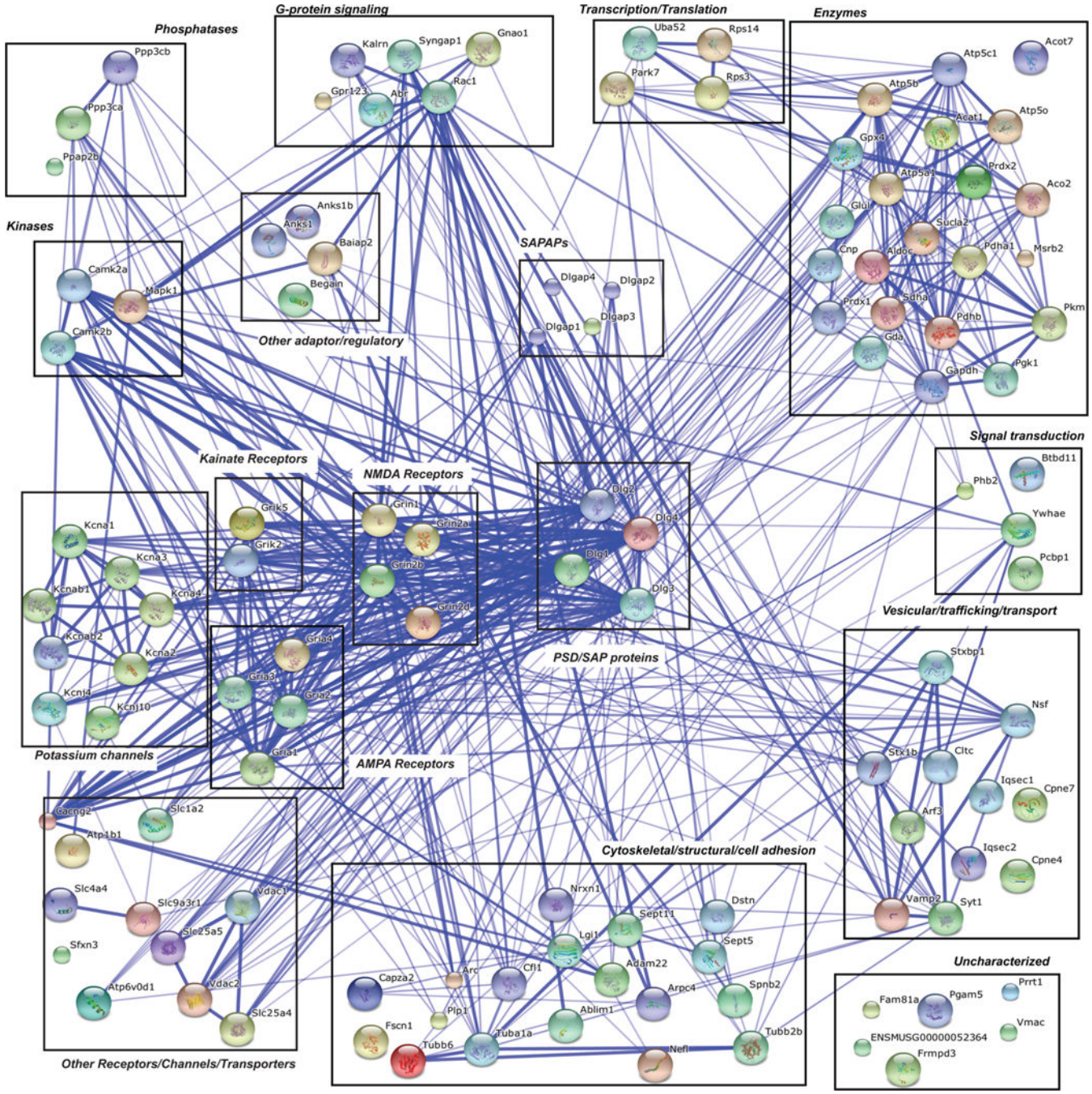

Figure 5.

Interconnectivity of the PSD-95 interactome. PSD-95 interacting proteins identified using a tandem-affinity purification approach[127] were input into the string database (http://string-db.org/) to visualize interactions between the identified components. 118 proteins were evaluated. We set parameters to only detect interactions that were found by multiple sources (see text). The thickness of the line corresponds to the confidence of interaction (thin lines, >0.4; medium lines, >0.7; thick lines, >0.9).[31]